NOTTINGHAM POLYTECHNIC
CLIFTON CAMPUS
CLIFTON LANE
NOTTINGHAM NG11 8NS

NOTTINGHAM POLYTECHNIC
CLIFTON CAMPUS
CLIFTON LANE
NOTTINGHAM NG11 8NS

NOTTINGHAM POLYTECHNIC
CLIFTON CAMPUS
CLIFTON LANE
NOTTINGHAM NG11 8NS

ProQuest Number: 10290182

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.



ProQuest 10290182

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

THE ROLE OF TRANSGLUTAMINASE IN STIMULUS-SECRETION COUPLING IN THE PANCREATIC β-CELL

A dissertation submitted to

The Council for National Academic Awards

by

Robin Antony OWEN

In partial fulfilment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

OCTOBER 1988

Department of Life Sciences

Trent Polytechnic

Nottingham

NOTTINGHAM POLYTECHNIC LIBRARY

LS.PhD 54

DECLARATION

This work has not been accepted in substance for any other degree and is not concurrently being submitted in candidature for any other degree.

This is to certify that the work here was carried out by the candidate himself. Due acknowledgement has been made of all assistance received.

Signed .....

(Candidate)

Signed .....

(Director of Studies)

I dedicate this thesis, to my wife Sue for all the love and support she has given me through these studies and always, and to my parents for their caring support throughout my education.

With love, Robin.

ACKNOWLEDGEMENTS

I am indebted to Dr. Martin Griffin for his resilience in guiding me through these studies and particularly his hard work during the presentation of my thesis (it has been character building stuff!).

I am additionally grateful to Dr. Peter Bungay for the dilligent and patient way he started me off on the project and for his friendship, his efforts are greatly appreciated. I would also like to thank Dr. Peter Walton for helpful discussions throughout the studies.

Dr. Sheila MacNeil and Rebecca Dawson aided me considerably with the calmodulin studies and I offer my warmest thanks to them. In addition I thank Dr. Ian Coutts and Dr. G. Michael Blackburn for the synthesis of test compounds.

Mrs Jane Kemp and Dr. Bernard Scanlon have benefited me with their friendly advice and technical assistance, and in helping me keep my sanity, for which I thank them.

I would like to express my gratitude to the technical staff of the Biochemistry Section, particularly Janet, Karen, Angela, Gill, and Josie, and additionally Chris, Louise, Nigel, Paul, and Siobhan, along with Mick and Maggs of the Physiology Section.

Mandy Richards, John Richardson and Dr. John Crookes of the radiobiology section provided invaluable assistance during these studies, particularly those connected with the ^{32}P studies.

My gratitude goes to Mr Martin Hutchings for the provision of the photographic material presented in this thesis and for assistance with publication material.

I have shared my little section of bench in the 'sardine can' with several characters, Ann the Sales Reps Dream, Door-slammer Dave, Iqbal, Social-conscience Jon, Fermenter Jean, Lighter-than-she-looks Karen, Immigration-Tax Kieran, Mike the Mouth, and latterly Ros and Mark, thanks for tolerating me and all my practical jokes, good luck for the future.

Miss Sharon Miller has suffered more than most over the last few months, I am sure she never wants to type the words transglutaminase, stimulus-secretion coupling, or glucose-stimulated insulin release ever again, I thank her for her excellent typing and patience; and also thanks to Mrs Joan Witter for additional assistance.

The Sir Halley Stewart Trust has funded these studies and my attendance at the conference in Miami, I am grateful to the trustees for their support.

Finally thanks to all the other members of Trent Polytechnic who have aided me in these studies but are too numerous to mention.

ABSTRACT

The Role of Transglutaminase in Stimulus-Secretion Coupling in the Pancreatic β-Cell.

Robin Antony OWEN, 1988.

The sensitivity of rat pancreatic islet transglutaminase to activation by Ca^{2+} was investigated using a modified assay incorporating dephosphorylated N,N'-dimethylcasein as substrate protein. The Km for Ca^{2+} obtained (3μM approx.) was an order of magnitude lower than previously reported for the islet enzyme (Bungay *et al.*, 1984). Magnesium (2mM) was found to have little effect on the sensitivity of the enzyme to Ca^{2+}.

A number of primary amine inhibitors were tested for the specificity of their action as inhibitors of transglutaminase activity. Monodansylcadaverine (IC_{50} = 43μM ; Ki = 9.8μM) was found to be a specific inhibitor of transglutaminase activity. Monodansylcadaverine (30-100 μM) significantly inhibited glucose-stimulated insulin release, but had no effect on basal insulin release. Monodansylcadaverine (75μM) inhibited the first phase of glucose-stimulated insulin release and greatly diminished the second phase. The control compound, dimethyl-monodansyl-cadaverine, inhibited neither transglutaminase activity or glucose-stimulated insulin release. These studies strongly implicate transglutaminase in the process of glucose-stimulated insulin release. The test compound N-(8-amino-octyl)-5-iodo-naphthalene -1-sulphonamide was found to be a specific antagonist of calmodulin.

Incubation of intact islets in the presence of [^{32}P]Pi and stimulatory levels of glucose followed by separation of phosphorylated islet proteins by SDS-polyacrylamide gel electrophoresis revealed the presence of a high molecular weight phosphopolymer which did not traverse a 3% (w/v) acrylamide gel. This polymer was found to be predominantly composed of protein. The majority of this phosphopolymer (70% approx.) was present in the 600g av. sedimented fraction of islet homogenates. Incubation of islet homogenates, obtained from intact islets previously incubated with [^{32}P]Pi and stimulatory levels of glucose, under conditions that activated the islet transglutaminase resulted in an increase in the amount of phosphopolymer present in the 600g av. sedimented fraction. Inhibitors of transglutaminase activity which are known to inhibit glucose-stimulated insulin release led to a significant reduction in the fraction of phosphopolymer present in the glucose-stimulated intact islet. These findings suggest that protein cross-linking and phosphorylation reactions may be closely linked in the pancreatic β-cell.

PUBLICATIONS

S. MAC'NEIL, R.A. DAWSON, M. GRIFFIN, R.A. OWEN, A.M. COOKE, N.J. PETTETT, & G.M. BLACKBURN.
Biochem. Soc. Trans. (1986) 14 627-628 (616th Meeting, London).
Calmodulin antagonists of improved specificity and potency.

P.J. BUNGAY, R.A. OWEN, I.C. COUTTS, & M. GRIFFIN.
Biochem. J. (1986) 235 269-278.
A role for transglutaminase in glucose-stimulated insulin release from the pancreatic β-cell.

R.A. OWEN, M. HUSSAIN, P.J. BUNGAY, & M. GRIFFIN.
Proceedings of Noble Conference in Cellular & Molecular Biology: Transglutaminase & Protein Cross-linking Reactions (March, 1987).
Transglutaminase-catalysed cross-linking of proteins may be modulated by protein phosphorylation.

S. MAC'NEIL, M. GRIFFIN, A.M. COOKE, N.J. PETTETT, R.A. DAWSON, R.A. OWEN, & G.M. BLACKBURN.
Biochem. Pharmacol. (1988) 37(9) 1717-1723.
Calmodulin antagonists of improved potency and specificity for use in the study of calmodulin biochemistry.

R.A. OWEN, P.J. BUNGAY, M. HUSSAIN, & M. GRIFFIN.
Biochim. Biophys. Acta. (1988) 968 220-230.
Transglutaminase-catalysed cross-linking of proteins phosphorylated in the intact glucose-stimulated β-cell.

CONTENTS

		Page
1.	INTRODUCTION	1
1.1	INTRODUCTION TO THE TRANSGLUTAMINASES	1
1.1.1.	Definition of transglutaminase	1
1.1.2.	Classification of transglutaminases	1
1.2.	GENERAL CATALYTIC FEATURES OF TRANSGLUTAMINASES	5
1.2.1.	Reactions catalysed by transglutaminase	5
1.2.2.	The mechanism of action of transglutaminase	8
1.2.3.	Energetic considerations	9
1.2.4.	Substrate specificity of transglutaminases	11
1.2.4.1.	The amide site	12
1.2.4.2.	The extended glutamine binding site	12
1.2.4.3.	The amine site	13
1.2.4.4.	Conclusions	14
1.3.	THE BIOLOGICAL ROLE OF THE TRANSGLUTAMINASES	16
1.3.1.	Factor XIIIa	16
1.3.2.	Hair follicle transglutaminase	18
1.3.3.	Epidermal transglutaminase	20
1.3.4.	Prostate transglutaminase	22
1.4.	THE BIOLOGICAL ROLE OF TISSUE TRANSGLUTAMINASE	26
1.4.1.	Receptor mediated endocytosis	26
1.4.2.	Cell activation and differentiation	31
1.4.3.	Tumour growth	36
1.4.4.	Tissue transglutaminase and polyamines in normal and neoplastic growth	38
1.4.5.	Cell ageing	44

1.5.	AIM OF THESIS	47
1.6.	MECHANISM OF GLUCOSE-STIMULATED INSULIN RELEASE	49
1.6.1.	Insulin	49
1.6.2.	Cellular pathway of insulin biosynthesis	49
1.6.3.	Stimuli leading to insulin release	50
1.6.4.	Elevation of Ca^{2+} and cAMP levels	52
1.6.5.	Mode of action of Ca^{2+} and cAMP on the insulin release mechanism	56
1.6.6.	Margination	57
1.6.7.	Exocytosis	60
1.6.8.	Conclusion	61

2.	MATERIALS AND METHODS	62
2.1.	MATERIALS	62
2.2.	ANIMALS	65
2.3.	CHEMICAL DETERMINATIONS	66
2.3.1.	Protein assay : method of Lowry	66
2.3.2.	Determination of radioactivity	66
2.3.2.1.	Filter papers	67
2.3.2.2.1.	Polyacrylamide gels : scintillation counting	67
2.3.2.2.2.	Polyacrylamide gels : autoradiography	67
2.3.2.3.	Determination of counting efficiency	68
2.4.	FRACTIONATION PROCEDURES	68
2.4.1.	Homogenisation	68
2.4.1.1.	Potter-Elvejhem	68
2.4.1.2.	Homogenisation of ^{32}P-labelled islets	69
2.4.1.3.	Ultrasonication	69
2.4.2.	Subcellular fractionation of tissues by differential centrifugation	69
2.4.3.	SDS-polyacrylamide gel electrophoresis	70
2.4.3.1.	Solutions	72
2.4.3.2.	Sample preparation	73
2.4.3.3.	Procedure	73
2.4.3.4.	Fixing, staining and destaining of gels	75
2.4.3.5.	Sectioning	76
2.4.3.6.	Preparation of gels for autoradiography	76
2.5	TRANSGLUTAMINASE ASSAY	76
2.5.1.1.	Standard transglutaminase assay	76
2.5.1.2.	Assay for the effect of test compounds on islet transglutaminase activity	78
2.5.1.3.	Measurement of the Ca^{2+}-sensitivity of transglutaminase	78

2.6	ASSAY FOR PROTEIN KINASE C ACTIVITY	79
2.7.	ASSAY FOR CALMODULIN-STIMULATED PHOSPHODIESTERASE	80
2.8	MARKER ENZYME STUDIES	82
2.8.1.	5'Nucleotidase assay	82
2.8.2.	Cytochrome C oxidase assay	83
2.9.	ISLET STUDIES	83
2.9.1.	Physiological media	84
2.9.1.1.	Media used in the collagenase digestion incorporating shaking by hand	84
2.9.1.2.	Media for collagenase digestion utilising flask shaker	86
2.9.2.	Siliconisation of glassware	87
2.9.3.	Isolation of islets of Langerhans	88
2.9.3.1.	Excision of pancreas	88
2.9.3.2.	Collagenase digestion by hand	88
2.9.3.3.	Collagenase digestion by flask shaker	90
2.9.4.	Treatment of islets	91
2.9.4.1.	Apparatus for islet incubations	92
2.9.4.2.	Grouping of islets	92
2.9.5.	Studies on insulin release	93
2.9.5.1.	Batch incubations of pancreatic islets	93
2.9.5.2.	Perifusion of pancreatic islets	94
2.9.5.3.	Radioimmunoassay for immunoreactive insulin	96
2.9.5.3.1.	Radioimmunoassay using ethanol precipitation	96
2.9.5.3.2.	Radioimmunoassay using Wellcome binding reagent	98
2.9.6.	Measurement of islet glucose oxidation	101
2.9.7.	Determination of islet total protein synthesis	103

2.9.8.	Measurement of pancreatic islet lipid methylation	104
2.9.9.	Phosphorylation studies	105
2.9.9.1.	Phosphorylation of peptides in intact cells	105
2.9.9.2.	Treatment of phosphorylated-islet homogenates	105
2.9.9.2.1.	Digestion with RNAase and DNAase	106
2.9.9.2.2.	Digestion with proteases	106
2.9.9.2.3.	Digestion of phosphorylated pancreatic membranes with phospholipase and pronase	106
2.9.9.3.	Effect of test compounds on phosphorylation pattern of pancreatic peptides	107
2.9.9.4.	Incorporation of [^{32}P]-phosphorylated islet peptides into high molecular weight aggregates	108
2.10.	TEST COMPOUNDS	108
2.10.1.	Synthesis of test compounds	108
2.10.2.	Addition of test compounds	110
2.11.	STATISTICAL ANALYSIS	111
2.11.1.	Standard error	111
2.11.2.	Students' t-test	111

3.	RESULTS	113
3.1.	SENSITIVITY OF THE ISLET TRANSGLUTAMINASE TO ACTIVATION BY Ca^{2+}	114
3.1.1.	Discussion	117
3.2.	DEVELOPMENT OF SPECIFIC PROBES FOR ISLET TRANSGLUTAMINASE	119
3.2.1.	Determination of the potency of test compounds for the inhibition of transglutaminase activity	120
3.2.2.	Determination of the effect of test compounds on glucose-stimulated insulin release in the pancreatic β-cell	145
3.2.3.	Effects of test compounds on glucose-stimulated glucose oxidation	152
3.2.3.1.	Discussion	155
3.2.4.	Effects of test compounds on the activity of the phospholipid- and Ca^{2+}- dependent kinase (protein kinase C)	160
3.2.4.1	Assay for protein kinase C in rat spleen extracts	160
3.2.4.2.	Ca^{2+} and phospholipid requirement of rat spleen protein kinase C	162
3.2.4.3.	Effect of test compounds on protein kinase C activity	164
3.2.5.	Effect of test compounds on calmodulin-stimulated bovine heart phosphodiesterase	169
3.2.6.	Effect of MDCD on total islet lipid methylation	176
3.2.7.	Discussion	178

3.2.8.	Effect of MDCD on the dynamics of glucose-stimulated insulin release	182
3.2.8.1.	Discussion	184
3.3.	SUBSTRATE STUDIES	186
3.3.1.	Presence of a high molecular weight phosphorylated protein polymer in glucose-stimulated pancreatic islets	188
3.3.2.	Treatment of phosphorylated islet protein homogenates with proteolytic enzymes	193
3.3.3.	Extraction of phosphorylated islet protein homogenates with ethanol-ether following T.C.A. precipitation and subsequent digestion with pronase	195
3.3.4.	Digestion of phosphorylated islet membranes with phospholipase A_2/phospholipase C and pronase	199
3.3.5.	Inhibition of glucose-stimulated formation of phosphopolymer in intact islets by inhibitors of transglutaminase activity	202
3.3.6.	The cross-linking of phosphorylated islet proteins into high molecular weight protein aggregates by islet transglutaminase	205
3.3.7.	Subcellular distribution of the high molecular weight phosphopolymer found in the glucose-stimulated islet	207
3.3.8.	Further experiments undertaken on the characterisation of the phosphopolymer	213
3.3.9.	Discussion	217

4. GENERAL DISCUSSION 222

5. REFERENCES 235

LIST OF FIGURES

		Page
1.1.	Reactions known to be catalysed by transglutaminase *in vivo*	7
1.2.	The mechanism of action of transglutaminase	10
1.3.	Pathway for the biosynthesis and interconversion of polyamines	39
2.1.	Fractionation scheme	71
2.2.	Perifusion system	95
2.3.	Insulin standard curve - ethanol precipitation	99
2.4.	Insulin standard curve - Wellcome binding reagent	102
3.1a & b.	Calculation of the apparent Km for Ca^{2+} of transglutaminase in the presence and absence of Mg^{2+} ions	116
3.2a & b. 3.3a & b. 3.4a & b.	Inhibition of rat liver transglutaminase by MDCD, 2-NSA and tosyl-SA. Calculation of K_i values using secondary plots of Lineweaver-Burk graph	121
3.5a & b- 3.19a & b.	Effect of test compounds on rat liver and rat pancreatic islet transglutaminase activity	125
3.20a & b.	Glucose-response curve for insulin release from isolated rat pancreatic islets	146

3.21.	The effect of enzyme concentration on the time dependent incorporation of ^{32}P into histone H1 by rat spleen protein kinase C	161
3.22.	Effects of Ca^{2+} and phospholipid on the incorporation of ^{32}P into histone H1 by rat spleen extracts	163
3.23, 24 & 25	The effect of test compounds on protein kinase C	166
3.26a & b. 3.27a & b.	Inhibition of calmodulin-stimulated phosphodiesterase activity by naphthalene-sulphonamides	171
3.28.	Glucose-stimulated insulin release profile in the presence of 75μM MDCD	183
3.29.	Protein phosphorylation pattern found in glucose-stimulated intact pancreatic islets and the effect of RNAase and DNAase upon this pattern	189
3.30.	Summary of the phosphorylation pattern obtained with islet homogenates pre-treated with RNAase and DNAase prior to electrophoresis	190
3.31.	Effect of T.C.A. precipitation and ethanol-ether extraction, and subsequent pronase digestion on the phosphorylation pattern of islet proteins	198
3.32.	The time dependent incorporation of phosphorylated islet proteins into high molecular weight protein aggregates	206

3.33.	Distribution of phosphopolymer, marker enzymes and transglutaminase in islet subcellular fractions	211
4.1.	Modification of cytoskeletal network	230
4.2.	Hypothetical cycle for the making and breaking of ε(γ-glutamyl)-lysine bonds	233

LIST OF TABLES

		Page
1.1.	Hydrolysis and aminolysis reactions catalysed by transglutaminase	6
3.1.	Summary of effect of test compounds on transglutaminase activity	140
3.2.	Chemical structures of test compounds	141
3.3.	Effect of test compounds on glucose-stimulated insulin release	148
3.4.	Effect of test compounds on basal insulin release	150
3.5.	Effect of test compounds on glucose-stimulated glucose oxidation in the pancreatic β-cell	153
3.6.	Effect of the mode of presentation of phosphatidylserine and 1,2-diolein on protein kinase C activity present in rat spleen extracts	165
3.7.	Summary of the effect of test compounds on protein kinase C activity	168
3.8.	IC_{50} values for the inhibition of calmodulin-stimulated phosphodiesterase by a range of test compounds	173
3.9.	Effect of MDCD, 2-NSA, dimethyl-MDCD and dimethyl-2-NSA on basal phosphodiesterase activity	175
3.10.	Effect of MDCD on total islet lipid methylation	177

3.11.	Summary of the phosphorylation pattern data obtained with islet homogenates pretreated with RNAase and DNAase prior to electrophoresis	191
3.12.	Effect of proteolytic enzymes on the protein phosphorylation pattern of ^{32}P-labelled islets	194
3.13.	Effect of T.C.A. precipitation and ethanol-ether extraction, and subsequent pronase digestion on the phosphorylation pattern of islet proteins	196
3.14.	Effect of phospholipase A_2/C and pronase on phosphorylated islet membranes	200
3.15.	Effect of transglutaminase inhibitors and their respective control compounds on glucose-stimulated insulin release and the protein phosphorylation pattern in glucose-stimulated intact cells	203
3.16.	Subcellular distribution of the high molecular weight phosphopolymer found in the glucose-stimulated islet	208
3.17.	Subcellular distribution of the high molecular weight phosphopolymer found in the glucose-stimulated islet after pre-incubation of islet homogenates in Ca^{2+}-containing medium	210

ABBREVIATIONS

The following abbreviations have been used in this thesis:

AMP	: adenosine 5'-monophosphate
cAMP	: adenosine 3':5'-cyclic monophosphate
ATP	: adenosine 5'-triphosphate
DNA	: deoxyribonucleic acid
DNAase	: deoxyribonuclease
DMSO	: dimethyl sulphoxide
EDTA	: ethylenediaminetetra-acetic acid
EGTA	: ethylene glycol-bis(beta-aminoethyl ether) N,N,N',N',-tetra-acetic acid
HBSS	: Hanks balanced salt solution
HEPES	: N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
i.r.	: infra red
KRB	: Krebs Ringer bicarbonate medium
MES	: 2-(N-Morpholino)ethanesulphonic acid
n.m.r.	: nuclear magnetic resonance
PMSF	: phenylmethylsulphonyl fluoride
RNA	: ribonucleic acid
RNAase	: ribonuclease
SDS	: sodium dodecyl sulphate
TCA	: trichloroaceitic acid
TEMED	: N,N,N',N',-tetramethylethylenediamine
t.l.c.	: thin layer chromatography
Tris	: 2-amino-2-hydroxymethyl-1,3-propandiol

1. INTRODUCTION

1.1. INTRODUCTION TO THE TRANSGLUTAMINASES

1.1.1. Definition of transglutaminases.

Preliminary reports describing a Ca^{2+}-dependent transamidating activity present in the liver and in some other tissues of the guinea pig were made by Waelsch and co-workers in the late 50's and early 60's (Sarkar et al., 1957; Clarke et al., 1959; Mycek and Waelsch, 1960). Since these early investigations, the group of enzymes termed transglutaminases have been the subject of considerable experimentation and the cause of a number of reviews (Chung, 1972, 1975; Folk & Chung, 1973; Lorand & Stenberg, 1976; Folk & Finlayson, 1977; Folk, 1980; Williams-Ashman & Canellakis, 1980; Folk, 1983; Lorand & Conrad, 1984; Conrad, 1985).

Transglutaminases are currently defined as calcium dependent enzymes with an active site thiol which catalyse an acyl transfer reaction between peptide-bound glutamine residues and primary amine groups; the result of this reaction is the incorporation of amine into protein or the formation of ε-(γ-glutamyl) lysine cross-links between proteins if the primary amine is peptide-bound lysine. Since it is not free glutamine which serves as the enzyme substrate, but rather compounds with ε-glutaminyl residues occupying endopositions, the name transglutaminase is a misnomer. Hence the Enzyme Commission recommends the systematic name R-glutaminyl-peptide:-amine-ε-glutamyl transferase (E.C. 2.3.2.13.).

1.1.2. Classification of transglutaminases.

Transglutaminases are so widely distributed that it is generally thought that their occurrence will eventually be found to be ubiquitous in animal cells and tissues, they are found both

intracellularly and extracellularly (Folk & Finlayson, 1977; Folk, 1980; Conrad, 1985). However the term transglutaminases refers to a group of enzymes that differ according to their localisation and their physical, chemical, immunochemical and catalytic properties (Chung, 1975). On the basis of these considerations the following groups of transglutaminase have been described:

i) Factor XIII.

This species of transglutaminase is the final link in the cascade of reactions in the blood coagulation system. To date factor XIII activity has been defined in plasma (Lorand et al., 1968), placenta (Bohn & Schwick, 1971), platelets (Schwartz et al., 1971), and in the white blood cell types, monocytes, macrophages and granulocytes (Berntorp et al., 1985).

ii) Hair follicle transglutaminase.

This enzyme has been localised to the inner root sheath and medulla of the hair follicle (Harding & Rodgers, 1972; Chung & Folk, 1972). The latter workers were the first to isolate this type of transglutaminase and it has subsequently been shown to be immunologically distinct from the other types of transglutaminase (Chung, 1975).

iii) Epidermal (type I) transglutaminase.

Epidermal transglutaminase has been localised to the granular layer of the epidermis where it catalyses the crosslinking of a soluble cytoplasmic precursor to form the cornified inner membrane of the mature keratinocyte in the stratum corneum (Buxman & Weupper, 1974; 1975). More recent studies have additionally found epidermal transglutaminase activity in the upper digestive tract and the lower female genital tract of the rat (Parenteau et al., 1986), and in rabbit tracheal epithelium (Jetten & Shirley, 1986).

iv) Tissue (type II) transglutaminase.

Tissue transglutaminase is the most widely distributed of the transglutaminase species and has been found in the cytosol of a large number of tissues including liver, spleen, muscle, kidney, lung, adrenal gland, brain, testis, pancreas, erythrocytes and the uterus (Chung, 1972; Lorand and Stenberg, 1976; Conrad, 1985). There are reports of this enzyme coexisting with other types of transglutaminase in the same cell, thus Chung & Folk (1972) detected both tissue and hair follicle transglutaminases occurring in the hair follicle; whilst both tissue and epidermal transglutaminase have been detected in keratinocytes (Lichti et al., 1985).

However, more recent reports have introduced speculation about the classification of some transglutaminases. The recent discovery of a particulate transglutaminase in rat liver (Sliffe et al., 1985, 1986, 1987; Chang & Chung, 1986; Tyrrell et al., 1986; Juprelle-Soret et al., 1988) and lung (Cocuzzi & Chung, 1986) in addition to a tissue (type I) transglutaminase has generated much interest and discussion, particularly with respect to the differential roles that the two enzymes may play in the same cell. This novel particulate enzyme has been shown to be immunologically distinct from factor XIII and the tissue transglutaminase, and in the liver it has been shown to be specifically associated with the plasma membrane.

Another novel transglutaminase has been reported which is regulated by calmodulin (Puskin et al., 1985, 1987) in platelets, red blood cells and the chicken gizzard. Of particular note was the finding that the activity of this transglutaminase was not inhibited by iodoacetamide, and that these transglutaminases are associated

with and strongly implicated in the cross-linking of membrane proteins associated with the cytoskeleton.

A further area of controversy exists as to the transglutaminases found in the prostate gland of rodents (c.f. section 1.3.4.).

With the development of more sophisticated modes of analysis including immunohistochemical techniques, amino acid sequencing, and the application of molecular genetics, a clearer definition of the differing types of transglutaminase should eventually emerge.

1.2. GENERAL CATALYTIC FEATURES OF TRANSGLUTAMINASES

1.2.1. Reactions catalysed by transglutaminase.

The range of aminolytic and hydrolytic reactions catalysed by transglutaminases are presented in Table 1.1. Of these seven reactions the most important are reactions 1 and 2, the incorporation of amines into proteins and the cross-linking of proteins, since they represent the covalent post-translational modification of proteins. The other reactions catalysed by transglutaminase (reactions 3-7 in Table 1.1.) involving the aminolysis or hydrolysis of an ester or amide have not been shown to be catalysed by transglutaminase *in vivo*.

Reactions 1 and 2 form the basis of the physiological role of the transglutaminases whose biological function has been determined. Thus, when the substrates are protein bound transglutaminase catalysis can result in protein cross-linking by the formation of ε-(γ-glutamyl) lysine bonds (Figure 1.1.a.); the formation of N,N-bis-(γ-glutamyl) polyamine derivatives resulting in the cross-linking of proteins (Figure 1.1.c.); and the incorporation of small primary amines into the protein substrate (Figure 1.1.b.).

Where P and P' are the same protein species a resulting cross-linked protein product will be homologous, whereas if they are differing protein species the product will be heterologous.

The potential clearly exists for a branched product of protein cross-linking where any single protein possesses either two separate protein-bound glutamine residues that can undergo catalysis or alternatively two protein-bound lysine residues.

The incorporation of small primary amines into protein substrates (Figure 1.1.b.) forms the basis for the most commonly used assay for transglutaminases of all types. The basis of the assay is

Table 1.1 Hydrolysis and Aminolysis Reactions catalysed By Transglutaminase

1. Hydrolysis of peptide-bound glutamine.



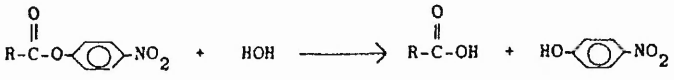
2. Hydrolysis of amides.



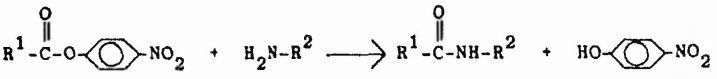
3. Aminolysis of amides.



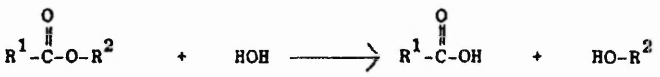
4. Hydrolysis of p-nitrophenol esters.



5. Aminolysis of p-nitrophenol esters.



6. Hydrolysis of aliphatic esters.



7. Aminolysis of aliphatic esters.

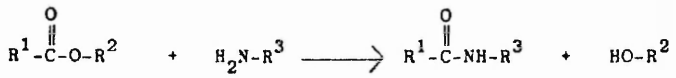
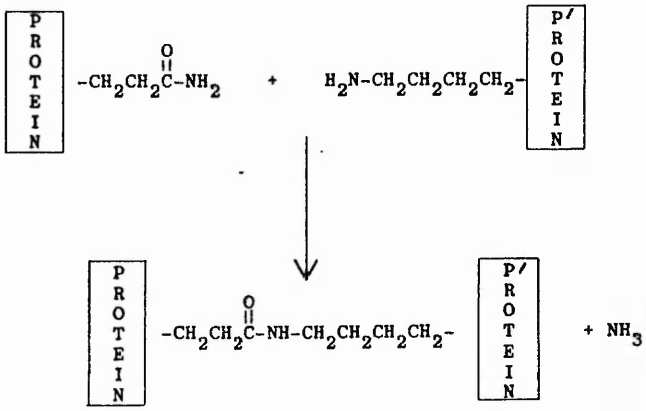


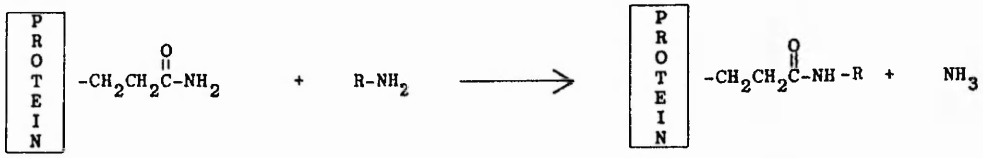
Figure 1.1 Reactions Known to be Catalysed by Transglutaminase In Vivo

A. Formation of ε(γ-glutamyl)lysine bonds : the crosslinking of proteins :

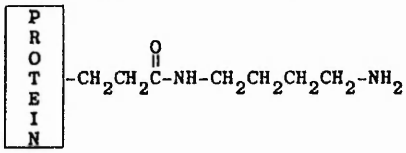


ε(γ-glutamyl)lysine crosslink

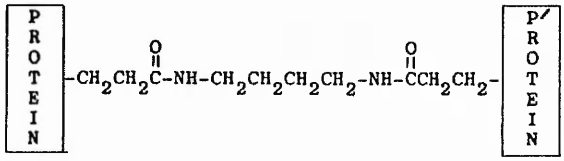
B. Incorporation of primary amines into proteins :

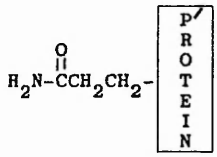


C. Formation of diamine crosslinks through the incorporation of polyamines (* reaction product from B with putrescine as the primary amine) :



N-(γ-glutamyl)putrescine





N,N-bis-(γ-glutamyl)putrescine crosslink

the measurement of the Ca^{2+}-dependent incorporation of isotopically labelled amines (eg. putrescine, histamine, methylamine) into protein substrates, the degree of protein labelling as determined by counting trichloroacetic acid-precipitable proteins (Clarke et al., 1959) is proportional to the enzyme activity. This basic method has undergone various improvements, particularly the blocking of the ε-lysine residues of test proteins (eg. casein, β-chain of insulin) so that no self-crosslinking of the protein substrate can occur. The most commonly used method to achieve this is dimethylation (Curtis and Lorand, 1976), although acetylation, succinylation, and guanidylation have also been employed. Filter paper (Lorand et al., 1972) and chromatographic techniques (Lorand and Campbell, 1971) have been employed to precipitate protein-bound radioactivity and to separate if from free amine.

More recently an ELISA system has been developed by Fesus and Arato (1986) to quantitate transglutaminase levels with an assay sensitivity of less than 1.0 ng/mg cellular protein.

1.2.2. The mechanism of action of the transglutaminases.

The currently accepted model for the mechanism of catalysis by transglutaminases is the binding of the glutamine substrate to the enzyme to form a binary complex involving a thioester bond with the active site cysteine-SH group, an acyl-enzyme intermediate being formed with the concurrent release of ammonia. The acyl-enzyme intermediate then undergoes nucleophilic attack either by water, forming peptide bound glutamic acid, or by a primary amine such as putrescine, forming peptide-bound γ-glutamyl-putrescine. A thorough review of the evidence supporting

this model is given by Folk (1983). The kinetic mechanism for this modified double-displacement model is presented in Figure 1.2.

Reactions catalysed by transglutaminases are dependent on calcium ions for activation, this activation is thought to bring about small conformational changes in protein structure which expose the enzyme active site (Folk, 1983). It has also been reported that other divalent cations such as strontium or manganese can activate the enzyme to a lesser extent (Folk et al., 1967). Recently the sequencing of Factor XIII (Putnam et al., 1987; Ichinose & Davie, 1987) and the partial sequencing of a tissue (type II) transglutaminase (Stein et al., 1987) by c-DNA cloning techniques have indicated that these transglutaminases have partial sequence homology with other calcium binding proteins such as calmodulin.

The following criteria may therefore be employed as useful properties for the characterisation of transglutaminase activity:

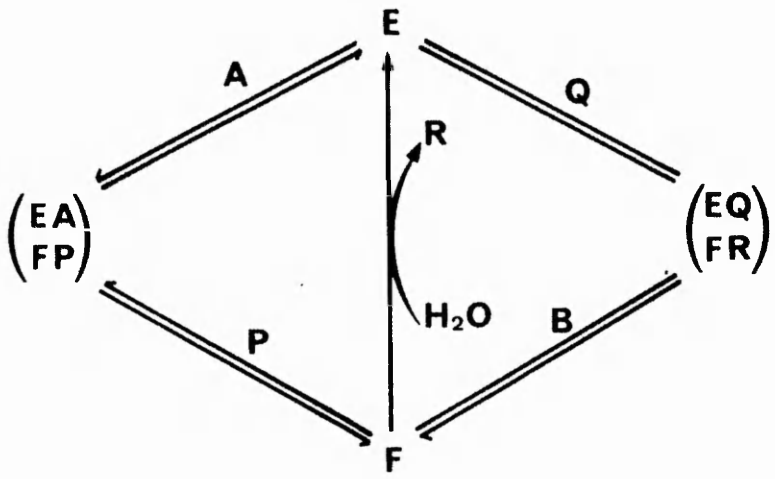
i) Calcium ion-dependence as judged by the inhibition of enzyme activity by metal-chelating agents such as EDTA or EGTA.

ii) Thiol-dependence as judged by the inhibition of enzyme activity by thiol-oxidants such as iodoacetamide, p-chloromercurobenzoic acid, and 5,5'-dithiobis (2-nitrobenzoic acid) (Folk & Cole, 1966; Connelan & Folk, 1969).

1.2.3. Energetic considerations.

The reactions presented in Figure 1.1. should be considered reversible as there would be very little change in free energy since for every γ-amide bond broken a γ-amide bond is formed. In vivo the ammonia leaving group would undergo immediate protonation

Figure 1.2 The Mechanism of Action of Transglutaminase



Key : Reaction 1.1b from Table 1.1 as an example

E = free enzyme

F = acyl-enzyme intermediate

A= peptide-bound glutamine

P = ammonia

R = peptide bound glutamic acid

B = primary amine

Q = peptide bound γ-glutamyl-amine

which would tend to promote the reaction to the right (Lorand & Conrad, 1984; Conrad, 1985).

However, it has proven very difficult to demonstrate the reversibility of protein crosslinking due to the crosslinked product undergoing clotting or phase transitions. Actual reversal has been reported only for factor XIIIa-catalysed dimerisation of fibrin with α_2-plasmin inhibitor (Ichinose & Aoki, 1982). The reversibility of amine incorporation can more easily be shown, Campbell-Wilkes (1973) was able to show [^{14}C]-histamine release subsequent to its incorporation into β-lactoglobulin.

1.2.4. Substrate specificity of transglutaminases.

Comprehensive substrate specificity studies have only been undertaken on the guinea-pig liver transglutaminase (type II) and with factor XIIIa from plasma. These two enzymes cannot be considered as totally representative of all types of transglutaminase. None-the-less studies on their substrate specificity serve to illustrate the factors which may be of importance to the biological role of transglutaminase. These factors are covered in the review of Folk (1983).

Substrate specificity in the case of transglutaminases may be viewed in respect of:

i) the requirements of the amide site where the acyl-enzyme intermediate forms;

ii) the extended glutamine site where peptides containing glutamine bind to the enzyme;

iii) the amine site where nucleophiles interact with acyl-enzyme intermediates.

1.2.4.1. The amide site.

Studies by Chung et al. (1970), Gross and Folk (1973), and Chung (1975) investigated the requirements for amides to act as substrates for the incorporation of amines by guinea-pig liver transglutaminase. These studies ratified the earlier findings of Waelsch and co-workers (Waelsch, 1962; Neidle and Acs, 1961), in that only L-glutamine residues in which the α-carbon NH_2 and COOH groups were peptide-bound could effectively participate in catalysis, whereas peptide-bound asparagine did not act as a substrate. In addition, the enzyme was found to have a preference for substrates with uncharged groups at or near to the glutamine residue (Folk and Cole, 1965, 1966), suggesting that a hydrophobic domain existed near the active site.

1.2.4.2. The extended glutamine binding site.

The amino acid side-chains in the vicinity of the glutamine residue are also thought to interact with the enzyme active site. This intereaction is a potential further aspect of substrate specificity of transglutaminases.

In a study on guinea-pig liver transglutaminase and factor XIIIa undertaken by Chung (1972), differences in the substrate specificity of the two enzymes were described; the liver enzyme could utilise benzoxycarbonyl-glutaminylglycine as a substrate, whereas factor XIIIa could not. Whilst Folk & Chung (1973) found that the patterns of cross-linking of fibrin were different for the two enzymes. These overall observations indicated a greater substrate specificity of factor XIIIa, and the possibility exists that these differences are related to the biological function of the two enzymes.

The substrate specificity of liver transglutaminase was further investigated by the variation of the amino-acid sequence around the glutamine residue in synthetic substrates (Gross et al., 1975). The evidence suggested that the enzyme intereacted with its substrate over a sequence of at least five amino acid residues. Further studies using a peptide fragment of β-casein with varying amino acid substitutions indicated that certain amino acids greatly affected the affinity of the peptide for factor XIIIa, but had a less pronounced effect upon the liver enzyme (Gorman & Folk, 1980; 1981). These studies also indicated that the active sites of the two enzymes corresponded to peptide fragments of up to 9 or 10 amino acid residues and supported the idea that uncharged or hydrophobic amino acids are important in substrate specificity.

1.2.4.3. The amine site.

Primary amines are the nucleophiles that result in the lysis of the thioester bond (see Section 1.2.2.), the specificity of transglutaminase for different structural primary amines has been investigated in a number of studies. Folk and Cole (1966) determined that the nucleophile must be strong enough to cause lysis of the thioester linkage, and in the case of aminolysis, the amine must be in the uncharged form. Whilst Chung et al. (1970) indicated that the acyl-enzyme intermediate itself may influence the specificity of the particular transglutaminase for its substrate amine.

The substrate specificity of a wide variety of primary amines was determined as a function of their effectiveness in inhibiting the incorporation of [^{14}C]-putrescine into N,N'-dimethylcasein by guinea-pig liver transglutaminase (Lorand et al., 1979). The best substrates possessed a large hydrophobic moiety attached to a 5-carbon alkylamine side chain. Using a

different assay, Gross et al. (1977), showed a preference of guinea-pig liver transglutaminase for aliphatic amines that were either unbranched or where the branching occurred at a distance of at least 5 carbon atoms from the amine group. As a result of these findings, which indicated a preference of the enzyme for amine substrates that had structural similarities to the side chain of lysine, studies were undertaken on a series of α-ω-diamino-carboxylic acid peptide derivatives. These studies revealed the L-lysine analogue to be the most reactive with a stereospecific preference for L-lysine isomers. These studies therefore gave a clear indication of a preference for L-lysine as a second substrate for transglutaminases.

In their studies Gross et al. (1977), also investigated the effect of altering the amino acid residues in the locus of lysine using synthetic lysine containing peptides, they found that only those amino acids adjacent to the lysine residue influenced any intereaction with the acyl-enzyme intermediate. However, a single L-leucine residue on the N-terminal side of the lysine residue significantly increased the specificity of these synthetic peptides, again indicating the hydrophobicity of the enzyme active site (Schrode & Folk, 1979).

1.2.4.4. Conclusions.

The studies outlined with guinea-pig liver transglutaminase and factor XIIIa indicate the common specificity of the two enzymes for peptide-bound glutamine and lysine, but a difference in the specificity of the extended glutamine site. The possession of an extended glutamine binding site by transglutaminases allows for these differences in specificity.

However, these conclusions upon the mechanism and specificity of the transglutaminases have resulted largely through the use of small model substrates. Few conclusions have been made about the manner in which the enzymes operate on macromolecular substrates in biological systems. Clearly, these intereactions must be complex when it is considered that the two protein substrates and the enzyme must become associated in a highly oriented and conformation-dependent fashion at some stage of the catalytic process. The current knowledge of these intereactions between the transglutaminases and their macromolecular substrates in biological systems is discussed in the next section.

1.3. THE BIOLOGICAL ROLE OF THE TRANSGLUTAMINASES

The evidence for the proposed biological roles of some of the transglutaminases is discussed in the following section, it should be pointed out however that some of these roles are still not conclusive. The more speculative roles of tissue transglutaminase are considered in the subsequent section.

1.3.1. Factor XIIIa.

Plasma Factor XIIIa exists as an inactive zymogen, Factor XIII, and when activated brings about the stabilisation of fibrin clots by catalysing cross-link formation between the peptide chains. As such, it represents the final link in the cascade of reactions in the blood coagulation system. The existence of the factor was documented as early as 1944 (Robbins), but it was not until 1968 that Loewy and Metacic reported this factor to be a transglutaminase. Although there have been some updates, the role of Factor XIIIa is most extensively reviewed by Folk and Finlayson (1977).

The zymogen, Factor XIII, has a molecular weight of $\simeq$ 300,000 and is composed of two identical catalytic subunits of $\simeq$ 75,000 Mr. (a-chains) and two identical non-catalytic subunits of $\simeq$ 80,000 Mr. (b-chains) (Schwartz *et al.*, 1971; 1973; Takagi & Konishi, 1972; Chung, 1972; Chung *et al.*, 1974). As a result of limited proteolysis catalysed by thrombin, the a-chains are modified by the removal of a single polypeptide from the amino terminal end (Takagi & Doolittle, 1974). Thus the zymogen, a_2b_2, becomes modified to a'_2b_2, the resultant modified a'-chains have molecular weights of $\simeq$ 70,000 (Chung *et al.*, 1974), and the modification results in an unmasking of the active site cysteine. In the presence of Ca^{2+} the modified enzyme a'_2b_2 reversibly dissociates into the active dimer

a'_2 of ≃ 150,000 Mr. and an inactive b_2 dimer (Chung et al., 1974), the Ca^{2+} also induces some a'-chain conformational changes. Factor XIIIa therefore carries out its catalysis as a dimer, a'_2.

The principal physiological substrate of Factor XIIIa is fibrin, which is derived from a precursor, fibrinogen, present in the plasma. Fibrinogen comprises of three polypeptide chains Aα of ≃ 70,000 Mr., Bβ of ≃ 60,000 Mr. and γ of ≃ 50,000 Mr. which are each present in duplicate (Mossesson & Finlayson, 1976). Fibrin is formed as a result of the clevage of the fibrinopeptides A and B as catalysed by thrombin, thus fibrinogen, $(A\alpha\, B\beta\, \gamma)_2$, is converted to fibrin, $(\alpha\, \beta\, \gamma)_2$. The resultant fibrin polymers assemble in an uncatalysed reaction to form a gel and it is this fibrin gel that Factor XIIIa then cross-links. Initially the γ-subunits are cross-linked to form a hemostatic plug, which is then further stabilised by the cross-linkage of the α-subunits (Finlayson & Aronson, 1974). The β-subunits do not appear to be cross-linked.

The formation of the active enzyme, Factor XIIIa, and the substrate fibrin in the plasma occurs predominantly at the site of injury and hence represents one mode of control upon clot formation, but various other intricate controls exist. Fibrinogen, the precursor of fibrin, has been found to have a regulatory effect on the thrombin catalysed conversion of Factor XIII, a_2b_2, to the modified tetramer, a'_2b_2 (Janus et al., 1983), and to play a further regulatory role by lowering the Ca^{2+} requirement of the dissociation of the modified tetramer resulting in a'_2 formation, down to ≃ 1.5 mM (Credo et al., 1978).

Whilst fibrin is the principal substrate of Factor XIIIa, fibrinogen has also been found to be cross-linked to the fibrin polymer. The result of this incorporation being an

enhancement in the mechanical and chemical resistance of the fibrin clot (Kamykowski et al., 1981) and to improve the adherence of the clot to cells (Grinnell et al., 1980). Fibrinogen has also been found to be cross-linked to collagen by Factor XIIIa (Mosher, 1984), which may play an additional role in cell adhesion. Two other substrates of Factor XIIIa are fibronectin and α_2-plasmin inhibitor which are cross-linked specifically to the α-chains of fibrin (Tamaki & Aoki, 1981; Ichinose & Aoki, 1982). The incorporation of α_2-plasmin inhibitor has been shown to significantly inhibit naturally occuring fibrinolytic processes (Sakata & Aoki, 1982).

Factor XIIIa has also been found in platelets (Schwartz et al., 1971), placenta (Bohn & Schwick, 1971), granulocytes, monocytes and macrophages (Berntorp et al., 1985). However the subunit composition of the zymogen in placenta and platelets differs in that there are no b-chains, only a-chains. The zymogen, a_2, with a molecular weight of ≈ 150,000 then undergoes thrombin activation to yield the active Factor XIIIa, a'_2, whilst the granulocyte enzyme does not display any thrombin dependence (Berntorp et al., 1985). The hypothesis is that these types of Factor XIIIa may either supplement the plasma enzyme or play specialised roles in aiding wound healing.

More recent studies have led to determination of the protein structure of human placental Factor XIIIa and other Factor XIIIa enzymes (Putnam et al., 1987; Ichinose & Davie, 1987; Grundmann et al., 1987).

1.3.2. Hair Follicle Transglutaminase.

As early as 1959 the presence of transamination and transpeptidation in wool had been established (Rogers & Springell, 1959), and Rogers (1962) subsequently went on to describe a

citrulline-rich protein located in the inner root sheath of hair follicles and medulla of hair. This protein was found to differ from the keratin proteins of hair in that it had a very high glutamic acid and low cysteine content. A putative intracellular precursor to this citrulline-rich protein has been discovered and named trichohyalin, it has been shown to be cross-linked *in vitro* into a higher molecular weight form (Rogers *et al.*, 1977). The important finding was made of the presence of ε(γ -glutamyl)-lysine cross-links in the citrulline-rich protein (Harding & Rogers, 1970) localised to the medulla and inner root sheaths of hair. Harding and Rogers (1972) reported an enzyme in hair follicle homogenates that formed ε(γ -glutamyl)-lysine bonds. Ultimately a transglutaminase was isolated from the guinea-pig hair follicle (Chung & Folk, 1972). They found two different transglutaminases in homogenates of inner root sheaths, one was similar to the liver enzyme and the other characterised as being distinct from the known transglutaminases. This enzyme was found to have a molecular weight of 54K and to dissociate into two distinct subunits of 27K, it had no esterase activity and did not cross-react with antibodies to the liver enzyme. The enzyme was found to be Ca^{2+}-dependent and was not present in a zymogenic form.

Later studies indicated that the enzyme was also immunologically distinct from epidermal transglutaminase (Buxman & Wuepper, 1976; 1978; Ogawa & Goldsmith, 1977).

In conclusion, the proposed role of the hair follicle transglutaminase is that it cross-links a precursor (possibly trichohyalin) or precursors into higher molecular weight polymers in the inner root sheath and medulla of hair, and thereby confers upon hair its characteristic properties of insolubility, rigidity, and

chemical resistance. However, limited progress has occurred in this field due to the difficulties in isolating the enzyme and in investigating its substrates.

1.3.3. Epidermal Transglutaminase.

As a consequence of the investigations upon the hair follicle transglutaminase, Buxman and Wuepper (1974; 1975) investigated epidermis free of hair and in cow snout epidermis found transglutaminase activity. They determined that this epidermal transglutaminase was immunologically distinct from both bovine and human Factor XIIIa.

Extensive work on the enzyme indicated its activity to be present in a range of cells making up the epidermis, with major activity localised to the stratum corneum and granular layer. The enzyme was immunologically distinct from other transglutaminases, and had a molecular weight for the enzyme of approximately 54,000 (Goldsmith et al., 1974; Goldsmith & Martin, 1975; Buxman & Wuepper, 1976; Ogawa & Goldsmith, 1976; 1977). Goldsmith et al. (1974) further demonstrated the presence of $\varepsilon(\gamma$-glutamyl)-lysine bonds in cow snout epidermis, a finding which was indicative of transglutaminase activity.

The discovery of an epidermal transglutaminase led to investigations into the substrates of the enzyme and characterisation of its functional role in the epidermis. Substrates of the enzyme were investigated through their labelling by the enzyme in a Ca^{2+}-dependent manner using monodansylcadaverine. By this method two substrates were identified in bovine epidermal extracts (Buxman et al., 1976), a soluble substrate with a molecular weight of 150,000 and a highly insoluble substrate with a high serine, glutamic acid and lysine content. It was proposed that the soluble precursor

protein was the natural substrate which was cross-linked by transglutaminase into large polymers which formed the envelope of the keratinocyte; these findings were subsequently confirmed by Hanigan and Goldsmith (1978). Buxman et al. (1979) then went on to indicate the importance of the epidermal transglutaminase as a marker for terminal differentiation.

Rice and Green (1979) switched their studies to epidermal keratinocyte cultures and isolated a soluble precursor of the cross-linked envelope. This precursor had a molecular weight of 92,000 when measured by sodium dodecyl sulphate - gel electrophoresis. It was immunologically and biochemically distinct from keratin and was found to be localised in the cytoplasm in the upper epidermal layers, but membrane bound in the envelope in the stratum corneum. The precursor had a very high glutamine content of 46% and was given the name involucrin.

Later studies by Buxman et al. (1980), employing an antibody to the 150,000 Mr. soluble precursor they had previously reported (Buxman et al., 1976), identified a low molecular weight precursor of 36,000 Mr. In the presence of epidermal transglutaminase and Ca^{2+} this precursor was converted to higher molecular weight polymers of Mr. 75,000 - 200,000 in addition to the formation of an insoluble polymer. They detected the $\varepsilon(\gamma$-glutamyl)-lysine dipeptide in the higher molecular weight products but not in the 36,000 Mr. soluble precursor. The composition of the 36,000 Mr. precursor was similar to that of the envelope protein, and the two proteins were indistinguishable immunologically.

The proposal was made that the 36,000 Mr. protein was the precursor protein which was subsequently cross-linked to form the epidermal envelope. Zettergren et al., (1982) extended these studies

to include the human epidermis, and confirmed the presence of the 36,000 Mr. precursor. It was found that the precursor dissociated to give a further precursor of molecular weight 6,000 which, when incubated with transglutaminase and Ca^{2+}, formed a range of higher molecular weight polymers. However no polymer was observed that corresponded to the 92,000 Mr. precursor observed by Rice and Green (1979) in keratinocyte cultures; these findings were largely confirmed by Kubilis and Baden (1984). Anti-serum to involucrin did not cross-react with human epidermal extracts of the 36,000 Mr. precursor from epidermis, which was subsequently named keratolinin. The evidence suggests that if involucrin does exist in the epidermis, it is distinct from keratolinin and the two proteins are both likely to participate in the formation of the epidermal cell envelope.

The proposed action of epidermal transglutaminase is therefore to cross-link soluble precursors into an insoluble polymer which is membrane bound and corresponds to the formation of the cornified envelope that lines the inner membrane of keratinocytes in the stratum corneum. In the epidermis the soluble precursor is keratolin, whereas in keratinocyte culture the soluble precursor is involucrin, involucrin has not been found in the epidermis. The stability and strength of the skin are, to a major part, attributable to the formation of the cornified envelope.

1.3.4. Prostate Transglutaminase.

Studies investigating the role of prostate transglutaminase in the formation of a post-coital plug have centered largely on the rat and guinea-pig, since rodent semen coagulation had been determined to be Ca^{2+}-dependent by Gotterer et al. (1955; 1957).

In the guinea-pig the main precursor of the coagulation product was determined to be a basic protein of molecular weight 17,900, lacking in proline, cysteine and tyrosine (Notides & Williams-Ashman, 1967). Based upon their findings that : the precursor had an amino-acid composition similar to that of the enzymatically formed polymer; was particularly rich in glutamate and lysine; that free ammonia was released during polymerisation; and that the reaction was both Ca^{2+}- and thiol-dependent; they hypothesised that the reaction was catalysed by transglutaminase. Subsequently came the detection of ε(γ-glutamyl)-lysine bonds in the coagulem (Williams-Ashman et al., 1972), which were found to be absent in the unclotted vesicular secretion (Pabalan, 1975).

A considerable amount of controversy then arose as to the nature of the transglutaminase activity catalysing the coagulation. Chung (1977) reported that the human and guinea-pig prostrate gland containd two distinct enzyme activities, one a zymogen with similar properties to the platelet enzyme Factor XIIIa, and a second which cross-reacted immunologically with the liver enzyme. Whilst other workers also reported two distint enzymes in the guinea-pig prostrate gland (Wing, 1979; Tong, 1980), these enzymes were found to be different from both the liver and Factor XIIIa enzyme.

The coagulation system in the rat was found to differ from that in the guinea-pig. ε(γ-glutamyl)-lysine bonds were found only in the clotted vesicular proteins, but the results suggested the clot resulted from the cross-linking of several different vesicular secretory proteins and not a single precursor as in the guinea-pig (Higgins et al., 1976; Bradshaw & Wolfe, 1977; Williams-Ashman et al., 1977, 1980). The studies of Williams-Ashman et al. (1980)

indicated that the cytosol of the rat coagulation gland and the secretion of the gland contained at least two forms of transglutaminase not detectable in other organs and differing from those found in the guinea-pig gland.

Interest arose as to whether polyamines might modulate or participate in transglutaminase-mediated clotting of seminal fluid, since polyamines are present in high concentrations in seminal plasmas of several mammalian species; for example human seminal plasma contains 3mM spermine. Subsequent studies indicated the presence of both γ-glutamyl polyamines and N,N-bis-γ-glutamyl-polyamine bridges in the copulatory plug (Schrode & Folk, 1978; Folk, 1980).

It was postulated that the explanation for these observations was that polyamines served to prevent the formation of the insoluble polymer in the penile urethra by controlling the degree of polymerisation (Williams-Ashman & Conellakis, 1980). Later studies on the rabbit indicated that transglutaminase, in conjunction with the protein uteroglobulin which is present in both seminal secretions and the female reproductive tract, supressed the antigenicity of spermatozoa (Mukherjee et al., 1983; Munjunath et al., 1984). This proposal was supported by Panoessa et al. (1984) who showed that transglutaminase catalysed the incorporation of polyamines into a major protein secreted from the rat seminal vesicle epithelium resulting in the formation of a modified protein which bound to the rat sperm cell.

More recently Porta et al. (1986) have detected transglutaminase activity free in the human seminal plasma and also bound to the spermatozoon surface, and have demonstrated the binding of polyamines to the spermatozoon membrane. They have postulated

that semen transglutaminase plays a role in supressing sperm antigenicity.

Thus, whilst some controversy encompasses the precise nature and identity of the prostrate transglutaminase(s), strong evidence indicates the role of these enzymes in forming the post-coital plug in some mammalian species, and thereby aiding fertilisation. There is further evidence to suggest a role of prostrate transglutaminase(s) in reducing the antigenicity of the spermatozoa.

1.4. THE BIOLOGICAL ROLE OF TISSUE TRANSGLUTAMINASE

Tissue transglutaminase is generally considered to be ubiquitous in mammalian organisms having been identified in a very large number of mammalian tissues. The enzyme has been found to be a non-zymogenic monomer of molecular weight 70-90,000. Comparitive studies which were based on chemical, physical, immunological and enzymatic properties indicated that the enzyme from different tissues in the same organism is identical (Chung, 1972; 1975). It is therefore probable that the enzyme plays an essential role in the cell. Since Ca^{2+} acts as a common trigger in a diverse range of biological events, the Ca^{2+}-dependence of the enzyme is therefore extremely significant in determining the cellular role of tissue transglutaminase.

The next section of the Introduction will therefore be concerned with those systems where a role for tissue transglutaminase in a particular cellular event has been implicated.

1.4.1. Receptor-Mediated Endocytosis.

Receptor-mediated endocytosis is the process whereby certain proteins bind to receptors on the surfaces of particular animal cells and are rapidly internalised before they can dissociate from their receptors. These ligands include serum proteins, low density lipoprotein, α_2 -macroglobulin, several lysosomal hydrolases, insulin and epidermal growth factor (EGF). For all ligands so far studied, internalisation occurs over clathrin-coated regions of the membrane. The unoccupied receptors of insulin and EGF are diffusely distributed over the cell surface, but cluster over clathrin-coated sections after ligand binding (Willingham et al., 1979).

The proposed involvement of transglutaminase in this process came as a result of observations that primary amines and

ammonium ions inhibited the clustering and internalisation of EGF and α_2-macroglobulin by cultured fibroblasts, and that the aggregation process required Ca^{2+} ions (Maxfield, 1979a; 1979b). Further studies indicated that the potency of a variety of compounds in inhibiting aggregation and internalisation of rhodamine-labelled α_2-macroglobulin and EGF by fibroblasts, Chinese hamster ovary cells and normal rat kidney cells, could be strongly correlated to their potency as inhibitors of tissue transglutaminase activity (Folk & Finlayson, 1977; Davies et al., 1980; Levitzki et al., 1980). The compounds tested included monodansylcadaverine, primary alkylamines and bacitracin, and it was noted that only compounds which could diffuse across the cell membrane had an inhibitory effect. The proposal was made that transglutaminase was involved in the covalent linkage of ligand-receptors to each other or to unidentified cellular components at the clathrin-coated sites.

However, subsequent studies by other workers challenged these conclusions. The effect of primary alkylamines such as methylamine and monodansylcadaverine was found to be a decrease in the actual number of receptors expressed at the cell surface (van Leuven et al., 1980; Hazum et al., 1979; McKenna et al., 1979; King et al., 1980; 1981). The studies of van Leuven indicated that the reduction in the number of receptors expressed at the cell surface by inhibitors was time and concentration dependent and fully reversible. It was further detailed that α_2-macroglobulin receptor recycling was occuring in the absence of ligand binding and that this process was inhibited by inhibitors of transglutaminase. As a result of this evidence the proposal was made that transglutaminase was involved in the internal recycling of cell surface receptors.

This hypothesis was consistent with the studies of Kaplan and Keogh (1981) who found that whilst one round of internalisation of α_2-macroglobulin could occur in the presence of primary amines, subsequent rounds of internalisation were inhibited.

Maxfield et al. (1981), reported that amines did not alter the mobility of ligand-receptor complexes in the membrane. Whilst Dickson et al. (1981) determined the presence of two classes of α_2-macroglobulin receptor, a small pool of high affinity receptors and a larger pool of low affinity receptors. The pre-incubation of fibroblasts with monodansylcadaverine resulted in the removal of high affinity ligands and transglutaminase might therefore be involved in the conversion of low affinity ligands into high affinity ones.

The idea that the observed inhibition of α_2-macroglobulin internalisation by primary amines was due to lysomotrophic effects were largely dispelled by studies which indicated that very similar analogues of monodansylcadaverine such as N,N'-dimethyl-monodansylcadaverine inhibited neither the internalisation of α_2-macroglobulin nor transglutaminase activity (Dickson et al., 1982).

It had been reported that transformed cells had very low levels of transglutaminase (Birckbichler et al., 1977; Baldwin et al., 1980; Novogrodsky et al., 1980). Therefore Davies et al. (1984) investigated the ability of cells with differing levels of transglutaminase to internalise ligands and studied the effect of primary amines on this internalisation. They found that cells completely devoid of transglutaminase activity were fully competent to bind and internalise protein ligands by receptor-mediated endocytosis. Hence they concluded that the enzyme was not a required component of receptor-mediated endocytosis.

The original observations of Davies et al. (1980) that concentrations of monodansylcadaverine below 200μM inhibited EGF internalisation were strongly refuted. Studies on the effect of primary amines on the internalisation of ^{125}I-labelled-EGF concluded that methylamine had no effect and that monodansylcadaverine only blocked internalisation at much higher concentrations of 400-500 μM (King et al., 1980; Haigler et al., 1980a; 1980b; Yarden et al., 1981). The results of Davies et al. (1980) using fluorescence microscopy were criticised as only being sensitive enough to yield qualitative data. The discrepancies between the inhibitory levels of monodansylcadaverine were ultimately resolved with the discovery that the EGF preparations used by Davies contained contaminating peptides which resulted in falsely high levels of inhibition (Haigler et al., 1980; Willingham & Pastan, 1981).

Since Davies had indicated that transglutaminase activity was not required for receptor-mediated endocytosis of ligands, he resolved that the effect of monodansylcadaverine (400-500 μM) on EGF internalisation was commensurate with the levels of the drug required to antagonise calmodulin-mediated steps in endocytosis (Davies et al., 1984). Therefore there is no apparent evidence for transglutaminase involvement in the internalisation of EGF.

However the concentration of monodansylcadaverine required for the inhibition of the receptor-mediated endocytosis of α_2-macroglobulin excluded any effect of this compound on calmodulin-mediated events. Further it was observed that monodansylcadaverine only inhibited endocytosis and receptor down-regulation in cells with high levels of transglutaminase (Baldwin et al., 1980). Using immunofluorescence techniques, Davies

et al. (1984) indicated that monodansylcadaverine became covalently bound to endocytic vesicles in Chinese hamster ovary cells, which was concurrent with the localisation of rhodamine-labelled α_2-macroglobulin. It was concluded that the localisation of monodansylcadaverine to endocytic vesicles represented abnormal products of transglutaminase activity. Therefore whilst transglutaminase has not been shown to be intrinsically involved in receptor-mediated endocytosis, it may mediate the effect of some drugs that can interfere with ligand internalisation.

The use of bacitracin as an inhibitor of receptor-mediated endocytosis has also been discounted in relation to the involvement of transglutaminase, since bacitracin has been shown to act as a competitive inhibitor of the binding of α_2-macroglobulin to its receptor (van Leuven et al., 1981; Dickson et al., 1981).

Evidence does however exist for the involvement of the tissue transglutaminase in a number of other endocytic processes where ligands are internalised and where primary amines have been shown to inhibit this process. These ligands include neurotransmitters and hormones such as catecholamines by frog erythrocytes (Chuang, 1981; 1984), acetylcholine (Higuchi et al., 1982; Hucho and Bandini, 1986) and tri-iodothyronine (Cheng et al., 1980); peptide hormones and growth factors such as insulin (Pastan & Willingham, 1981; Draznin & Towbridge, 1982); bacterial toxins such as Pseudomonas toxin (Seglen & Gordon, 1980), Shigella toxin (Keusch, 1981) and diptheria (Kaplan & Nielson, 1979); transport of proteins such as low density lipoproteins (Tucciarone & Lanclos, 1981; Kojima et al., 1987); and of viruses including Semliki forest virus (Leppla et al., 1980), vesicular stomatis virus (Fitzgerald et al., 1980).

However, more detailed studies are required to resolve these observations, particularly since the same inhibitory effects as those of primary amines may be produced in some systems by lysomotrophic drugs such as chloroquine (Davies & Murtaugh, 1984).

It is apparent from the studies of Davies (1984) that transglutaminase is not directly involved in the mechanism of receptor-mediated endocytosis. However the evidence does indicate that some links between transglutaminase activity and the process have been established in the case of some ligands which clearly requires more investigation. For example transglutaminase may only be involved in the mechanism of receptor-mediated endocytosis where the receptor is recycled as is the case for α_2-macroglobulin, but not in those cases where the receptor is degraded as with the EGF-receptor (Herzog, 1981).

1.4.2. Cell activation and differentiation.

The possible involvement of tissue transglutaminase in cell activation and differentiation was first suggested by the studies of Novogrodsky et al. (1978) with the observation that transglutaminase activity was rapidly increased in human peripheral blood lymphocytes treated with phytomitogens. This effect was dependent upon the binding of the phytomitogens to the cell surface. Subsequent studies indicated that activated monocytes and macrophages also exhibited significantly raised levels of tissue transglutaminase (Fesus et al., 1981; Schroff et al., 1981; Leu et al., 1982; Murtaugh et al., 1983).

The proposal was further enhanced by studies on the effects brought about by inhibitors of transglutaminase activity. The primary amines monodansylcadaverine, methylamine and cystamine were shown to inhibit Fc receptor-mediated endocytosis of opsonised

erythrocytes by guinea-pig peritoneal macrophages (Fesus et al., 1981; Leu et al., 1982). The stimulation of β-lymphocytes by antigen (lactin) to produce antibody-secreting cells was inhibited by primary amines with a potency that paralleled their inhibition of transglutaminase activity (Gunzler et al., 1982; Julian et al., 1983).

Transglutaminase was proposed to be a marker for the degree of activation or differentiation of macrophages since the expression of transglutaminase was found to be dependent upon both the differentiation state and type of macrophage (Schroff et al., 1981).

The studies of Murtaugh et al. (1983) on the activation of mouse peritoneal macrophages culture in mouse serum indicated that the observed approximate 150 fold increase in transglutaminase activity was promoted by a heat labile constituent of plasma or serum. These heat labile constituents were subsequently identified as retinoids (Moore et al., 1984; Davies et al., 1985). The stimulation of transglutaminase activity was also found to be enhanced by interferon-γ in studies on human peripheral blood monocytes (Mehta, 1985). Interferon-γ has been documented to affect the differentiation of various cells of monocytic lineage (Steeg, 1982; Gayne et al., 1983; Perussia et al., 1983; Ball et al., 1984; Sztein et al., 1984; Verelizier, 1984). Both the effects of retinoids and interferon-γ were shown to be due to increased synthesis of transglutaminase and not due to the activation of existing transglutaminase (Mehta, 1985; Murtaugh et al., 1986). Studies by Mehta and Berestein (1986) observed that concomittant with a 20-50 fold increase in transglutaminase activity following treatment of cultured monocytic leukaemia cells with retinoic acid,

were distinct physiological changes including the adherence of cells to plastic and their ability to phagocytose. The induction of transglutaminase synthesis by retinoic acid was inhibited by pertussis-toxin, but the toxin had no effect upon the basal level of transglutaminase activity (Johnson & Davies, 1986). It was also observed that ADP-ribosylation of a 41,000 Mr. protein was induced by the toxin and therefore suggested that the effect of retinoids was mediated by a membrane receptor.

Recent studies by Mehta et al. (1987a) indicated that the differentiation of human peripheral blood monocytes into macrophages corresponded to an increase in tissue transglutaminase. These authors also showed that the level of transglutaminase activity in monocytes/macrophages could be induced by retinol and was augmented by interferon-γ via a mechanism involving increased binding of retinol.

In a second publication Mehta et al (1987b), studying mouse residential peritoneal macrophages, again reported an induction of transglutaminase activity by retinoids which could be augmented by interferon-γ. However, this study presented strong evidence to indicate that the increased level of transglutaminase corresponded to a decreased ability of mouse macrophages to effect a cytostatic activity upon murine adenocarcinoma (EMT-6) tumour cells. Monodansylcadaverine treatment resulted in a complete recovery of macrophages from retinoid supression of cytostatic activity. It was suggested that retinol-induced activation of transglutaminase resulted in post-translational changes in a tumour necrosis factor, a major cytotoxin released by activated macrophages. This study therefore suggested that induction of tissue transglutaminase activity may be associated with supression of macrophage activation.

No comment was made about how this contradicted the other studies undertaken by the authors which had suggested that increased transglutaminase activity was associated with an increased capacity for endocytosis (Fesus et al., 1981; Schroff, 1981; Leu, 1982; Mehta & Berestein, 1986). These differences clearly need to be resolved.

A study on the effect of glucosteroids, retinoids, 1,25-dihydroxy-vitamin D_3, and the tumour promoter phorbol myristate acetate (TPA) on the expression of transglutaminase activity in bone marrow-derived mouse and rat mononuclear phagocytes and human myeloid leukaemia cells was recently undertaken by Goldman (1987). Amongst various observations was the effect of retinoic acid, which induced transglutaminase activity in the rat cells, but supressed it in mouse cells. In contrast 1,25-dihydroxy-vitamin D_3 had little effect on transglutaminase expression in mouse cells but supressed expression in rat cells. These agents also had a reversed effect on the differentiation of macrophages from mouse and rat progenitor cells (Shezen & Goldman, 1983). Thus retinoic acid, which induced transglutaminase activity in rat monocytes, supressed macrophage formation from rat bone marrow progenitor cells. In the mouse cell system, retinoic acid which supressed transglutaminase activity in murine monocytes was found to enhance macrophage formation from mouse bone marrow progenitor cells. These results again present something of an enigma since they infer that transglutaminase induction is correlated to the supression of cell differentiation which contradicts other observations cited.

The retinoid-induced expression of tissue transglutaminase in normal and leukaemic myeloid cells was investigated using a cDNA probe to mouse tissue transglutaminase

(Davies et al., 1987) it was found that retinoic acid acts as an acute inducer of transglutaminase gene expression. The induced expression of the enzyme was potentiated by agents that increased intracellular cAMP levels, and was inhibited by agents that activated protein kinase C.

The effect of retinoic acid on rat hepatocytes in vitro revealed an increase in transglutaminase activity which was proceeded by a significant rise in putrescine (Piacentini et al., 1987), the treatment also resulted in the appearance of many large, unidentified cytoplasmic inclusions.

Other cells in which tissue transgultaminase has been implicated in activation and differentiation processes include platelets (Barnes et al., 1984), Chinese hamster ovary cells (Milhand et al., 1980; Scott et al., 1982), neurones (Gilad & Varon, 1985; Maccioni & Seeds, 1986), Friend erythroleukaemia cells (Hsu & Friedman, 1983), and embryonic cells (Cariello et al., 1984; Maccioni & Arechaga, 1986; Uhl & Schindler, 1987). Many of these activation processes are coupled to an increase in cytoplasmic concentration of Ca^{2+}, which is thought to be essential in triggering the further steps in development, and which would potentially result in the activation of transglutaminase.

In conclusion, tissue transglutaminase has been implicated in the differentiation/activation of many cells. However the intricacies of this involvement remain to be delineated, since the findings in some cases appear to be contradictory, especially as to whether the rise in transglutaminase activity is directly coupled to an increase in endocytic activity. It is possible that the enzyme may be involved generally in the events associated with the growth and proliferation of cells, and not specifically involved in endocytic or exocytic processes.

1.4.3. Tumour growth.

The findings of Yancey and Laki (1972) that inhibitors of fibrin clot stabilisation also inhibited the formation of the YPC-1 tumour in mice, and hence increased host survival, was one of the earliest reports of a link between transglutaminase and tumour growth. It was postulated that tissue transglutaminase activity was essential to tumour growth. Subsequently it was found that there was a direct correlation between the tissue transglutaminase content of the host recipient organ and the growth and metastasis of tumours (Laki et al., 1977). An inverse correlation was found between the mean survival time of mice and the tissue transglutaminase content of transplanted tumours.

However, contradictory observations were subsequently made by other workers in the field that tissue transglutaminase activity was essential to keep cells in a non-proliferating state. Novikoff hepatoma and primary hepatomas induced by 3'-methyl-4-dimethylaminoazobenzene were found to contain reduced tissue transglutaminase activity when compared to normal liver, furthermore the subcellular distribution of transglutaminase activity in these tumours was more particulate associated when compared to that in normal liver (Birckbichler et al., 1976). Several transformed cell lines were also shown to have lower levels of transglutaminase activity when compared to their normal counterparts (Bickbichler et al., 1977), an observation which was subsequently confirmed by Davies et al. (1984). Studies on virus transformed cells found that the content of $\varepsilon(\gamma$-glutamyl)-lysine cross-links was lower than in non-transformed cells. A role was suggested for transglutaminase-catalysed protein cross-linking in the maintenance

of cells in the non-proliferating state (Birckbichler et al., 1978; Birckbichler & Paterson, 1980), where the enzyme was thought to act by stabilising the membrane architecture. Subsequent studies further suggested a role for tissue transglutaminase as a primary regulator of proliferation with the observation that inhibition of tissue transglutaminase resulted in an increase in proliferation markers in WI-38 cells.

A decrease in ε(γ-glutamyl)-lysine cross-links was found in cultured L-cells grown in the presence of monodansyl-cadaverine or cystamine (Haughland et al., 1982), which was shown to correlate with an increase in membrane fluidity. These observations further enhanced the idea that tissue transglutaminase might confer stability on cell membranes by the formation of ε(γ-glutamyl)-lysine cross-links.

A reduction in transglutaminase activity was found in Yashida Ascites tumour cells and in Ascites tumour-bearing liver as compared to normal liver (Vanella et al., 1983), further supporting the findings of Birckbichler and co-workers, although the study only measured activity in the soluble fraction.

Tissue transglutaminase activity was found to be decreased in the sequence normal liver > tumour bearing liver > hepatocellular carcinoma using both diethylnitrosamine and 6-p-dimethylaminophenylazobenzothiazole as carcinogens (Barnes et al., 1984; 1985), and a redistribution of tissue transglutaminase activity to the particulate fraction was detected. A 4-7 fold reduction in tissue transglutaminase activity was found to occur in the primary tumour of rat sarcomata following detection of metastases to the lung (Barnes et al., 1985). This study was later expanded to show that this reduction in transglutaminase activity coincided

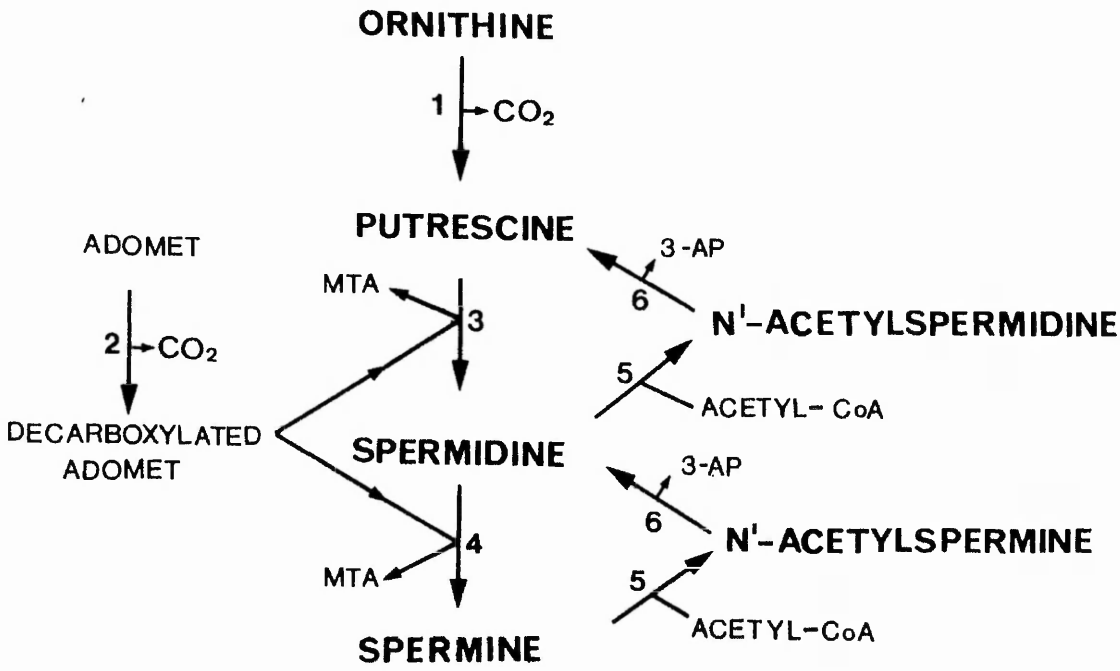
exactly with the onset of tumour metastasis and was parallel by a massive (20 fold) increase in free putrescine levels (Hand et al., 1987). Two subsequent studies have supported these findings, Delcros et al. (1986) found that in cloned rhabdomyosarcoma cells the tissue transglutaminase activity decreased in the sequence non-metastatic > weakly metastatic > strongly metastatic, whilst Roch et al. (1987) reported that in human colon tumours the tissue transglutaminase activity decreased in the sequence normal > benign > malignant.

There is therefore evidence to suggest an inverse correlation between tissue transglutaminase activity and tumour growth/metastasis, but further work is required to indicate whether these changes in tissue transglutaminase activity are involved in a causal manner in the development of the neoplastic phenotype, or whether they are parallel phenomena.

1.4.4. Tissue transglutaminase and polyamines in normal and neoplastic growth.

Polyamines are ubiquitous organic cations that are synthesised in large amounts by rapidly growing and dividing cells (for reviews see Tabor & Tabor, 1976; 1984; Russell & Durie, 1978; Heby, 1981; Goyns, 1982; Pegg et al., 1982a; Pegg & McCann, 1982; Pegg, 1986). The structure, biosynthesis and interconversion of the naturally occuring polyamines putrescine, spermidine and spermine is illustrated in Figure 1.3. Ornithine, which is formed by the action of arginase, is converted to putrescine by the action of L-ornithine decarboxylase (ODC). Putrescine is converted to spermidine by the action of an aminopropyltransferase called spermidine synthase. A second aminopropyltransferase, spermine synthase, adds an additional propylamine moiety to spermidine, forming spermine. The source of these propylamine groups is decarboxylated S-adenosylmethionine which is produced by the action of its decarboxylase; the other product of

Figure 1.3 Pathway for the Biosynthesis and Interconversion of Polyamines.



The enzymes involved are : 1. ornithine decarboxylase (E.C. 4.1.1.17); 2. S-adenosylmethionine decarboxylase (E.C. 4.1.1.50); 3. spermidine synthase (E.C. 2.5.1.16); 4. spermine synthase (E.C. 2.5.1.22); 5. spermidine/spermine-N^1-acetyltransferase; 6. FAD-dependent polyamine oxidase. Abbreviations : ADOMET, S-adenosylmethionine; 3-AP, 3-acetamido propanal; MTA, 5'-methylthioadenosine.

the aminopropyltransferase reactions is 5'methylthioadenosine. The interconversion pathway results from the action of two enzymes, spermidine/spermine-N-acetyltransferase and polyamine oxidase (FAD-dependent), however the importance of these cycles is not known. It is noteworthy that ODC may be down-regulated by transamidation reactions involving the covalent incorporation of putrescine into the enzyme (Haddock-Russel, 1981).

Most of the studies on the importance of polyamines in cell metabolism have been investigated by the use of specific inhibitors of ODC, which is the rate-limiting enzyme in the pathway, particularly favoured has been the enzyme-activated irreversible inhibitor α-difluoromethylornithine (DFMO), (Metcalf et al., 1978).

The function of polyamines in mammalian cells remains obscure although there is general agreement from studies with inhibitors and mutants, that polyamines are essential for cell growth. DFMO was found to exert a cystostatic rather than cytotoxic effect on mammalian cells (Mamont et al., 1980; 1982; 1984; Pegg & McCann, 1982; Pegg et al., 1982b; Pegg, 1984), and the reduction in cell growth was completely overcome by the addition of putrescine, spermidine or spermine.

The combination of DFMO and spermine synthase inhibitor did result in a cytotoxic effect (Pegg et al., 1982), as was observed in other studies where spermine levels were significantly reduced (Casero et al., 1984; Porter, 1985; Mc'Govern, 1986). Coupled with these observations were reports that the cellular functions of polyamines could be singularly fulfilled by spermine or spermidine (Mamont et al., 1980; Rudkin et al., 1984; Pegg, 1984; Pegg & Coward, 1985).

The precise reason for the reduction in cell growth resulting by inhibition of polyamine synthesis is not fully understood, DNA synthesis is known to be affected but the earliest effect observed is on protein synthesis (Rudkin et al., 1984). It is possible that the changes in DNA synthesis are secondary to a reduction in the rate of protein synthesis, these observations were supported by other studies (Pegg & Mc'Cann, 1982; Takermoto et al., 1983; Tabor & Tabor, 1984). The cytostatic effect of polyamine biosynthesis-inhibitors could therefore be due to the reduced rate of synthesis of key metabolic enzymes. Subsequent loss of cell viability accompanied by complete depletion of polyamines may be due to cellular requirements for polyamines to maintain normal chromatin structure, since mutants lacking ODC or treatment of cells with DFMO results in major chromosome aberrations. Other cellular perturbations include alterations in nuclear morphology, loss of actin filaments and loss of microtubules (Pohjanpelto & Knautila, 1982; Knautila & Pohjanpelto, 1983; Pohjanpelto et al., 1985).

Polyamines possess a primary amine group and, as discussed in Section 1.2.1., may act as substrates for transglutaminase (c.f. Figure 1.1. b and c). The ability of putrescine, spermidine and spermine to become incorporated into proteins via transglutaminase catalysis has been demonstrated in extracellular and intracellular systems (for review see Williams-Ashman & Cannelakis, 1980). The evidence for covalent polyamine incorporation into the seminal fluid clot has been discussed in Section 1.3.4. Reports of the incorporation of radiolabelled polyamines into proteins in intracellular systems have included HTC cells (Cannelakis et al., 1981), WI-38 cells (Paterson et al., 1982), the giant neuron of Aplysia californica (Ambron &

Kremzner, 1982), and activated human lymphocytes (Folk et al., 1980). Concurrent with lymphocyte activation is a rise in the intracellular levels of polyamines (Fillingame & Morris, 1973), the activity of the enzymes of polyamine synthesis (Bachrach et al., 1981) and transglutaminase activity as discussed in section 1.4.2. Due to these and other observations that transglutaminase activity is linked to cell growth and proliferation (Birckbichler et al., 1980; Haddock-Russel, 1981), it is tempting to suggest that transglutaminase mediated polyamine incorporation into proteins may be important in these growth states.

However many studies have not shown the involvement of γ-glutamyl-polyamine linkage and therefore lack sufficient evidence that the incorporation of polyamines is transglutaminase-mediated. Further, the majority of studies involving radiolabelling of cellular proteins when cultured in the presence of radiolabelled polyamines was found to correspond to hypopusine formation (Chen, 83; Cooper et al., 1983). Hypopusine is formed from lysine by an addition which originates from spermidine (Cooper et al., 1983; Park et al., 1984), and is a component amino-acid of the translocation initiation factor elf-4D which is present in all cells.

Beninati et al. (1985) conclusively demonstrated the transglutaminase-mediated incorporation of polyamines into protein in the rat liver, kidney and testis by the identification of bis-(γ-glutamyl)-polyamine derivatives. Beniati and Folk (1987) subsequently suggested that polyamines modulate ε(γ-glutamyl)-lysine protein cross-links through their competition with lysine for transglutaminase-catalysed γ-glutamyl-bond formation. In addition these workers noted that the most heavily labelled protein was of a very high molecular weight, these findings were supported by Porta et al. (1987). It is also of particular interest that the induction

of tissue transglutaminase activity by retinoic acid (c.f. Section 1.4.2.) was proceeded by a significant rise in putrescine levels, but with no significant change in spermidine or spermine (Piacentini et al., 1987).

Work on experimental tumours has produced extensive evidence for the accumulation and enhanced synthesis of polyamines in tumours (Noguchi et al., 1976; Kallio et al., 1977; Janne et al., 1978; Kaminski, 1983). Increased levels of polyamines in erythrocytes were related to the growth of fibrocarcinomas, but the polyamine levels of the tumours themselves did not correlate with the growth rate of the tumours (Grossie et al., 1986). However, studies with a metastasising rat sarcomata indicated a 20 fold increase in the levels of putrescine during the onset of metastasis which was accompanied exactly by a marked decrease in transglutaminase activity; these changes were accompanied by a decrease in the levels of covalently bound putrescine (Hand et al., 1987). In a parallel study by these authors, a non-metastasising rat sarcomata did not result in any significant changes in the levels of transglutaminase activity or polyamine levels during tumour growth, suggesting that the observed changes were related to the onset of metastasis.

There have been attempts to employ polyamine-biosynthesis inhibitors as anti-tumoral agents (reviewed by Sjoerdsma & Schechter, 1984; Janne et al., 1983), although their use seems limited due to the non-specific cytostatic action of such compounds. However some studies have suggested that treatment of tumour cells with polyamine-biosynthesis inhibitors sensitises the cells to killing by certain anti-tumour agents (Martin et al., 1983; Sano et al., 1984).

The function of tissue transglutaminase and polyamines in stimulus-secretion coupling in the pancreatic β-cell will be discussed in Section 1.5.

1.4.5. Cell ageing.

Tissue transglutaminase has been implicated in cross-linking of the cytoskeletal matrix and membrane proteins through the formation of ε(γ-glutamyl)-lysine bonds resulting in the formation of rigid structures has been suggested as a general pattern in cells undergoing terminal differentiation in the process of ageing and senescence. The potential involvement of the epidermal transglutaminase in the terminal differentiation of epidermal keratinocytes has been discussed in Section 1.3.3.

Another model for the involvement of tissue transglutaminase in cell ageing is the Ca^{2+} loaded erythrocyte. The incubation of erythrocytes with Ca^{2+} and the Ca^{2+} ionophore A23187 results in a loss of membrane deformability (Smith et al., 1981), permanent shape change (White, 1976), and a concomitant change in membrane polypeptide content including the cross-linking of proteins into high molecular weight aggregates. Tissue transglutaminase activity was detected in erythrocytes (Brenner & Wold, 1978), and subsequently implicated in the cross-linking mechanism when it was found that primary amines could inhibit the process (Lorand et al., 1976; Anderson et al., 1977). It was further found that radiolabelled primary amines were specifically incorporated into membrane proteins of the erythrocytes (Lorand et al., 1975). Of particular importance in the studies resulting from this model was the appearance of the high molecular weight protein polymers which contained appreciable amounts of the ε(γ-glutamyl)-lysine dipeptide in Ca^{2+}-loaded erythrocytes (Lorand et al., 1978; Seifring et al.,

1978). Subsequent analysis of the high molecular weight polymer indicated that it was comprised of a number of proteins including spectrin, ankyrin (band 2.1), bands 3 and 4.1, and some haemoglobin (Bjerrum et al., 1981; Lorand & Conrad, 1984). More recent studies employing dansyl-antibody labelling techniques have revealed band 3 to be the predominant γ-glutamyl containing substrate in inside-out erythrocyte vesicles (Lorand et al., 1986), whilst studies with competitive inhibitors of tissue transglutaminase have confirmed the incorporation of radiolabel into band 3, band 4.1, spectrin and ankyrin (Lorand et al., 1987).

These results have led to the suggestion that some of the membrane changes occuring in aged erythrocytes, irreversibly sickled erythrocytes, and certain hereditary erythrocyte disorders may be linked to the activation of tissue transglutaminase. In the erythrocyte the normal Ca^{2+} concentration is maintained below 1×10^{-6}M by an outwardly directed Ca^{2+}/ATPase-pump, but failure of this pump directly or indirectly may result in the Ca^{2+} concentration being sufficiently raised to cause activation of tissue transglutaminase. As a consequence, cross-linking of membrane proteins may result.

Similarly a tissue transglutaminase has been identified in the mammalian lens (Lorand et al., 1981; Hsu et al., 1981) and been shown to be involved in the formation of β-crystallin polymers (Berbers et al., 1983). It has been proposed that the uncontrolled formation of these polymers in the ageing lens may contribute to cataract formation.

Additionally, Selkoe et al. (1982), have indicated the possible participation of transglutaminase in cross-linking cytoskeletal proteins to form paired helical fragments and

neurofibrillary tangles in ageing neurones possibly due to a rise in intracellular Ca^{2+} levels. Such paired fragments have been identified in the brains of Alzheimer patients.

1.5. AIM OF THE THESIS

The evidence so far discussed concerning the cellular role of the tissue transglutaminase associates the enzyme with cell membrane-mediated phenomena. On the basis of this association, previous studies from this laboratory (Bungay et al., 1984) investigated the potential involvement of tissue transglutaminase in the exocytotic mechanism of insulin release from the pancreatic β-cell, a process involving considerable endomembrane flow and which is thought to be mediated by a rise in intracellular Ca^{2+} levels.

This model was selected as being a suitable model for the involvement of transglutaminase in stimulus-secretion coupling because:

i) the problems previously discussed in Section 1.4.1. of the interference of drugs with receptor-binding do not exist in pharmacological studies of glucose-stimulated insulin release, since glucose itself is the metabolic signal;

ii) Ca^{2+}-mediated stimulus-secretion coupling is relatively easy to measure in studies using intact islets of Langerhans;

iii) islets of Langerhans which can be readily isolated, comprise predominantly of the insulin secreting β-cell.

Furthermore the exocytotic release of insulin from the pancreatic β-cell is known to be a Ca^{2+}-dependent process involving a number of cell membrane and cytoskeletal mediated events however the precise cellular targets of Ca^{2+} which ultimately result in the exocytotic process are still unknown. Therefore the possibility existed that transglutaminase might be a Ca^{2+}-target in the β-cell which, upon activation, facilitated insulin release by the post-translational modification of proteins through cross-linking reactions. Such reactions may play an important role

in the membrane-mediated events that occur during the secretory process.

The main conclusions from these earlier studies were that rat pancreatic islets contained a Ca^{2+}- and thiol- dependent transglutaminase enzyme, and that primary amines inhibited this transglutaminase activity with a potency that equalled their ability to inhibit glucose-stimulated insulin release, thus suggesting a role for the islet tissue transglutaminase in the mechanism of glucose-stimulated insulin release (Bungay et al., 1982, 1984). These studies were largely coroberated by Malaisse and his group (Gomis et al., 1983), although later studies challenged the use of primary amines as specific inhibitors of transglutaminase (Sener et al., 1984).

The ultimate aim of this study is to elucidate the role of the islet transglutaminase in the Ca^{2+}-mediated events leading to the secretion of insulin in the pancreatic β-cell. One of the major approaches taken was to develop specific inhibitors of islet tissue transglutaminase activity, and to employ these specific inhibitors as pharmacological probes inorder to indicate more fully the involvement of the islet enzyme in the secretory process. It is therefore pertinent to give a brief overview of the mechanism of glucose-stimulated insulin release from the pancreatic β-cell.

1.6. MECHANISM OF GLUCOSE-STIMULATED INSULIN RELEASE

1.6.1. Insulin.

Insulin is a polypeptide hormone which plays a fundamental role in glucose homeostasis in mammals. The secretory response of the β-cell is regulated by fluctuations in the extracellular concentrations of several hormones and neurotransmitters and a variety of nutrients such as glucose, amino acids, fatty acids, and ketone bodies. However, the major physiological stimulus is glucose as presented to the β-cell via the blood, and the β-cell is acutely sensitive to changes in the extracellular glucose concentration in the physiological range 3-10 mM.

Insulin affects the metabolism and function of most tissues, although the key target tissues appear to be liver, muscle and adipose tissue. At the cellular level insulin increases the membrane transport of glucose and amino acids, increases glycogen synthesis and glycolysis whilst decreasing glycogenolysis and gluconeogenesis, increases lipogenesis whilst decreasing lipolysis and fatty acid oxidation, and increases protein metabolism. Insulin thereby promotes the rapid uptake and utilisation or storage of fuels and so maintains fuel molecules in the blood at concentrations appropriate to the competing needs of all tissues. On a long-term basis, insulin has a regulatory effect on DNA and RNA synthesis, cell growth and division. The absence of insulin production in humans leads to the metabolic condition diabetes mellitus.

1.6.2. Cellular pathway of insulin biosynthesis.

Insulin is synthesised by way of a complex pathway which involves at least two intermediates and several cellular organelles. Insulin is synthesised initially as preproinsulin by polyribosomes associated with the rough endoplasmic reticulum

(Patzelt et al., 1978). The preregion of preproinsulin intereacts with the lipid-rich endoplasmic reticulum and hence enters the cisternal space, the concurrent removal of the signal sequence yields proinsulin, which then undergoes rapid peptide-chain folding. The proinsulin is then transported to the Golgi apparatus within microvesicles (Orci, 1974; 1982; 1985). At the Golgi apparatus proinsulin is packaged into secretory granules and the proteolytic maturation to yield insulin occurs (Steiner et al., 1974; 1976). Evidence recently reviewed by Orci (1986) has indicated that newly formed secretory granules containing proinsulin are clathrin coated, which then become non-coated mature secretory granules upon proinsulin conversion to insulin. With the conversion of proinsulin to insulin the immature granules with a low uniform density change in morphology to mature granules with a dense inclusion of crystalline zinc-insulin; the cleaved C-peptide is thought to occupy the cytoplasm of the granule. The conversion of proinsulin to insulin has been proposed to be catalysed by a trypsin-like and carboxypeptidase B-like pair of enzymes which cleave the C-peptide fragment, recently Davidson and Hutton (1987) reported that an enzyme carboxypeptidase H present in the pancreatic β-cell of rats was involved in proinsulin conversion.

1.6.3. Stimuli leading to insulin secretion.

The mechanism by which secretagogues such as glucose are able to mobilise the secretory apparatus is not understood in any great detail. The area has been the subject of extensive investigation since the development of methods for the isolation of intact, functional islets of Langerhans (reviewed by Hedeskov, 1980). Islets are not a pure preparation of β-cells, however they do provide a suitable unit for experimental manipulation into stimulus-secretion coupling.

Secretagogues which stimulate insulin release can generally be classified as either primary stimuli which promote insulin release directly, or secondary stimuli which alter the response of the β-cell to a primary stimulus but do not directly affect the secretory rate themselves. Additionally there are inhibitors which inhibit the response to both primary and secondary stimuli. These fundamental differences between the two classes of stimuli indicates a difference in their mode of action on the release process. It is possible that this results as a consequence of primary stimuli generally resulting in the production of a Ca^{2+} signal, whilst secondary stimuli frequently generate cAMP.

In general the response of the β-cell to all physiological stimuli depends upon the maintenance of intracellular ATP and cAMP levels, on the presence of extracellular Ca^{2+}, and on the presence of a functional microtubular and microfilamentous system.

It is now well established that the metabolism of glucose by the β-cell is a prerequisite for glucose to elicit the secretory response (Ashcroft et al., 1980). The overall currently accepted hypothesis is that a trigger substance(s) that stimulates insulin release is formed in the β-cell during the metabolism of fuel molecules (the 'Fuel Hypothesis', Malaisse et al., 1979). This hypothesis has been further reviewed by Sener and Malaisse (1984) with a view to the metabolism of nutrients in the β-cell, the specific metabolic fate of glucose in the β-cell has been extensively reviewed by Meglasson and Matchinsky, 1986.

The mechanism for the coupling of metabolic events to distal events in the secretory sequence remains a matter of debate and has recently been reviewed by Malaisse et al. (1984). It is possible that specific metabolic intermediates may be involved,

considerable research has emphasised the participation of high-energy phosphate intermediates, protons, and of reducing equivalents in the coupling process.

1.6.4. Elevation of Ca^{2+} and cAMP levels.

An increase in the concentration of Ca^{2+} within the cytoplasmic compartment of the β-cell, induced by stimuli, is thought to trigger the secretory mechanism (Hedeskov, 1980; Wollheim & Sharp, 1981; Prentki & Wollheim, 1984; Rasmussen & Barett, 1984). In the absence of extracellular Ca^{2+}, glucose and other secretagogues are incapable of eliciting insulin release, whilst increased Ca^{2+} uptake occurs in response to glucose (Wollheim & Sharp, 1981).

The mechanism by which glucose and other secretagogues elicit a rise in the intracellular concentration of Ca^{2+} is not fully understood. The factors controlling the intracellular Ca^{2+} concentration are: the removal of Ca^{2+} from the cell by an ATPase-dependent Ca^{2+} pump and a Na^{+}/Ca^{2+} countertransport driven by the inward Na^{+} gradient (Blaustein & Nelson, 1982; Schatzmann, 1982); the intracellular sequestration of Ca^{2+} by organelles, predominantly the endoplasmic reticulum and additionally the mitochondria and possibly the secretory granules, and by cytoplasmic proteins including calmodulin which bind Ca^{2+} with a high affinity; and the entry of extracellular Ca^{2+} which is thought to occur predominantly via voltage-dependent Ca^{2+} channels and to a much lesser extent by diffusion along the inwardly directed electrochemical gradient.

Glucose metabolism, via the generation of an unknown signal, is thought to depolarise the membrane by closing a plasma-membrane K^{+} channel which leads to the gating of voltage-dependent Ca^{2+} channels (Wollheim & Sharp, 1981; Ashcroft et al., 1984).

Since the observation that receptor agonists which use Ca^{2+} as an intracellular messenger also promote the turnover of phosphatidylinositol (Hodkin & Hodkin, 1953), much attention has been devoted to this potential mechanism of raising intracellular Ca^{2+}. Carbohydrate secretagogues were found to promote phosphatidylinositol and phosphatidyl-4-5-diphosphate hydrolysis (Fex & Lernmark, 1972; Freinkel et al., 1975; Clements & Rhoten, 1976; Axen et al., 1983; Laychock, 1983; Dunlop & Larkins, 1984; Rana et al., 1985). Phosphatidylinositol metabolism was also found to be promoted by muscarinic cholinergic receptors (Best & Malaisse, 1984; Morgan et al., 1985; Wollheim & Biden, 1986). The specific nature of the signal resulting from glucose metabolism which promotes phosphatidylinositol turnover has not been delineated, although a link with NAD(P)H levels has been suggested, since concentration response curves for NAD(P)H levels in response to glucose closely parallel those for ^{32}P labelling of phosphatidylinositol in response to glucose for islets pre-labelled with ^{32}P before stimulation (Malaisse et al., 1979; Best & Malaisse, 1983).

The first step in phosphatidylinositol turnover has been shown to be the hydrolysis of phosphatidylinositol-4, 5-diphosphate, a derivative of phosphatidylinositol, by phospholipase C in the plasma membrane (Michell et al., 1981; Berridge & Irvine, 1984) resulting in the generation of two intracellular mediators, inositol-1,4,5-triphosphate and diacylglycerol. Inositol-1,4,5-triphosphate has been demonstrated to generate the mobilisation of Ca^{2+} from the endoplasmic reticulum (Streb et al., 1983; Berridge & Irvine, 1984; Biden et al., 1984; Prentki et al., 1984). Recently Wolf et al. (1986) indicated that glucose-6-phosphate attenuates inositol-1,4,5-triphosphate induced Ca^{2+} release from the endoplasmic

reticulum. Since it is known that phosphatidylinositols can bind divalent ions, phosphatidylinositol turnover could further generate intracellular Ca^{2+} by the release of these ions which in turn could result in a change in the ion permeability of the plasma membrane.

The formation of diacylglycerol may increase both membrane fluidity (Hawthorne, 1982) and fusion (Allan et al., 1976), and may explain the observation that glucose increases membrane fluidity (Deelers et al., 1981). A possible result of the raised levels of diacylglycerol and Ca^{2+} may be the activation of a protein kinase which is Ca^{2+} and diacylglycerol dependent, protein kinase C (Nishizuka, 1984). The involvement of protein kinase C in the process of insulin secretion is suggested by observations that the diacylglycerol analogue 12-0-tetradecynoyl-phorbol-13-acetate (TPA) which is a strong potentiator of glucose-stimulated insulin release (Malaisse et al., 1980; Virji et al., 1978; Yamamoto et al., 1982; Hii et al., 1986) is also a potent activator of protein kinase C (Hubinont, et al., 1984).

Diacylglycerol can also be metabolised to phosphatidic acid which is then converted to arachidonic acid by the action of phospholipase A_2. The ability of phosphatidic acid to act as a Ca^{2+} ionophore is paralleled by its stimulatory effect on glucose-stimulated insulin release (Dunlop et al., 1982). Arachidonic acid has also been implicated in promoting glucose-stimulated insulin release (for review see Robertson, 1986). However studies have indicated that Ca^{2+} mobilisation is predominantly generated by inositol-1,4,5-triphosphate and associated derivatives and the idea that arachidonic acid promotes any significant Ca^{2+} mobilisation during glucose-stimulated insulin release has now been refuted (Morgan et al., 1987). The latter authors demonstrated that carbacol generated a dose-dependent increase in inositol-1,4,5-

triphosphate and subsequently increased the intracellular concentration of Ca^{2+}, whereas mellitin which activates phospholipase A_2 and hence the generation of arachidonic acid did not lead to any significant increase in Ca^{2+} levels nor was glucose-stimulated insulin release raised over basal levels.

The latter results also indicated that a rise in Ca^{2+} levels resulting from the mobilisation of intracellular stores is not the sole second messenger in glucose-stimulated insulin release, since treatment with carbacol in the absence of glucose did not result in insulin release. Therefore, in the biphasic pattern of insulin release (Wollheim & Sharp, 1981) the first phase does not appear to result specifically from a rise in Ca^{2+} levels from intracellular stores. It is possible that the initial burst of insulin release may result from secretory granules primed immediately at the plasma membrane.

The evidence strongly indicates a possible regulatory role for phosphatidylinositol metabolism in glucose-stimulated insulin release (for review see Best et al., 1984).

In addition to Ca^{2+}, cyclic-3',5'-adenosine monophosphate (cAMP) also plays a role as a positive modulator of glucose-stimulated insulin release (Montague & Howell, 1976; Hedeskov, 1980; Wollheim & Sharp, 1981; reviewed by Malaisse & Malaisse-Lagae, 1984). The pancreatic β-cell contains all the components of the cAMP system, including the plasma membrane associated adenylate cyclase, soluble and particulate cyclic nucleotide phosphodiesterase activities, soluble and particulate cAMP-dependent protein kinases.

Studies using either forskolin (an activator of adenylate cyclase) (Malaisse et al., 1984; Wiedenkeller & Sharp, 1983; Henquin & Meissner, 1984; Hermansen, 1985), and

isobutylmethylxanthine (IBMX) or theophylline (inhibitors of cyclic nucleotide phosphodiesterase activity) (Brisson & Malaisse, 1973; Campinelli & Malaisse, 1980), have indicated that a rise in the intracellular cAMP level enhances glucose-stimulated insulin release. However there is some debate as to whether the stimulatory effect of glucose on insulin release results in a significant change in cAMP, or whether cAMP levels are principally modified by secondary stimuli to augment the insulin secretory effect of glucose.

1.6.5. Mode of Action of Ca^{2+} and cAMP on the insulin release mechanism.

The result of increased intracellular levels of Ca^{2+} and cAMP in response to extracellular stimuli represents the link between stimulus perception and the activation of the secretory mechanism. The precise nature of this link is therefore of extreme importance in understanding the process of glucose-stimulated insulin release. The potential targets of Ca^{2+} are Ca^{2+}-dependent and Ca^{2+}-calmodulin dependent enzymes and proteins, including protein kinases. The predominant target of cAMP appears to involve the activation of cAMP-dependent protein kinases. These protein kinases through phosphorylation may potentially alter the functional activity of components involved in the secretory mechanism. This consideration that protein phosphorylation might be a common mode of action of both Ca^{2+} and cAMP in controlling insulin release was first proposed by Ashcroft (1980) and has recently been reviewed by Harrison et al. (1984). The protein kinases identified in pancreatic islets include : cAMP-dependent protein kinase in guinea-pig islets (Montague & Howell, 1972), two cAMP-dependent protein kinase isoenzymes in rat islets (Sugden et al., 1979); two Ca^{2+}-calmodulin-dependent protein kinases, phosphorylase kinase (Harrison et al., 1984) and myosin light chain kinase (Harrison & Ashcroft, 1982;

MacDonald & Kowluru, 1982; Penn et al., 1982; Landt et al., 1982); and a Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C) (Lord & Ashcroft, 1984; Tanigawa et al., 1982). As with other aspects of stimulus-secretion coupling, there is some controversy as to the relevant importance of the respective kinases in the secretory process, for example Harrison et al. (1986) presented evidence to suggest that Ca^{2+}-calmodulin-dependent protein kinase plays a central role in the insulin-secretory process, whilst protein kinase C and cAMP-dependent protein kinase played only a secondary role; the role of protein kinase C in glucose-stimulated insulin release was further questioned by Hii et al. (1987). Extensive studies are still therefore required to discover and identify the endogenous substrate proteins of these kinases.

The question arises as to how the consequences of β-cell stimulation as outlined result in the ultimate secretion of insulin. The release mechanism has two major components, the movement of insulin-storage granules from the cytoplasm to the plasma membrane (margination), and the release of the granule contents by fusion of the granule membrane with the plasma membrane (exocytosis).

1.6.6. Margination.

The intracellular movement of secretory granules in the β-cell, as with a variety of other cell types, is thought to be associated with the network of microtubules and microfilaments (for reviews see Howell, 1984; Howell & Tyhurst, 1984; 1986). Colchicine, vinblastine and other drugs which disrupt microtubules were also found to inhibit insulin release, although only the second phase of insulin release was found to be inhibited (Yaseen et al., 1982). This suggests that the first phase of insulin release might be due to the release of insulin from granules already primed at the plasma membrane, thus adding to the hypothesis that microtubules are

involved in the movement of granules over relatively long distances from the general cytoplasmic pool to the plasma membrane. Lacy et al. (1972) reported that Ca^{2+} played a direct role in regulating the intereaction between secretory granules and microtubules, such intereactions have also been reported by Pipeleers et al. (1979) and Suprenant and Dentler (1982). Microtubules are polymerised structures in a state of dynamic equilibrium with a pool of a-tubulin and b-tubulin subunits. In the unstimulated β-cell 30% of tubulin is polymerised into microtubules, whereas under conditions of glucose-stimulated insulin release or an increase in cAMP levels 70% of tubulin is polymerised (Montague et al., 1976). It has also been found that drugs which stabilise microtubule formation such as taxol also inhibit insulin release, and therefore it has been postulated that a dynamic turnover of microtubules is a requirement for margination. It was suggested that the effect of cAMP was mediated by a cAMP-dependent protein kinase, since phosphorylation of tubulin or proteins closely associated to microtubules had been demonstrated in response to cAMP (Colca et al., 1983). Thus Colca et al. (1983) described the phosphorylation of proteins of molecular weight 54,000 and 57,000 which they proposed to be microtubule subunits, a 57,000 molecular weight protein was also found to be phosphorylated by a cAMP-dependent protein kinase in a rat insulinoma tissue (Brocklehurst & Hutton, 1983). However, Harrison et al. (1984) pointed out the problems in interpreting this data.

Cytochalasin B, an agent which disrupts microfilaments, was found to increase insulin release (Van Obberghan et al., 1973), and therefore it was proposed that microfilaments prevented granules fusing with the plasma membrane. Further studies with phalloidin, an agent which polymerises actin (the principal component of microfilaments), on permeabilised islets resulted in

enhanced insulin release (Stutchfield & Howell, 1984). The suggestion was made that under stimulation the contraction of microfilaments brought granules into contact with the plasma membrane. Ca^{2+} is thought to play a role in controlling the contractile activity, since Ca^{2+} controls the activity of other actin-containing contractile systems. Microfilaments are composed largely of F-actin produced by polymerisation of G-actin, during secretion there is an increased conversion of G-actin into F-actin, whilst storage granules have been shown to intereact with F-actin and not G-actin (Howell & Tyhurst, 1979).

Myosin has also been identified as a major cellular protein in islet cells and resembles that found in smooth muscle. The intereaction of actin and myosin to form actinomyosin is an important component in many contractile systems and may be involved in granule movement in the β-cell. The intereaction of actin and myosin and therefore of contractile activity is dependent upon the phosphorylation of myosin carried out by myosin light chain kinase, a Ca^{2+}-calmodulin-dependent protein kinase. Therefore Ca^{2+} may regulate the movement of granules by controlling the contractile activity of actinomyosin complexes. Secretory granules have also been demonstrated to intereact with actinomyosin, this association was found to be ATP-dependent (Howell & Tyhurst, 1982).

It is possible that microtubules and microfilaments play a cooperative role in insulin release. In other cell systems they only intereact in the presence of microtubule associated proteins (MAP) (Griffith & Polland, 1978). Whilst Nishida *et al.* (1981) found that microtubule-actin intereactions could be inhibited by the phosphorylation of MAP *in vitro*. The phosphorylation of MAP *in vivo* by cAMP- or Ca^{2+}-dependent kinases could therefore result in the temporal dissociation of microtubules from microfilaments and

this might be associated with secretory granule movement. Of additional interest are a range of calmodulin and Ca^{2+} binding proteins that can intereact with components of the tubulin, actin and myosin cytoskeleton (Kakiuchi & Sabue, 1983).

As an overall putative model for these intereactions Howell & Tyhurst (1986) suggested that increases in Ca^{2+} and cAMP enhanced the formation of microtubules and F-actin, and that these components together with phosphorylated myosin coordinated the margination of secretory granules. However, whilst there are a multitude of observations a cohesive theory has yet to be demonstrated.

1.6.7. Exocytosis.

The final event in insulin release requires the fusion of the insulin storage granule with the plasma membrane, but the membranes must first undergo biochemical modification to minimise the repellant effect of the highly charged head groups on the membrane phospholipids. Both Ca^{2+} and polyamines have been implicated in membrane fusion either by partially neutralising these charges or by acting as bridges linking phospholipids in the two membranes. However it is more probable that the removal of the head groups of the phospholipids with their conversion into 1,2-diacylglycerols is the event which triggers membrane fusion, since diacylglycerols promote membrane fluidity and promote membrane fusion. This conversion is catalysed phospholipase C, which is a Ca^{2+} regulated enzyme. The exocytotic process has to be counteracted by endocytosis to maintain the magnitude of the plasma membrane. An alternative route to that of exocytosis for the insulin of the secretory granule is for it to be degraded intracellularly.

1.6.8. Conclusion.

The Introduction has outlined the salient points in the process of glucose-stimulated insulin release. It is therefore the aim of this study to investigate a role for the Ca^{2+}-activated islet transglutaminase in this process and possibly to define a point of involvement of the enzyme in the secretory events.

2. MATERIALS AND METHODS

2.1. MATERIALS

All of the work undertaken in this thesis was carried out in the laboratories of the Department of Life Sciences, Trent Polytechnic, except for the studies undertaken on the calmodulin dependent activation of bovine heart phosphodiesterase which were undertaken in the laboratories of the Department of Medicine, Clinical Sciences Centre, Northern General Hospital, Sheffield.

Chemicals used for electrophoresis were of Electran grade, chemicals used for the determination of the Ca^{2+}-sensitivity of transglutaminase were of Aristar grade, chemicals used for buffers and physiological media were of Analar grade, other chemicals were of General Purpose Reagent grade.

The list below indicates the sources of chemicals used:

Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K. ;glycine methyl ester

Amersham International plc., Little Chalfont, Buckinghamshire, U.K.;

[8-^{3}H] adenosine 3',5'- cyclic phosphate (20-30 Ci/mmol)

[2-^{3}H] adenosine 5'monophosphate (10-20 Ci/mmol)

adenosine 5'- [-^{32}P]-triphosphate (0.5-3.0 Ci/mmol)

D - [U-^{14}C]-glucose (>230 mCi/mol); [^{125}I]-insulin (>50 μCi/μg)

L - [4,5-^{3}H]-leucine (0.5-1.0 Ci/mmol)

L - [methyl-^{3}H]-methionine (2-10 Ci/mmol)

phosphorus - 32 (as orthophosphate) (10 mCi/ml)

[1,4-^{14}C]-putrescine dihydrochloride (100-200 mCi/mmol)

calmodulin, (from bovine brain)

B.C.L., Lewis, E. Sussex, U.K.; phosphodiesterase (calmodulin deficient from bovine heart), trifluoroperazine dihydrochloride.

British Drug House, Poole, Dorset, U.K.; acetic acid, acrylamide, ammonium persulphate, decon 90, N,N'-dimethyl casein, dimethyl sulphoxide, E.D.T.A., glycine, glycerol, hydrogen peroxide 100 vols. (30% $^{w}/v$), N,N'-methylenebisacrylamide, PAGE blue 83, phenylmethanesulphonyl fluoride, sodium dodecyl sulphate, N,N,N,N'-tetramethylethylenediamine.

Gibco Bio-cult Diagnostics, Paisley, U.K.; Hanks balanced salt solution.

Flow Laboratories, Irvine, U.K.; Hepes buffer (1M).

Fluka AG, Buchs, Switzerland; monodansylcadaverine, sarcosine methyl ester.

Fosters Air Supplies, Nottingham, U.K.; 95% O_2 in CO_2.

L.K.B., Croydon, Surrey; Optiphase Safe liquid scintillation fluid.

Novo, Bagsvaerd, Denmark; porcine [^{125}I]-insulin, anti-porcine insulin guinea-pig serum, rat insulin.

Sarstedt, W. Germany; microfuge tubes, plastic scintillation-vial inserts.

Serva Feinbiochemica, Heidleberg, W. Germany; collagenase from Clostridium histolyticum.

Sigma, Poole, Dorset; adenosine 3',5'-cyclic monophosphate, adenosine 5'-monophosphate, alkaline phosphatase, collagenase from Clostridium histolyticum type V, cytochrome C type V (from bovine heart), 1,2-diolein, Dowex-1-chloride (8% crosslinkage, dry mesh 200-400), E.G.T.A., histone type V-S (from calf thymus), 2-mercaptoethanol, methylbenzenethonium hydroxide (pfs.) (1M), molecular weight standards for SDS-polyacrylamide gel electrophoresis, L- -phosphatidyl-L-serine, phospholipase A_2 (from

porcine pancreas), phospholipase C (from B. cerus), Sigmacote (siliconising fluid), Ophiophagus hannah snake venom, Trizma base.

Wilfred Smith Ltd., Edgware, Middlesex, U.K.; bovine serum albumin (fraction V).

Wellcome Reagents Ltd., Beckenham, Kent, U.K.; Wellcome insulin binding reagent.

Whatman, Maidstone, Kent, U.K.; 3MM filter paper.

The following equipment was used throughout the experiments of the thesis:

Measurement of pH was undertaken using both a Pye-Unicam PW9409 pH meter (Pye Unicam, Cambridge, U.K.), and a Beckman Φ71 pH meter.

Absorbance measurements were made using a Pye-Unicam SP-400 ultra-violet, variable wavelength spectrophotometer.

Enzyme assays and kinetic analysis involving absorbance changes were undertaken using a Beckman DU7 Spectrophotometer.

Sonication was undertaken with an M.S.E. Soniprep 150 sonicator (M.S.E. Scientific Instruments, Crawley, Sussex).

A Gallenkamp Flask Shaker was used in the collagenase digestion of rat pancreati.

For the detection of radioisotope emissions, a Packard TriCarb 300C Liquid Scintillation Counter was used to determine β - emissions and a Packard Auto-Gamma 500C with a 3 inch crystal to determine γ- emissions.

Bench monitoring following ^{32}P experimentation was undertaken with a mini-monitor g.m. meter type 5.10, a New England PCM5 with a New England Dual Probe DP2R/4, and an Eberline RO2 Dose

Meter (Ion Chamber). Personal monitoring was additionally undertaken with a thermo-luminescent dose meter and a film badge which were measured on a monthly basis.

Electrophoretic analysis of protein samples was undertaken using a LKB 2001 Vertical Electrophoresis Unit.

Centrifugation was undertaken in the centrifuges listed below which were fitted with the rotors and used at the relative centrifugal forces indicated:

Centrifuge	Rotor	Relative centrifugal force (g av.)
M.S.E. Superspeed 50	8 x 25ml angle ($r_{av.}$ = 5.84cm)	71,000g and 80,000g
	8 x 25ml angle fitted with 3ml adaptors ($r_{av.}$ = 6.22cm)	71,000g
M.S.E. Chillspin	swing-out 4-place universal with multitube carriers	600g - 1830g max
M.S.E. Micro Centaur	12 x 1.5ml	11,600g max

2.2. ANIMALS

Pancreatic tissue for the isolation of islets of Langerhans, liver and spleen tissue were all obtained from Sprague-Dawley rats of either sex weighing 250-350g.

2.3. CHEMICAL DETERMINATIONS

2.3.1. Protein assay : method of Lowry.

The protocol undertaken was a modification of the method of Lowry *et al.* (1951), and used SDS at a final concentration of 0.8% (w/v) to solubilise otherwise insoluble proteins.

Stock solution A comprised of 4% (w/v) NaOH, 2% (w/v) Na_2CO_3, and 0.02% sodium tartrate; stock solution B comprised of 0.5% $CuSO_4.5H_2O$; both solutions were prepared in distilled water. The sample protein was diluted to come within the bovine serum albumin range and duplicate 100µl aliquots were mixed with 100µl aliquots of 10% (w/v) SDS. The stock solutions were then mixed at a ratio of 12.25 A : 0.25 B and 1.0ml aliquots of this solution added to the protein samples in SDS. The samples were then vortexed and incubated for 20 min. at room temperature, then 0.1ml of Folin-Ciocalteau reagent (freshly diluted 1:1 with distilled water) was added. The samples were vortexed and incubated a further 20 min. at room temperature before the measurement of absorbance at 750nm. Standards of bovine serum albumin in the range 50-1000µg per ml were treated similarly.

2.3.2. Determination of radioactivity.

The radioisotopes ^{14}C and ^{3}H were determined by liquid scintillation counting, ^{32}P was determined by both liquid scintillation counting and by Cerenkov counting. ^{125}I samples were counted directly in plastic stoppered gamma tubes in a gamma counter.

For liquid scintillation counting the following sample preparation procedures were undertaken, dependent upon the type of sample.

2.3.2.1. Filter papers.

Samples deposited on Whatman 3MM filter paper were counted in Optiphase Safe (L.K.B.) liquid scintillation counting cocktail which was suitable for dry heterogeneous samples. Squares of filter paper (1 cm^2) derived from either transglutaminase assays or protein kinase C assays were placed in 5ml polythene scintillation vial inserts (Sterlin Ltd.) and counted in 2.0ml of scintillation cocktail.

2.3.2.2.1. Polyacrylamide gels : scintillation counting.

Sections of polyacrylamide gels from ^{32}P labelled islet proteins obtained as described in the methodology for electrophoresis were placed in plastic scintillation vials (Sterlin Ltd) with 10ml of distilled water and counted by Cerenkov counting on a channel setting of 0 - 20 Kev.

2.3.2.2.2. Polyacrylamide gels : autoradiography.

Dried polyacrylamide gels prepared as described in the methodology for electrophoresis were placed centrally in a film cassette holder and in the absence of any light a sheet of X-ray film (Fugi RX X-ray film) was placed over the dried gel and the film cassette holder sealed. The X-ray film was first sensitised with a flash gun such that, upon developing, it had an absorbance of 0.15 above that of unexposed film when measured at a wavelength of 540nm.

The cassette was placed at $^{-}70^{o}C$ for 3-6 days (dependent upon the activity of the ^{32}P). The exposed X-ray film was then placed in developer for 2 min., followed by a brief wash in water and then 1 min. in fixative, and a second wash in water.

2.3.2.3. Determination of counting efficiency.

For dried filter paper samples, 'total count' samples (10µl) were taken from vials containing a known amount of radioactivity, air dried on filter papers and counted. For ^{14}C, the counting efficiency was regularly found to be 75-85%; for ^{32}P the counting efficiency was regularly found to be greater than 90%.

The Quench Indication Parameter of the Packard 460C liquid scintillation counter was used for most other determinations of counting efficiency. A quench correction curve was prepared by using a set of vials containing the same known amount of radioactivity with varying amounts of the quenching agent chloroform which were counted in the scintillation counter according to the manufacturers' instructions. The scintillation counter then utilised the data to calculate the counting efficiency of each unknown sample.

2.4. FRACTIONATION PROCEDURES

2.4.1. Homogenisation.

2.4.1.1. Potter-Elvejhem.

Immediately following excision the tissue (usually liver or spleen) was washed in ice-cold homogenisation medium consisting of 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4, then blotted dry and weighed. The tissue was then chopped and homogenised in this medium at a volume ratio of 3:1 medium:tissue by 6 passes of a glass/teflon Potter-Elvejhem homogeniser (clearance 0.61mm) mechanically driven at 1700 r.p.m.. This whole procedure was undertaken on ice.

Islets of Langerhans were homogenised similarly at a ratio of 2-3 pancreatic islets per µl of homogenisation medium.

2.4.1.2. Homogenisation of ^{32}P-labelled Islets.

Because of the high risk of contamination due to aerosol formation, ^{32}P-radiolabelled pancreatic islets could not be homogenised by a mechanically driven Potter-Elvejhem, and therefore a hand-held glass-glass 1.0ml homogeniser was employed, homogenisation was achieved by 15 passes of the hand-held homogeniser.

2.4.1.3. Ultrasonication.

This method of disrupting cells was found to be suitable for homogenising islets of Langerhans where the homogenate was to be used in transglutaminase assays, but not for subcellular fractionation studies.

Islets of Langerhans isolated as described in Section 2.9.3. were placed in 1.5ml auto analyser vials and excess medium removed using a finely drawn Pasteur pipette. The islets were then washed 5 times with ice-cold sucrose medium (0.25M sucrose, 1mM Tris-HCl, 1mM EDTA, pH 7.4) to remove all traces of incubation or isolation medium. To the islets was then added sufficient homogenisation medium to give a ratio of 2-3 islets of Langerhans per µl. Islets of Langerhans were then homogenised in this buffer by sonication with two bursts of 5s each at 3µ peak-to-peak with a 10s interval between bursts.

The probe of the sonicator was cooled to 4^{o}C prior to use and the whole procedure undertaken on ice. The homogenate was stored at either 4^{o}C if required on the same day or at $^{-}$70^{o}C if required on subsequent days.

2.4.2. Subcellular fractionation of tissues by differential centrifugation.

Different degrees of fractionation were undertaken dependent upon the degree of purity required for the experiment. The

majority of the work required only a high speed pellet (P) and/or the resultant particle-free supernatant (S) obtained from homogenates centrifuged at 71,000 g av. for 45 min. at 4°C. The high speed pellet (P) contained all cellular debris, cell membranes and organelles. Where the particle-free supernatant was used, care was taken not to incorporate any of the lipid layer which frequently occurred at the top surface of the preparation. The preparation of particle-free supernatants from homogenates of islets of Langerhans was undetaken in 3ml adaptors for an 8 x 25ml rotor due to the small volume of homogenate, generally <1.0ml.

Where fractionation of islet homogenates into a 600g low speed pellet (N), an 80,000g high speed pellet (P) and a particle-free supernatant (S), was required a modification of the methods of Christie & Ashcroft (1985) was employed.

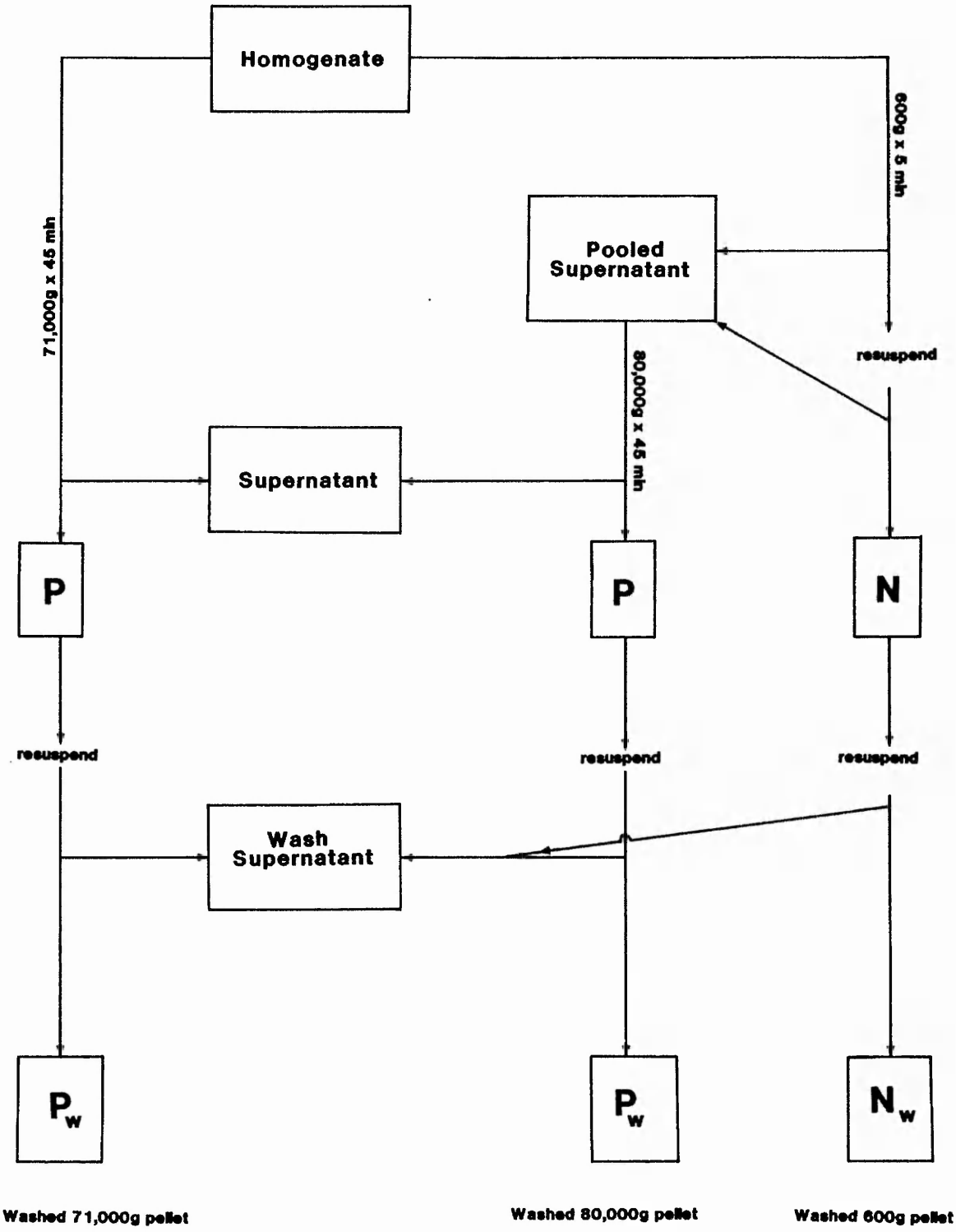
The pellets obtained from islet homogenates were resuspended by ultrasonication in 50-100µl of sucrose medium. All the procedures above were carried out at 4°C and all fractions stored on ice.

These fractionation schemes are represented diagramatically in Fig. 2.1..

2.4.3. SDS-Polyacrylamide gel electrophoresis.

The method employed was a modification of that described by Laemmli (1979) for use with a vertical slab gel apparatus which is widely used for separation of proteins from tissue or cell extracts.

Figure 2.1 Fractionation scheme



2.4.3.1. Solutions.

The solutions used were:

Tris-glycine electrode buffer (25mM Tris, 192mM glycine)	15.2g Tris base 72.0g glycine 5.0g SDS pH 8.5 in 5000ml.
Tris-SDS stock solution, pH 6.8 (0.25M Tris)	3.03g Tris base 0.2g SDS pH 6.8 in 100ml.
Tris-SDS stock solution, pH 8.8 (0.75M Tris)	9.08g Tris base 0.2g SDS pH 8.8 in 100ml.
Sample buffer	25ml Tris-SDS stock, pH 6.8 2g SDS 10ml glycerol 5ml 2-mercaptoethanol 0.1ml 1% bromophenol blue made up to 100ml.
Acrylamide stock solution	30g acrylamide 0.8g N'-N-methylene-bisacrylamide made up to 100ml.
Ammonium persulphate	2% (w/v) in distilled water (freshly prepared).

2.4.3.2. Sample preparation.

Samples containing 50-100µg of protein were dissolved at the appropriate ratio of sample buffer : sample with the appropriate strength sample buffer such that a final sample buffer strength of 1X resulted, (62.5mM Tris, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.01mg/ml bromophenol blue), e.g. 25µl of 4X strength sample buffer was added to 75µl of sample. Upon addition of sample buffer the mixture was boiled for 5 min and stored at $^{-}20^{o}C$, prior to electrophoresis the sample was boiled a second time for 5 min before loading on the gel inorder to ensure the dissolution of proteins.

2.4.3.3. Procedure.

Glass plates were first cleaned with detergent and then rinsed with distilled water and filtered ethanol. The gel mould apparatus for vertical slab gel electrophoresis (L.K.B.) was then assembled according to the manufacturer's instructions to produce 1.5mm thick gels.

The resolving gel was poured first by combining 19.8ml of acrylamide stock solution, 30ml of Tris-SDS stock solution pH 8.8 and 10.2ml distilled water in a Buchner flask for two gels or multiples of these ratios for additional gels. This mixture was de-gassed for 30 min. with a vacuum pump. Immediately before pouring the gel 1.5ml of freshly made 2% (w/v) ammonium persulphate solution and 20µl TEMED were added and the solution thoroughly mixed without introducing air bubbles. The gels were then poured to a height of approximately 12cm such that at a later date a stacking gel of 1.5cm could be poured on top. The gel solution was overlayed with butan-2-ol to exclude air and to create a flat surface to the gel. The gel was then allowed to polymerise overnight.

The stacking gel was prepared by mixing 2ml of acrylamide stock solution, 10ml Tris-SDS stock solution, pH 6.8 and 8ml distilled water. This mixture was similarly de-gassed for 10-15 min. during which time the butan-2-ol was removed from the top of the resolving gel with several washes of Tris-SDS stock solution, pH 8.8, and any remaining solution removed by absorption onto filter paper. 1ml of 2% (w/v) ammonium persulphate solution and 10µl TEMED were then added to the de-gassed stacking gel solution with careful thorough mixing and this solution poured onto the resolving gel with the 10 well, 1.5mm width, combs in place. The gels were 'flicked' repeatedly to free any adherent air bubbles and the frame filled to the top. An over-lay of butan-2-ol was employed to exclude air and the gel left to polymerise for at least 90 min. The combs were carefully removed and the resultant wells washed 3-4 times with electrode buffer prior to being filled with electrode buffer ready for sample loading.

The gels so prepared had the following compositions:

	resolving	stacking
acrylamide	10% (w/v)	3% (w/v)
N',N methylenebisacrylamide	0.27% (w/v)	0.08% (w/v)
SDS	0.1% (w/v)	0.1% (w/v)
Tris	0.375M	0.125M

Samples were added to the bottom of each of the wells with a Hamilton 100µl syringe. The rest of the electrophoresis apparatus was assembled according to the manufacturer's instructions and connected to a suitable power supply. Electrophoresis was then carried out at 10-15^{o}C and at a constant current of 20mA per gel until the samples had passed through the stacking gel, the current was then raised to 30mA per gel, the total time of electrophoresis

was 4-5h at which time the bromophenol blue marker was within 1cm of the end of the gel. Standards of proteins of known molecular weights (Sigma) were also electrophoresed beside the samples on the same gel to allow calibration for determination of protein molecular weights. The protein standards used were myosin (205,000), β-galactosidase (116,000), phosphorylase B (97,400), bovine albumin (66,000), egg albumin (45,000) and carbonic anhydrase (29,000).

In some ^{32}P-phosphopolymer studies (Section 3.3.8.) a further layer of 0.8% (w/v) agarose was added on top of the stacking gel. This gel was made by dissolving 0.8g of agarose in 100ml of Tris-SDS stock solution, pH 6.8 by autoclaving. The agar solution was allowed to cool to 50^{o}C and poured into the mould with the comb in place, the molten agarose was poured to come half-way up the teeth to ensure the easy removal of the comb without damage to the wells. To accommodate this extra layer the resolving gel was made 1.5cm shorter. Thus a gel was formed of 0.8% (w/v) agarose on a stacking gel of 3% (w/v) acrylamide and a resolving gel of 10% (w/v) acrylamide.

2.4.3.4. Fixing, staining and destaining of gels.

After electrophoresis, the migration distance of the dye front of bromophenol blue was recorded and the whole gel carefully transferred from the mould to a clean glass dish containing 750ml of a fixative solution comprising of 10% (w/v) TCA and 3.4 (w/v) sulphosalicylic acid and 'fixed' for a minimum of 30 min.

The staining solution comprised of 1.25g Page 83 (B.D.H.), 18% (v/v) methanol and 5% (v/v) glacial acetic acid. This was prepared freshly by first dissolving the Page 83 in the methanol and filtering it, the glacial acetic acid was then added and the volume made up to 1 litre with distilled water. The fixed gels were

then transferred to the staining solution and left to stain overnight.

Destaining of gels was accomplished by immersion of the gels in a solution of 18% (v/v) methanol and 5% (v/v) glacial acetic acid. For studies involving [^{32}P]Pi the destain also contained 2mM sodium phosphate.

2.4.3.5. Sectioning.

After destaining the gels were photographed and then each lane was sectioned every 1cm apart from the stacking gel which was sectioned into two components, the first 5mm and the rest of the stacking gel. Radioactivity was then determined as described in Section 2.3.2.2.

2.4.3.6. Preparation of gels for autoradiography.

When gels were required for autoradiography the destained gels were placed in 10% (v/v) glycerol overnight and then placed in sodium salicylate solution for exactly 30 min. The sodium salicylate solution comprised of salicylic acid (1M), NaOH (1M) and 10% (v/v) glycerol adjusted to pH 7.0 with NaOH. The gel was then placed carefully on a sheet of Whatman 3MM filter paper and covered with a sheet of cling-film and dried for at least 3h on a gel dryer under vacuum (L.K.B. 2003 slab gel dryer). The gel was then allowed to cool prior to removal of the vacuum and then used for autoradiography as described in Section 2.3.2.2.2.

2.5. TRANSGLUTAMINASE ASSAY

2.5.1.1. Standard Transglutaminase Assay.

Transglutaminase activity was measured by [^{14}C]-putrescine incorporation into N,N'-dimethylcasein by using a modification of the method of Lorand et al. (1972).

The standard incubation mixture at 37°C consisted of 30mM Tris/HCl, pH 7.4, 3.85mM dithiothreitol, 2.5mM $CaCl_2$ or 5mM EDTA, 1.2mM [^{14}C]-putrescine (3.96 Ci/mmol), N,N'-dimethylcasein (5mg/ml). The assay components were in a final volume of either 100µl or 50µl after the addition of 45µl or 22.5µl respectively of enzyme sample. Generally a final volume of 50µl was employed for assays on islet transglutaminase due to the limited volume of enzyme available.

The incubations were carried out in 0.8ml microcentrifuge tubes in a water bath at 37°C and the reaction started by addition of enzyme samples. Samples (10µl) were taken for the counting of trichloroacetic acid-insoluble radioactivity at appropriate time intervals and carefully deposited on 2H pencil-labelled squares (1cm^2) of Whatman 3MM filter paper which were immediately placed into a beaker of stirred ice-cold 10% (w/v) TCA for a minimum of 10 min. Unbound radioactivity was then removed from the filter squares by a washing procedure that involved:

3 x 10 min wash in ice-cold 5% (w/v) TCA;

1 x 5 min wash in acetone/ethanol, 1:1 (v/v);

1 x 5 min wash in acetone.

The ratio of washing media in millilitres to the number of filter paper squares was at least five fold. The washing apparatus comprised of a perforated inner beaker containing the filter squares and an outer larger beaker containing a magnetic stirrer and the washing medium. This allowed for the rapid processing of large numbers of samples. The filter papers were air-dried overnight and counted for radioactivity (Section 2.3.2.1.). Background was determined by taking blank filter squares through exactly the same procedure. Rates of Ca^{2+}-dependent incorporation of

putrescine were then calculated after the subtraction of control values obtained from EDTA control vials. A unit of enzyme activity is one nmol putrescine incorporated per h under the conditions of the assay.

2.5.1.2. Assay for the effect of test compounds on islet transglutaminase activity.

Test compounds were added to the assay after serial dilution to give the desired final concentration. Control incubations contained the same final concentration of dimethyl sulphoxide (if used to dissolve the test compounds) as the incubations containing the test compounds. Where any test compounds were a particular salt, eg. hydrabromide salts, the appropriate sodium salt was added to control incubations so that the same final concentration of ions was present in control and test incubations. All test concentrations were undertaken in triplicate.

2.5.1.3. Measurement of the Ca^{2+}-sensitivity of transglutaminase.

Islet supernatants were prepared as described in Section 2.4.1.3., except that 1mM EGTA was substituted for EDTA in the homogenisation buffer. The islet supernatant extract was dialysed for a minimum of 2h at $4^{o}C$ against 50mM Tris/HCl (pH 7.4 at $37^{o}C$)/1mM EGTA.

The native N,N'-dimethylcasein in the assay was substituted with dephosphorylated N,N'-dimethylcasein. N,N'-dimethylcasein was dephosphorylated according to the method of Cooke and Holbrook (1974).

A solution of approximately 50mg/ml (10-15ml total volume) N,N'-dimethylcasein was prepared in 50mM Tris/HCl, pH 7.4 at $37^{o}C$ to which $ZnSO_4$ was added to a final concentration of 10μM.

Alkaline phosphatase (Sigma) (20mg/ml) was added to give 0.8µg of enzyme per mg of N,N'-dimethylcasein and left at room temperature for 2.5h. The solution was then transferred to a small dialysis bag and dialysed for 2.5h against 500ml of 50mM Tris/HCl (pH 7.4 at 37°C) containing 10µM $ZnSO_4$ at room temperature. Dialysis was continued for a further 36h at 4°C with two changes of the dialysis medium. Finally the dephosphorylated N,N'-dimethylcasein was dialysed for 24h at 4°C against 500ml 50mM Tris/HCl (pH 7.4 at 37°C) with one change of medium to remove traces of $ZnSO_4$. The solution of dephosphorylated N,N'-dimethylcasein was then assayed for protein and made up to 25mg/ml and frozen in small aliquots ready for use.

For calcium-sensitivity measurements the final assay volume was 50µl and the reaction was started by the addition of 20µl of enzyme preparation which gave a final concentration of EGTA of 0.4mM in 50mM Tris/HCl. Different amounts of $CaCl_2$ were added to the assay medium such that the final desired concentration of free Ca^{2+} was obtained, ranging from 3.2 to 50.6 µM. For calculation of the free Ca^{2+} concentrations, an association constant at pH 7.4 of $10^{7.12}$ was used (Schatzman, 1973).

2.6. ASSAY FOR PROTEIN KINASE C ACTIVITY

Rat spleen was used as a source of protein kinase C. Tissue was homogenised with a Potter-Elvehjem homogenizer in 3 volumes of 20mM Tris/HCl (pH 7.4), 2mM EGTA, 50mM 2-mercaptoethanol. The homogenate was centrifuged at 71,000g for 45 min at 4°C and the supernatant retained, this preparation was diluted 1:10 with 20mM Tris/HCl (pH 7.4) just prior to use in the assay. The standard reaction mixture (100µl) contained the following final concentration of components: 20mM Tris/HCl (pH 7.4), 5mM magnesium acetate, 200µg

of histone H1 per ml, 10μM [γ-^{32}P] ATP (0.6 Ci/mmol), 98μM $CaCl_2$, 48μM EGTA, 1.2mM 2-mercaptoethanol, 16μg phosphatidylserine per ml, 2.4μg per ml of 1,2-diolein and the appropriate concentration of test compound; this gave a free Ca^{2+} concentration of 50μM. Reactions were started by the addition of 24μl of spleen extract (approximately 25μg protein). The incorporation of ^{32}P from [γ-^{32}P] ATP into histone H1 was measured by the modified procedure of Lord and Ashcroft (1984) whereby phosphorylated proteins were recovered by precipitation with 5% (w/v) trichloracetic acid containing 0.25% (w/v) sodium tungstate.

Ca^{2+}/phospholipid-dependent protein kinase activity was calculated by subtracting the phosphorylation obtained in the presence of Ca^{2+} alone from that obtained in the presence of Ca^{2+}, 1,2-diolein and phosphatidylserine over a period of 2 min incubation at 37°C. Addition of phospholipid and 1,2-diolein stimulated enzyme activity by approximately 60%. Under these conditions the incorporation of ^{32}P was linear with time.

The effect of test compounds upon protein kinase C was determined by comparing the Ca^{2+}/phospholipid-dependent activity to the same activity obtained in the presence of a range of concentrations of the test compound.

2.7. ASSAY FOR CALMODULIN-STIMULATED PHOSPHODIESTERASE

Calmodulin-dependent bovine heart phosphodiesterase (Boehringer) activity was assayed by the method of MacNeil et al. (1984). The reaction mixture at 37°C contained, in a final volume of 400μl : 40mM Tris/HCl (pH 7.0), 4mM 2-mercaptoethanol, 5mM $MgCl_2$, cyclic [^{3}H] AMP (2×10^5 c.p.m. per tube) (Amersham), 100μM cyclic

AMP, 25μM $CaCl_2$, 1.5 mUnits of enzyme per tube and 12.5ng of calmodulin (Amersham).

Incubations of 15 min at 37°C were commenced by the addition of enzyme and terminated by transferring tubes to a boiling water bath for 15s. Tubes were then transferred to ice. The 5'-AMP formed during the incubation with phosphodiesterase was then converted to adenosine by the addition of 100μl of cobra snake venom (Ophiophagus hannah) (Sigma) (1mg/ml) which contains 5'-nucleotidase and the tubes incubated for 10 min at 37°C before returning the tubes to ice.

Product nucleosides were then separated from unreacted substrate using Dowex anion exchange resin. A 1ml slurry of 1 part activated Dowex-1-X8 (200-400 mesh) anion exchange resin (Sigma) to 4 parts methanol was added to each tube and the contents vortexed prior to centrifugation at 1000g for 10 min. The supernatant, containing adenosine, was decanted from each tube and counted for tritiated adenosine by scintillation counting in Optiphase Safe (L.K.B.).

To serve as an internal check on the assay control, tubes were always included in each assay containing no calmodulin, 2.5ng, 12.5ng and 200ng of calmodulin. 12.5ng of calmodulin was regularly found to stimulate 60-80% of maximal phosphodiesterase activity. Controls containing no enzyme were taken through the assay from the boiling stage onwards and subtracted from all test values.

Snake venom was reconstituted in distilled water and stored at 4°C for several weeks. Calmodulin-deficient bovine heart phosphodiesterase was reconstituted in a storage buffer comprising of 5mM MES, 30% (v/v) ethyleneglycol, 1mM dithiothreitol, 5% (w/v) glucose, 15mM sodium acetate, 50mM NaCl, 20μM PMSF and stored at -20°C for several weeks.

The effect of test compounds upon the calmodulin-dependent activation of phosphodiesterase was undertaken by comparing the affect of 12.5ng of calmodulin upon the enzyme in the absence of any test compound to that obtained in the presence of a range of concentrations of the test compound.

2.8. MARKER ENZYME ASSAYS

2.8.1. 5'Nucleotidase Assay.

5'Nucleotidase activity was measured by the method of Christie and Ashcroft (1985) which involved monitoring the conversion of [^{3}H] 5'-AMP to [^{3}H]-adenosine by separating free adenosine from unconverted AMP using ion exchange chromatography.

The reaction mixture, contained in 0.8ml microcentrifuge tubes, comprised of 50mM Tris/HCl (pH 8.0), bovine albumin (0.1mg/ml), 3mM $MgCl_2$, 2mM adenosine monophosphate and 2-[^{3}H] adenosine monophosphate (0.1µ Ci). The final volume was 100µl after the addition of 50µl of enzyme sample (islet fractions diluted 1 in 2 to 1 in 5).

Samples in duplicate were incubated for 1h at 30^{o}C and the reaction terminated by placing the reaction mixture in a boiling water bath for exactly 30s. To each sample 0.4ml of 0.1mM unlabelled (carrier) adenosine was then added and the sample applied to 0.75ml columns of QEAE-A25 Sephadex equilibriated with 20mM ammonium formate. Elution was carried out with 1.5ml ammonium formate and the eluate containing [^{3}H]-adenosine was counted for radioactivity in 20ml of Optiphase Safe. Controls containing boiled-enzyme were taken through the same procedure for each of the diluted enzyme samples. Values obtained from these controls were used for background subtraction in the calculation of enzyme activity.

2.8.2. Cytochrome C oxidase assay.

Cytochrome C oxidase activity was measured by monitoring the oxidation of reduced cytochrome C. The reaction mixture comprised of 41µM reduced cytochrome C, 1mM EDTA, 50mM potassium phosphate (pH 7.4). The reduced cytochrome C was prepared by titration of freshly made cytochrome C solution with sodium dithionite (1.5mg/ml) until approximately 90% of the cytochrome C was reduced which correlated to an absorbance of approximately 0.8 at 550nm.

Reactions were started by the addition of 50µl of enzyme sample (diluted if necessary) to 600µl of the reaction mixture, and the decrease in absorbance at 550nm was monitored over 10 min. The molar extinction coefficient of cytochrome C was taken as 19.7×10^3/M/cm.

2.9. ISLET STUDIES

During the course of the research study a change was made in the procedure used for the isolation of pancreatic islets. This involved a change from collagenase digestion where shaking was by hand to a collagenase digestion utilising a flask shaker (Galenkamp). This also involved a change from using a combination of Hanks Balanced Salt Solution (Hanks and Wallace, 1949) and Krebs Ringer Bicarbonate Medium (Krebs and Henseleit, 1932) in those experiments where pancreatic islets were isolated by the former method to Gey and Gey (1936) medium used in all experiments on pancreatic islets isolated by utilising the flask shaker. This change in isolation method yielded a greater number of pancreatic islets and allowed for multiple isolations when large numbers of islets were required. Insulin release experiments were undertaken to

ensure comparability in the secretory function of the pancreatic islets from the two types of isolation. Therefore the following methodology includes a description of both modes of isolation and the media used.

2.9.1. Physiological Media.

2.9.1.1. Media used in the collagenase digestion incorporating shaking by hand.

Two physiological media were used: Hanks Balanced Salt Solution (HBSS) used in the isolation of pancreatic islets and Krebs Ringer Bicarbonate Medium (KRB) used during the subsequent experimental incubation of islets. Both media were used in forms modified from those originally described.

HBSS (Hanks and Wallace, 1949) was prepared from a concentrated stock solution (10x without addition of phenol red or sodium bicarbonate) in the following manner:

100ml of 10x concentrated HBSS was placed into a 1 litre beaker and the solution made up to approximately 900ml with distilled water at $4^{o}C$. To this solution 200mg of bovine serum albumin (essentially fatty acid free) was added and when dissolved the solution was adjusted to pH 7.4 with approximately 3ml of 7.5% (w/v) sodium bicarbonate. The HBSS was then made up to 1 litre in a volumetric flask and stored at $4^{o}C$ until used. The medium had the following composition:

	mg/l	mM
NaCl	8000	137
$CaCl_2$	140	1.27
KCl	400	5.37
KH_2PO_4	60	0.44
$MgSO_4.7H_2O$	100	0.41

$NaHCO_3$	350	4.17
$Na_2HPO_4.2H_2O$	60	0.34
glucose	1000	5.55
bovine albumin	200	0.003

HBSS was not used if more than 2 days old. The above quantities were halved if less medium was required.

The KRB medium (Krebs and Henseleit, 1932) was prepared as follows:

A stock solution of KRB was made by combining the following salt solutions in a screw-capped glass bottle:

0.9% (w/v) NaCl	100ml
1.3% (w/v) $NaHCO_3$	21ml
1.15% (w/v) KCl	4ml
1.22% (w/v) $CaCl_2.2H_2O$	2ml
3.82% (w/v) $MgSO_4.7H_2O$	1ml

This solution was gassed with 95% O_2 in CO_2 for a minimum of 30 min after which 640mg bovine serum albumin (essentially fatty acid free) was added together with 1.28ml of 1.0M HEPES (pH 7.4). Glucose-containing solutions used in the incubations were prepared from this stock solution by the further addition of the appropriate volume of 50mg/ml (280mM) glucose in KRB stock solution and 100mM sodium phosphate buffer (pH 7.4) so that the final concentration of glucose was either 2.8, 30.5mM or any value particularly required between these minimum and maximum values used, the phosphate concentration was 1mM.

The final composition of KRB medium was:

	mg/l	mM
NaCl	6962	119.0
$CaCl_2.2H_2O$	189	1.29
KCl	356	4.77
$MgSO_4.7H_2O$	295	1.24
$NaHCO_3$	2111	25.14
$NaH_2PO_4.2H_2O$	144	0.81
$Na_2HPO_4.2H_2O$	30	0.19
HEPES	-	10.0
glucose	variable	various
bovine albumin	5000	0.076

KRB medium was prepared fresh daily and stored at 37°C prior to use.

2.9.1.2. Media for collagenase digestion utilising flask shaker.

Gey and Gey medium (1936) was used for both the islet isolation procedure and for subsequent incubations of pancreatic islets. The only alteration made was the addition of glucose to the incubation medium.

Gey and Gey medium was made by combining the volumes of the stock salt solutions indicated below:

			Gey and Gey Medium	
salt	stock g/l	volume added to 1 litre (ml)	g/l	mM
NaCl	65.0	100	6.5	111.2
$NaHCO_3$	22.7	100	2.27	27.0
KCl	37.0	10	0.37	4.96
$MgCl_2.6H_2O$	20.0	10	0.20	0.98

$MgSO_4.7H_2O$	6.9	10	0.69	0.28
KH_2PO_4	3.0	10	0.03	0.22
Na_2HPO_4	11.3	10	0.11	0.80
$CaCl_2.2H_2O$	29.4	10	0.29	2.00

(added whilst gassing)

The volumes indicated were placed in a 1 litre volumetric flask and distilled water added to approximately 950ml and the solution gassed with 95% O_2 in CO_2 for 20 min at 37°C. Whilst the solution was being gassed 10ml of $CaCl_2$ stock solution was added.

After gassing the solution was made up to volume and 1g of bovine albumin (essentially fatty acid free) added and the solution then checked to ensure its pH was 7.4 ± 0.2. The Gey and Gey solution was stored at 37°C before use and was made freshly each day. Glucose-containing solutions used in the incubations were prepared by adding the appropriate volume of a 50mg/ml (280mM) glucose solution in Gey and Gey medium so that the final concentration of glucose was either 2.8, 30.5mM or any value required between these values.

2.9.2. Siliconisation of glassware.

Inorder to prevent the adherence of pancreatic islets, the surface of all glassware was siliconised. This was achieved by coating the glass surface with siliconising fluid (Sigmacote, Sigma) for a few seconds. The glassware was then thoroughly dried in a warm cabinet. Prior to use all siliconised glassware was thoroughly rinsed with distilled water to remove any acidic traces resulting from the drying of the siliconising fluid.

2.9.3. Isolation of islets of Langerhans.

The method of isolation of intact pancreatic islets by collagenase digestion was essentially that described by Lacy et al., (1972) which is itself a refinement of the earlier method of Lacy and Kostianovsky (1967).

2.9.3.1. Excision of pancreas.

Sprague-Dawley rats of both sexes weighing 250-350g were used. For each isolation two rats were sacrificed by cervical fracture and the abdomens opened. The duodenum was clamped with a pair of Spencer-Wells forceps at the point of entry of the common bile duct into the duodenum. A small incision was then made in the bile duct just prior to the bifurcation point of the duct to the liver, using a small pair of fine pointed scissors. Into this incision was placed a cannula of 0.6mm diameter plastic tubing attached to a 31 x 0.6mm needle. The pancreas was then slowly inflated by attachment of cannula to a reservoir of isolation medium via a peristaltic pump until the tail of the pancreas appeared turgid, (approx 20ml of medium per pancreas).

The cannula and Spencer-Wells clamp were removed and any attached fat tissue carefully cut away from the turgid pancreas. The inflated pancreas was then excised by carefully cutting it away from its point of attachment to the duodenum and large intestine, its attachment to the stomach and liver, and then finally lifted to a petri dish via the attached spleen. The spleen was then removed along with any additional fat tissue. The clean pancreas was then placed in a scintillation vial and the second pancreas excised.

2.9.3.2. Collagenase digestion by hand.

Excess HBSS was poured off from the combined pancreati which were then chopped continuously in the residual HBSS

(approximately 10ml) with sharp scissors for exactly 10 min. The chopped tissue was washed five times with ice-cold HBSS and all excess HBSS carefully removed with a finely-drawn siliconised pasteur pipette after the final wash. To release the pancreatic islets from this tissue, 8mg of collagenase (Serva Feinbiochimica) was added to the prepared tissue in the scintillation vial which was then tightly capped. This mixture was then shaken by hand with an acquired semi-circular motion at a rate of 60-70 strokes per min in a water bath at 37^{o}C. The time of this digestion was varied according to the activity of the batch of collagenase : thus for 0.58 Units per mg collagenase, 10 min was the optimum time; for 0.79 Units per mg collagenase, 8 min was the optimum time. The digestion was stopped by the addition of approximately 20ml ice-cold HBSS which served to cool the digestion mixture and dilute out the collagenase. The mixture was allowed to settle for 4-5 min and the supernatant removed. This washing procedure was repeated twice more to ensure that the digestion had ceased. Samples of the sedimented material (approximately 1ml each) were dispersed in approximately 30ml HBSS contained in a plastic petri dish which was painted black on the outer surface. Intact pancreatic islets released by digestion were then visualised with a dissecting microscope at a magnification of 20x under incident illumination from a light source internal to the dissecting microscope. The pancreatic islets then appeared as opaque white spherical or ovoid objects. The pancreatic islets were then collected by use of a finely drawn-out pasteur pipette and placed in another blackened petri dish containing HBSS whilst leaving behind the connective and acinar tissue debris. Using this procedure, usually some 200-300 islets could be otained from a pair of

pancreati, however considerable variations either side of this range occurred.

For studies involving the incubation of intact islets in physiological media the pancreatic islets were incubated for 45 min in KRB medium containing 2.8mM glucose in an atmosphere of 95% O_2 in CO_2 before grouping for experimental incubations. For experiments using islet homogenates, the pancreatic islets were transferred a second time to a petri dish and then finally to a 0.8ml autoanalyser vial. These two transfers were carried out with a fine drawn-out Pasteur pipette connected to a mouth aspirator which allowed for more precise manipulation of the pancreatic islets so that no debris was transferred with them.

2.9.3.3. Collagenase digestion by flask shaker.

The two pancreati were first washed with Gey and Gey medium and the excess medium decanted off. The pancreati were then chopped using a pair of fine pointed, long bladed scissors for 75s. The chopped tissue was then transferred to a plastic conical centrifuge tube ($15cm^3$) and rapidly accelerated to 1,500 rev/min (M.S.E. Centaur bench centrifuge) and then immediately decelerated. The supernatant layer with fat was decanted, this washing procedure was repeated once or twice more until the supernatant appeared clear. This preparation was then transferred to a small glass quick-fit conical flask (50ml.).

Buffered medium was added or removed from the flask to give an approximately equal volume ratio of chopped tissue : medium. Release of pancreatic islets was then achieved by adding 10mg of Serva collagenase and 2mg of Sigma collagenase (previously weighed out into an autoanalyser vial). A Suba-seal stopper was then inserted into the flask and the flask attached to the flask shaker.

The shaker was then switched on at a setting and for a time duration which by experimentation was found to be optimum for the particular batch of Serva collagenase, thus for Serva collagenase of 0.73 Units/mg a setting of 8.5 for 8.75 min was found to be optimum, whilst for Serva collagenase of 0.67 Units/mg a setting of 8.75 for 9.25 min gave optimum islet release. To minimise the need for experimentation Serva collagenase was ordered in 1g aliquots which was sufficient for 100 isolations. After this period the digestion was examined visually and if any strands could be visualised on the flask, it was shaken vigorously for a further 15-45s until they were removed.

The digest was then transferred with rinsing into a conical plastic centrifuge tube and rapidly accelerated to 1,500 rev per min and immediately decelerated. The upper third to half of the supernatant was discarded down to a point where the opacity increased, any remaining digest from the flask was rinsed into the centrifuge tube and additional clean medium added to the tube. Using a pasteur pipette cut-off at the point where the neck narrowed, the pellet was vigorously resuspended throughout the medium and the centrifugation step repeated. This process was undertaken a total of 5-7 times until the supernatant ceased to be cloudy.

The intact pancreatic islets released by digestion were collected as described for the collagenase digestion where shaking was by hand. The collected islets were either grouped as described below or pooled together in an autoanalyser vial.

2.9.4. Treatment of islets.

Both methods of isolation resulted in either an autoanalyser vial containing clean islets which were subsequently homogenised as described in Section 2.4; or a black petri dish

containing isolated pancreatic islets. The subsequent experiments were undertaken on the intact islets either in KRB medium if the 'hand method' of isolation had been employed, or in Gey and Gey medium if the 'shaker method' of isolation had been employed. For the purposes of description these two media are simply referred to as 'incubation medium' since their experimental function was the same.

2.9.4.1. Apparatus for islet incubations.

For studies on insulin release and glucose utilisation, incubation of islets was undertaken in 10ml glass beakers which were subsequently stoppered with rubber bungs (Suba-seal No 49) and incubated at 37^{o}C in a constant temperature water bath. The rubber bungs allowed for the addition of medium and gassing of chamber atmosphere without requiring the removal of the stoppers. Thus, it was possible to make additions of medium with either 1ml syringes or microlitre syringes through the septum of the rubber bung. Gassing with 95% O_2 in CO_2 was achieved by fitting the gassing inlet line with a syringe needle which could thereby penetrate the rubber bung, a second needle was inserted as an outlet to prevent the build-up of pressure. Gassing was undertaken for 6-8 minutes and then the two needles removed to leave an atmosphere of 95% O_2 in CO_2. When a large number of incubations were undertaken in one experiment, a gassing manifold with several gas outlets was employed.

2.9.4.2. Grouping of islets.

Throughout the experiments on intact islets it was necessary to obtain groups of varying sizes from groups of 5 islets for insulin release experiments to groups of 60 islets for ^{32}P

phosphorylation studies. These groups of pancreatic islets were carefully attained from the petri dish so that each group was representative of the total population.

2.9.5. Studies on insulin release.

2.9.5.1. Batch incubation of pancreatic islets.

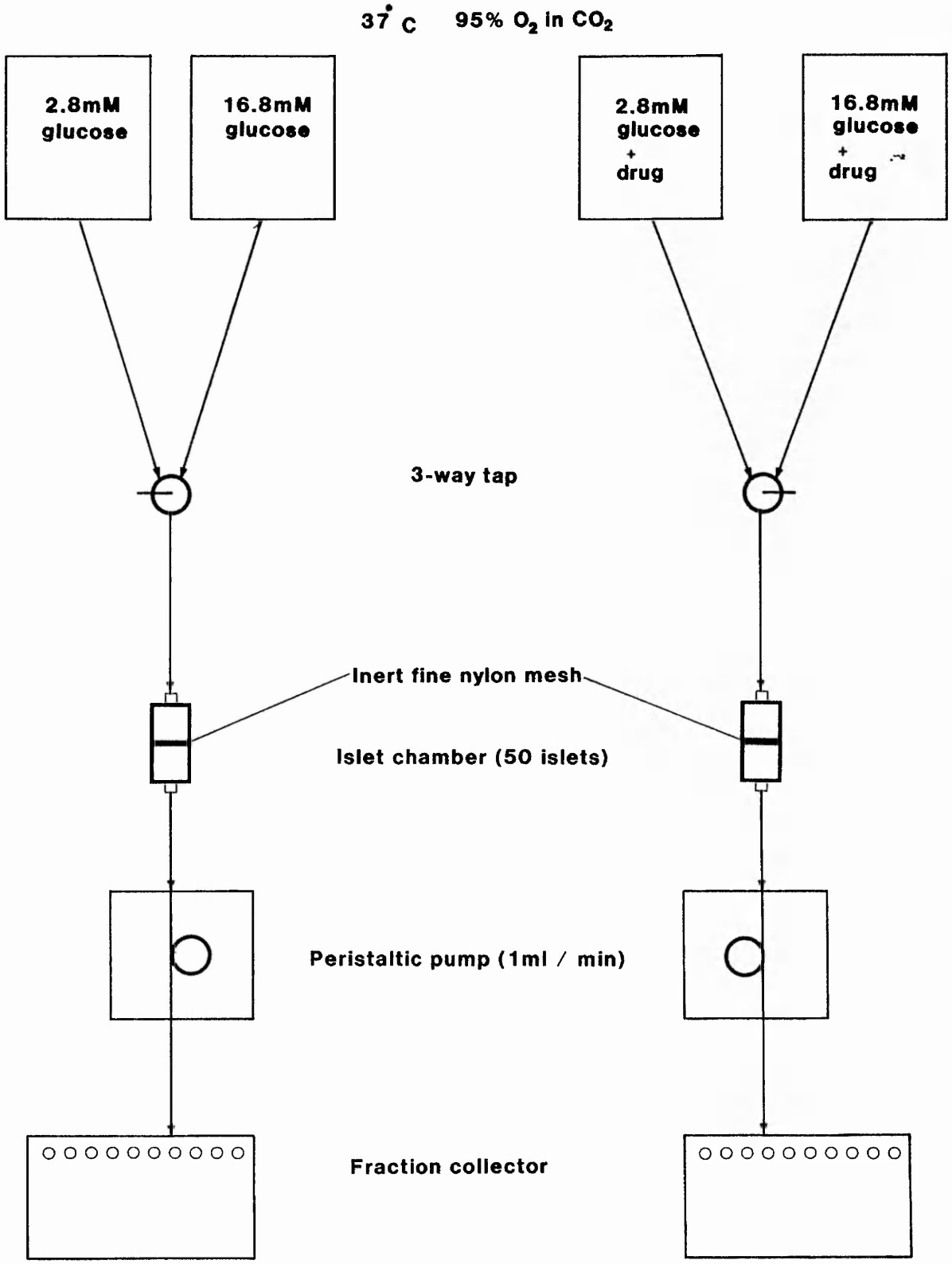
Batches of 5 islets were transferred into glass 10ml beakers with a minimum of medium using a finely drawn siliconised pasteur pipette and excess medium then removed. Immediately, 0.5ml of incubation medium containing 2.8mM glucose was added. Where a compound was to be tested for its effect upon glucose-stimulated insulin release then 2.8mM glucose medium with the desired concentration of test compound was added instead. On multiple incubations the time of commencement of each incubation was staggered to allow the later processing of each treatment. The beakers were stoppered and gassed as described in Section 2.9.4.2. The islets were incubated for 45 min at this basal level of glucose to allow the entry of test compounds into the islets prior to the stimulation of insulin secretion by raising the glucose concentration from 2.8mM glucose to 16.8mM glucose. Following this pre-incubation period 0.5ml of incubation medium containing 30.5mM glucose and the appropriate concentration of test compound (if required in a particular incubation) was added through the suba seal to give a final glucose concentration of 16.8mM glucose and a final volume of 1.0ml. For the effect of test compounds on basal release, a further 0.5ml of medium containing 2.8mM glucose together with the desired concentration of test drug was added. Control incubations carried out in the absence of the test compound were treated by exactly the same procedure but the test compound was omitted. Dose response curves to glucose were attained by adding 0.5ml of glucose solutions

(concentrations between 2.8mM and 30.5mM) to obtain final glucose concentrations in the incubation mixture between 2.8mM - 16.8mM. Following a further 60 min incubation at 37°C aliquots of medium were carefully sampled ensuring that no islets were removed with the medium, and these samples stored at -70°C until radioimmunoassay was performed to determine insulin concentrations.

2.9.5.2. Perifusion of pancreatic islets.

A perifusion system was set up to study the dynamics of insulin secretion and the effect of test compounds upon this process. The perifusion system comprised of chambers in which the pancreatic islets were placed, connected by 0.5mm plastic tubing to reservoirs of incubation medium and to a fraction collector via a peristaltic pump as shown in Figure 2.2. By means of a 3-way tap each chamber was linked to both basal (2.8mM) glucose-containing Gey and Gey medium and stimulatory (16.8mM) glucose-containing Gey and Gey medium. The whole system was primed prior to the commencement of an experiment with the exclusion of all air. Batches of 50 islets were then introduced into each of the chambers using a drawn out pasteur pipette and the chambers closed again with the exclusion of air. The chambers (Swinex - 13, Millipore S.A., France) included an inert fine nylon mesh membrane onto which the islets settled (10μm pore size). Incubation medium containing 2.8mM glucose was then drawn over the islets for 45 minutes; when the medium contained a test compound this time period was employed to allow entry of the test compound into the β-cells. The flow rate of medium over the pancreatic islets was kept constant at 1.0 ml per min. Samples were then collected for 4 minute periods for 20 minutes, these samples were used to measure the basal level of insulin release. By means of the 3-way tap the medium was then switched from

Figure 2.2 **Perifusion system**



2.8mM to 16.8mM glucose and samples were collected at the following time periods: 0.5 min for 5 min, then every 1 min for 10 min, every 2 min for 10 min, and finally every 4 min period for 40 min. The total collection period was therefore 20 min of 2.8mM stimulation and 65 min of 16.8mM stimulation. A single experiment comprised of two control chambers where there was no test compound present in the Gey and Gey medium, and two chambers where the test compound was present at the desired concentration. All media were kept at 37°C throughout the experiment, and the atmosphere over the media was 95% O_2 in CO_2. The samples collected were then frozen at -70°C for subsequent measurement of insulin levels by radioimmunoassay.

2.9.5.3. Radioimmunoassay for immunoreactive insulin.

Two different types of radioimmunoassay were used in the duration of the project. This change was made for a number of reasons. The new method employed used Wellcome insulin binding reagent, this meant fewer dilutions of unknown samples were required since the range of standard concentrations that occurred on the linear portion of the calibration was larger, and because it allowed for a more rapid processing of a large number of unknown samples without any loss in the accuracy of the assay.

2.9.5.3.1. Radioimmunoassay using ethanol precipitation.

The amount of immunoreactive insulin present in samples was determined using an ethanol precipitation method (Heding, 1972). For each assay standard samples containing known amounts of rat insulin standard (Novo) and suitably diluted unknown samples were taken through the same procedure. The standards of rat insulin were prepared in NaFAM buffer which comprised of 40mM sodium phosphate (pH 7.4), 6% (w/v) human albumin, 0.6% sodium chloride, and 0.24 mg/ml sodium merthiolate. Unknown samples were prepared in

duplicate over a range of dilutions such that some of the dilution values fell on the linear portion of the calibration curve. Both unknown samples and standard samples were diluted with the NaFAM buffer and 100µl aliquots of the diluted preparations were placed in suitably labelled 12 x 75mm disposable glass test tubes and kept on ice.

To all of these samples 100µl of anti-porcine guinea pig serum (Novo) prepared in FAM buffer was added. The FAM buffer comprised of 40mM sodium phosphate (pH 7.4), 1mg/ml human albumin and 0.24 mg/ml sodium merthiolate. The working solution of anti-porcine guinea pig serum had a binding capacity of approximately 170µU of insulin per ml of serum. The tubes were then vortexed and incubated at $4^{o}C$ for 20h. Following this incubation, 100µl of ^{125}I-labelled porcine insulin (20-30 µU per ng and 20-30 mCi per mg) prepared in FAM buffer was added to each sample and to three more tubes containing 100µl each of FAM and NaFAM to serve as total-count controls. After vortexing, the tubes were incubated at $4^{o}C$ for an additional 4h. The tubes were then placed in ice and 1.6ml of 95% (v/v) ethanol (kept at room temperature) added to each tube, and the tubes subsequently vortexed. The tubes were then centrifuged at 1800g av. and $4^{o}C$ and the supernatant carefully decanted into suitably labelled plastic gamma-counting tubes, which were then capped and counted for radioactivity. The data obtained from the rat insulin standard samples was used to plot a calibration graph of counts per minute versus insulin concentration. The values for unknown samples which corresponded to the linear region of the graph were then extrapolated to give an insulin concentration. For each sample, an average value

for insulin concentration in the undiluted sample was then calculated from the values obtained.

A typical calibration graph for this type of radioimmunoassay is shown in Figure 2.3. Typically, samples from batch incubations with basal glucose (2.8mM) were diluted 1 in 5 and 1 in 10, whilst samples from incubations at high glucose (16.8mM) were dilute 1 in 20 and 1 in 50, and samples with a test compound present in the incubation were diluted 1 in 10, 1 in 20 and 1 in 50.

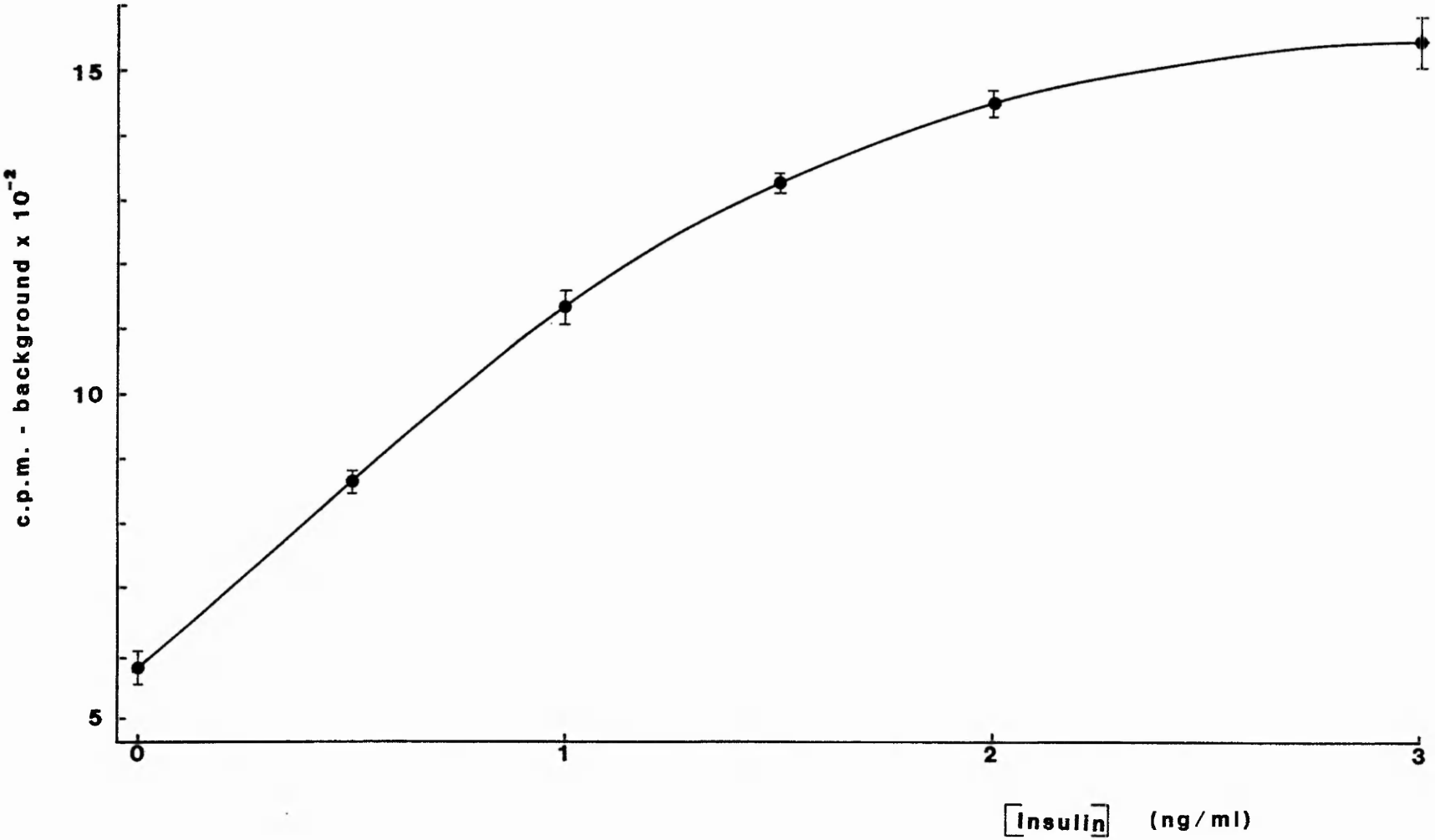
2.9.5.3.2. Radioimmunoassay using Wellcome Insulin Binding Reagent.

The Wellcome radioimmunoassay separated antibody bound insulin from free insulin by a double antibody procedure employing a pre-precipitated antibody based on the method of Hales and Randle (1963). The insulin binding reagent consisted of a mixture of guinea pig anti-insulin serum and rabbit anti-guinea pig globulin serum in a buffer which comprised of 40mM sodium phosphate (pH 7.4), 20mM EDTA, 0.1% (w/v) sodium azide, 0.5% (w/v) bovine serum albumin. This reagent had been prepared by allowing the anti-insulin serum and the precipitating serum to react overnight with the resultant formation of a microscopic precipitate which retained insulin binding properties.

Each assay comprised of a 'blank zero' tube to which no unlabelled insulin was added, a set of standard insulin tubes in triplicate, a suitably diluted unknown sample in duplicate, totals to which only unlabelled insulin was added, and 'reagent blank' to which no insulin binding reagent was added.

Rat insulin (Novo) was used to prepare a range of standards (0-20 ng insulin per ml, 0-500 μU per ml). Standards and unknown samples were prepared with diluent buffer of the following

Figure 2.3 **Insulin standard curve - Ethanol precipitation**



composition, 40mM sodium phosphate (pH 7.4), 0.9% (w/v) NaCl, 0.5% bovine albumin, 0.1% sodium azide which was prepared freshly on a weekly basis. Unknown samples from batch incubations containing 2.8mM glucose were taken undiluted and diluted 1 in 2, samples from incubations at high glucose (16.8mM) were diluted 1 in 4 and 1 in 8, whilst samples with a test compound present in the incubation were diluted 1 in 2 and 1 in 4. Samples from perifusion experiments with basal glucose (2.8mM) were used undiluted, samples from the first 10 minutes of stimulation with 16.8mM glucose were diluted 1 in 5 and 1 in 10, and subsequent samples were diluted 1 in 2 and 1 in 4. Radio-iodinated insulin (Novo and Amersham), (Novo : 20-30 mCi/mg; Amersham 60-100 mCi per mg) was diluted with diluent buffer such that 50µl corresponded to approximately 10,000 counts per minute.

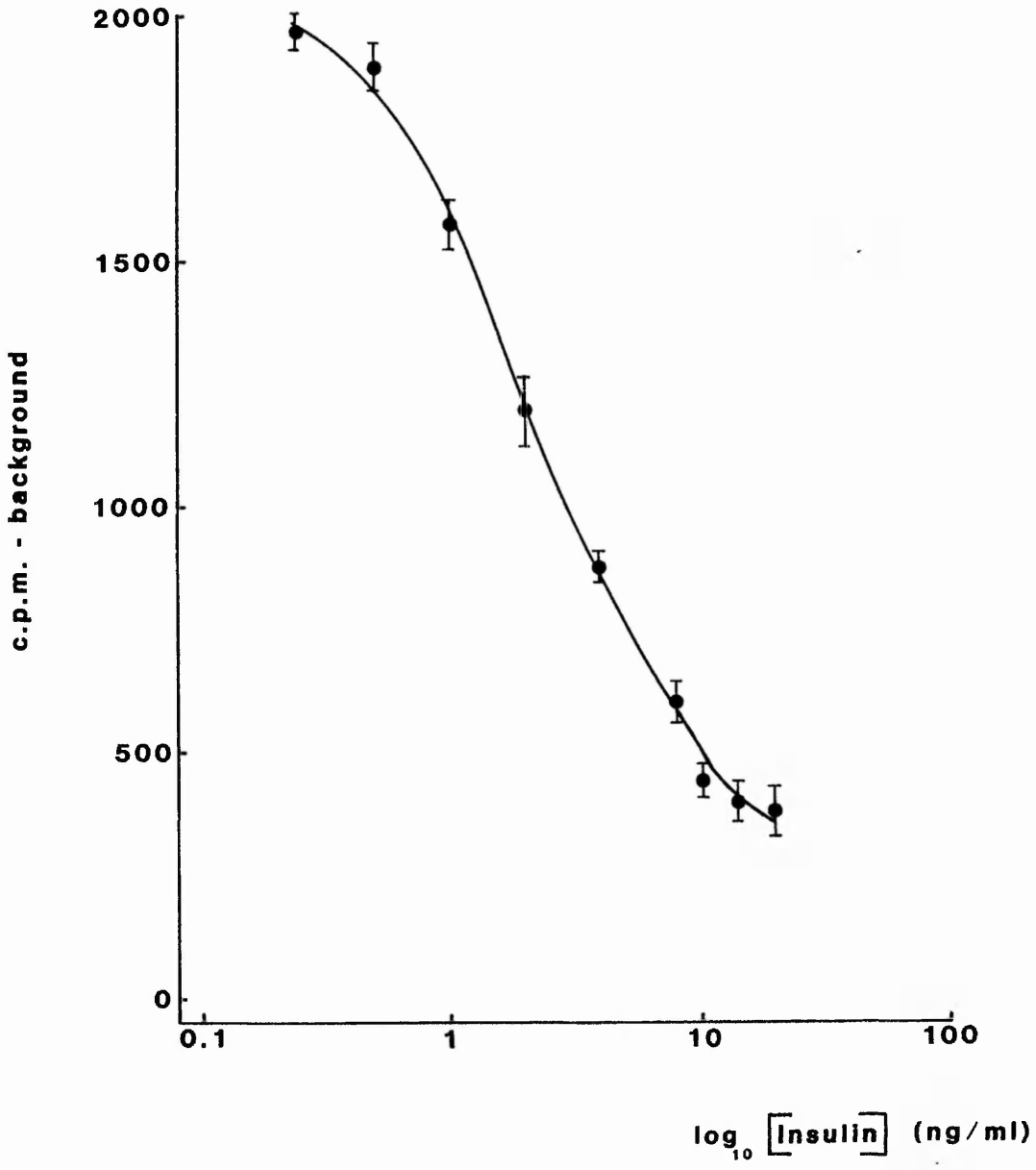
Having placed 50µl of the appropriate insulin standard or test sample (50µl of diluent buffer for the zero and blank tubes) into plastic LP3 tubes, 50µl of insulin binding reagent was added to all tubes except for the 'reagent blank' and total tubes using a repeat dispensor. The samples were then vortexed and incubated at $4^{o}C$ for 6h. Radio-iodinated insulin (50µl) was then added to all tubes which were vortexed again before returning to a refridgerator at $4^{o}C$ for a further 18h. The precipitate containing the antibody-bound tracer was then separated from unbound tracer by the careful but vigorous (sufficient to swirl up the contents of the tube) addition of 500µl of diluent buffer using a repeat dispensor, and the subsequent centrifugation of the tubes at 2000g av. for 30 min. The supernatants were discarded and the last traces of supernatant removed by touching the lip of the tube on a pad of absorbent paper. The tubes were then capped and the radioactivity present in the precipitates determined by gamma counting. From the

data obtained for the standard samples a graph was plotted of the log of the concentration of insulin versus the counts per minute; a typical calibration graph is shown in Figure 2.4. Using this graph the values of unknown samples were determined by extrapolation back to the y-axis.

2.9.6. Measurement of islet glucose oxidation .

Glucose-stimulated glucose oxidation by islets was determined by the method of Malaisse et al. (1974) by measuring the amount of $^{14}CO_2$ released from [U-^{14}C]-glucose. Groups of 20 islets were placed in autoanalyser vials and excess medium removed, 50µl of incubation medium containing 2.8mM glucose and the appropriate concentration of any test compound was then added. The vials were placed inside glass 10ml beakers which were closed with Suba-seal rubber bungs and the beakers then gassed to give an atmosphere of 95% O_2 in CO_2. The pancreatic islets were preincubated for 45 min at 37^{o}C before the addition of 45µl of incubation medium containing 34.2mM glucose ± the appropriate concentration of test compound and the addition of 5µl of [U-^{14}C]-glucose (Amersham) (1µCi) to give a final glucose concentration of 16.8mM. Basal levels of glucose utilisation were determined by the addition of 45µl of 3.1mM glucose giving a final glucose concentration of 2.8mM. The pancreatic islets were then further incubated for 60 minutes, after which time 200µl of hyamine hydroxide (methylbenzethonium hydroxide) was placed in the glass beaker to absorb any released $^{14}CO_2$ and 50µl of 0.2 N HCl added to the islet incubation medium, reducing the pH to below 1.0 inorder to release any $^{14}CO_2$ in the medium. The beakers were then incubated at 37^{o}C for a further 30 min. The vials were then lifted from the beakers and the bottom of the outer surface washed with 400µl of distilled water into the glass beaker. The contents of the beaker

Figure 2.4 Insulin standard curve - Wellcome binding reagent



were then transferred to a glass scintillation vial using a further 400µl rinse with distilled water. The diluted hyamine hydroxide (1.0ml) was mixed with scintillant (Optiphase) and counted for radioactivity. Background counts were determined by exactly the same procedure with the exclusion of pancreatic islets.

2.9.7. Determination of islet total protein synthesis.

Protein synthesis in pancreatic islets was determined by measuring the incorporation of [^{3}H]-leucine into trichloroacetic acid precipitable proteins. Groups of 20 islets were placed in autoanalyser vials and excess medium removed, 50µl of incubation medium containing 2.8mM glucose and the appropriate concentration of test compound was then added. The vials were placed inside glass 10ml beakers which were closed with Suba-seal rubber bungs and then gassed to give an atmosphere of 95% O_2 in CO_2. After a preincubation period of 45 min at 37^{o}C, 45µl of incubation medium containing 34.2mM glucose and the appropriate concentration of test compound and 5µl of [^{3}H]-leucine (5µCi) was added to the islets. This resulted in a final concentration of 16.8mM glucose and 0.9µM leucine. The vials were then incubated for a further 60 min at 37^{o}C after which the vials containing the pancreatic islets were removed and 100µl of ice-cold 20% (w/v) trichloroacetic acid added. After sonication of the islets at 4^{o}C by two bursts of 5s at 3µ the sonicate was transferred to 1.8ml microfuge tubes. The tubes were centrifuged for 4 min at 11,600g in a microfuge and the supernatant decanted. The pellet was then washed three times in ice-cold 5% (w/v) trichloroacetic acid containing 2mM 'cold' leucine by resuspension and centrifugation, then once in acetone/ethanol (1:1 v/v) and once in acetone. The precipitate was then counted for radioactivity.

Background counts were determined by exactly the same procedure with the exclusion of pancreatic islets.

2.9.8. Measurement of pancreatic islet lipid methylation.

Total islet lipid methylation was determined by measuring the incorporation of L-[methyl-^{3}H]-methionine into chloroform/diethyl ether-extractable material, essentially as described by Best et al. (1984). Groups of 80-120 islets were preincubated for 45 min at 37^{o}C in 500µl of incubation medium containing 2.8mM glucose with or without the desired final concentration of test compound. These incubations were undertaken in glass 10ml beakers closed with self-sealing rubber bungs in an atmosphere of 95% O_2 in CO_2. After this preincubation period, a further 500µl of incubation medium containing 30.5mM glucose with or without the test compound was added together with 10µ Ci of L-[methyl-^{3}H]-methionine (74 Ci/mol) to give final concentrations of 16.8mM glucose and 0.135µM methionine. After a further incubation of 60 min the supernatant was carefully removed from the islets, and then 1.0ml of ice-cold 10% (w/v) trichloroacetic acid was added to the islets. The resulting precipitate was washed twice with 10% trichloroacetic acid and then transferred to a 15ml glass centrifuge tube. To the precipitate was added 1.0ml of distilled water, and the aqueous mixture then extracted with 1.0ml of chloroform/methanol/11M HCl (200:100:1, by vol.). The lower organic layer was carefully transferred to a scintillation vial and the organic solvent evaporated with a stream of N_2. The residue was then dispersed in scintillation fluid and counted for radioactivity.

2.9.9. Phosphorylation Studies.

2.9.9.1. Phosphorylation of peptides in intact cells.

Batches of 60 islets were placed in siliconised plastic LP4 tubes and excess medium removed. To the islets 140µl of Gey and Gey medium containing 18.0mM glucose and 10µl of [^{32}P]Pi (10 mCi/ml carrier free) was added, resulting in a final volume of 150µl of medium containing 16.8mM glucose. The pancreatic islets were incubated for 90 min at 37^{o}C.

Following incubation excess medium was drawn off, using a disposable plastic pasteur pipette with a finely drawn tip, and deposited in plastic waste bottles held in thick lead pots. The islets were then transferred to microfuge tubes and washed twice by centrifugation and resuspension in homogenisation medium. The homogenisation medium comprised of 0.3M sucrose, 50mM sodium phosphate (pH 7.4), 5mM sodium fluoride, 1mM EGTA, 4mMATP and 1mM phenylmethanesulphonyl fluoride. The groups of 60 islets were then transferred to a glass-glass hand held homogeniser and homogenised in 50µl of homogenisation medium. The homogenate was transferred back to the microfuge tube and the homogenisation tube washed with 25µl of homogenisation medium which was then added to the microfuge tube. To this 75µl of homogenate 25µl of 4x strength electrophoresis sample buffer was added, a small pressure release outlet was made in the top of the microfuge tube, and the sample was then boiled for 5 minutes before being stored at $^{-}$20^{o}C ready for electrophoretic analysis.

2.9.9.2. Treatment of phosphorylated islet homogenates.

A variety of treatments of the phosphorylated islet homogenates were undertaken to investigate the nature of the radiolabelled material found in them.

2.9.9.2.1. Digestion with RNAase/DNAase.

Islet homogenates were treated with RNAase and DNAase to investigate whether any of the radiolabelled material was nucleic acid rather than protein. Homogenates were prepared as described in the above section, except that the final homogenate volume was 70µl. To this homogenate 5µl of a mixture of RNAase and DNAase both at a concentration of 750µg/ml was added, giving a concentration in the homogenate of 50µg/ml. The homogenates were then incubated for 5 min at 37°C prior to the addition of 25µl of 4x strength electrophoresis sample buffer and subsequent boiling for 5 min and storage at ⁻20°C ready for electrophoretic analysis.

2.9.10.2.2. Digestion with proteases.

Islet homogenates were treated with the proteases pronase and trypsin. In these studies, after the 90 min incubation period the excess medium was drawn off and discarded, and the batches of 60 pancreatic islets then pooled in a single microfuge tube before being washed in homogenisation medium (without addition of phenylmethanesulphonyl fluoride).

The pancreatic islets were then homogenised at a ratio of 2-3 pancreatic islets per µl. Aliquots of the homogenate (50µl) were then first treated with RNAase and DNAase (both at a concentration of 50µg/ml) for 5 min at 37°C. These samples were then further digested with the particular protease (at a concentration of 50µg/ml) for 30 min at 37°C before being dissolved in electrophoresis sample buffer as previously described.

2.9.9.2.3. Digestion of phosphorylated pancreatic membranes with phospholipase and pronase.

Following the homogenisation of pooled phosphorylated islets in homogenisation medium (without addition of phenylmethane-

sulphonyl fluoride) the homogenate was centrifuged at 80,000g av. for 45 min. The pellet was then resuspended in the same volume of incubation medium as the volume of the original homogenate. The incubation medium in which the pellet was resuspended comprised of 50mM sodium phosphate, 1mM EGTA, 0.3M sucrose, 4mM ATP, 5mM NaF, 3mM $CaCl_2$ and 100μM $ZnCl_2$ at pH 7.4; this gave a free calcium concentration of 2mM.

Control samples of the resuspended membrane pellet (50μl) were treated for 15 min at 37°C with RNAase and DNAase (50μg/ml) and were then dissolved in electrophoresis sample buffer; test samples of the resuspended membrane pellet (50μl) were treated for 15 min at 37°C with a combination of RNAase and DNAase (50μg/ml), phospholipase C (5U) and phospholipase A_2 (5U); some of these samples were then treated for a further 15 min at 37°C with pronase (50μg/ml), both sets of samples were subsequently dissolved in electrophoresis sample buffer.

2.9.9.3. Effect of test compounds on in vivo phosphorylation pattern of pancreatic peptides.

A preincubation period was used when test compounds were to be included in the incubations of pancreatic islets with 16.8mM glucose and [^{32}P]Pi. Batches of 60 islets were placed in siliconised plastic LP4 tubes and the excess medium removed. To the islets 100μl of Gey and Gey medium containing 2.8mM glucose and the appropriate concentration of test compound was added and the mixture incubated for 45 min at 37°C. After this period a further 40μl of Gey and Gey medium containing 56mM glucose and [^{32}P]Pi (10μl of a 10mCi per ml carrier free solution), was added, resulting in a final volume of 150μl of Gey and Gey medium containing 16.8mM glucose and 100μCi [^{32}P]Pi. The pancreatic islets were incubated for

90 min at 37°C and then processed as described in Section 2.9.10.1.

2.9.9.4. Incorporation of [^{32}P]-phosphorylated islet peptides into high molecular weight aggregates.

Pooled batches of 60 islets were homogenised at a ratio of 2-3 islets per µl in medium which comprised of 0.3M sucrose, 50mM sodium phosphate (pH 7.4), 5mM sodium fluoride, 1mM EGTA, 4mM ATP, and 1mM phenylmethanesulphonyl fluoride. The incubation mixture for investigating the cross-linking of phosphorylated islet proteins by the islet transglutaminase consisted of the components already present in the homogenisation medium plus a calculated 5mM free Ca^{2+} or 5mM EGTA in a total volume of 70µl after the addition of 50µl of islet homogenate. Multiples of these volumes were used according to the total volume of homogenate available. Incubation was undertaken at 37°C. Samples of 70µl were taken at 0, 15, 30 and 45 minutes and treated for 5 min with DNAase/RNAase (50µg/ml). These samples were then dissolved in electrophoresis sample buffer as previously described.

2.10. TEST COMPOUNDS

Test compounds were synthesised either by Dr I. C. Coutts, Department of Physical Sciences, Trent Polytechnic, Nottingham, or by Dr G. M. Blackburn, Department of Chemistry, University of Sheffield.

2.10.1. Synthesis of test compounds.

Monodansylcadaverine, glycine methyl ester and sarcosine methyl ester were purchased from the appropriate chemical company as detailed in Section 2.1.

The NN'-dimethylcadaverine sulphonamides N-(5-dimethylaminopentyl)-2-napthalenesulphonamide and N-(5-diethylaminopentyl)-5-dimethylamino-1-naphthalenesulphonamide (free bases) were made by reaction of the appropriate naphthalenesulphonyl chloride in pyridine with NN'-dimethylcadaverine which was prepared from 5-chlorovaleronitrile as detailed in Lott and Krapcho (1957). For the N-(5-aminopentyl)-2-naphthalenesulphonamide (hydrabromide salt) 6-(2-naphthalenesulphonamide)-hexanamide was treated with bromine in methanol as described by Lorand et al. (1979) and the resulting carbarnate was hydrolysed with a saturated solution of HBr in acetic acid (Wani et al. 1972). The purity of compounds was assessed by t.l.c., mass spectrometry, i.r. spectroscopy and n.m.r.

2,4,6-isopropyl benzene sulphonamide was prepared as described by Lorand L. et al (1979).

W7 (5-chloronaphthalene-1-sulphonyl chloride) was prepared as described by Beattie and Whitmore (1933) as dark brown crystals from glacial acetic acid mp. 95°C.

C_6-Iodo-W7 and C_8-iodo-W7 were prepared from 5-iodonaphthalene-1-sulphonic acid by reacting its anhydrous sodium salt with phosphoryl chloride at 80°C. The product was crystalised from glacial acetic acid as yellow crystals mp. 115°C.

3-trifluoromethylbenzenesulphonyl chloride was prepared according to the method of Hempel & Klauke (1971) by the chlorosulphonylation of benzotrifluoride to give an oil bp. 103.8°C. The product was further characterised by treatment with liquid ammonia to give 3-trifluoromethylbenzenesulphonamide mp. 106.8°C.

The purity and structure of the above compounds eminating from the laboratory of Dr Blackburn (Sheffield) were

confirmed by determination of melting points and spectroscopic analysis using n.m.r. and mass spectrometry.

2.10.2. Addition of test compounds.

Monodansylcadaverine was initially dissolved in 100µl of dilute HCl, made up to half the final volume with incubation medium, the pH carefully adjusted with dilute NaOH to pH 7.4, and made up to volume with additional incubation medium to give a stock solution of 10mM. Due to its instability in light all media containing monodansylcadaverine were protected from light.

Glycine methyl ester and sarcosine methyl ester were dissolved in incubation medium and then added directly to the incubation mixture.

All other test drugs were dissolved in the minimum volume of dimethyl sulphoxide required and then made up to the required concentration using incubation medium. The highest concentration of dimethyl sulphoxide used in stock solutions was 10% (v/v) in a 10mM stock solution and the highest concentration of dimethyl sulphoxide used in an enzyme assay or islet incubation mixture was 0.5% (v/v).

Control incubations contained the same final concentration of dimethyl sulphoxide as the incubations containing test compounds. For hydrabromide salts of test compounds NaBr was added to control incubations so that the same final concentration of Br^- was present in control and test incubations.

2.11. STATISTICAL ANALYSIS

2.11.1. Standard Error (S.E.).

The standard error of data were calculated using the formula:

$$S.E. = \frac{x}{\sqrt{n}}$$

where x = the sample mean

n = the number of observations

2.11.2. Students t-test.

A two-sample t-test was employed to determine the difference between the means of separate sets of observations. The test statistic t was calculated using the formula:

$$t = \frac{(x_1 - x_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

where x_1, x_2 = the sample means of the two sets of observations.

s_1, s_2 = the corresponding standard deviations of the populations.

n_1, n_2 = the number of observations in each group.

In addition the degrees of freedom (d.f.) was calculated using the formula:

$$d.f. = \frac{((s_1^2/n_1) + (s_2^2/n_2))^2}{\frac{(s_1^2/n_1)^2}{(n_1 - 1)} + \frac{(s_2^2/n_2)^2}{(n_2 - 1)}}$$

; n_1, n_2, s_1 and s_2 being as described previously.

Using the t and d.f. value,, the confidence limit for the data was determined from a t-table. Confidence limits of 0.05 and below were taken as statistically significant.

This analysis was undertaken using the MINITAB System (Minitab Inc, Enterprise Drive, State College, PA16801, USA) on the computer system of the Department of Computing, Trent Polytechnic.

3. RESULTS

The main aim of this project was to further investigate the involvement of transglutaminase in the mechanism of glucose-stimulated insulin release from the pancreatic β-cell.

This aim was achieved by pursuing three principal objectives:

1. Characterisation of the islet transglutaminase;
2. Development of specific pharmacological probes for transglutaminase;
3. Determination of transglutaminase substrates in pancreatic islets.

These avenues of investigation are considered separately in Sections 3.1, 3.2 and 3.3 respectively.

3.1. SENSITIVITY OF THE ISLET TRANSGLUTAMINASE TO ACTIVATION BY Ca^{2+}

Previous investigations (Bungay et al., 1982; 1984; Gomis et al., 1983) indicated the rat islet transglutaminase to be a Ca^{2+}-activated and thiol-dependent enzyme with an apparent K_m for Ca^{2+} in the range 40-90 μM. However, since concentrations of Ca^{2+} as high as these are unlikely to be found in the pancreatic β-cell during periods of stimulated insulin release, the accuracy of these findings was re-assessed. Such studies were important since in order to implicate a role for transglutaminase in Ca^{2+}-mediated stimulus-secretion coupling the enzyme should be active at the concentrations of Ca^{2+} (0.1 - 0.2 μM approx., Wollheim and Pozzan, 1984; Deelers et al., 1985) reported to be found in the β-cell (using fluorescent dyes) during the glucose-stimulated response.

Previous work in the laboratory (Hand et al., 1985) indicated that the use of dephosphorylated N,N'-dimethylcasein in the enzyme assay was essential in order to obtain a true reflection of the activation of transglutaminase by Ca^{2+}, since significant amounts of Ca^{2+} could be sequestered by protein-bound phosphate groups.

Cell extracts of isolated rat islets were therefore incubated with dephosphorylated N,N'-dimethylcasein and concentrations of free Ca^{2+} in the range 3.2 - 50.6 μM. Additional experiments were also undertaken to investigate the effects of Mg^{2+} on the activation of the enzyme by Ca^{2+}, in these experiments incubations also contained 2mM $MgCl_2$. Graphs were plotted of the time dependent incorporation of [^{14}C]-putrescine into N,N'-dimethylcasein at the varying Ca^{2+} concentrations in the presence or absence of 2mM $MgCl_2$. From these graphs the initial

rates of putrescine incorporation were determined for each of the Ca^{2+} concentrations and re-plotted as a double reciprocal plot after the method of Lineweaver and Burk (1934) (Figures 3.1a. and b.). From these plots the apparent K_m for Ca^{2+} in the presence and absence of Mg^{2+} ions was determined.

Under these conditions the apparent K_m for Ca^{2+} in the absence of Mg^{2+} ions was found to be 3.2μM, a factor of 10 or so lower than values obtained previously (Bungay et al., 1982; 1984; Gomis et al., 1983) and in keeping with the values obtained by Hand et al. (1985) for rat liver transglutaminase. In the presence of 2mM Mg^{2+} ions the apparent K_m for Ca^{2+} was calculated to be 2.2μM, thus indicating that Mg^{2+} has little effect on the Ca^{2+}-sensitivity of the islet transglutaminase. This absence of effect of Mg^{2+} on the sensitivity of the islet transglutaminase to Ca^{2+} further parallels the findings of Hand et al. (1985), who found no effect of Mg^{2+} ions on the Ca^{2+}-sensitivity of the rat liver transglutaminase.

Figure 3.1a. and 3.1b.

Calculation of the apparent K_m for Ca^{2+} of transglutaminase in the presence and absence of Mg^{2+} ions.

Islets were sonicated as described in Section 2.4.1.3. in medium comprising of 0.25M sucrose, 1mM Tris/HCl and 1mM EGTA, pH 7.4.. Supernatant extracts were then prepared as described in Section 2.4.2.. The supernatant was then assayed for transglutaminase activity as described in Section 2.5.1.3. in the presence of free Ca^{2+} concentrations ranging from 3.2μM to 50.6μM. From the resultant data the initial rates of putrescine incorporation into the dephosphorylated N,N'-dimethylcasein at varying concentrations of Ca^{2+} were determined. A reciprocal plot of $1/V_o$ ($Units^{-1}$) against $1/[Ca^{2+}]$ was made, where the intercept on the x-axis represented the negative reciprocal of the apparent K_m for Ca^{2+} of islet transglutaminase in the absence of Mg^{2+} ions (Figure 3.1a.) and in the presence of 2mM Mg^{2+} ions (Figure 3.1b.). One Unit of enzyme activity was equal to 1 nmol of putrescine incorporated/h. Each point is the mean (± S.E.M.) for three determinations.

Figure 3.1a

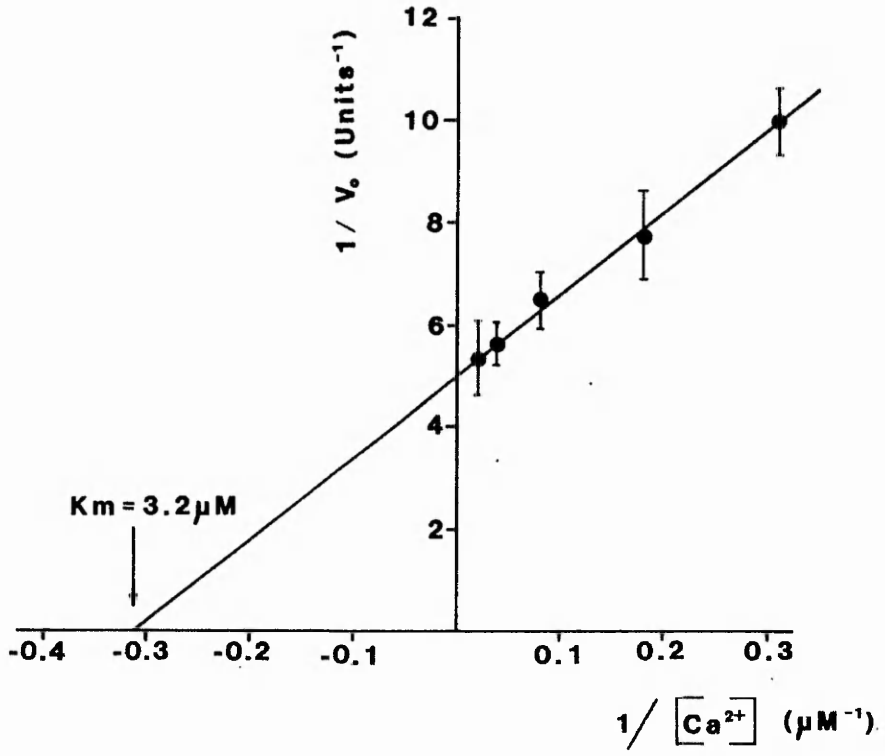
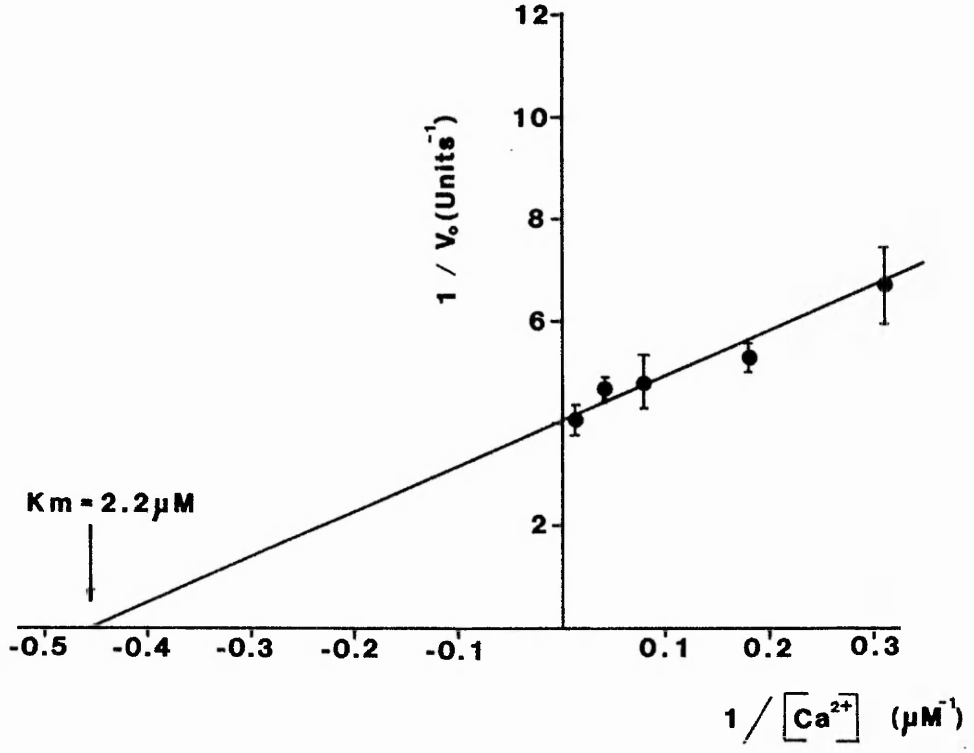


Figure 3.1b



3.1.1. DISCUSSION

Considerable evidence now exists to indicate that a rise in the cytosolic concentration of Ca^{2+} is required to elicit the secretory response of the pancreatic β-cell to glucose (Prentki and Wollheim, 1984). The magnitude of this rise in intracellular $[Ca^{2+}]$ has been estimated using the fluorescent indicator quin-2 to be in the order of 0.1μM so that the concentration of free Ca^{2+} in the stimulated β-cell may reach approx. 0.2μM (Wollheim and Pozzan, 1984; Deleers et al., 1985). Consequently the islet transglutaminase must be sensitive to small changes in the free $[Ca^{2+}]$ in the β-cell in order to act as a Ca^{2+}-receptor protein during β-cell activation. This requirement for the enzyme has not been substantiated by previous studies on the islet transglutaminase which have suggested that half-maximal enzyme activity may occur at Ca^{2+} concentrations of 40-90 μM (Bungay et al., 1982; 1984; Gomis et al., 1983). However, these estimates were based on assays conducted with native N,N'-dimethylcasein as the protein substrate, under which conditions the actual free $[Ca^{2+}]$ may be overestimated as a result of the binding of the metal cation to protein-bound phosphate groups (Hand et al., 1985). At low Ca^{2+} concentrations there may also be competition between Ca^{2+} and other metal ions such as Mg^{2+} for the available binding sites (phosphate groups) on the protein substrate. As a result of such competition, changes in the availability of Ca^{2+} for activation of the enzyme may result. Such competition will also present difficulties in assessing the effects of other cellular metal cations such as Mg^{2+} on the sensitivity of transglutaminase to Ca^{2+}. Under the more controlled conditions of employing dephosphorylated N,N'-dimethylcasein as the substrate, the

islet transglutaminase was found to be at least an order of magnitude more sensitive to Ca^{2+} than had been previously demonstrated, with an apparent K_m for Ca^{2+} of 2-3 μM. In addition it was found that the presence of Mg^{2+}, which is present in cells at millimolar concentrations, is unlikely to affect the sensitivity of islet transglutaminase to activation by Ca^{2+}. The sensitivity of islet transglutaminase to Ca^{2+} is comparable with that measured for the phospholipid- and Ca^{2+}- dependent protein kinase (protein kinase C) obtained from islets, which possesses an apparent K_m for Ca^{2+} of 3.9μM (Lord & Ashcroft, 1984), and from bovine heart, for which an apparent K_m for Ca^{2+} of 5μM was determined (Wise et al., 1982). The results therefore suggest that islet transglutaminase may be sufficiently sensitive to activation by Ca^{2+} for the enzyme to directly act as a receptor for Ca^{2+} in the stimulated pancreatic β-cell.

3.2. DEVELOPMENT OF SPECIFIC PHARMACOLOGICAL PROBES FOR ISLET TRANSGLUTAMINASE

This study aimed to investigate the possible involvement of transglutaminase in the mechanism of glucose-stimulated insulin release by investigating whether specific inhibitors of transglutaminase, as judged by investigations carried out *in vitro*, were inhibitors of glucose-stimulated insulin release from the pancreatic β-cell. The problem with previous investigations of this nature was the lack of defined specificity of the pharmacological 'probes' employed.

Many of the compounds selected for the study had already been implicated as competitive inhibitors/substrates of transglutaminase activity (Lorand *et al.*, 1979). Additional compounds tested were modifications of these compounds tested by Lorand in order to improve both the potency and specificity of the compounds in inhibiting transglutaminase activity.

Initial investigations examined the potency of the test compounds on the activity of transglutaminase by an *in vitro* assay. These test compounds were subsequently investigated for their inhibitory effect on glucose-stimulated insulin release. Potentially promising test compounds were then checked for their specificity by testing their effect on other possible intracellular targets whose inhibition may also result in the inhibition of glucose-stimulated insulin release.

3.2.1. DETERMINATION OF THE POTENCY OF TEST COMPOUNDS FOR THE INHIBITION OF TRANSGLUTAMINASE ACTIVITY

A range of test compounds, the majority of which contained an available primary amine group, were screened for their effect on the activity of transglutaminase. During the course of the study the mode of screening used for these test compounds was varied slightly to improve upon the efficiency of the screening process.

Initial studies involved the screening of test compounds for their effect on rat liver transglutaminase activity. This approach was undertaken because the rat liver enzyme was more readily available and because, as discussed later in this section, it was found to be very similar in its catalytic properties to the enzyme found in the rat pancreatic islet.

The effect of the test compounds on rat liver transglutaminase activity is presented in Figures 3.2a, 3.3a and 3.4a. From these plots the mean apparent Km for putrescine incorporation into N,N'-dimethylcasein for the rat liver transglutaminase was found to be 0.31 ± 0.04μM (n = 3). The average V_{max} value for putrescine incorporation into N,N'-dimethylcasein was 148.4 ± 15.6 nmol putrescine incorporated/h per mg protein (approx.).

All these plots were found to intersect the $1/V_o$ axis at $1/V_{max}$, indicating that MDCD, 2-NSA and tosyl-SA were all acting as competitive inhibitors/substrates of transglutaminase activity under the conditions of the assay.

From the secondary plots constructed of the calculated Km' against the concentration of inhibitor (Figures 3.2b, 3.3b and 3.4b) the most potent inhibitor of rat liver transglutaminase activity was found to be MDCD with a calculated K_i value of 4.8μM. The K_i values calculated for tosyl-SA and 2-NSA were 16μM and 32μM respectively, indicating them to be less potent than MDCD.

Figures 3.2a and b, 3.3a and b, 3.4a and b.

Inhibition of rat liver transglutaminase activity by MDCD, 2-NSA and tosyl-SA. Calculation of K_i values using secondary plots of the Lineweaver-Burk graph.

Rat liver extracts (71,000g supernatants) were assayed for transglutaminase activity as described in Section 2.5 in the presence of varying concentrations of [^{14}C]-putrescine (1.2, 0.9 and 0.6 mM) and of the test compound as indicated in the Figures. The test compounds were prepared in 50mM Tris, pH 7.4 at 37^{o}C, and serially diluted in the same buffer. Initial rates of putrescine incorporation into N,N'-dimethylcasein (5mg/ml) were calculated following the subtraction of background counts which were obtained from samples incubated in the presence of 5mM EDTA. Reciprocal plots of initial velocity, V_o, (nmol putrescine incorporated/h) against substrate concentration, S_o, (mM putrescine) were constructed for each concentration of test compound.

Key to symbols used in figures:

Figure 3.2a	concentration of MDCD	5μM ●
		10μM ◆
		20μM ▼
Figure 3.3a	concentration of 2-NSA	10μM ●
		20μM ◆
		50μM ▼
Figure 3.4a	concentration of tosyl SA	20μM ●
		50μM ◆
		70μM ▼

▲ represents the control system without addition of test compound.

From these reciprocal plots the V_{max} value was calculated from the intercept on the $1/V_o$ axis, and the apparent K_m, K_m', was calculated from the point of intercept of each graph with the $1/S_o$ axis which gave the $\bar{K}_m'$ value.

Secondary plots were then made for MDCD, 2-NSA and tosyl-SA of the K_m' value against the concentration of inhibitor: this data is presented in Figures 3.2b, 3.3b and 3.3c respectively. The point of intercept of these plots on the inhibitor concentration axis was taken as the $\bar{K}_i$ value.

Figure 3.2a

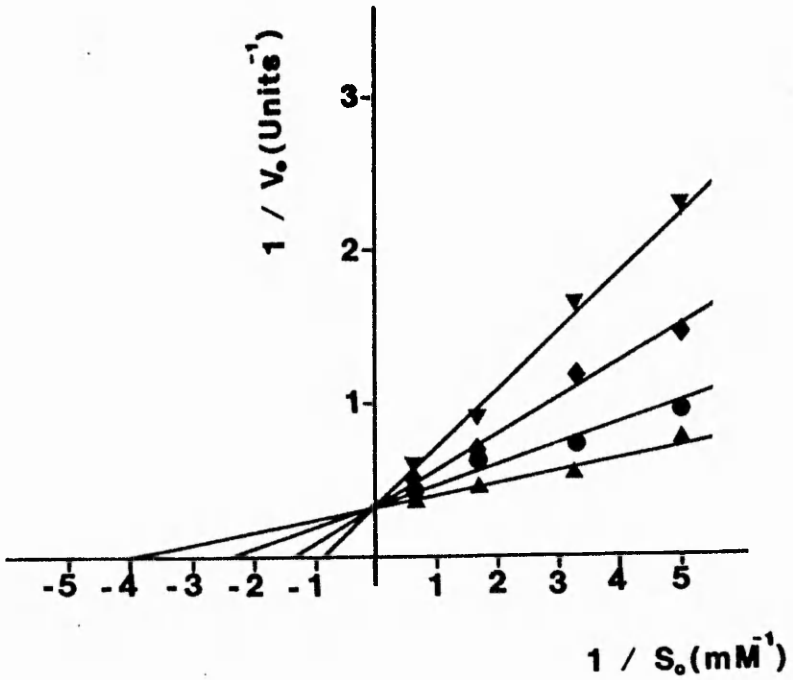


Figure 3.2b

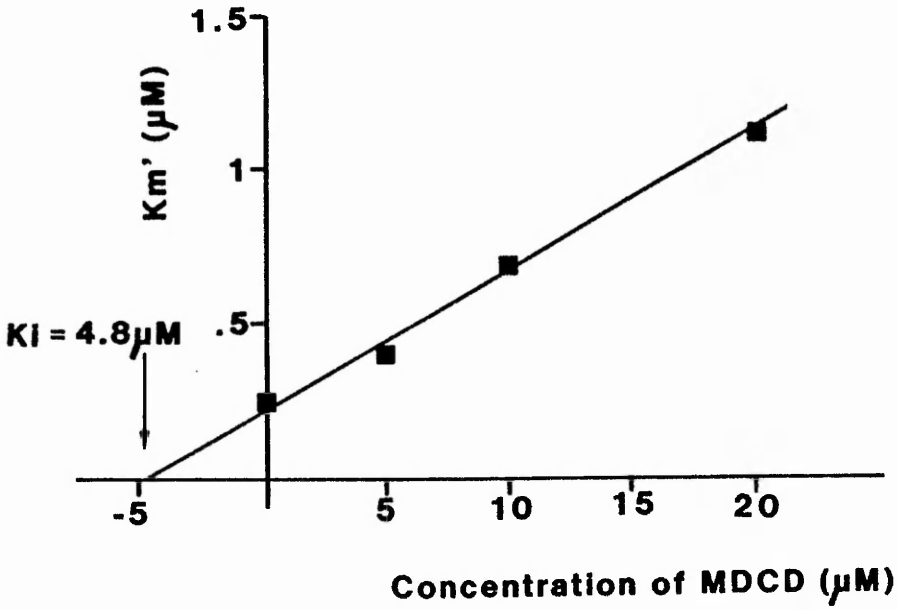


Figure 3.3a

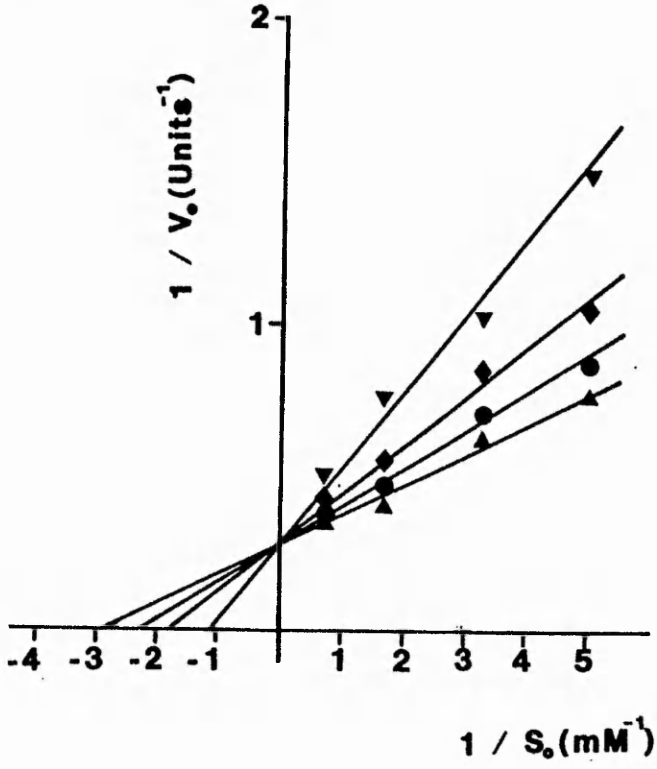


Figure 3.3b

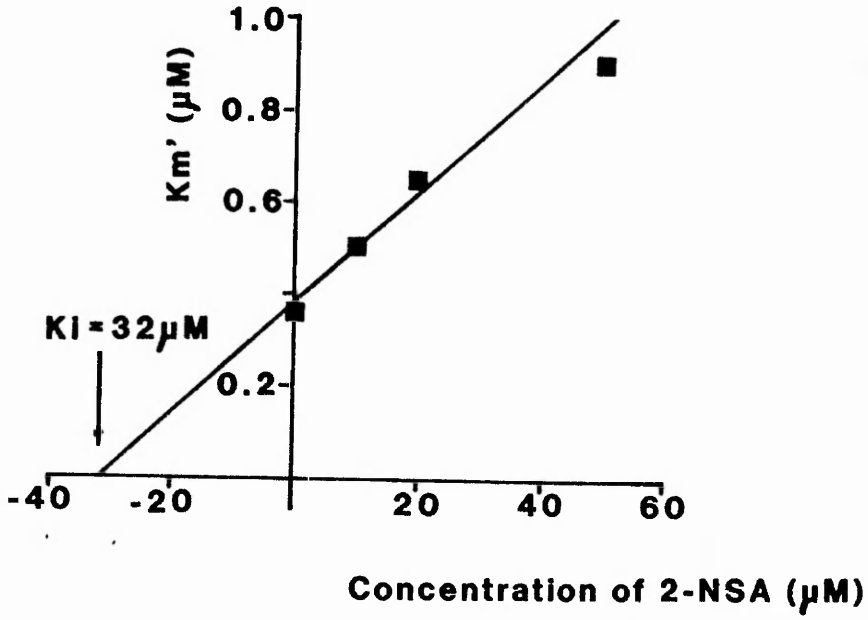


Figure 3.4a

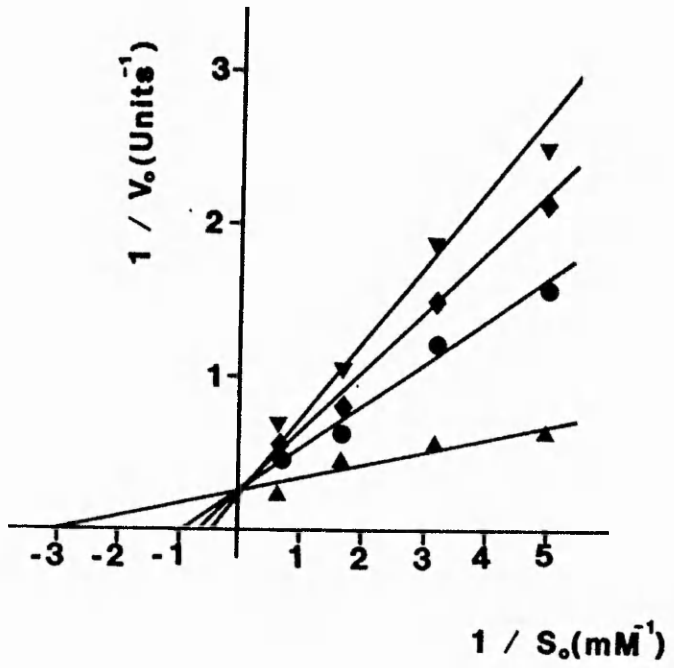
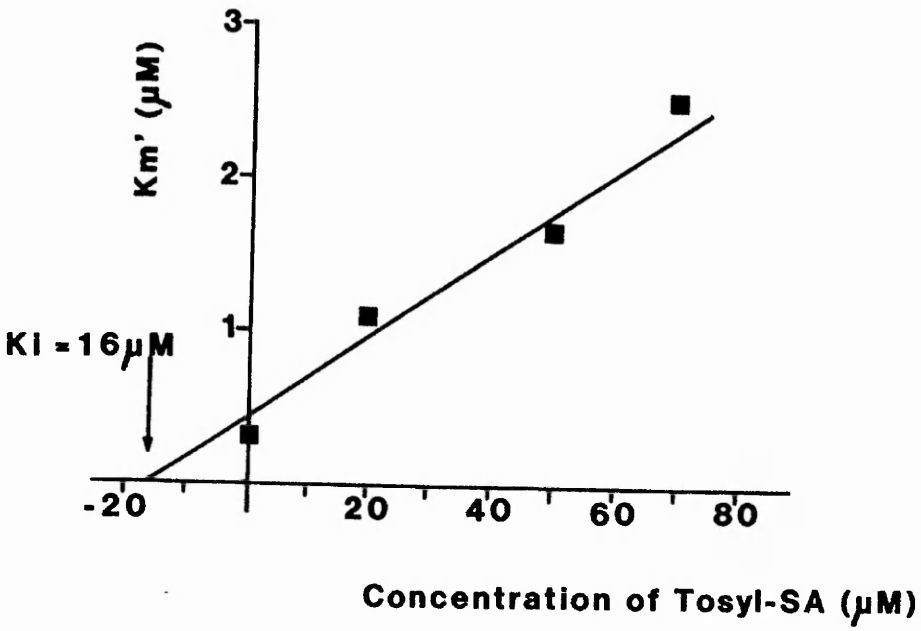


Figure 3.4b



During the course of this study, owing to the large number of test compounds to be assayed, it was decided to modify the mode of assessment of the potency of the compounds as inhibitors of transglutaminase activity. Because of the ready availability of rat liver transglutaminase it was decided to employ the rat liver enzyme as the initial test enzyme. Those compounds which proved to be potent inhibitors of rat liver transglutaminase were subsequently tested by the same procedure for their inhibitory effect on the activity of rat pancreatic islet transglutaminase. The modified procedure involved testing the effect of a range of concentrations of the compound on transglutaminase activity (as described in the legend to Figures 3.5 - 3.19) and expressing this effect as a percentage of the control transglutaminase activity in the absence of any inhibitor.

From these plots the concentration of test compound that resulted in a 50% inhibition of transglutaminase activity (IC_{50} value), and the concentration of test compound that resulted in half-maximal inhibition ($[I]_{0.5}$) were determined, and the inhibition constant K_i calculated according to the method of Davies et al. (1980). These values are summarised in Table 3.1. The chemical structure of the respective test compounds are presented in Table 3.2.

Of the compounds tested MDCD was found to be one of the most potent inhibitors (competitive substrate) of transglutaminase activity (IC_{50} = 43μM, K_i = 9.8μM for the islet enzyme). Confirmation that this was due to a competitive effect by the primary amine group of the molecule was indicated by the lack of inhibition shown by the dimethylated control analogue, dimethyl-MDCD.

Figures 3.5a and b - 3.19a and b.

Effect of test compounds on rat liver and rat pancreatic islet transglutaminase activity.

Extracts from rat liver and rat pancreatic islets (71,000g supernatants) were assayed for their transglutaminase activity in the presence of a range of concentrations of each compound, (as detailed in the respective Figures), using a constant N,N'-dimethylcasein concentration of 5mg/ml and a constant [^{14}C]putrescine concentration of 1.2mM as described in Section 2.5.

The degree of inhibition of transglutaminase activity in the presence of each concentration of test compound was then determined by comparison to control values obtained in the absence of any test compound. Plots were then made of the percentage inhibition of transglutaminase activity (rat liver or rat pancreatic islet as indicated in the Figures) against the $\log_{10}$ of the concentration of test compound. Typical plots for each compound tested are presented in Figures 3.5a - 3.19a. Each point represents the mean (±S.E.) of three separate tests within the same experiment.

From these plots the concentration of test compound that resulted in a 50% inhibition of transglutaminase activity (IC_{50} value) was determined.

Secondary, Eadie-Hofstee, plots were constructed by plotting the percentage inhibition against the percentage inhibition/[compound]. Typical plots are presented in Figures 3.5b - 3.19b for the test compounds indicated. From these figures the concentration of test compound which resulted in half-maximal inhibition ([I] 0.5 value) was determined as the gradient of the plotted line.

These [I]0.5 values were then used to calculate the inhibition constant, K_i, for each of the test compounds on rat liver or rat pancreatic islet transglutaminase by substitution into the equation

$$K_i = \frac{[I]_{0.5}}{1 + (S/K_m)}$$ (Davies et al., 1980)

; where S = concentration of putrescine in the assay (1.2mM)

K_m = Michaelis-Menton constant for putrescine;

= 0.31mM for rat liver transglutaminase as determined earlier in this section;

= 0.33mM for rat pancreatic islet transglutaminase (Bungay, 1984).

Figure 3.5a **Effect of MDCD on rat liver transglutaminase**

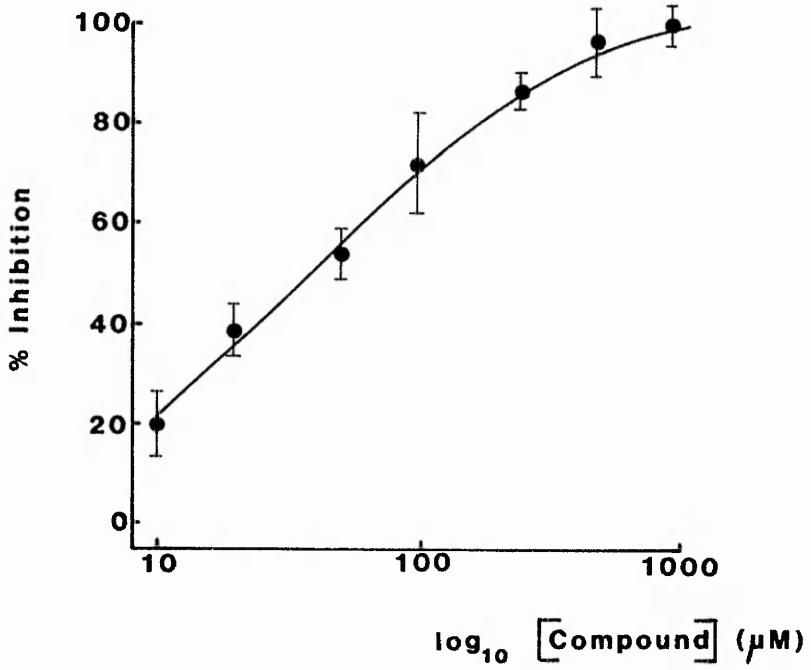


Figure 3.5b

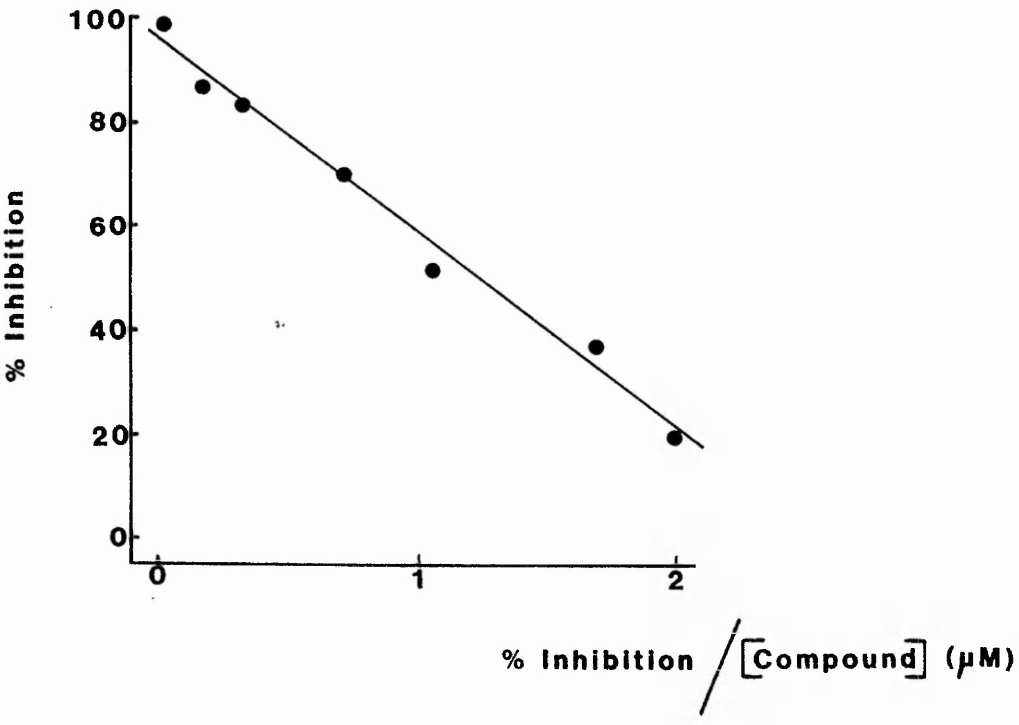


Figure 3.6a Effect of 1-NSA on rat liver transglutaminase

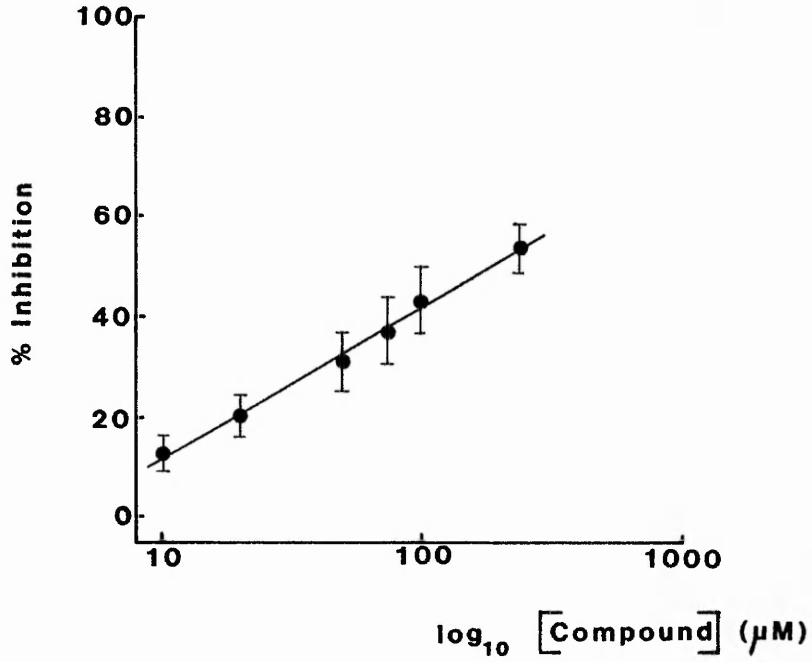


Figure 3.6b

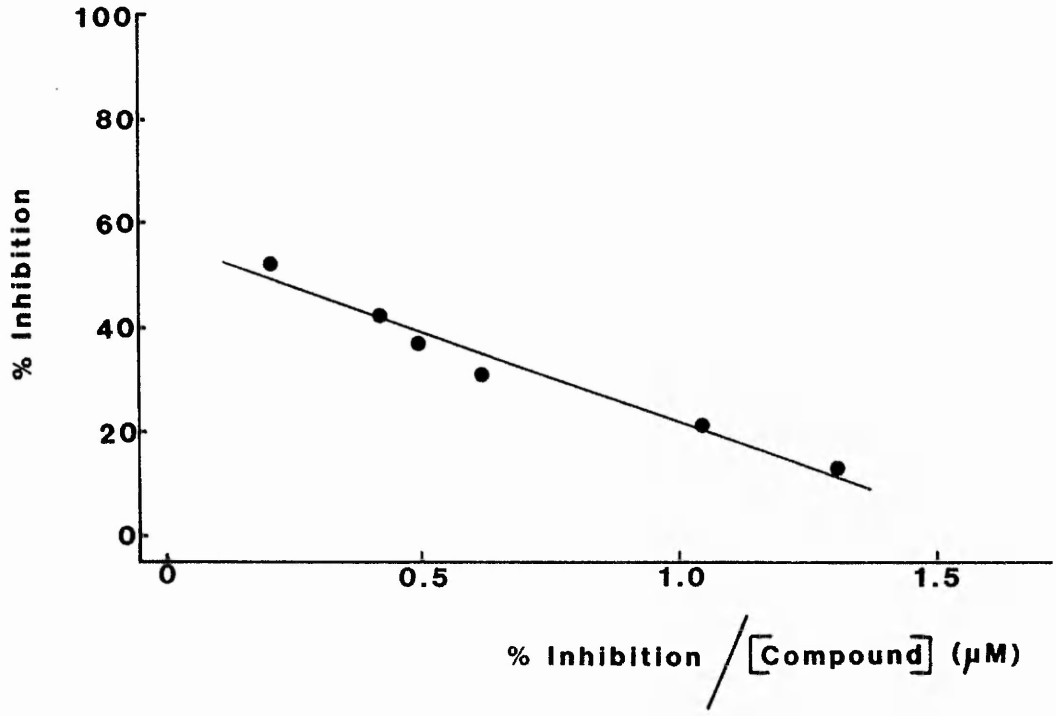


Figure 3.7a Effect of 2-NSA on rat liver transglutaminase

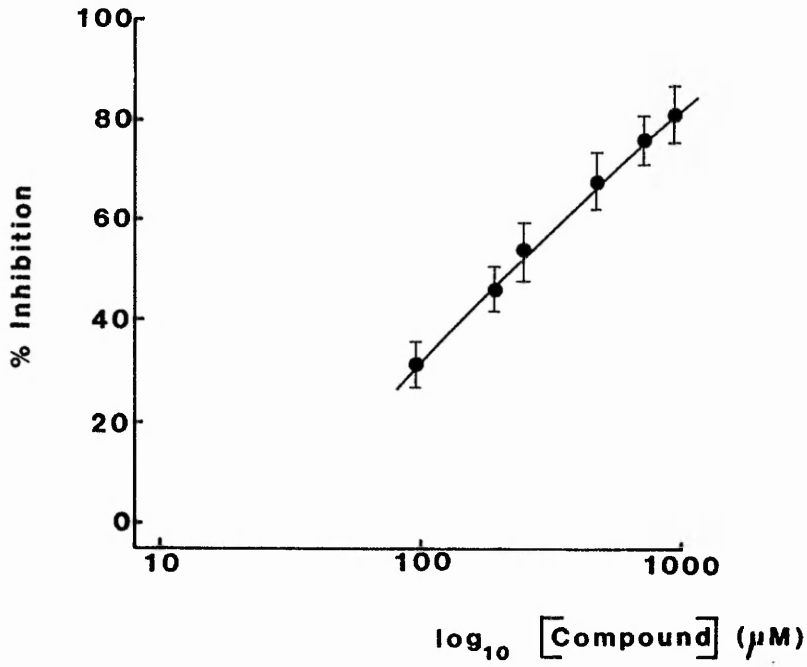


Figure 3.7b

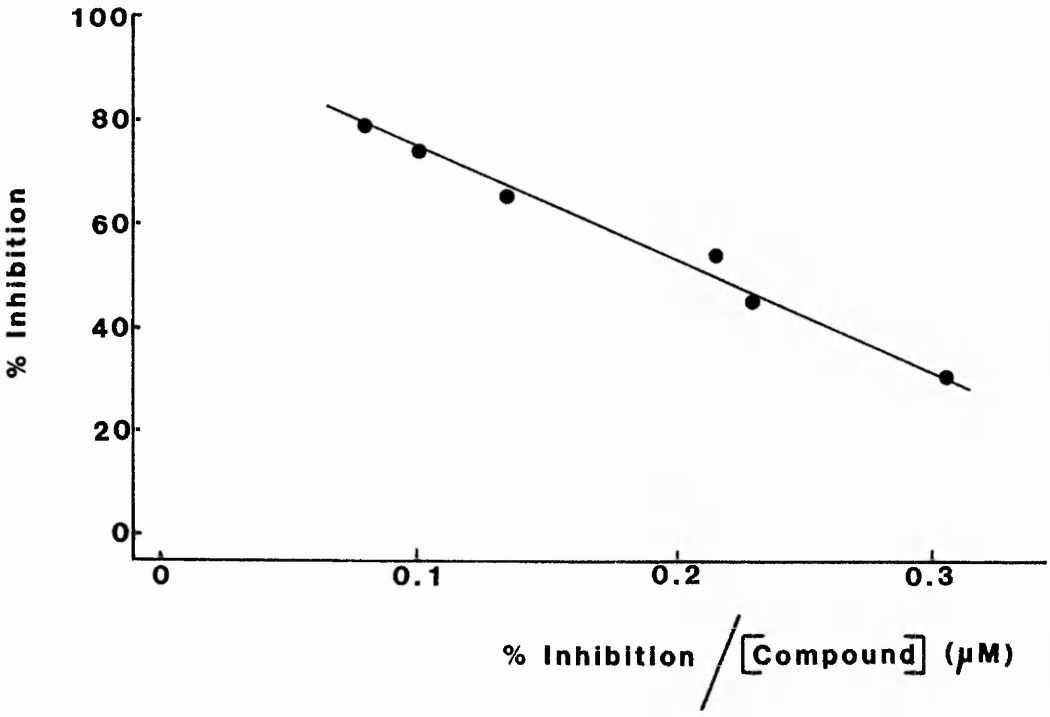


Figure 3.8a Effect of 1-NCA on rat liver transglutaminase

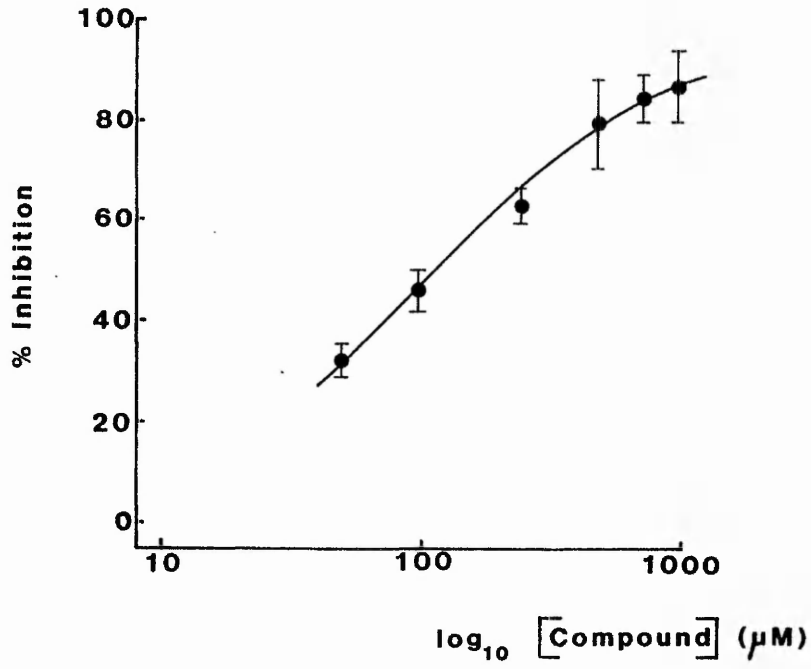


Figure 3.8b

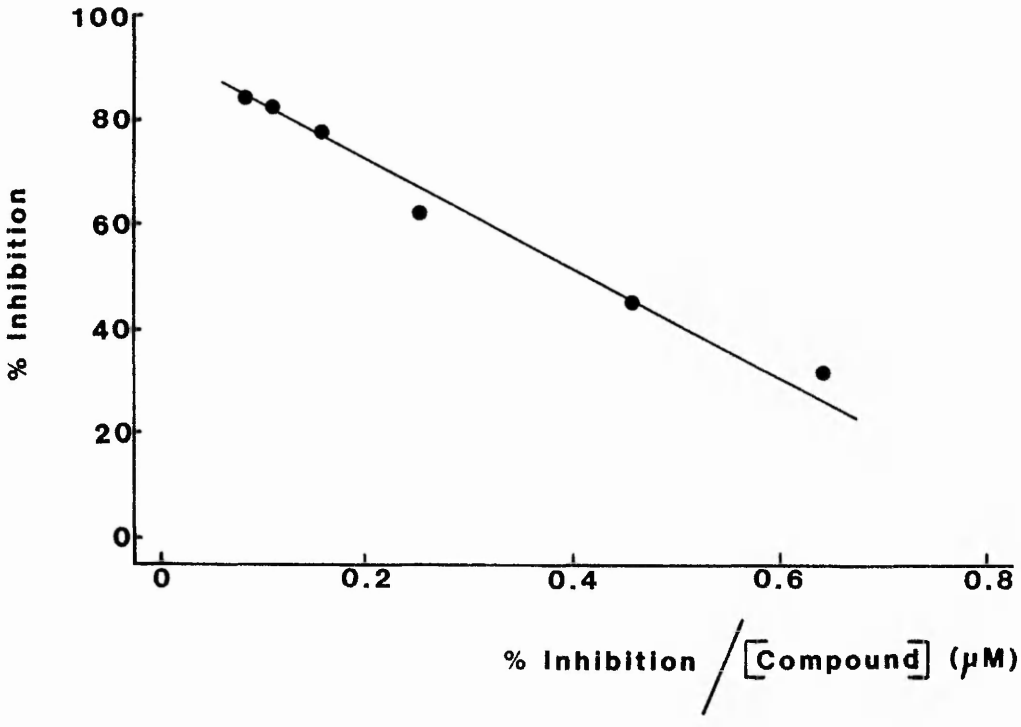


Figure 3.9a Effect of TMB-SA on rat liver transglutaminase

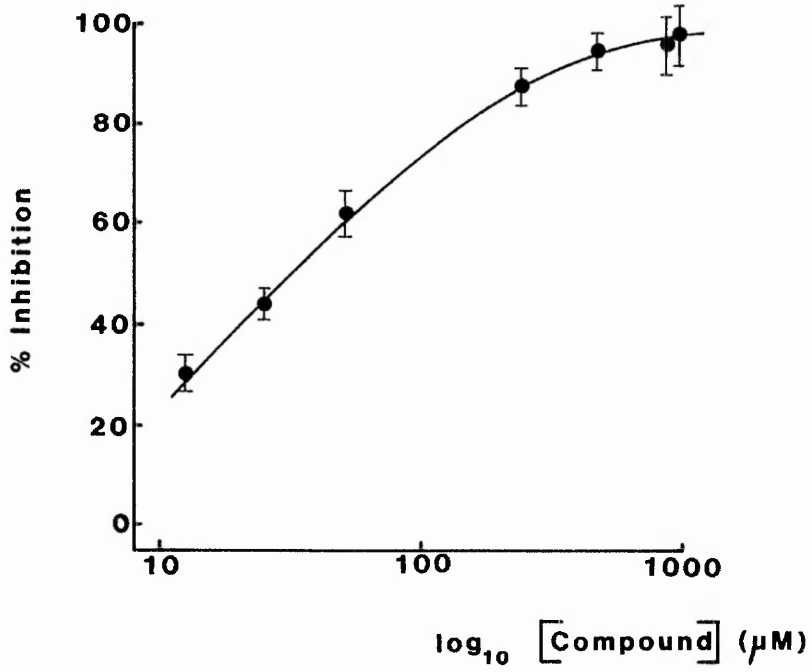


Figure 3.9b

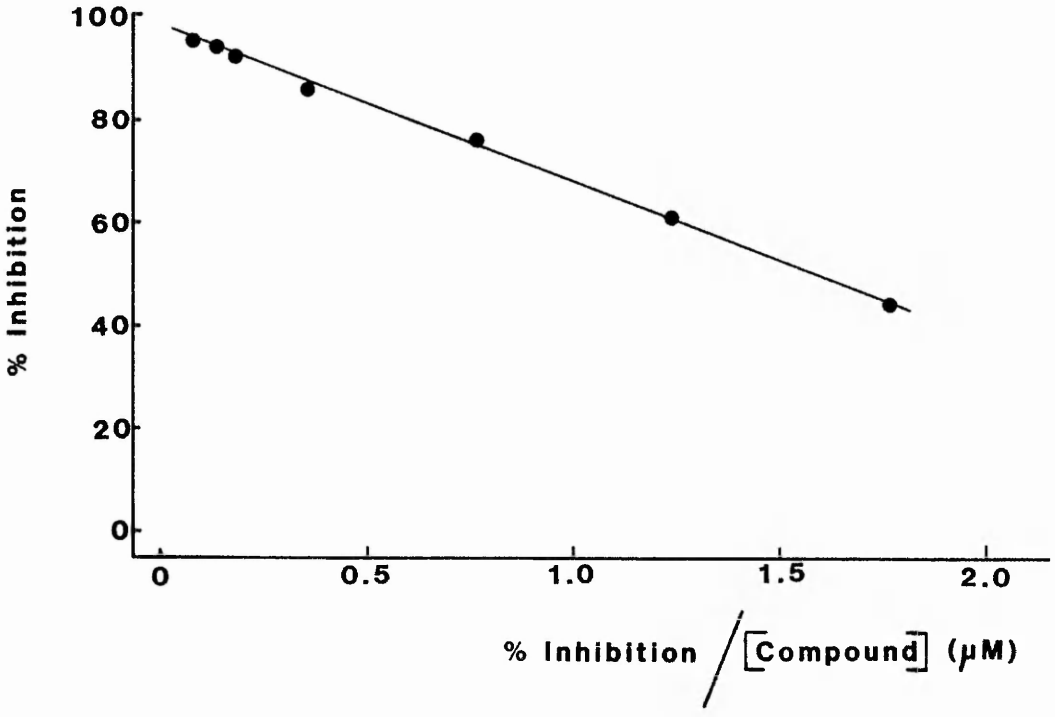


Figure 3.10a Effect of Tosyl-SA on rat liver transglutaminase

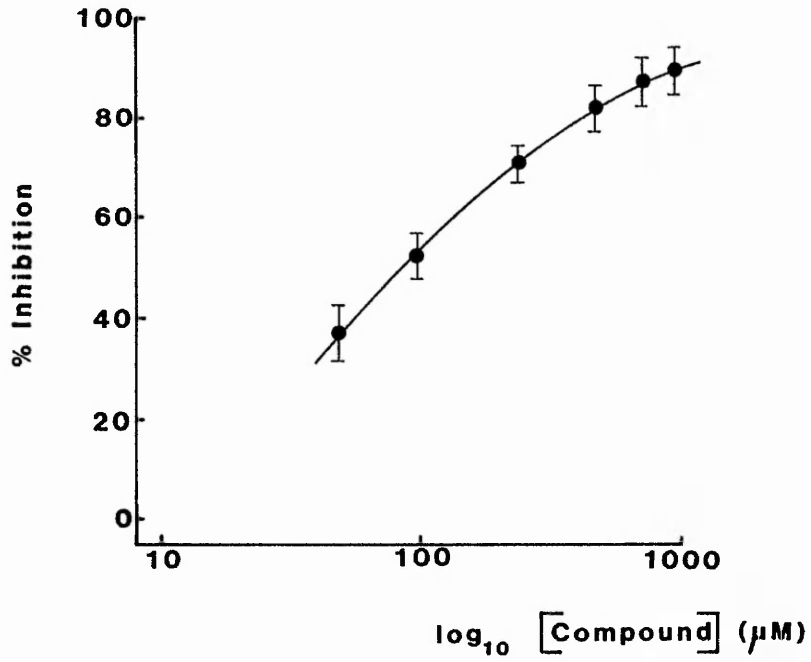


Figure 3.10b

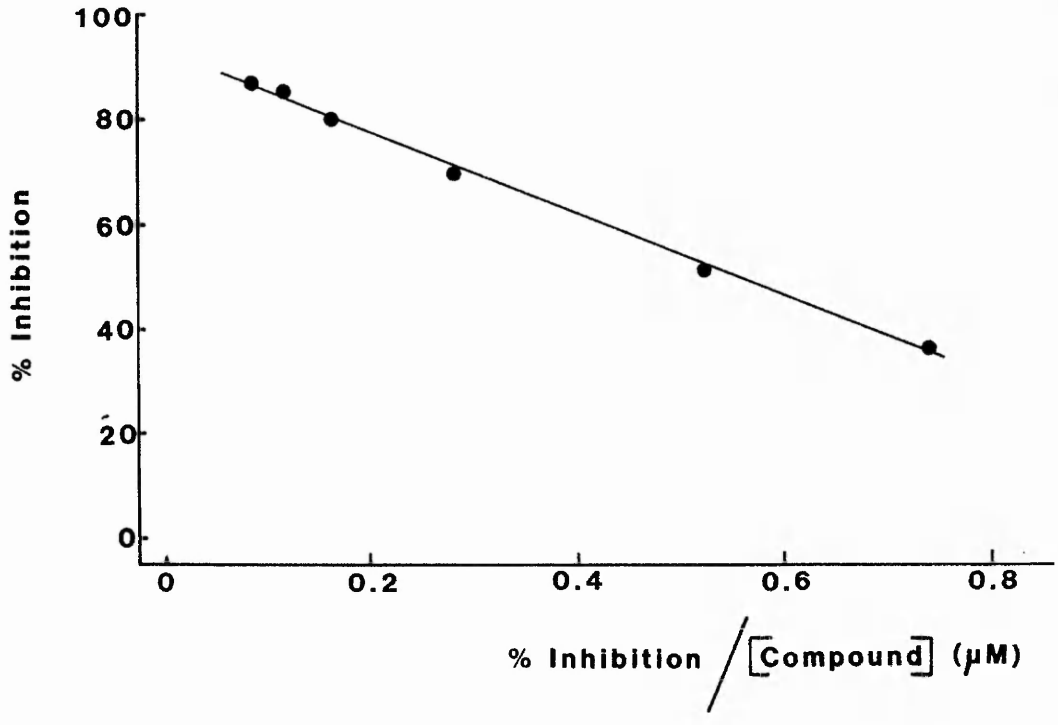


Figure 3.11a Effect of Bromobenzyl-SA on rat liver transglutaminase

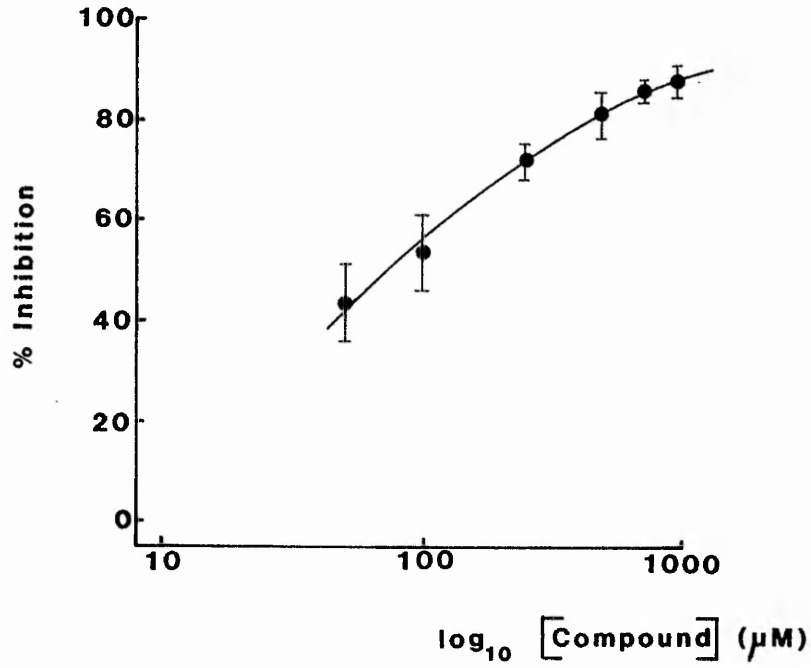


Figure 3.11b

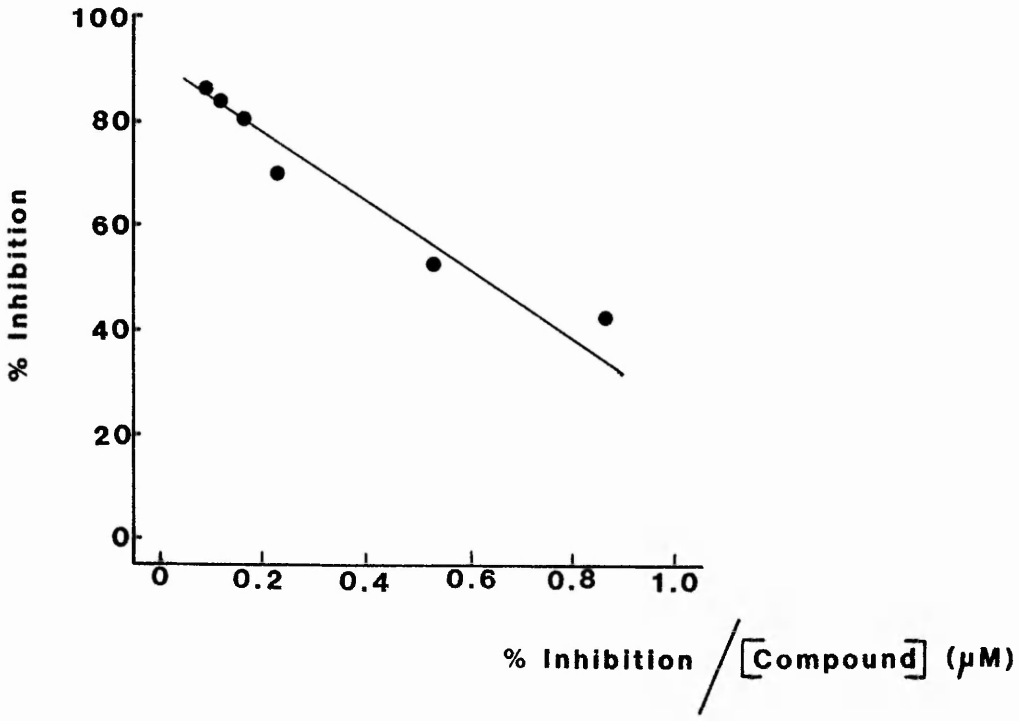


Figure 3.12a **Effect of W7 on rat liver transglutaminase**

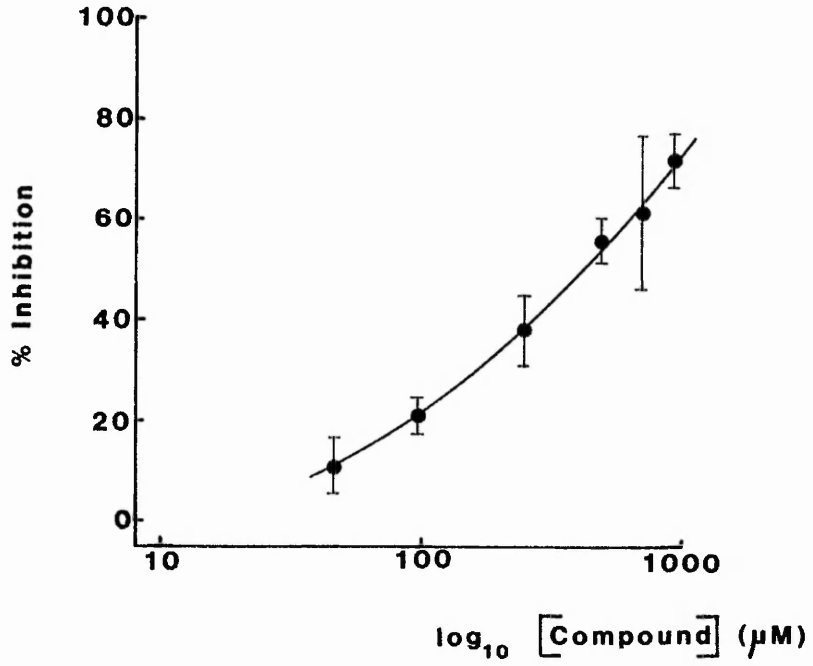


Figure 3.12b

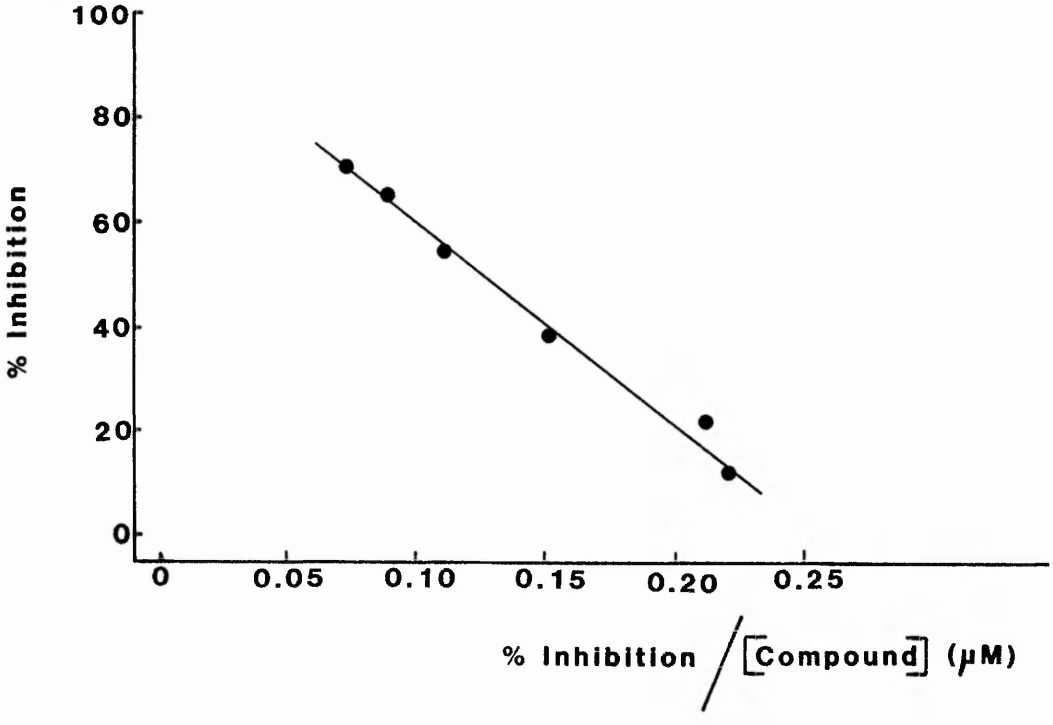


Figure 3.13a Effect of ● C_6-Iodo-W7 & ■ C_8-Iodo-W7 on rat liver transglutaminase

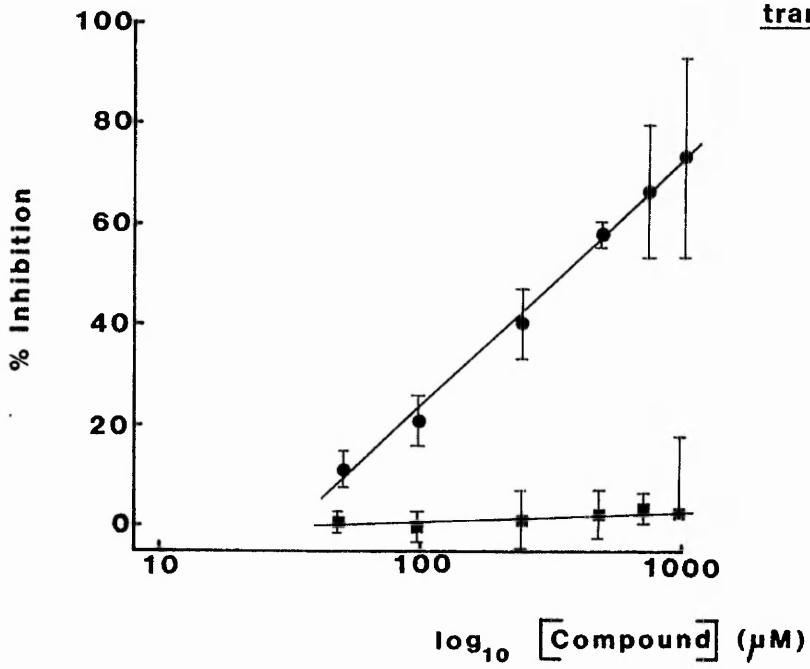


Figure 3.13b

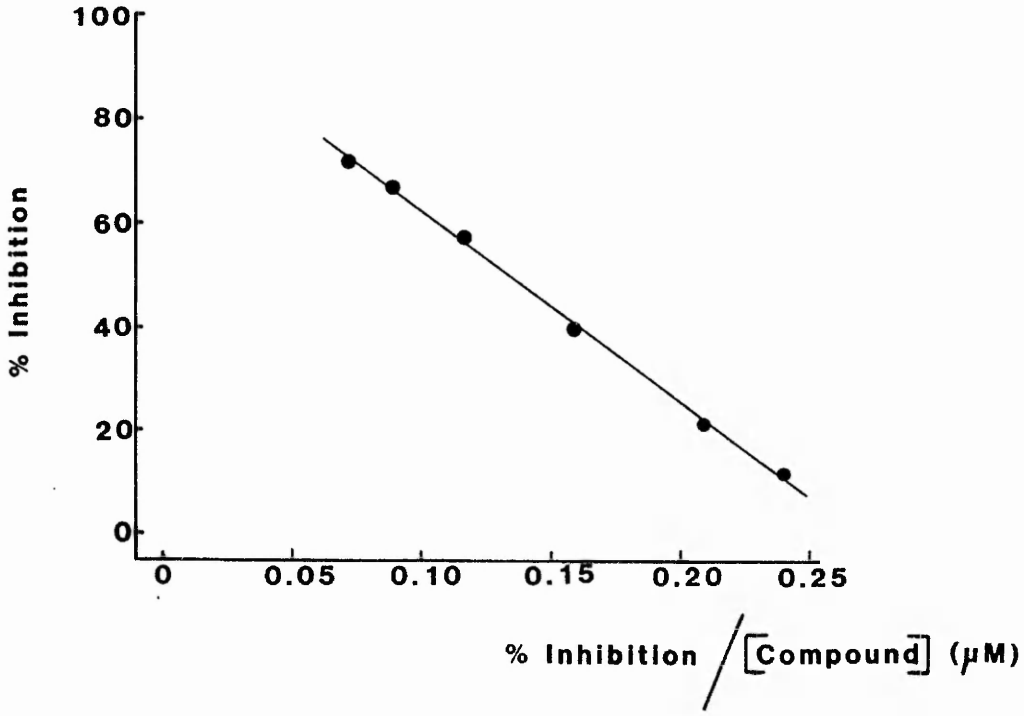


Figure 3.14a Effect of TFMB-SA on rat liver transglutaminase

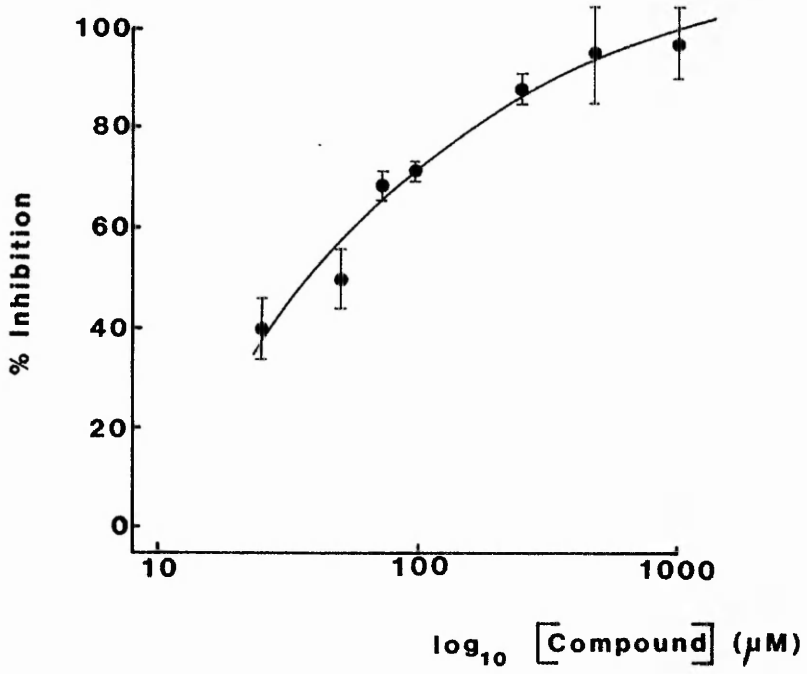


Figure 3.14b

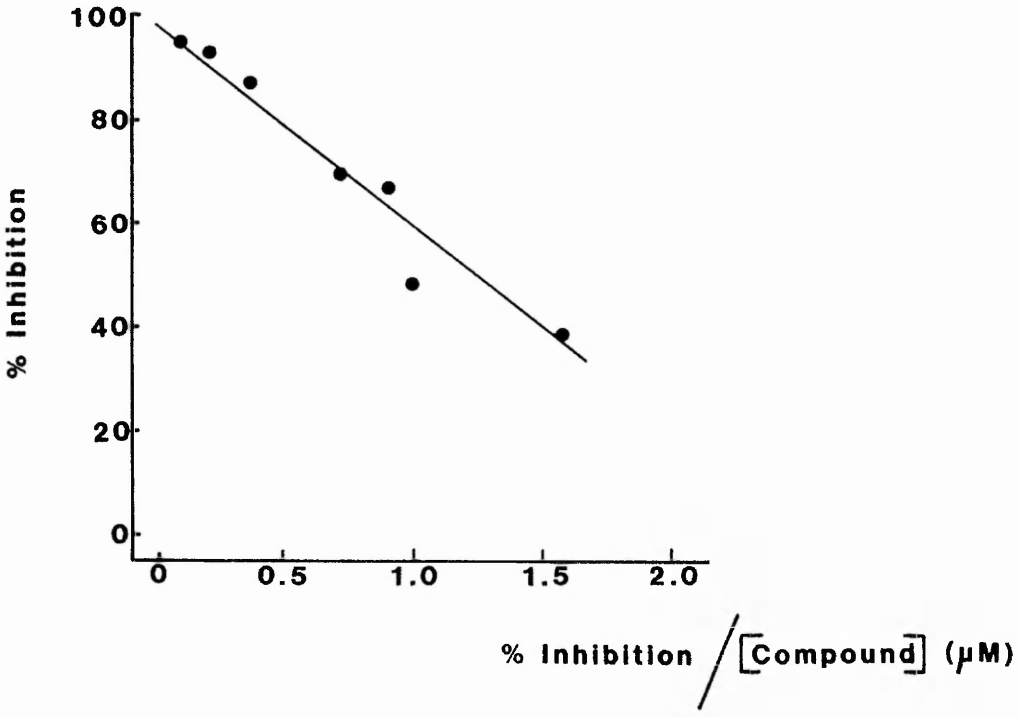


Figure 3.15a Effect of ● MDCD & ■ diMe-MDCD on rat pancreatic islet transglutaminase

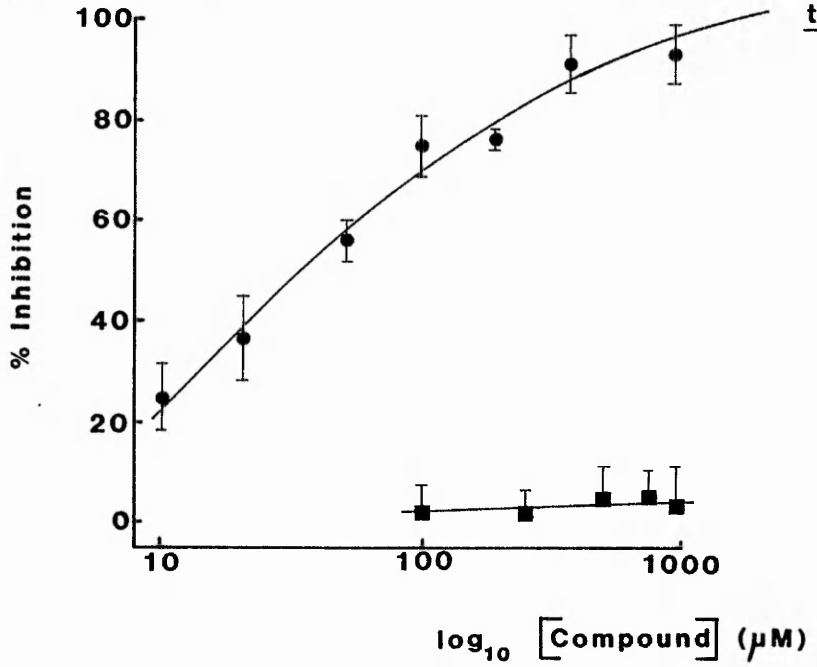


Figure 3.15b

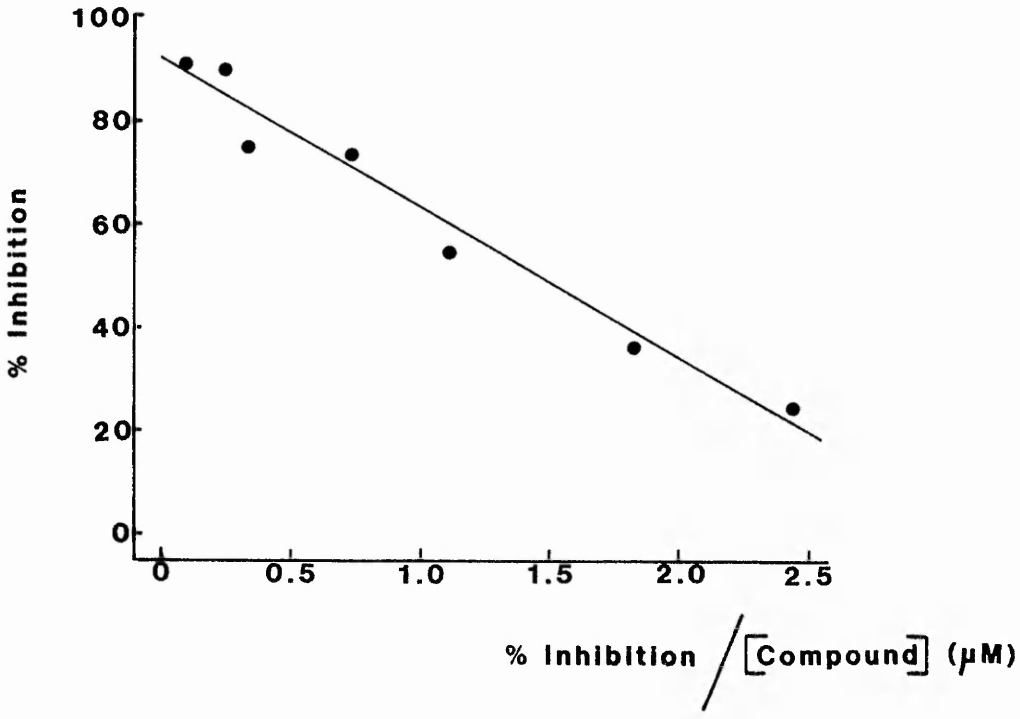


Figure 3.16a **Effect of 1-NSA on rat pancreatic islet transglutaminase**

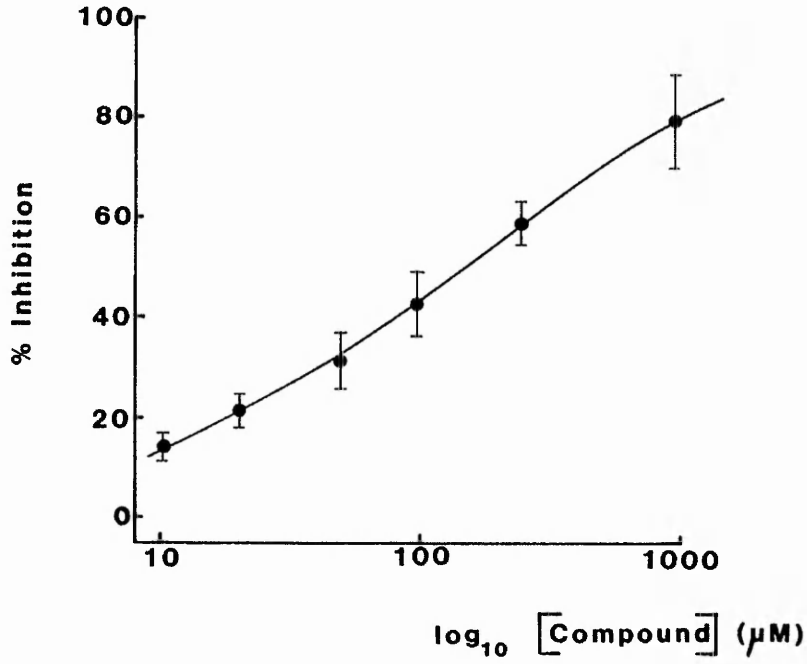


Figure 3.16b

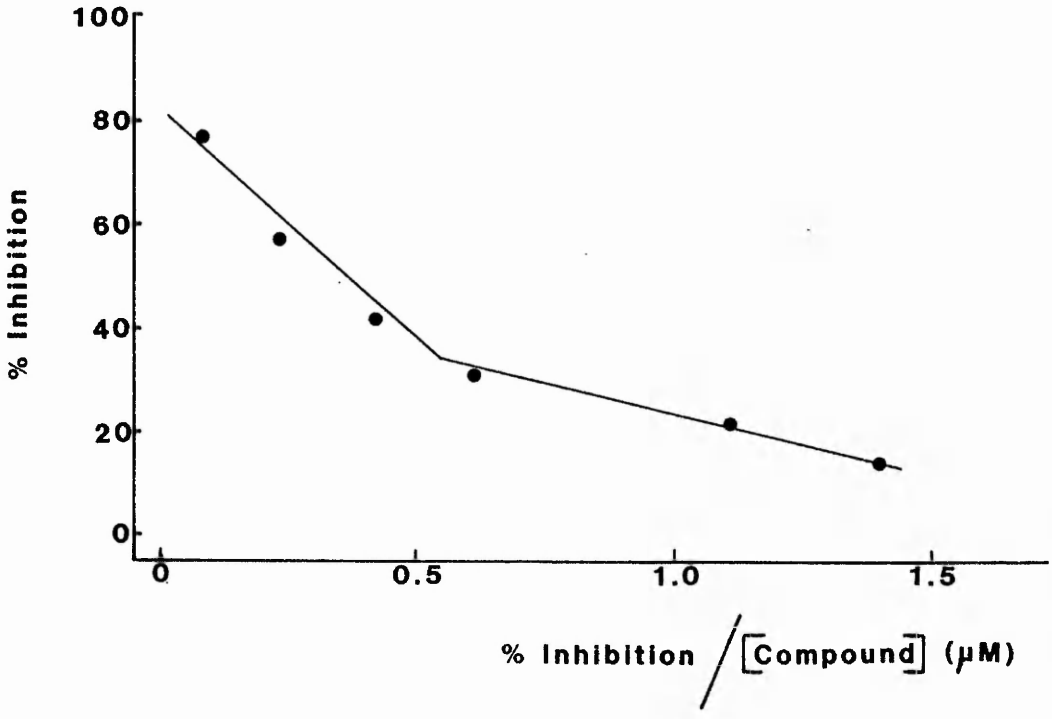


Figure 3.17a Effect of ● 2-NSA & ■ diMe-2-NSA on rat pancreatic islet transglutaminase

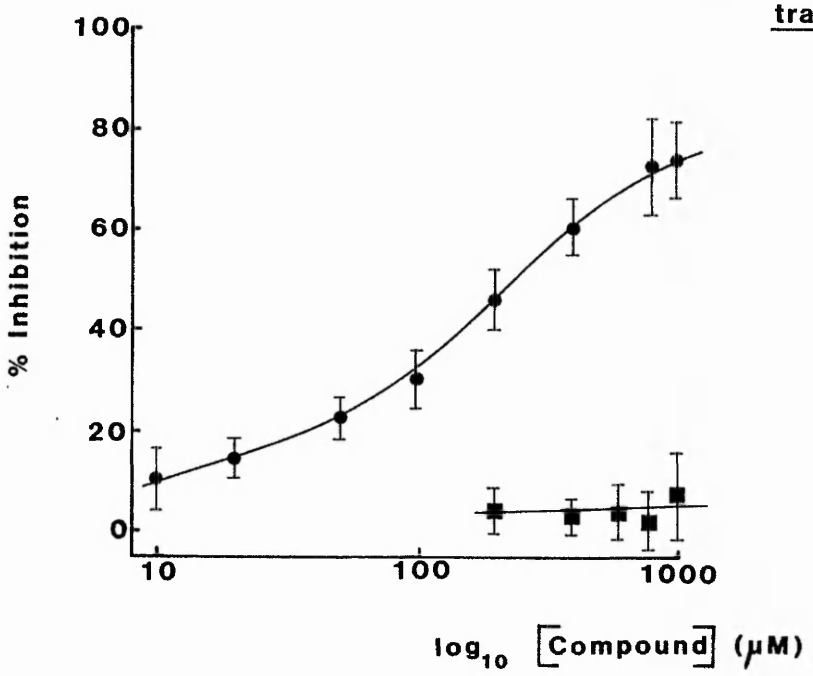


Figure 3.17b

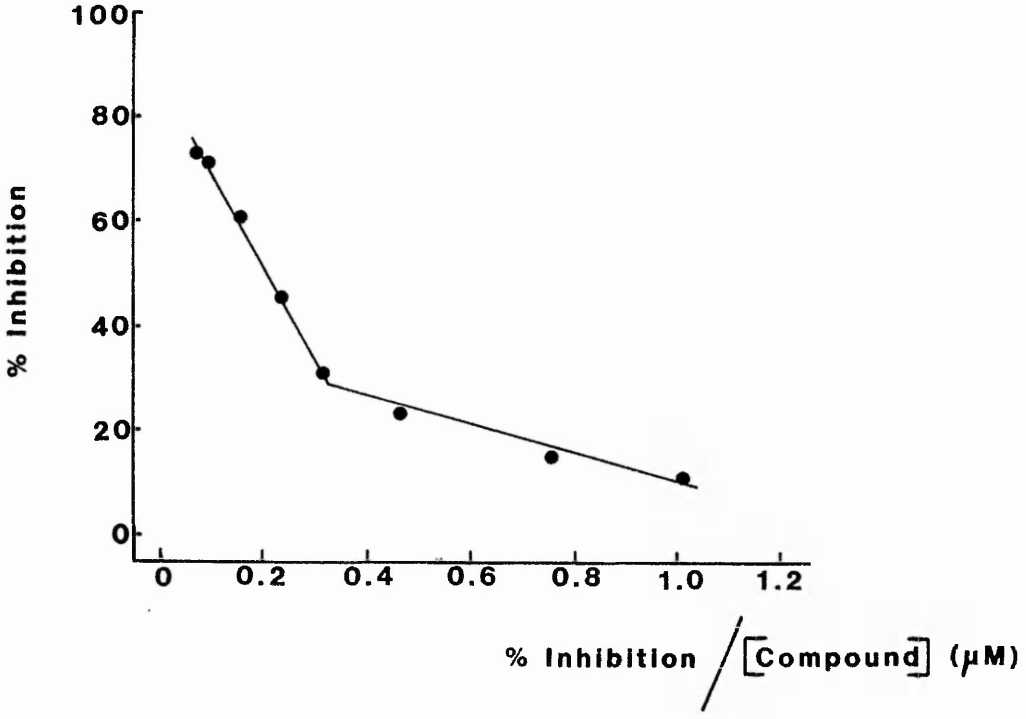


Figure 3.18a Effect of TMB -SA on rat pancreatic islet transglutaminase

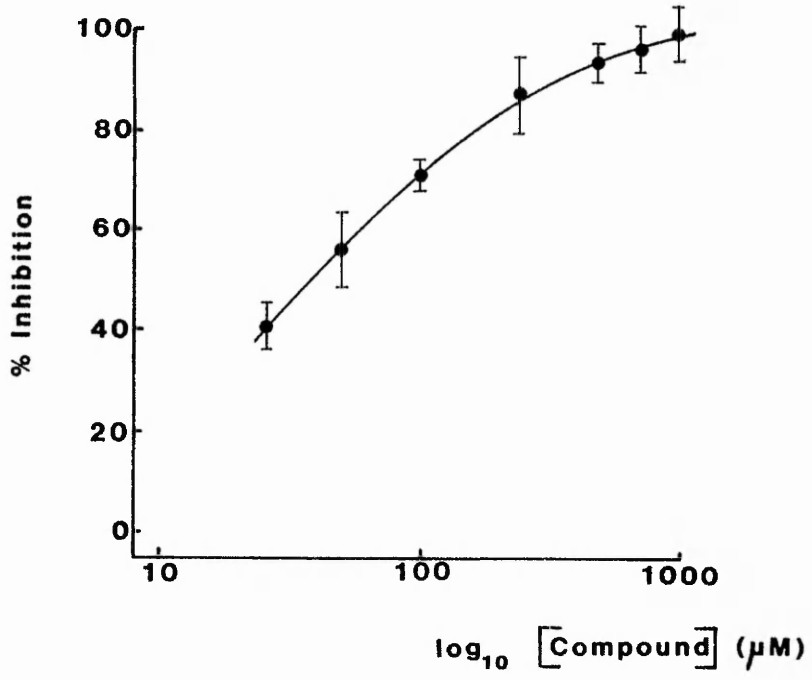


Figure 3.18b

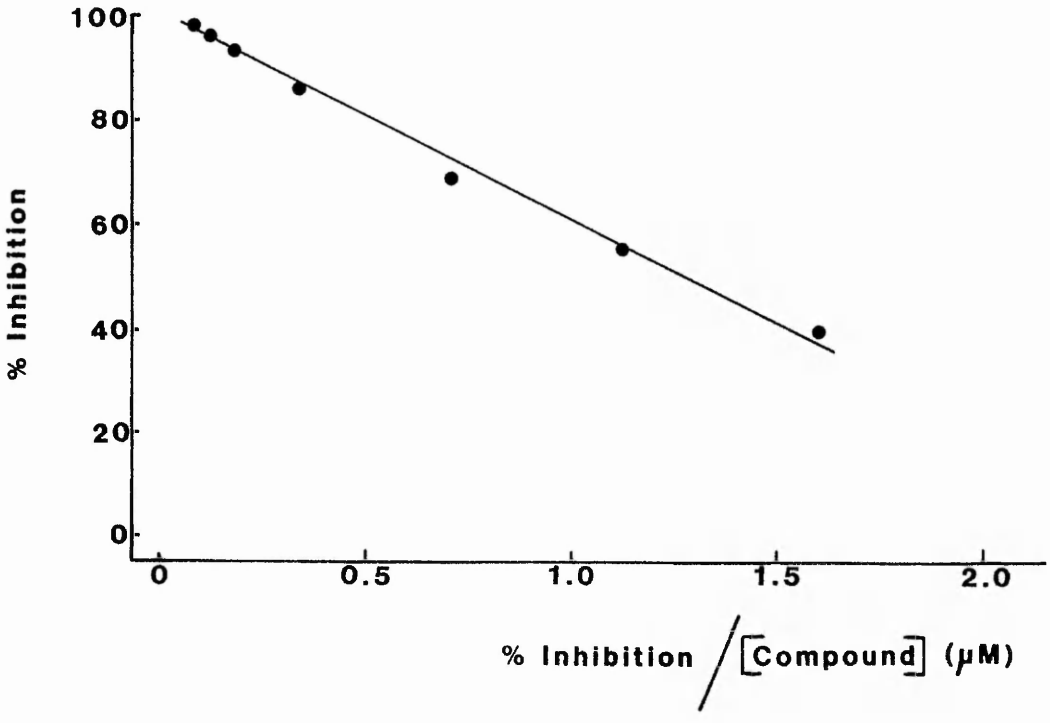


Figure 3.19a Effect of TIP-SA on rat pancreatic islet transglutaminase

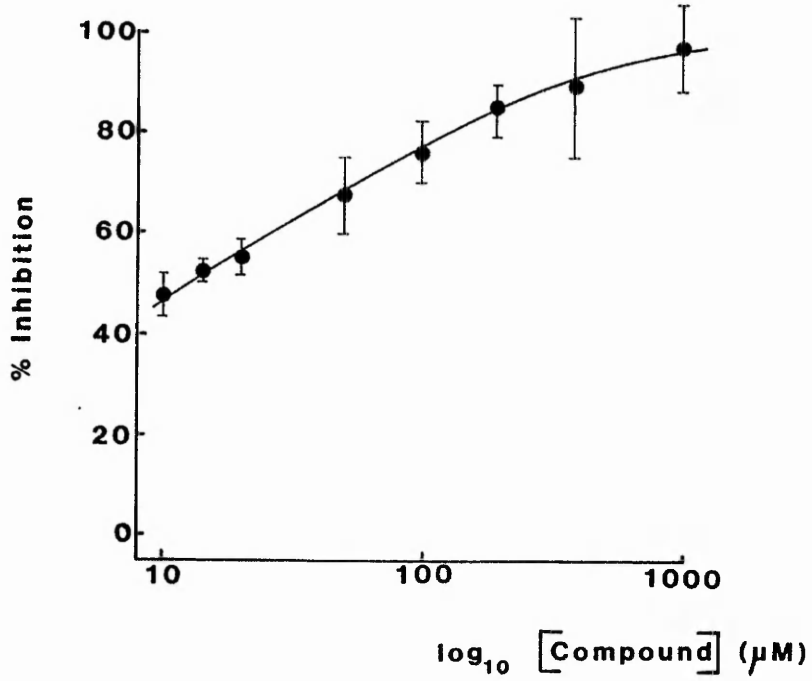


Figure 3.19b

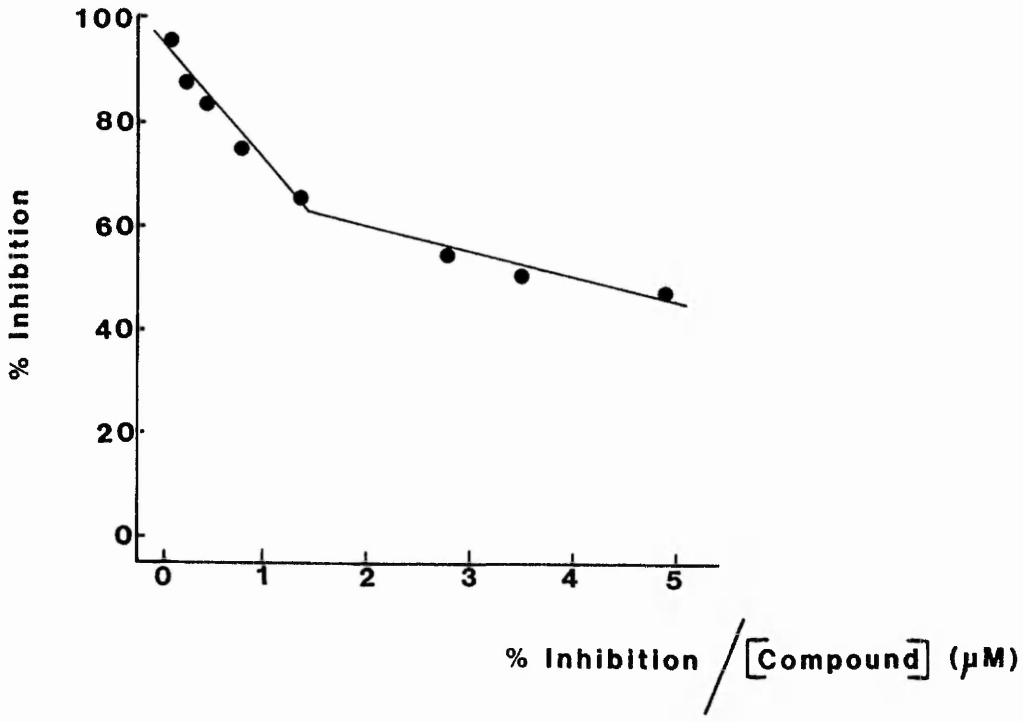


Table 3.1.

Summary of effect of test compounds on transglutaminase activity

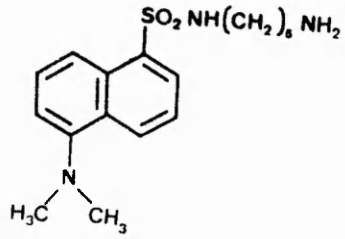
The table represents the mean IC_{50} and K_i values obtained from repeat experiments of the form exemplified in Figs. 3.5. - 3.19. The data is from 3 replicate experiments unless otherwise indicated in parentheses. Where 50% inhibition was not obtained, the % inhibition obtained at the highest concentration tested is indicated in parentheses. TFP = trifluoroperazine.

Table 3.1.

Drug	Rat liver enzyme		Pancreatic islet enzyme	
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
MDCD	40 ± 9	7.6 ± 1.7	43 ± 7	9.8 ± 1.5
diMeMDCD	>1mM (8%) (2)	-	>1mM (6%) (2)	-
1-NSA	162 ± 19	16.8 ± 1.9	171 ± 15	17.6 ± 1.5
2-NSA	215 ± 11	36.5 ± 1.8	238 ± 27	62.5 ± 7.1
diMe-2-NSA	>1mM (11%) (11%) (2)	-	>1mM (10%) (2)	-
1-NCA	117 ± 34	24.1 ± 7.0	-	-
TMB-SA	31 ± 5	6.4 ± 1.0	39 ± 7	8.2 ± 1.4
TIP-SA	-	-	20 ± 2	2.5 ± 0.3
Tosyl-SA	89 ± 13	15.5 ± 2.3	-	-
Bromobenzyl-SA	72 ± 15	15.4 ± 3.2	-	-
TFMB-SA	>0.5mM (11%) (2)	-	-	-
W7	362 ± 93	73.9 ± 18.9	-	-
C_6-Iodo-W7	398 ± 71	76.6 ± 13.7	-	-
C_8-Iodo-W7	>1mM (5%) (2)	-	-	-
TFP	>1mM (10%) (2)	-	-	-

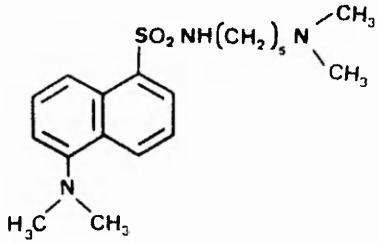
Table 3.2.

Chemical structure of test compounds.



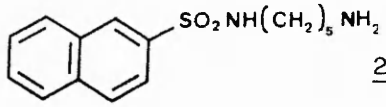
MDCD : monodansylcadaverine

: 5-dimethylamino-N-(5-aminopentyl)-naphthalene-1-sulphonamide.

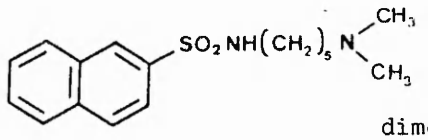


dimethyl-MDCD : dimethyl-monodansylcadaverine

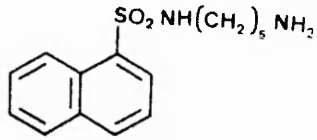
: 5-dimethylamino-N-dimethyl-(5-aminopentyl)-naphthalene-1-sulphonamide.



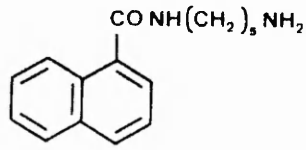
2-NSA : N-(5-aminopentyl)-naphthalene-2-sulphonamide.



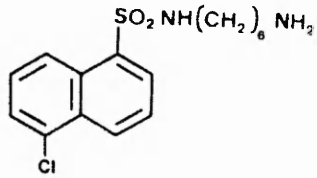
dimethyl-2-NSA : N-dimethyl-(5-aminopentyl)-naphthalene-2-sulphonamide.



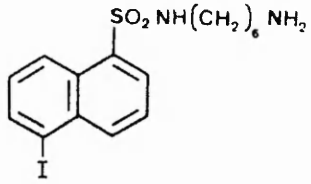
1-NSA : N-(5-aminopentyl)-naphthalene-1-sulphonamide.



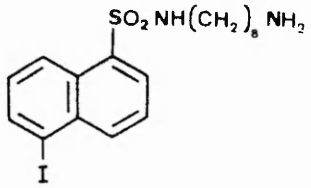
1-NCA : N-(5-aminopentyl)-naphthalene-1-carboxyamide.



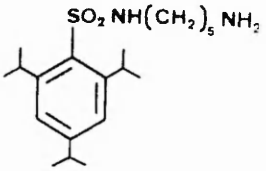
W7 : N-(6-aminohexyl)-5-chloro-naphthalene-1-sulphonamide.



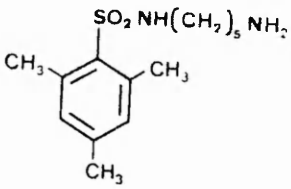
C_6-Iodo-W7 : N-(6-aminohexyl)-5-iodo-naphalene-1-sulphonamide.



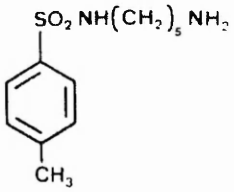
C_8-Iodo-W7 : N-(8-aminooctyl)-5-iodo-naphthalene-1-sulphonamide.



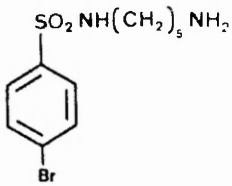
TIP-SA : N-(5-aminopentyl)-2,4,6-tri-isopropyl-benzene-1-sulphonamide.



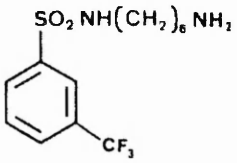
TMB-SA : N-(5-aminopentyl)-2,4,6-trimethyl-benzene-1-sulphonamide.



Tosyl-SA : N-(5-aminopentyl)-4-methyl-benzene-1-sulphonamide.



Bromobenzyl-SA : N-(5-aminopentyl)-4-bromo-benzene-1-sulphonamide.



TFMB-SA : N-(6-aminohexyl)-3-trifluoro-methylbenzene-1-sulphonamide.

The Eadie-Hofstee plots for 1-NSA and 2-NSA gave linear secondary plots for the liver enzyme (Fig. 3.6b and 3.7b respectively), but produced plots consisting of two components for the islet enzyme (3.16b and 3.17b respectively). A similar observation was made by Davies et al. (1980) for the inhibition of Chinese hamster ovary cell transglutaminase by methylamine. For the purpose of kinetic evaluation the $[I]_{0.5}$ value was determined from the initial inhibition line where the enzyme appeared more sensitive to inhibition by 1-NSA and 2-NSA. 1-NSA (IC_{50} = 171μM, K_i = 17.6μM) was found to be a slightly more potent inhibitor of pancreatic islet transglutaminase activity than 2-NSA (IC_{50} = 238μM, K_i = 62.5μM), but both compounds were found to be less inhibitory than MDCD. Dimethyl-2-NSA had no significant effect on either enzyme, confirming the competitive effect exerted by the primary amine group of these compounds. Altering the sulphonamide of 1-NSA to the carboxamide as found in 1-NCA (IC_{50} = 117μM, K_i = 24.1μM for the liver enzyme) did not yield any significant increase in potency.

TMB-SA (IC_{50} = 31μM, K_i = 6.4μM for the liver enzyme) was found to have a similar potency as an inhibitor of transglutaminase activity to that of MDCD. The use of a tri-methylated benzene ring instead of a naphthalene ring did not therefore result in a less effective inhibitor of transglutaminase activity. Furthermore, replacement of the methyl groups with isopropyl groups as found in TIP-SA (IC_{50} = 20μM; K_i = 2.5μM for the islet enzyme) resulted in a much more potent inhibitor of transglutaminase activity. The Eadie-Hofstee plot for TIP-SA produced a plot consisting of two components for the islet enzyme (Fig. 3.19a), analogous to those observed for 1-NSA and 2-NSA. The $[I]_{0.5}$ value was again determined from the initial (steeper) inhibition line.

Tosyl-SA (IC_{50} = 89μM, K_i = 15.5μM) and bromobenzyl-SA (IC_{50} = 72μM, K_i = 15.4μM) were found to have similar potencies for their inhibition of the rat liver transglutaminase activity. Surprisingly the other test compound based on a benzene ring, TFMB-SA (IC_{50} > 500μM), had very little effect on the activity of the liver enzyme.

The potency of W7 and some other closely related compounds was also investigated, W7 was found not be a potent inhibitor of rat liver transglutaminase activity (IC_{50} = 362μM, K_i = 73.9μM). Replacing the chlorine molecule for iodine at the 5-position of the naphthalene ring did not have any significant effect on the potency of this compound, since C_6 - Iodo - W7 (IC_{50} = 398μM, Ki = 76.6μM) had a similar potency to that of W7. However, increasing the chain-length of the sulphonamide moiety from C_6 to C_8 had a profound negative effect on the potency of the compound for inhibition of rat liver transglutaminase activity. C_8 - Iodo - W7 resulted in only a 5% inhibition of transglutaminase activity at the maximum concentration tested of 1mM.

Trifluoroperazine was found to have little effect on transglutaminase activity up to the maximum concentration tested of 1mM.

3.2.2. DETERMINATION OF THE EFFECT OF TEST COMPOUNDS ON GLUCOSE-STIMULATED INSULIN RELEASE IN THE INTACT PANCREATIC β-CELL

In order to implicate the involvement of transglutaminase in glucose-stimulated insulin release investigations were undertaken to determine whether test compounds found to act as inhibitors of transglutaminase activity *in vitro* also inhibited glucose-stimulated insulin release from isolated pancreatic islets.

Since two modes of islet isolation by collagenase digestion were employed in this project, initial investigations were undertaken to test the glucose-response of the isolated islets from the different preparations.

Batches of 5 islets were incubated for 1 hour in 1 ml of incubation media containing glucose concentrations in the range 2.8 - 16.8 mM. The insulin content of the supernatant medium was determined by radioimmunoassay as described in Section 2.9.5.3.

Typical dose-response curves of islets to glucose when isolated from the "hand isolation" method and the "flask-shaker isolation" method are shown in Figures 3.20a and 3.20b respectively. In general the response of islets from the two modes of isolation was found to be comparable. Typical rates of insulin release for islets isolated by the "hand method" were 6.7 ± 0.9 (n = 33) μUnits/islet/h for islets incubated at 2.8mM glucose and 94.0 ± 6.4 (n = 48) μUnits/islet/h for islets incubated at 16.8mM glucose; whilst for islets isolated by the "flask shaker" method typical rates of insulin secretion were 7.1 ± 1.4 (n = 21) μUnits/islet/h for islets incubated at 2.8mM glucose and 103.2 ± 8.1 (n = 38) μUnits/islet/h for islets incubated at 16.8mM glucose.

Figure 3.20a and b.

Glucose-response curve for insulin release from isolated rat pancreatic islets.

Batches of 5 islets isolated by collagenase digestion using either the "hand isolation" method (Section 2.9.3.2.) or the "flask-shaker" method (Section 2.9.3.3.) were incubated in 1 ml of incubation medium containing a range of concentrations of glucose for 1h in an atmosphere of 95% O_2/5% CO_2. The insulin content of the supernatant medium was determined by RIA (cf. Section 2.9.5.3.). Figure 3.20a represents a typical dose-response curve for islets isolated by the "hand method", and Figure 3.20b for islets isolated by the "flask-shaker" method.

Figure 3.20a

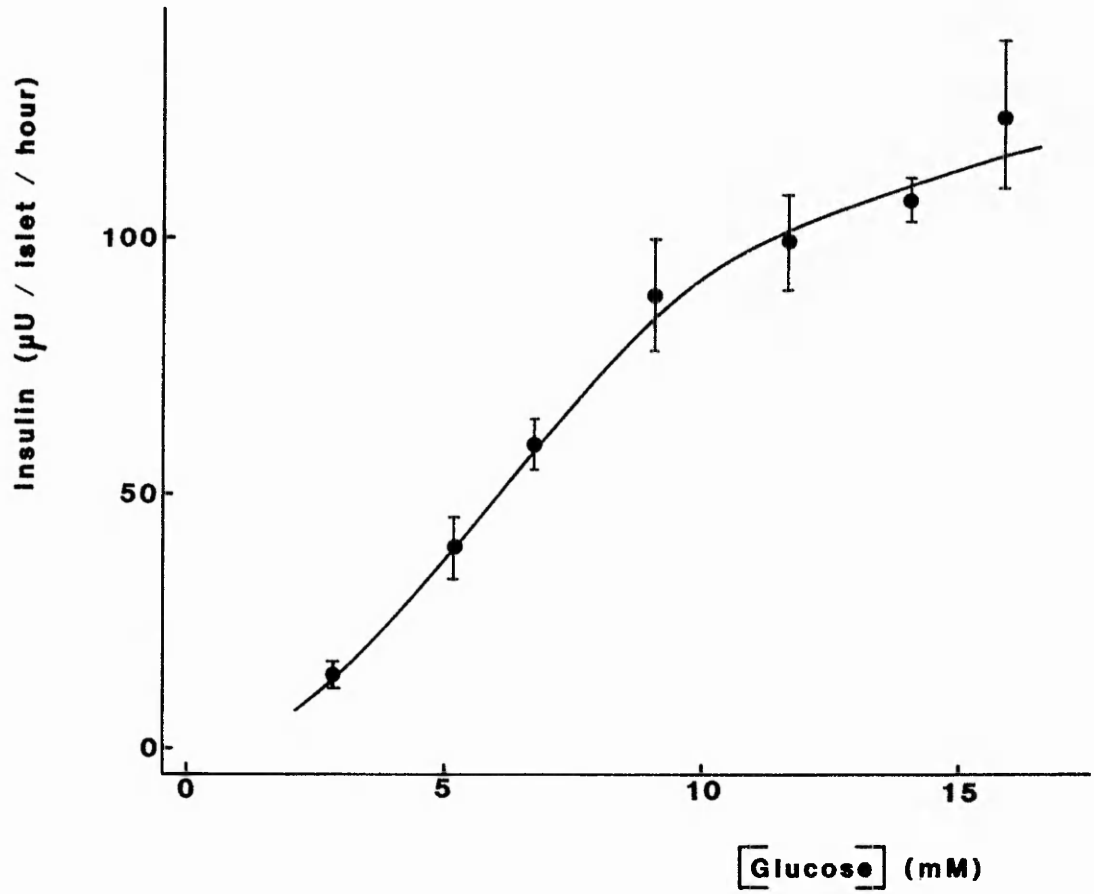
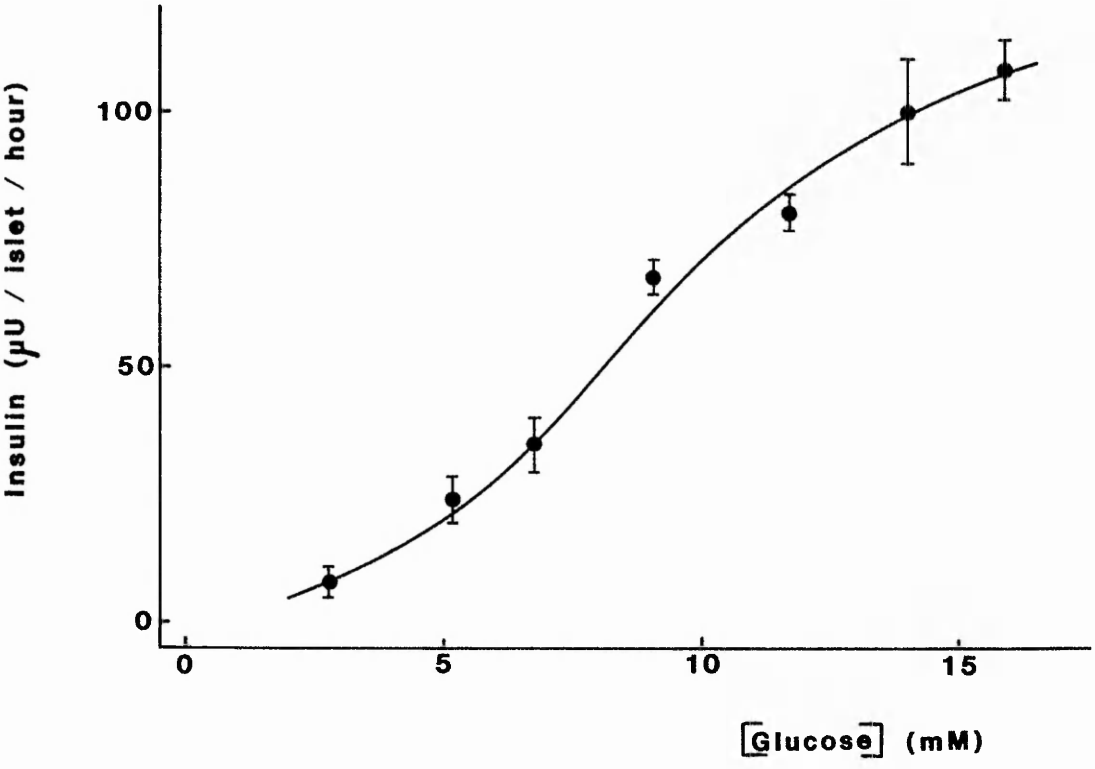


Figure 3.20b



Since these data showed no significant difference from each other it was concluded that islets isolated by the two different modes described were comparable and that direct comparisons could be made between data obtained from islets isolated by the two different methods. The subsequent data presented for the effect of test compounds on glucose-stimulated insulin release (Table 3.3) and on basal insulin release (Table 3.4) was obtained from islets isolated by both of the isolation methods.

The most potent inhibitor of glucose-stimulated insulin release was MDCD which was effective over the range 30-100 μM. The control compound dimethyl-MDCD however had no inhibitory effect up to the highest concentration tested of 400μM.

1-NSA was inhibitory over the range 75-200 μM, but less potent than MDCD. 1-NSA was more potent than 2-NSA at 100μM but slightly less potent at 200μM. As previously observed with MDCD, the control compound for 2-NSA, dimethyl-2-NSA was not inhibitory at these concentrations, but did evoke a 76% inhibition of glucose-stimulated insulin release at 400μM. 1-NCA when tested at 200μM caused an apparent doubling of glucose-stimulated insulin release.

Similar observations were made for the compounds TMB-SA and TIP-SA. TMB-SA was slightly inhibitory at 50μM (20 ± 6%), but caused an apparent increase in insulin secretion at 100μM and 150μM. TIP-SA caused an apparent increase in glucose-stimulated insulin release at a concentration of 100μM and the islets themselves appeared translucent rather than their normal white opaque appearance after the incubation period.

Table 3.3.

Effect of test compounds on glucose-stimulated insulin release.

Groups of 5 islets were incubated for 45 min with 2.8mM glucose in the presence of different concentrations of the test compound in order to facilitate entry of the drug into the cells. This was followed by a further 60 min incubation in the presence of 1 ml of medium containing 2.8mM or 16.8mM glucose. The concentration of the test compound was kept constant throughout both incubations.

Stimulation of insulin release over the 60 min period was determined by subtracting basal rates of insulin release obtained from islets maintained in incubation medium containing 2.8mM glucose incubated simultaneously over the same period of 45 + 60 min. The inhibition of glucose-stimulated insulin release for each compound tested was then expressed as a percentage (± S.E.M.) of the rate in control incubations without any test compound present. The figures in parenthesis refer to the number of batches of islets. The significance of difference from control incubations performed in each experiment was assessed using the Students' t-test. N.S. denotes no significant difference from controls.

Table 3.3.

Test compound	Concentration (μM)	Effect on glucose-stimulated insulin release (% of control)
MDCD	30	67 ± 6 (5) P < 0.05
	50	35 ± 11 (6) P < 0.002
	100	15 ± 8 (5) P < 0.001
Dimethyl-MDCD	200	76 ± 9 (6) N.S.
	400	91 ± 7 (6) N.S.
2-NSA	100	45 ± 9 (6) P < 0.001
	200	14 ± 4 (6) P < 0.01
Dimethyl-2-NSA	100	100 ± 4 (7) N.S.
	200	100 ± 6 (6) N.S.
	400	24 ± 9 (7) P < 0.01
1-NSA	50	100 ± 11 (6) N.S.
	75	63 ± 2 (5) P < 0.05
	100	59 ± 4 (5) P < 0.05
	200	35 ± 5 (5) P < 0.01
1-NCA	200	210 ± 27 (6) P < 0.01

TMB-SA	50	80 ± 6 (6)	P < 0.05
	100	112 ± 7 (5)	N.S.
	150	201 ± 10 (5)	P < 0.01
TIP-SA	100	> 300 (3)	P < 0.001
Tosyl-SA	200	73 ± 7 (6)	N.S.
	400	53 ± 5 (5)	P < 0.01
Bromobenzyl-SA	100	83 ± 10 (6)	P < 0.05

Table 3.4.

Effect of test compounds on basal insulin release.

Batches of 5 islets were incubated at 37°C for 45 min in 0.5 ml of medium containing 2.8mM glucose followed by 60 min in 1.0 ml of the same medium. Samples of medium were taken and their insulin content determined by RIA. The rates of insulin release over the total period of incubation were expressed as μU insulin/h/islet and then expressed as a percentage of control incubations in the absence of any test compound. Typical control rates of insulin release were 6.9 ± 1.2 μU insulin/h/islet (n = 42). Figures in parenthesis refer to number of batches of islets. The significance of difference from control incubations performed in each experiment was assessed using the Students' t-test. N.S. denotes no significant difference from controls.

Test Compound	Concentration (μM)	Effect on basal insulin release (% of control)
MDCD	100	97 ± 2 (5) N.S.
Dimethyl MDCD	400	98 ± 4 (3) N.S.
2-NSA	200	104 ± 6 (4) N.S.
Dimethyl-2-NSA	400	108 ± 6 (3) N.S.
1-NSA	200	93 ± 8 (4) N.S.
1-NCA	200	124 ± 5 (4) $p < 0.01$
TMB-SA	150	118 ± 4 (3) $p < 0.01$
TIP-SA	100	142 ± 9 (4) $p < 0.001$
Tosyl-SA	400	104 ± 7 (3) N.S.
Bromobenzyl-SA	100	89 ± 10 (3) N.S.

1-NCA, TMB-SA and TIP-SA were also found to have a stimulatory effect on basal glucose (2.8mM) levels of insulin release (Table 3.4), whereas all the other compounds tested had no significant effect on basal insulin release.

The compounds tosyl-SA and bromobenzyl-SA were only weak inhibitors of glucose-stimulated insulin release (27% at 200μM and 17% at 100μM respectively).

3.2.3. EFFECTS OF TEST COMPOUNDS ON GLUCOSE-STIMULATED GLUCOSE OXIDATION

The secretion of insulin from the β-cell is thought to be intimately related to the metabolism of glucose by the β-cell, and a variety of inhibitors of glucose metabolism have been shown to inhibit glucose-stimulated insulin release. Therefore to ensure the specificity of test compounds as inhibitors of transglutaminase activity it was important to show they did not inhibit glucose-stimulated glucose oxidation in the pancreatic β-cell. Since if compounds were inhibiting glucose oxidation this could result, in part, in the observed inhibition of glucose-stimulated insulin release. Compounds that appeared to be toxic to the islet cell were also tested inorder to elucidate their possible mode of toxicity.

The results of the studies undertaken are presented in Table 3.5. MDCD was found to have no effect on glucose-stimulated insulin release at 100μM, whilst the control compound dimethyl-MDCD was not inhibitory up to 400μM. 2-NSA caused a significant inhibition at 200μM (40%) but was not inhibitory at 100μM, whilst 1-NSA was not inhibitory at 200μM. The control compound dimethyl-2-NSA did not significantly inhibit glucose oxidation at 400μM. However when the sulphonamide was changed to a carboxamide as in 1-NCA a significant inhibition of glucose-stimulated glucose oxidation resulted, (65% at 200μM).

TMB-SA did not manifest an inhibitory effect on glucose utilisation at a concentration of 100μM. However substitution of the three methyl groups for isopropyl groups as in TIP-SA resulted in a very marked inhibitory effect on glucose oxidation (57% at 100μM, 73% at 150μM). Tosyl-SA caused a 47% inhibition of insulin release at a concentration of 400μM and was

Table 3.5.

Effects of test compounds on glucose-stimulated glucose oxidation in the pancreatic β-cell.

Glucose oxidation was determined for groups of 10-20 islets by measuring $^{14}CO_2$ release from [U - ^{14}C]glucose (>230 mCi/mol) as described in Section 2.9.6. Incubations were carried out for 1h after the addition of a stimulatory concentration (16.8mM) of glucose. For each concentration of test compound control rates were determined for untreated islets from the same isolated batch. The inhibition of glucose oxidation by test compounds was expressed as a mean percentage (± S.E.M.) relative to control incubations, with the number of observations shown in parenthesis. The significance of difference from controls was determined by Students' t test. Typical rates of glucose-stimulated glucose oxidation were 344 ± 35 pmol/h per 10 islets (n = 17), which represented an approximate 10 fold increase above the basal level of glucose oxidation. N.S. denotes no significant difference from the controls.

Table 3.5.

Test compound	Concentration (μM)	Effect on glucose-stimulated glucose oxidation (% of control)
MDCD	100	106 ± 4 (4) N.S.
Dimethyl-MDCD	400	96 ± 3 (5) N.S.
2-NSA	100	108 ± 6 (4) N.S.
	200	60 ± 7 (5) $P < 0.005$
Dimethyl-2-NSA	400	82 ± 3 (3) N.S.
1-NSA	200	100 ± 3 (5) N.S.
1-NCA	200	35 ± 7 (5) $P < 0.005$
TMB-SA	100	101 ± 11 (4) N.S.
TIP-SA	100	43 ± 6 (5) $P < 0.001$
	150	27 ± 8 (6) $P < 0.001$
C_8-Iodo-W7	10	86 ± 10 (4) N.S.
	50	32 ± 11 (4) $P < 0.05$
	100	21 ± 7 (3) $P < 0.5$
Tosyl-SA	300	56 ± 4 (6) $P < 0.01$
	400	49 ± 9 (5) $P < 0.01$

therefore less toxic than TIP-SA.

The W7 analogue C_8-Iodo-W7 was found to be a potent inhibitor of glucose oxidation at 50μM (68%) and 100μM (79%), but caused no significant inhibition at a test concentration of 10μM.

3.2.3.1. DISCUSSION

The data summarised in Table 3.1 further indicates the close kinetic similarity between the islet and liver transglutaminases. In general, where compounds were screened against both enzymes, the resultant IC_{50} and K_i values were comparable. The only significant difference observed was the effect of 1-NSA and 2-NSA on both enzymes. The observation of two separate linear components for the Eadie-Hofstee plots for the effect of 1-NSA and 2-NSA on the rat pancreatic islet enzyme (Figures 3.16b and 3.17b respectively) is difficult to explain. These results may suggest minor differences between the catalytic properties of the rat liver and rat pancreatic islet enzyme. However, Section 3.1 also highlighted further kinetic similarities between the two enzymes with respect to their sensitivity to activation by Ca^{2+}. The determined Km' value of the rat liver enzyme (0.31 ± 0.04μM) for putrescine incorporation into N,N'-dimethylcasein also compared favourably with that previously determined for the rat pancreatic islet enzyme (0.33 ± 0.08μM) (Bungay, 1984).

The two different protocols for calculating the inhibition constant, K_i, for test compounds were found to give similar results. For MDCD the K_i values were 4.8μM (1st method) and 7.6μM (2nd method); for tosyl-SA the values were 16μM (1st method) and 15.5μM (2nd method); for 2-NSA the values were 32μM (1st method) and 36.5μM (2nd method). These results tend to suggest a favourable comparability between the two methods employed.

Of the compounds tested as inhibitors of transglutaminase activity, MDCD evolved as a compound that could be used as a potential probe for the action of this enzyme in pancreatic islets. Thus MDCD was found to be a potent inhibitor of the

pancreatic islet transglutaminase with an IC_{50} of 43μM and a K_i of 9.8μM. This inhibitory effect was found to be dependent upon the possession of a primary amine group since the control analogue dimethyl-MDCD was not inhibitory to the pancreatic islet transglutaminase. In good agreement with the potency of MDCD as an inhibitor of islet transglutaminase activity was its potency as an inhibitor of glucose-stimulated insulin release (33-86% inhibition) in the concentration range 30-100 μM. In contrast the control analogue dimethyl-MDCD was found not to inhibit glucose-stimulated insulin release up to the maximum test concentration of 400μM, thus further suggesting the specificity of MDCD as a probe for the action of the islet transglutaminase in the pancreatic islet.

Important to the use of MDCD as a potential probe for transglutaminase activity was the finding that this compound significantly inhibited glucose-stimulated insulin release at concentrations for which the compound did not inhibit glucose-stimulated glucose oxidation. Thus, at low concentrations of the drug (30-50 μM) glucose-stimulated insulin release was inhibited in the range 33-65% a concentration of 100μM MDCD did not inhibit glucose-stimulated glucose oxidation.

2-NSA was found to be a less potent inhibitor of rat islet transglutaminase activity *in vitro* (IC_{50} = 238μM; Ki= 62.5μM) than MDCD and was correspondingly found to be a less potent inhibitor of glucose-stimulated insulin release(55% inhibition at 100μM). The dependence of the molecule carrying a primary amine group in order to be effective was again illustrated by the control analogue dimethyl-2-NSA which was not found to be an inhibitor of either transglutaminase activity or glucose-stimulated insulin release at concentrations up to 200μM. However, at a concentration of 400μM, glucose-stimulated insulin release was inhibited by 76%, an

observation worthy of further investigation. 2-NSA inhibited glucose-stimulated glucose oxidation by 40% at 200μM and therefore the inhibition of glucose-stimulated insulin release (86%) at this concentration cannot be singularly attributed to the inhibition of islet transglutaminase activity. However, 2-NSA did not affect glucose-stimulated glucose oxidation at 100μM and it may therefore be a useful probe for transglutaminase activity at this concentration.

1-NSA (IC_{50} = 171μM) was also found to be a less potent inhibitor of transglutaminase activity than MDCD (IC_{50} = 43μM). The only difference between MDCD and 1-NSA is the N-dimethyl group on the 5-position of the naphthalene ring of MDCD. It is possible that this group may make MDCD a more effective competitive inhibitor of transglutaminase through it binding to sites adjacent to the enzyme active site and therefore increasing the affinity of the enzyme for MDCD. 1-NSA was also slightly more potent than 2-NSA (IC_{50} = 238μM) for the inhibition of transglutaminase activity, this may similarly relate to enzyme binding phenomena. It is difficult to explain why 1-NSA did not have any inhibitory effect on glucose-stimulated glucose oxidation at 200μM whereas 2-NSA caused 40% inhibition at this concentration. However, this result suggests that the observed inhibitory effect of 1-NSA on glucose-stimulated insulin release over the range 75-200 μM (37-65% inhibition) may be due to the inhibition of transglutaminase activity.

Although alteration of the sulphonamide of 1-NSA to a carboxamide as found in 1-NCA, resulted in an increased potency of the compound as an inhibitor of transglutaminase activity, the stimulatory effect of 1-NCA on both glucose-stimulated and basal insulin release suggested that this compound may be toxic to the islet β-cell. This indication was corroborated by the inhibitory

effect of 1-NCA on glucose-stimulated glucose oxidation making this compound an unsuitable probe for studying the involvement of transglutaminase in glucose-stimulated insulin release from the pancreatic β-cell.

The similar potencies of TMB-SA (IC_{50} = 39μM) and MDCD (IC_{50} = 43μM) indicated that the employment of a tri-methylated benzene ring instead of a naphthalene ring did not result in a less effective inhibitor. However TMB-SA had a stimulatory effect on both glucose-stimulated and basal insulin release and was inhibitory to glucose-stimulated glucose oxidation at a concentration of 100μM and therefore could not be employed as a specific probe for transglutaminase activity in islets. An increased potency for transglutaminase was achieved by replacing the methyl groups in TMB-SA with isopropyl groups as found in TIP-SA (IC_{50} = 20μM). The more bulky and hydrophobic isopropyl groups therefore appear to increase the affinity of this compound for transglutaminase and hence increase its effectiveness as a competitive inhibitor. However, TIP-SA was found to inhibit glucose-stimulated glucose oxidation (57% at 100μM) and this observed toxicity may partially explain why TIP-SA caused a stimulation of both glucose-stimulated insulin release and basal insulin release.

Tosyl-SA and bromobenzyl-SA were found to have similar potencies for their inhibition of transglutaminase activity with IC_{50} values of 89μM and 72μM respectively for the liver enzyme. Both benzene-ring based test compounds were therefore much less potent than either TIP-SA or TMB-SA. The presence of a single bromine or methyl group on the benzene nucleus therefore appears to be less effective than either a tri-isopropyl or trimethyl substitution in effecting the inhibition of transglutaminase activity. However neither tosyl-SA or bromobenzyl-SA inhibited glucose-stimulated

insulin release with any degree of potency. This may be due to an impaired entry of the compounds into the islet. Further, the inhibitory effect of tosyl-SA was not specifically due to the inhibition of transglutaminase activity since at a concentration of 400μM this compound also inhibited glucose-stimulated glucose oxidation.

TFMB-SA (IC_{50} > 0.5mM) had very little inhibitory effect on the activity of the liver enzyme. This result was surprising when compared to the results obtained with the other test compounds based on the benzene ring, and may relate to the close proximity of the trifluoro-methyl group on the 3-position of the benzene ring to the 1-position of the sulphonamide chain, thereby inhibiting the binding of the molecule by the enzyme.

The decreased potency of C_8-iodo-W7 on the inhibition of transglutaminase activity as compared to C_6-iodo-W7 and W7 was in agreement with the data of Lorand et al. (1979) which indicated that a C5 chain length had an optimum binding affinity for the enzyme, and that increasing the chain length resulted in a decrease in this affinity.

3.2.4. EFFECTS OF TEST COMPOUNDS ON THE ACTIVITY OF THE PHOSPHOLIPID- AND Ca^{2+}- DEPENDENT KINASE (PROTEIN KINASE C)

An additional target of the test compounds whose inhibition may result in the inhibition of glucose-stimulated insulin release is the phospholipid- and Ca^{2+}- dependent protein kinase, protein kinase C (Wise et al., 1982b). For example the latter authors reported that the naphthalene sulphonamide W7 was inhibitory towards the activity of protein kinase C.

3.2.4.1. Assay for protein kinase C in rat spleen extracts.

The inhibitory potency of test compounds on protein kinase C was investigated by determining their effects on the incorporation of ^{32}P from [γ - ^{32}P] ATP into histone H1 by a modification of the procedure of Lord and Ashcroft (1984), as detailed in Section 2.6.

Rat spleen was chosen as a source of protein kinase C since this organ has been demonstrated to contain significant levels of the soluble form of the enzyme and hence is relatively easy to obtain (S.J.H. Ashcroft, personal communication). Initial studies were undertaken to first validate the assay by investigating the dependence of the reaction rate on enzyme concentration, and the linearity of the reaction rate with time. A range of dilutions of the rat spleen enzyme preparation (71,000g supernatant) were assayed as detailed in the legend to Figure 3.21.

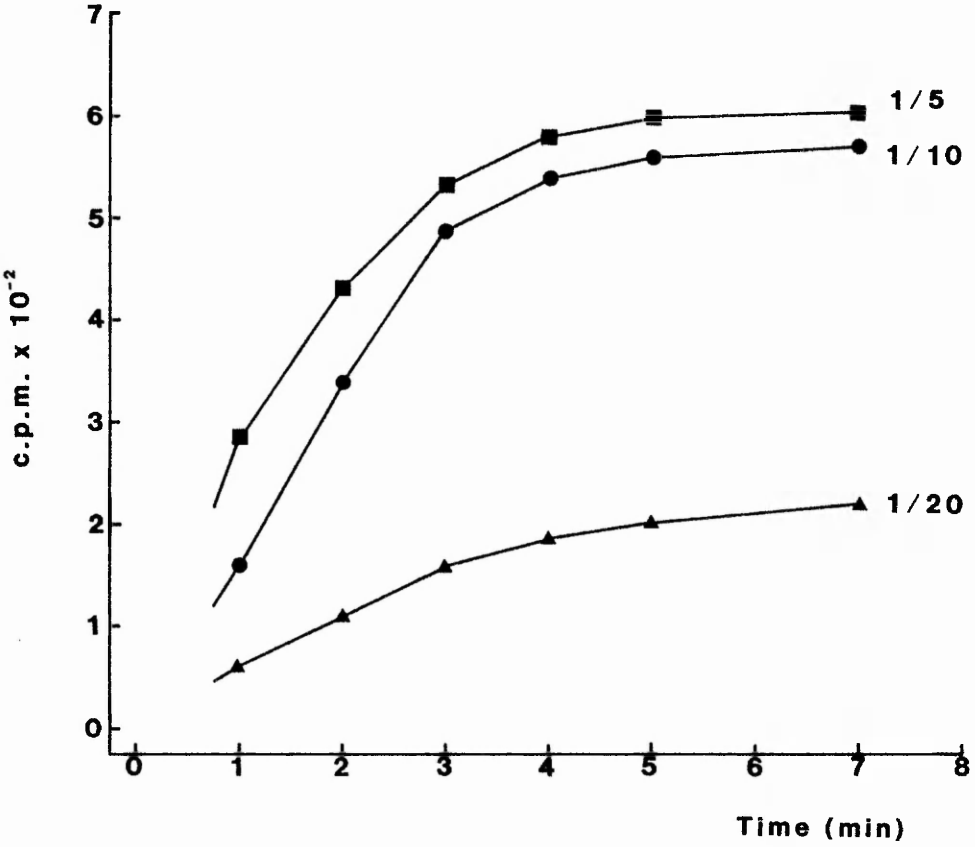
The results presented in Figure 3.21 indicate that the reaction rate increased with increasing concentration of enzyme present. The dilutions of 1 in 10 and 1 in 20 were found to be linear with respect to the incorporation of ^{32}P into histone H1 over the first 3 min, whilst the 1 in 5 dilution was found to deviate from linearity over the same time period. All three dilutions of the

Figure 3.21.

The effect of enzyme concentration on the time dependent incorporation of ^{32}P into histone H1 by rat spleen protein kinase C.

Protein kinase C activity in rat spleen extracts (71,000g supernatant) was determined by measuring the incorporation of ^{32}P from [γ - ^{32}P] ATP into histone H1, as detailed in Section 2.6. Activity was calculated by subtraction of the phosphorylation obtained in the presence of Ca^{2+} alone from that obtained in the presence of Ca^{2+}, 1,2-diolein and phosphatidylserine when samples were taken at the time points indicated. Determinations were undertaken with samples of the rat spleen 71,000g supernatant diluted 1 in 5, 1 in 10 and 1 in 20 with the original homogenisation medium. Each point represents the mean of 2 separate determinations.

Figure 3.21



spleen enzyme preparation (71,000g supernatant) tended towards a maximum incorporation of ^{32}P into histone H1 between 3 and 7 min.

On the basis of these results a 1 in 10 dilution of the enzyme preparation was selected as being suitable for the assay using a sample time of 2 min, which was well within the linear portion of the reaction. Further investigation of the Ca^{2+}- and phospholipid- dependence of the enzyme was undertaken using these criteria.

3.2.4.2. Ca^{2+} and phospholipid requirement of rat spleen protein kinase C.

The requirement of the rat spleen enzyme for Ca^{2+} and phosphatidylserine/1,2-diolein was determined by their separate omission from the incubation mixture. The resultant data is presented in Figure 3.22.

Under all three incubation conditions the time course for the incorporation of ^{32}P into histone H1 was linear over the first 3 min as previously observed, and tended towards a maximum after 3 min. Under the conditions utilised the presence of phospholipid alone resulted in the lowest activity, whilst the presence of 50μM Ca^{2+} alone gave a 1.7 fold increase in the activity as compared to phospholipid alone when measured at the 2 min sampling point. The presence of both Ca^{2+} and phospholipid resulted in a 2.8 fold increase in activity as compared to phospholipid alone, and a 1.7 fold increase in activity as compared to Ca^{2+} alone.

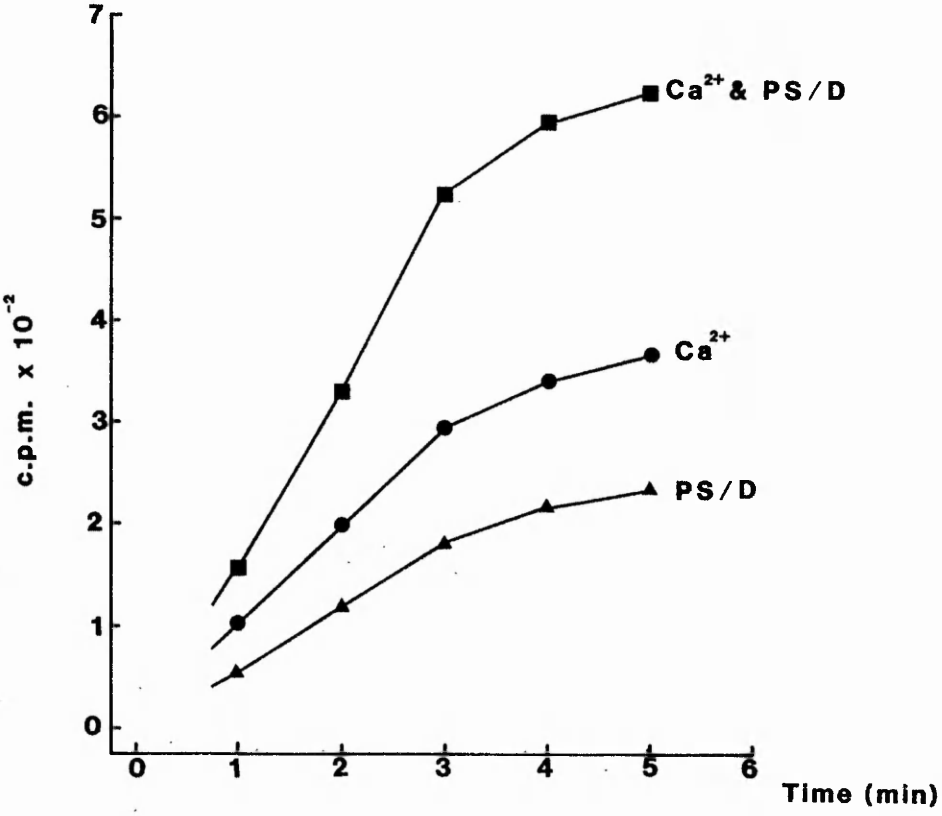
Further studies on the assay procedure were undertaken to optimise the ^{32}P labelling of histone H1 by incubation with various preparations of phosphatidylserine and 1,2-diolein. As described in Section 2.6, phosphatidylserine was prepared by dissolution in chloroform and aliquots of this solution placed in sterile bijou bottles, the chloroform was subsequently evaporated

Figure 3.22.

Effects of Ca^{2+} and phospholipid on the incorporation of ^{32}P into histone H1 by rat spleen extracts.

The time course for the incorporation of ^{32}P from [γ - ^{32}P] ATP into histone H1 was determined for rat spleen extracts (71,000g supernatant) diluted 1 in 10 as detailed in Section 2.6. Each point represents the mean of 2 separate determinations made in the presence of : ■, 50μM Ca^{2+}, diolein (2.4 μg/ml) and phosphatidylserine (16 μg/ml); ●, 50μM Ca^{2+} alone; ▲, diolein (2.4 μg/ml), phosphatidylserine (16 μg/ml) and 5mM EGTA.

Figure 3.22



under a stream of nitrogen leaving a dried coating of phosphatidylserine on the glass. The phosphatidylserine was then emulsified by addition of Tris/HCl buffer (pH 7.4) followed by ultrasonication. The 1,2-diolein was prepared separately by dissolution in Tris/HCl (pH 7.4) buffer. The effect of the nature of the preparation of phosphatidylserine and diolein on protein kinase C activity is presented in Table 3.6.

The maximum increase in ^{32}P incorporation into histone H1 was obtained when phosphatidylserine was emulsified by a single 45s burst and 1,2-diolein was added separately to the assay system. This mode of addition to the assay was therefore adopted for all subsequent studies on protein kinase C activity.

3.2.4.3. Effect of test compounds on protein kinase C activity.

Table 3.7 summarises the effect of the test compounds assayed on protein kinase C activity as determined by their effect upon Ca^{2+} and phospholipid dependent ^{32}P labelling of histone H1 from [γ - ^{32}P] ATP. Typical data obtained for the test compounds TIP-SA, W7 and trifluoroperzine are presented in Figures 3.23, 3.24 and 3.25 respectively.

The majority of the test compounds did not inhibit protein kinase C dependent phosphorylation of histone H1 by values greater than 30% over the range of concentrations tested and therefore no IC_{50} value could be calculated.

The three test compounds which were found to inhibit protein kinase C activity in excess of 50% were TIP-SA (IC_{50} = 200 ± 22μM), W7 (IC_{50} = 342 ± 47μM) and trifluoroperazine (IC_{50} = 74 ± 7μM).

Table 3.6.

Effect of the mode of presentation of phosphatidylserine and 1,2-diolein on protein kinase C activity present in rat spleen extracts.

The standard protein kinase C assay as described in Section 2.6 was modified by altering the presentation of phosphatidylserine and 1,2-diolein added to the reaction mixture as detailed in the table.

The data represents the factor by which the incorporation of ^{32}P into histone H1 increased when compared to the system in the absence of phosphatidylserine (PS) or 1,2-diolein (D). N.S. denotes no significant difference. The data shown are the mean value (± SE) with the number of determinations shown in parenthesis.

Table 3.6.

Mode of preparation of phosphatidylserine (PS) and 1,2-diolein (D).	Observed increase in the incorporation of ^{32}P into histone H1.
PS sonicated by 3 x 10s bursts D added separately	(3) 1.46 ± 0.05 P > 0.01
PS sonicated by 1 x 45s burst D added separately	(3) 1.58 ± 0.05 P > 0.01
PS and D combined and sonicated together by 3 x 10s bursts	(3) 1.41 ± 0.02 P > 0.01
PS sonicated by 3 x 10s bursts D sonicated separately by 3 x 10s bursts	(3) 1.53 ± 0.01 P > 0.01
PS sonicated by 3 x 10s bursts no D	(3) 1.35 ± 0.02 P > 0.05
D alone	(3) 1.09 ± 0.04 (N.S.)

Figures 3.23, 3.24 and 3.25.

The effect of test compounds on protein kinase C.

The effect of test compounds at a range of concentrations on protein kinase C activity in rat spleen extracts (71,000g supernatants) was determined by measuring the incorporation of ^{32}P from [γ - ^{32}P] ATP into histone H1, as detailed in Section 2.6. Protein kinase C activity, in the presence of test compounds, was calculated by subtraction of the amount of phosphorylation obtained with Ca^{2+} plus test compound from that obtained in the presence of Ca^{2+}, phosphatidylserine, 1,2-diolein and test compound over a 2 min incubation period. These values were then expressed as a percentage of the Ca^{2+}-phospholipid dependent phosphorylation in the absence of test compound.

The data presented in Figs. 3.23 (TIP-SA), 3.24 (W7) and 3.25 (trifluoroperazine) represent typical data from single experiments, each point being the mean of three determinations. Average control sample values are quoted in the legend to Table 3.7.

Figure 3.23

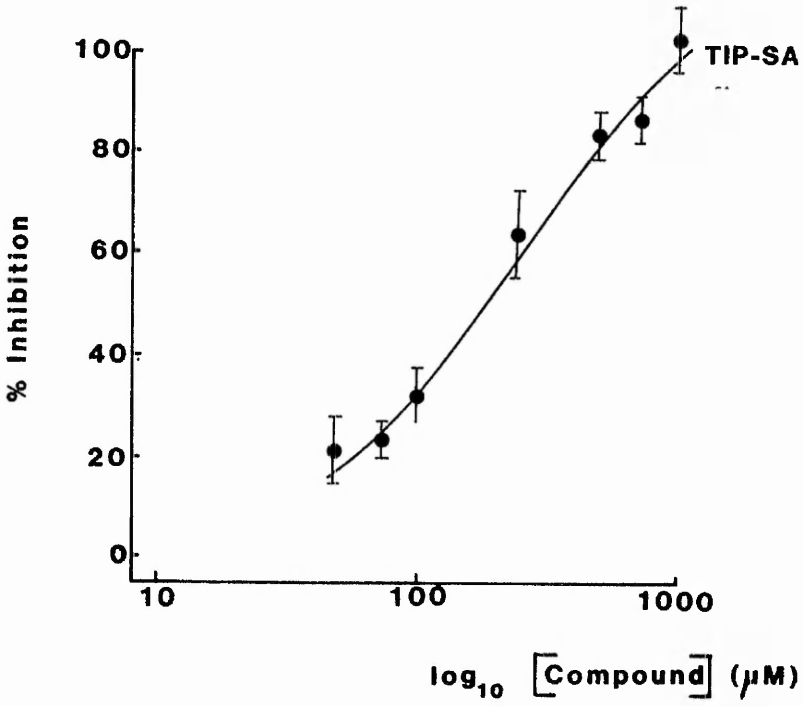


Figure 3.24

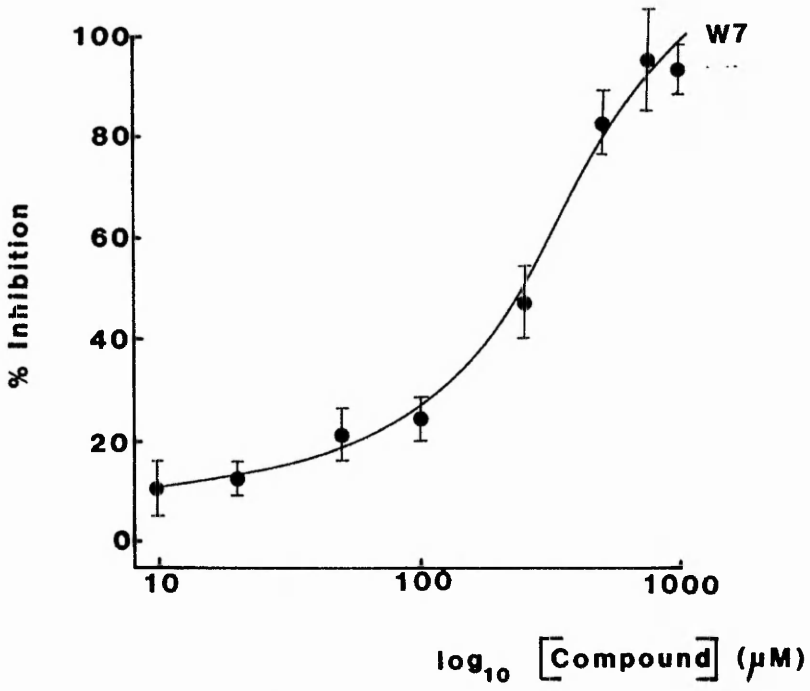


Figure 3.25

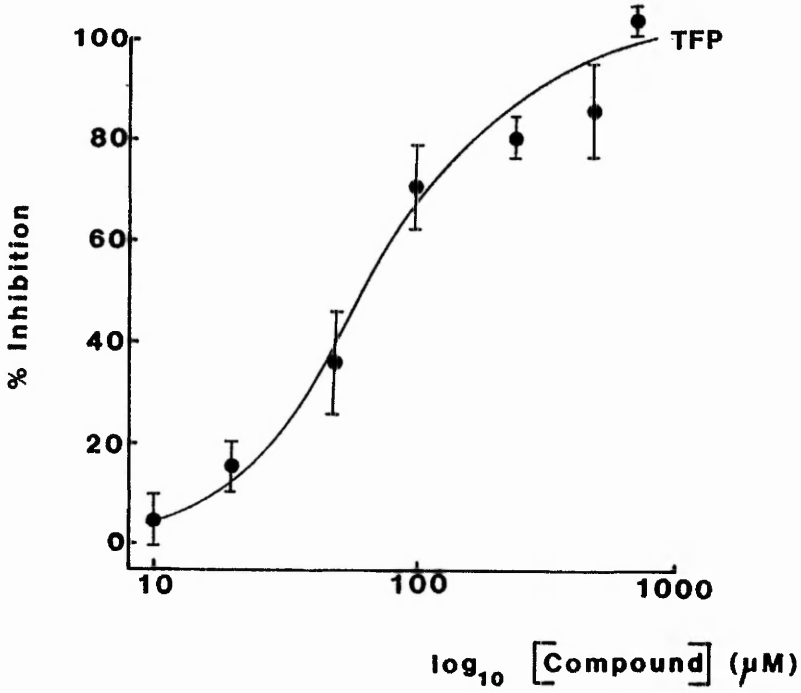


Table 3.7.

Summary of the effect of test compounds on protein kinase C activity.

The effect of test compounds on protein kinase C activity was undertaken as described in the legend to Figs. 3.23, 3.24 and 3.25. The values quoted represent the mean of three separate experiments of the type presented in Figs 3.23, 3.24 and 3.25.

The average ^{32}P incorporation values (c.p.m.) for control samples incubated with Ca^{2+} and phospholipid over a 2 min incubation period was 3167 ± 463 c.p.m. (n = 32). This represented an average increase of 63.2 ± 6.7% when compared to control samples incubated in the absence of phospholipid. The data in parenthesis represent the maximum percentage inhibition obtained at the concentration of test compound given.

Table 3.7

Test Compound	Concentration of compound resulting in 50% inhibition of enzyme activity, [IC_{50}]
MDCD	>1 mM (0%)
diMe MDCD	>1 mM (2%)
2-NSA	>2.5 mM (25%)
diMe-2-NSA	>1 mM (21%)
1-NSA	>1 mM (27%)
TMB-SA	>1 mM (20%)
TIP-SA	200 ± 22 μM
Tosyl-SA	>1 mM (30%)
Bromobenzyl-SA	>1 mM (6%)
W7	342 ± 47 μM
C_6-Iodo-W7	>250 uM (3%)
C_8-Iodo-W7	>1 mM (30%)
TFMB-SA	>1 mM (35%)
trifluoroperazine	74 ± 7 μM

3.2.5. EFFECTS OF TEST COMPOUNDS ON CALMODULIN-STIMULATED BOVINE HEART PHOSPHODIESTERASE

Calmodulin is a ubiquitous intracellular calcium-binding protein which has been identified in pancreatic islets (reviewed by Valverde and Malaisse, 1984). When calmodulin binds Ca^{2+}, it becomes modified to an active complex which is then able to activate a wide variety of target enzymes and proteins (reviewed by Tomlinson et al. 1984). In the pancreatic β-cell investigations have predominantly centred on the Ca^{2+}-calmodulin activation of cyclic nucleotide phosphodiesterase, adenylate cyclase and protein kinase(s) (reviewed by Valverde & Malaisse, 1984). Activation of these enzymes is considered to be implicitly involved in the events of stimulus-secretion coupling in the pancreatic β-cell as discussed in Sections 1.6.4. - 1.6.6. Whilst the exact details of the involvement of calmodulin in glucose-stimulated insulin release remain to be elucidated, it was still essential to test inhibitors of transglutaminase activity for their effects on the calmodulin activation of a chosen target enzyme. The target enzyme selected was the calmodulin-activated phosphodiesterase from bovine heart.

The protocol for investigating the effect of test compounds on calmodulin-activation of this phosphodiesterase has been outlined in Section 2.7. The assay was based on the incubation of calmodulin-deficient bovine heart phosphodiesterase with [^{3}H] cyclic AMP and a sufficient amount of calmodulin (12.5 ng) to give 60-80% activation of the enzyme. The resultant 5'-AMP formed was converted to adenosine by 5'-nucleotidase of cobra snake venom, nucleoside products were then separated from unreacted substrate using an anion exchange resin. The amount of adenosine formed, as measured by the radioactivity present, was proportional to the phosphodiesterase activity. The degree of inhibition of phosphodiesterase activation

in the presence of a range of concentrations of test compound were expressed as a percentage of the phosphodiesterase activity in the absence of any test compound.

Graphs were plotted of the percentage inhibition of calmodulin activation of phosphodiesterase against the concentration of drug present, and from these plots the concentration of drug required to evoke a 50% inhibition of enzyme activity, IC_{50}, was determined for each compound. Typical plots for the compounds MDCD and 2-NSA are presented in Figure 3.26a, and their respective control compounds dimethyl-MDCD and dimethyl-2-NSA in Figure 3.26b. The effect of W7 and the W7 analogue, C_8-iodo-W7, are presented in Figure 3.27a and b respectfully. A summary of all the IC_{50} values determined is presented in Table 3.8.

Of the compounds already found to have an inhibitory effect on transglutaminase activity, 2-NSA (IC_{50} = 105μM) was found to be the most potent inhibitor of the activation of phosphodiesterase by calmodulin. Dimethyl-2-NSA (IC_{50} = 277μM) was approximately one third as potent as 2-NSA and MDCD (IC_{50} = 387μM) was approximately one quarter as potent as 2-NSA. Dimethyl-MDCD was not a very potent inhibitor of the activation of phosphodiesterase by calmodulin (IC_{50} >500μM) when tested at concentrations up to 500μM.

Altering the position of the sulphonamide chain from the 2- to the 1- position resulted in an approximate 5 fold decrease in the potency of 1-NSA (IC_{50} = 570μM) as compared to 2-NSA (IC_{50} = 105μM). Alteration of the sulphonamide to a carboxamide resulted in a large change in potency, 1-NCA (IC_{50} > 2.5mM) was a factor of 5 times less potent than 1-NSA (IC_{50} = 570μM).

Chlorination of the naphthalene ring at the 5-position resulted in a very large increase in potency of compounds as antagonists of the activation of phosphodiesterase by calmodulin, W7

Figure 3.26a, b and 3.27a, b.

Inhibition of calmodulin-stimulated phosphodiesterase activity by naphthalenesulphonamides.

Calmodulin-stimulated phosphodiesterase activity was determined as described in the Experimental section (2.7) in both the presence and the absence of the test compounds shown. By reference to control rates of [^{3}H]adenosine production, results were expressed as a percentage inhibition of enzyme activity. The graphs presented are from single data points in an individual experiment and represent typical plots obtained. These experiments were repeated a minimum of 3 times to obtain a mean IC_{50} value (± SEM).

The mean phosphodiesterase activity (± SEM) for control incubations representing 80% (approx) stimulation of maximal activity was 32.0 ± 1.1 (n = 12) nmol. of cyclic AMP hydrolysed per h. per mU enzyme.

Figure 3.26a

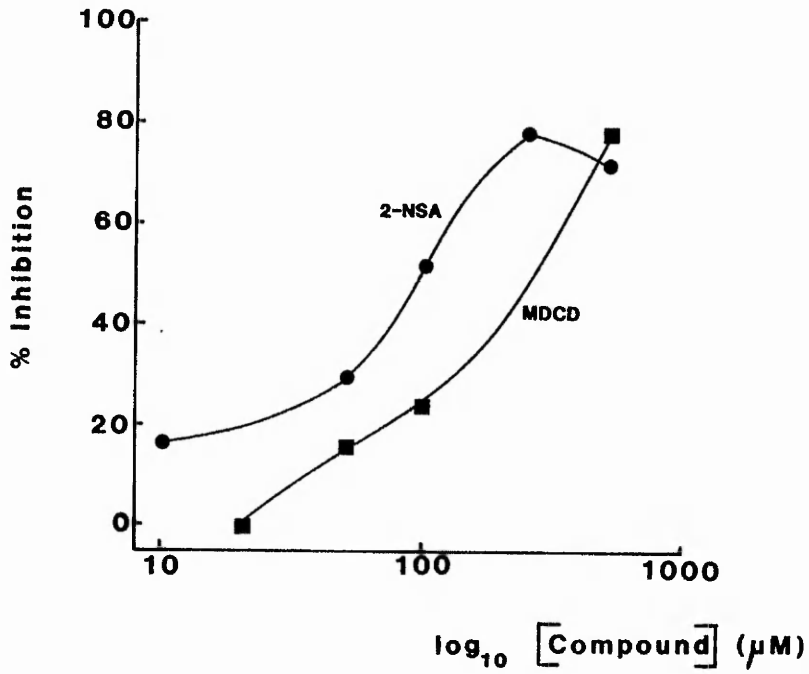


Figure 3.26b

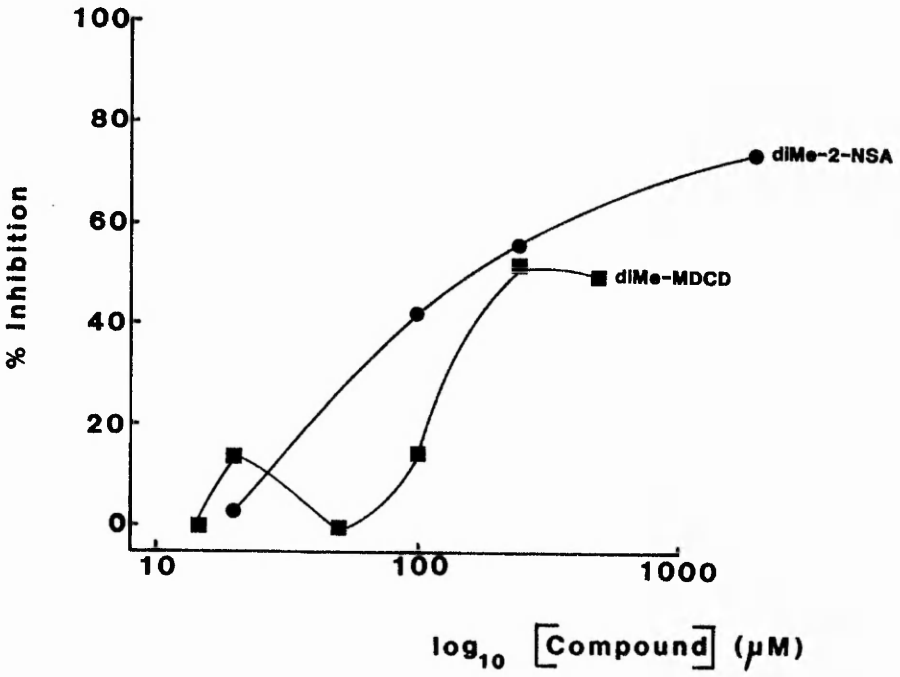


Figure 3.27a

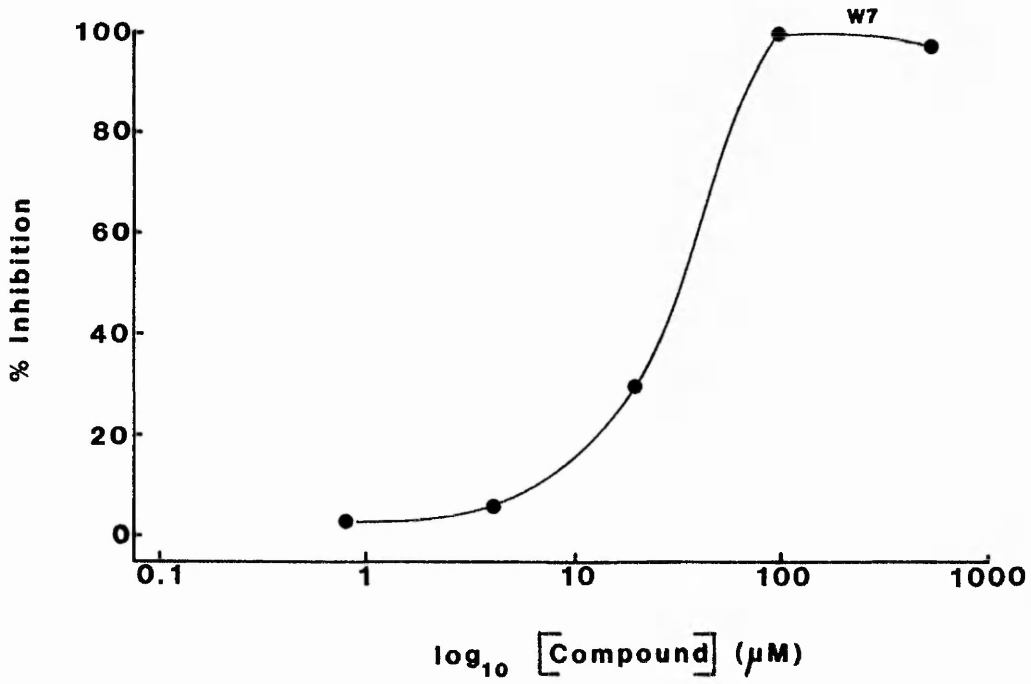


Figure 3.27b

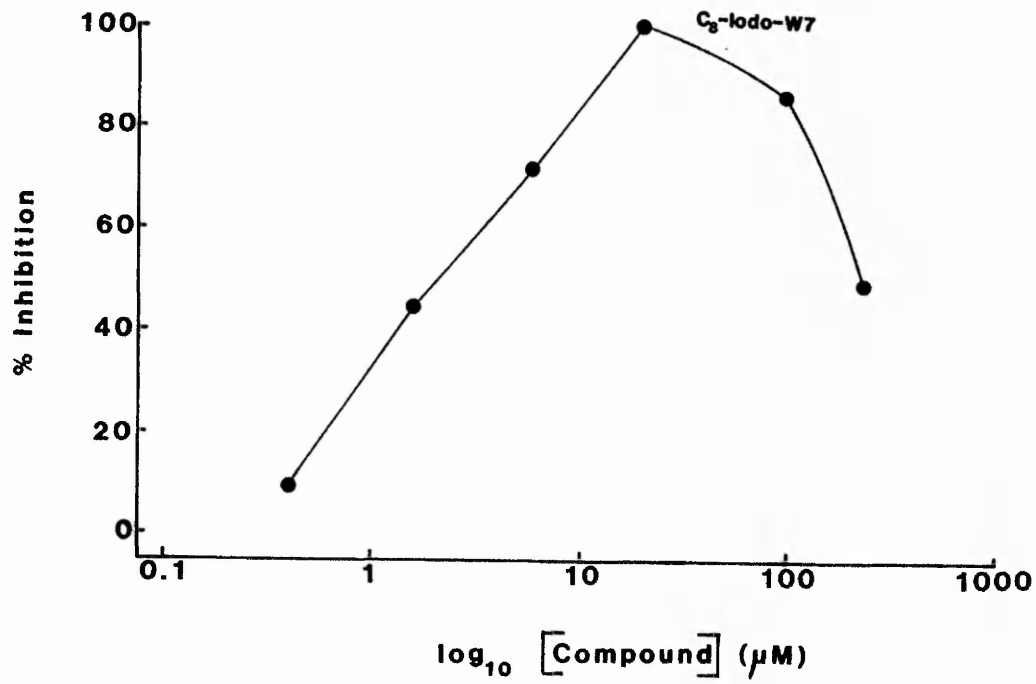


Table 3.8.

IC_{50} values for the inhibition of calmodulin-stimulated phosphodiesterase by a range of test compounds.

The IC_{50} values were determined for each compound from plots as illustrated in Figure 3.26a. The IC_{50} value for 1-NSA (570μM) is quoted from the literature value of Hidaka and Tanaka (1983). The number of experiments undertaken on each test compound are indicated in parentheses, the figures represent the mean IC_{50} value (± SEM) from the experiments. The mean control phosphodiesterase activity is quoted in the legend to Figure 3.26 and 3.27.

Test Compound.	Concentration of compound resulting in 50% inhibition of enzyme activity [IC_{50}] (μM).
MDCD	387 ± 9 (4)
diMe MDCD	>500 (3)
2-NSA	105 ± 18 (3)
diMe-2-NSA	277 ± 34 (3)
1-NSA	570 (Hidaka & Tanaka, 1983)
1-NCA	>2,500 (2)
TMB-SA	350 ± 21 (3)
TIP-SA	26 ± 15 (3)
Tosyl-SA	>500 (2)
Bromobenzyl-SA	1,800 ± 315 (3)
W7	29 ± 2 (3)
C_6-Iodo-W7	29 ± 6 (3)
C_8-Iodo-W7	3 ± 0.6 (4)
TFMB-SA	250 ± 62 (3)
TFP	4 ± 2 (3)

(IC_{50} = 29μM) was approximately 20 times more potent than 1-NSA (IC_{50} = 570μM). Changing the halide at the 5-position from chlorine in W7 to iodine in C_6-iodo-W7 did not result in any change in the potency of the compound, since both compounds had IC_{50} values of 29μM. However, increasing the chain-length of the sulphonamide moiety from C_6 to C_8 had a profound effect on the potency of the molecule, C_8-iodo-W7 (IC_{50} = 3μM) was approximately 10 times more potent than C_6-iodo-W7 (IC_{50} = 29μM).

TIP-SA was found to be a potent antagonist of the activation of phosphodiesterase by calmodulin (IC_{50} = 26μM). The other four compounds based on a benzene ring proved to be less potent antagonists than TIP-SA. A single methyl substituent in tosyl-SA (IC_{50} >500μM) or a bromine in bromobenzyl-SA (IC_{50} = 1.8mM) resulted in a reduction of the potency of these compounds as calmodulin antagonists under the conditions of the assay. A trimethyl substitution as found in TMB-SA (IC_{50} = 350μM) and the trifluoromethyl substitution in TFMB-SA (IC_{50} = 250μM) did however result in an increased potency of these compounds as antagonists, but not to the level of potency shown by tri-isopropyl substitution as found in TIP-SA.

The compounds MDCD, dimethyl-MDCD, 2-NSA and dimethyl-2-NSA were further tested for their potency against basal phosphodiesterase activity measured in the absence of calmodulin. The resultant data is presented in Table 3.9.

Basal activity was not significantly affected by concentrations of MDCD up to 500μM, of 2-NSA up to 200μM, and by dimethyl-MDCD and dimethyl-2-NSA up to 250μM. The two dimethylated analogues were found to stimulate basal phosphodiesterase activity between 2- and 4- fold at a concentration of 500μM.

Table 3.9.

Effect of MDCD, 2-NSA, dimethyl-MDCD and dimethyl-2-NSA on basal phosphodiesterase activity.

Basal phosphodiesterase activity was determined as described in the Experimental section (2.7), but with the absence of calmodulin from the assay compounds. By reference to control basal rates of [^{3}H]adenosine production, results were expressed as a mean percentage (± S.E.M.) inhibition of basal enzyme activity, with each value the mean of three separate determinations.

The mean basal rate (± S.E.M.) for phosphodiesterase activity was 3.6 ± 0.6 nmol of cyclic AMP hydrolysed per h per mU enzyme (n = 12).

Test Compound	Concentration (μM)	Effect on basal phosphodiesterase activity (% of control)
MDCD	250	98 ± 6 N.S.
	500	103 ± 9 N.S.
2-NSA	100	104 ± 12 N.S.
	200	92 ± 16 N.S.
dimethyl-MDCD	250	128 ± 34 N.S.
	500	216 ± 36 $p<0.01$
dimethyl-2-NSA	250	108 ± 12 N.S.
	500	429 ± 70 $p<0.01$

3.2.6. EFFECT OF MONODANSYLCADAVERINE (MDCD) ON TOTAL ISLET LIPID METHYLATION

It has been suggested that the toxic action of MDCD on cells may result from its inhibition of phospholipid methylation (Mato et al., 1983). Since phospholipid methylation has been shown to occur in pancreatic islets (Saceda et al., 1983; Best et al., 1984), it is possible that its inhibition may result in the inhibition of glucose-stimulated insulin release.

The effect of MDCD on total islet lipid methylation during glucose stimulation was therefore assessed as described in the legend to Table 3.10. Comparison of the incorporation of radiolabel measured in control incubations with the value obtained for islets incubated in the presence of 50μM MDCD indicated that no significant inhibition of islet lipid methylation was caused by MDCD ($p < 0.75$). Therefore inhibition of glucose-stimulated insulin release by MDCD is unlikely to be the result of inhibition of islet phospholipid methylation.

Table 3.10.

Effect of MDCD on total islet lipid methylation.

Total islet lipid methylation was determined by measuring the incorporation of L-[methyl-^{3}H]-methionine into chloroform/diethyl ether - extractable material from isolated pancreatic islets as described in Section 2.9.8. The isolated islets were initially incubated for 45 min with 2.8mM glucose in the presence or absence of 50μM MDCD, and then for a subsequent incubation period with 16.8mM glucose and 10μCi of L-[methyl-^{3}H]-methionine in the presence or absence of 50μM MDCD. Radiolabelled lipids were then extracted in chloroform/diethyl ether and counted for radioactivity. The data shown represents the mean values ± S.E.M. with the number of determinations shown in parentheses.

Statistical analysis was undertaken using the Students' t test; N.S. denotes no statistical significance from control data.

Incubation conditions	Total islet phospholipid methylation (d.p.m./h per 100 islets)
16.8mM glucose	29508 ± 4118 (5)
16.8mM glucose + 50μM MDCD	34315 ± 3321 (5) N.S. ($p < 0.75$)

3.2.7. DISCUSSION

The IC_{50} values calculated for the inhibition of protein kinase C activity for trifluoroperazine (74 ± 7μM) and W7 (342 ± 47μM) compare favourably with the literature values.Wise et al. (1982b) indicated an IC_{50} of trifluoroperazine for protein kinase C of 40μM and an IC_{50} of W7 for protein kinase C of 140μM.

MDCD was not found to be inhibitory to protein kinase C activity at the highest concentration tested of 1 mM. However, MDCD did act as an antagonist of calmodulin when measured in the standard assay system using the calmodulin-sensitive bovine heart phosphodiesterase. The potency of this effect was almost an order of magnitude lower than that measured for the effect of MDCD on islet transglutaminase activity.

Concentrations of MDCD can now therefore be defined (30-75 μM) which give rise to significant inhibition of glucose-stimulated insulin release, but which have little or no effect on calmodulin-stimulated phosphodiesterase activity and no effect on protein kinase C activity. Previous work in the laboratory (Bungay et al., 1984) indicated that MDCD was not inhibitory to the synthesis of total islet protein in glucose-stimulated islets at a concentration of 100μM. Bungay et al. (1984) further showed that inhibition of protein synthesis did not, under the conditions of the insulin release experiments used in this project, inhibit glucose-stimulated insulin release. In keeping with the specificity of MDCD as an inhibitor of transglutaminase activity was the finding that this compound did not significantly inhibit glucose-stimulated islet phospholipid methylation at a concentration of 50μM.

It may therefore be said that the observed inhibition of glucose-stimulated insulin release by MDCD over the range 30-75 μM is likely to be a result of the specific inhibition of the pancreatic islet transglutaminase enzyme.

Of the other compounds tested, 2-NSA did not inhibit protein kinase C activity, but it was found to be inhibitory to the biological activity of calmodulin as well as inhibiting glucose-stimulated glucose oxidation . Therefore 2-NSA could not be employed as a specific probe for transglutaminase activity in islets.

Dimethyl-2-NSA was found to inhibit the activation of phosphodiesterase by calmodulin, and since no other cellular targets have been identified, it is possible that the observed inhibition of glucose-stimulated insulin release at a concentration of 400μM may be via specific calmodulin antagonism in the β-cell.

1-NSA was similarly found to have an overlap in the concentrations at which it inhibited glucose-stimulated insulin release and those at which it inhibited the calmodulin dependent activation of bovine heart phosphodiesterase. The small structural difference between 1-NSA and 2-NSA resulted in a significant change in the potency of the compounds as calmodulin antagonists, with the substitution at the 2-position of the naphthalene ring being approximately 5-times more potent. Similar observations were made by Hidaka and Tanaka (1983) when comparing 2-NSA with 1-NSA, and when comparing W7 (IC_{50} = 26μM) and a W7 analogue where the sulphonamide group was altered to the 2-position (IC_{50} = 14μM). At lower concentrations of 1-NSA where its action in the islet may have been the specific inhibition of transglutaminase activity, only a low inhibition of glucose-stimulated insulin release was observed. Therefore 1-NSA was not found to be a sufficiently potent specific-inhibitor of transglutaminase activity.

1-NCA was found to be a less potent inhibitor of phosphodiesterase activation by calmodulin than 1-NSA, whereas the reverse was observed when comparing the potency of the two compounds against transglutaminase activity. However the toxic effects of 1-NCA on insulin release made it an unsuitable probe in the islet.

A profound effect on the biological activity of these compounds was observed by iodinating the naphthalene ring at the 5-position and extending the length of the sulphonamide chain from C_6 to C_8 as in C_8-iodo-W7. For example, C_8-iodo-W7 was found to be considerably more potent (10 fold) than W7 as an antagonist of the activation of phosphodiesterase by calmodulin. In contrast increasing the length of the alkyl chain had the opposite effect on the inhibition of transglutaminase activity, thereby greatly increasing the specificity of C_8-iodo-W7 for calmodulin antagonism. C_8-iodo-W7 was not found to be inhibitory to protein kinase C activity or to glucose-stimulated glucose oxidation at concentrations at which it inhibited activation of phosphodiesterase by calmodulin (<10μM). These findings suggest the possible employment of C_8-iodo-W7 as a specific probe for the role of calmodulin in insulin secretion from the β-cell.

A general trend was observed in the benzene-sulphonamides tested which indicated that an increase in the potency of these compounds as calmodulin antagonists may be correlated to the bulkiness of the hydrophobic side chains.

TIP-SA proved to be toxic to all of the cellular targets it was screened against. This overall toxicity to the cell probably explains the apparent increase in glucose-stimulated insulin release observed and the poor condition of the islets following incubation with this compound. TMB-SA could only be employed as a specific probe for transglutaminase activity at concentrations of

<50μM because of its inhibitory effect on the action of calmodulin when used at higher concentrations. At this low concentration the degree of inhibition of glucose-stimulated insulin release was unsatisfactory (20% at 50μM). Both tosyl-SA and bromobenzyl-SA were also unsatisfactory as probes for transglutaminase activity in islets due to their effects on glucose-stimulated glucose oxidation and because of their low potencies against glucose-stimulated insulin release.

In conclusion, MDCD evolved as a specific inhibitor of transglutaminase activity when used over the concentration range of 30-75μM. The specificity of MDCD may also be further tested by the parallel use of dimethy-MDCD as a control compound. Both C_8-Iodo-W7 and dimethyl-2-NSA may prove useful as specific probes for investigation of the involvement of calmodulin in cellular processes.

3.2.8. EFFECT OF MONODANSYLCADAVERINE ON THE DYNAMICS OF GLUCOSE-STIMULATED INSULIN RELEASE.

Having determined that MDCD was a specific inhibitor of islet transglutaminase activity and that in batch incubations MDCD inhibited glucose-stimulated insulin release, the effect of MDCD on the dynamics of glucose-stimulated insulin release was investigated.

This analysis was undertaken using a perifusion system as described in Section 2.9.5.2. . The resultant insulin-release profile in the presence and absence of 75μM MDCD from four separate experiments undertaken in duplicate is presented in Fig. 3.28, along with a single experiment with di-methyl-MDCD.

Insulin release in the presence of basal glucose remained at a constant level of 4-5μU per min., but upon stimulation with 16.8mM glucose a biphasic release pattern was observed which was preceeded by a small but consistent decrease in insulin release (1^{st} trough). Following this small trough a rapid increase in insulin release then occured over the next 5 min. averaging between 30-40 μU per min. (1^{st} phase), followed by a further decrease (2^{nd} trough) and then a more gradual second increase in the insulin release pattern (2^{nd} phase). The observed dynamics of glucose-stimulated insulin release were similar to those first reported by Grodsky et al. (1968) and subsequently observed by many other workers. A similar insulin release profile was obtained from the single experiment undertaken in the presence of 75μM di-methyl-MDCD, although further experiments would be required to confirm the absence of effect of this compound.

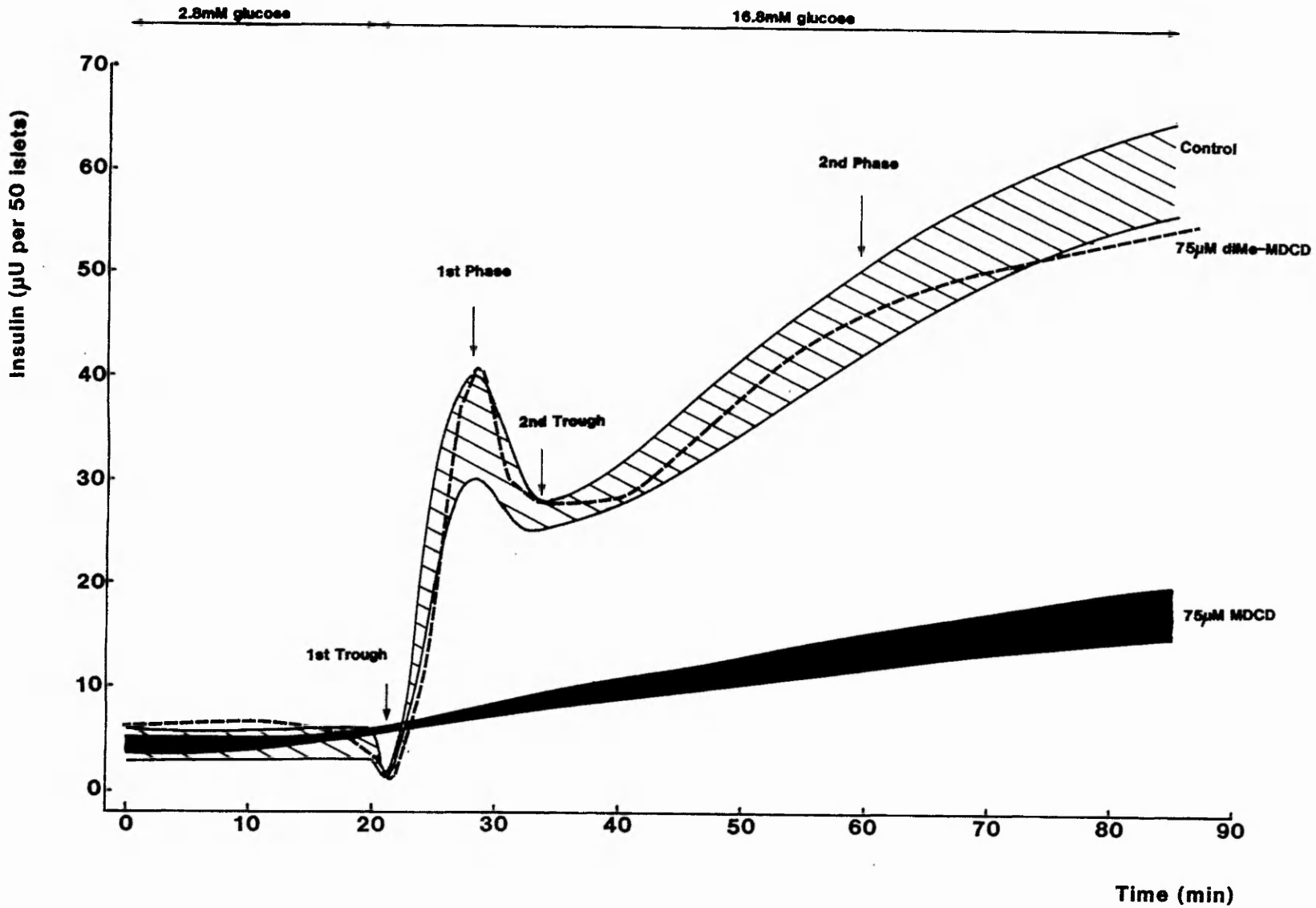
In the presence of 75μM MDCD the 1^{st} phase of insulin release appeared to be completely inhibited, interestingly the 1^{st} trough was not observed. A small but continual increase of insulin release was observed over the incubation period of 80 min. which amounted to 30% (approx.) of that seen in the absence of MDCD.

Figure 3.28.

Glucose-stimulated insulin release profile in the presence of 75μM MDCD.

Batches of 50 islets were perifused with medium containing 16.8mM glucose ± 75μM MDCD as described in Section 2.9.5.2. . The perifusate samples collected were then stored at -70°C for subsequent determination of the insulin content by RIA (cf. Section 2.9.5.3.). The control data and data for 75μM MDCD represent the results of four experiments where each treatment was undertaken in duplicate. A single experiment was undertaken with 75μM di-methyl-MDCD which is represented by the broken line.

Figure 3.28


2.8mM glucose
16.8mM glucose
Insulin (μU per 50 Islets)
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
Time (min)
Control
75μM diMe-MDCD
75μM MDCD
1st Phase
2nd Phase
1st Trough
2nd Trough

3.2.8.1. DISCUSSION.

The biphasic pattern of glucose-stimulated insulin release which is normally observed is thought to be linked to the intracellular Ca^{2+} concentration (cf. Section 1.6.4.). The 1^{st} phase corresponding to the glucose induced mobilisation of intracellular Ca^{2+} stores which rapidly become exhausted and hence the observed decrease of the 2^{nd} trough. The glucose-induced opening of voltage-gated Ca^{2+} channels (cf. Section 1.6.4.) is then thought to result in a more gradual and prolonged increase in cytosolic Ca^{2+} from extracellular sources and hence the observed 2^{nd} phase of insulin release (Wollheim & Sharp, 1981). However as outlined in Section 1.6.6. there is now evidence to suggest that the 1^{st} phase of insulin release corresponds to secretion from insulin granules which are particularly labile to glucose and already primed at or near the plasma membrane. The 2^{nd} more prolonged phase of insulin secretion corresponding to secretion from insulin granules that must first undergo margination from the cytoplasmic pool to the plasma membrane. This two-compartment model was first proposed by Grodsky et al. (1969).

The observed effect of MDCD on the biphasic pattern of insulin release indicates that the 1^{st} phase is totally removed, whilst the 2^{nd} phase is greatly diminished. This observation infers that transglutaminase is involved in the mechanism of insulin release associated with apposition of secretory vesicles with the plasma membrane, evidence which is enhanced by the lack of inhibitory effect of di-methyl-MDCD at the same concentration.

These initial studies form the basis for more investigation. The reversibility of the inhibition by MDCD could be investigated, if reversibility was detected this would infer that transglutaminase is involved in the dynamic turnover of some aspect

of the secretory apparatus. The effect of MDCD on insulin secretion stimulated by secretagogues other than glucose, such as K^+, cAMP potentiators such as forskolin, and activators of protein kinase C such as the diacylglycerol analogue TPA (12-O-tetradecynol-phorbol-13-acetate), may provide information as to the point of involvement of transglutaminase in the distal events of glucose-stimulated insulin release.

3.3. SUBSTRATE STUDIES

The studies so far presented strongly infer that the inhibition of glucose-stimulated insulin release by MDCD is effected by the specific inhibition of islet transglutaminase activity. Previous studies in the laboratory, concerned with the identification of the endogenous substrates of the islet enzyme, have indicated the specificity of the islet transglutaminase to be directed towards the cross-linking of endogenous membrane or membrane-associated proteins when undertaken with islet homogenates (Bungay et al., 1986). These studies indicated the presence of a large molecular weight radiolabelled polymer which was formed when islet homogenates were incubated with [^{14}C]-methylamine and Ca^{2+}. This polymer was not observed when homogenates were incubated in the presence of EGTA or iodoacetamide. However, owing to a number of technical difficulties the presence of radiolabelled proteins has not been detected in islet proteins when intact islets are incubated with [^{14}C]-methylamine and Ca^{2+} in the presence of stimulatory levels of glucose.

Another mechanism whereby islet proteins may be modified post-translationally so that they affect the secretory apparatus is through acting as substrates for specific protein kinases as discussed in Section 1.6. The prospect of protein substrates of transglutaminase being phosphorylated prior to their cross-linking would allow for the study of high molecular weight phosphorylated products of transglutaminase activity in the intact cell following the labelling of islet proteins using [^{32}P]Pi. Technically this could overcome the limitations of the previous studies with [^{14}C]-methylamine.

The possibility of additional control of transglutaminase activity by the phosphorylation of its substrates also seems an attractive model since to simply activate the enzyme by

Ca^{2+} levels alone could lead to a lack of refined control of the secretory process. It is unlikely that any additional control of the islet transglutaminase is achieved by a particular localisation of the enzyme to a specific cellular compartment since the islet transglutaminase appears to be predominantly cytosolic in nature. Hence it is more likely that either the enzyme or its substrates are modified to provide a further modulation of enzyme activity. It has also been reported that the preferred protein substrates of transglutaminase are shared by a number of other enzymes which cause post-translational modification of proteins including protein kinases (Martelo et al., 1970; Reimann et al., 1971). Whilst Iwanij (1977) found that phosphoproteins act as good substrates for the liver transglutaminase. There have also been very tentative suggestions that transglutaminase itself may be activated by phosphorylation (Gomis et al., 1986).

If protein kinases and transglutaminase activities play a role simultaneously in the intact β-cell during stimulus-secretion coupling it is likely that they are closely coordinated.

The following studies were therefore aimed to further demonstrate the presence of substrate proteins for transglutaminase in rat pancreatic islets, and to investigate the possible relationship between proteins which are phosphorylated and those which are modified by transglutaminase in the intact β-cell during stimulus-secretion coupling.

3.3.1. PRESENCE OF A HIGH MOLECULAR WEIGHT PHOSPHORYLATED PROTEIN POLYMER IN GLUCOSE-STIMULATED PANCREATIC ISLETS

To investigate a possible relationship between transglutaminase activity and protein phosphorylation in the glucose-stimulated pancreatic islet involving the cross-linking of phosphorylated islet proteins, intact islets were first radiolabelled using [^{32}P]Pi. Experiments were then undertaken to determine whether a high molecular weight phosphorylated protein could be detected in the glucose-stimulated islet. The phosphorylation pattern obtained for islet proteins following their separation by SDS-polyacrylamide gel electrophoresis is illustrated in Figure 3.29. Of the material which incorporated ^{32}P, approximately 15% was found to be present at the top of the 3% (w/v) stacking gel, making this the most heavily labelled component in the gel. Since high molecular weight ^{32}P material may be due to the presence of radiolabelled RNA and DNA or of nucleic acid - protein complexes, islet homogenates were treated with DNAase and RNAase prior to electrophoretic separation. This treatment led to a small reduction in the total amount of radiolabel present in the gel, especially that found in the first 2cm of the resolving gel. However no significant reduction occurred in the amount of radiolabel present at the top of the stacking gel, indicating that this component was not [^{32}P]-labelled nucleic acid or protein complexed to nucleic acid. A summary of the phosphorylation pattern obtained from a number of experiments (n = 18) where islet homogenates were pre-treated with RNAase and DNAase prior to electrophoresis is shown in Figure 3.30 and the data is tabulated in Table 3.11.

Figure 3.29.

Protein phosphorylation pattern found in glucose-stimulated intact pancreatic islets and the effect of RNAase and DNAase upon this pattern.

Batches of 60 islets were incubated in the presence of [^{32}P]Pi and stimulatory levels of glucose (16.8 mM) to promote maximum radiolabelling of ATP in the islet (Christie and Ashcroft, 1984) and phosphorylation of islet peptides during β-cell stimulation. Islets labelled in this way were homogenised and the phosphorylated proteins then analysed for radioactivity following their separation by SDS-gel electrophoresis. The radioactivity found in samples treated this way are given in column A. Identically treated islet homogenates were further treated with RNAase (50 μg/ml) and DNAase (50 μg/ml) for 5 min before electrophoretic analysis. The radioactivity found in samples treated in this way is given in column B. There was no visual difference in the protein stained electrophoretograms obtained from the two differing treatments. The migration distance of protein molecular weight markers treated identically to the protein samples are indicated.

The autoradiogram shown in column 2 was prepared by the methods outlined in Section 2.3.3.2.2. using islet homogenates that had been pre-treated with RNAase and DNAase prior to electrophoretic separation.

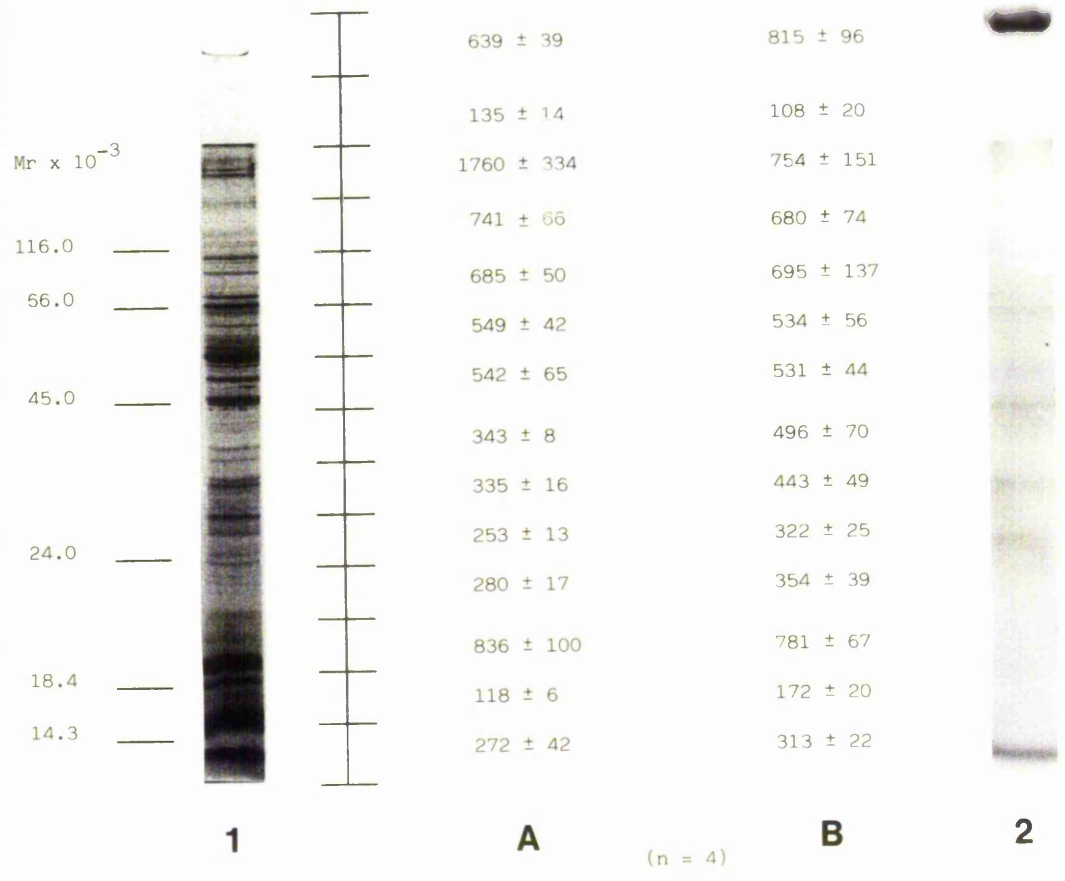

RADIOACTIVITY IN GEL (c.p.m.)
Mr x 10^{-3}
116.0
66.0
45.0
24.0
18.4
14.3
639 ± 39
135 ± 14
1760 ± 334
741 ± 66
685 ± 50
549 ± 42
542 ± 65
343 ± 8
335 ± 16
253 ± 13
280 ± 17
836 ± 100
118 ± 6
272 ± 42
815 ± 96
108 ± 20
754 ± 151
680 ± 74
695 ± 137
534 ± 56
531 ± 44
496 ± 70
443 ± 49
322 ± 25
354 ± 39
781 ± 67
172 ± 20
313 ± 22
1
A
(n = 4)
B
2

Figure 3.30.

Summary of the phosphorylation pattern obtained with islet homogenates pre-treated with RNAase and DNAase prior to electrophoresis

The histogram represents the average data obtained from a number of experiments (n = 18) of islet homogenates prepared as described in the legend to the previous figure and pre-treated with RNAase (50 µg/ml) and DNAase (50 µg/ml) for 5 min at 37^{o}C prior to electrophoresis.

The 0.5cm top section of the stacking gel comprised of approximately 15% of the total incorporated [^{32}P], as presented in Table 3.11.

The c.p.m. values for each separate set of control data were corrected for decay from the day of counting back to the activity date. The range of the total counts found in the gel varied from 4207 - 11206.

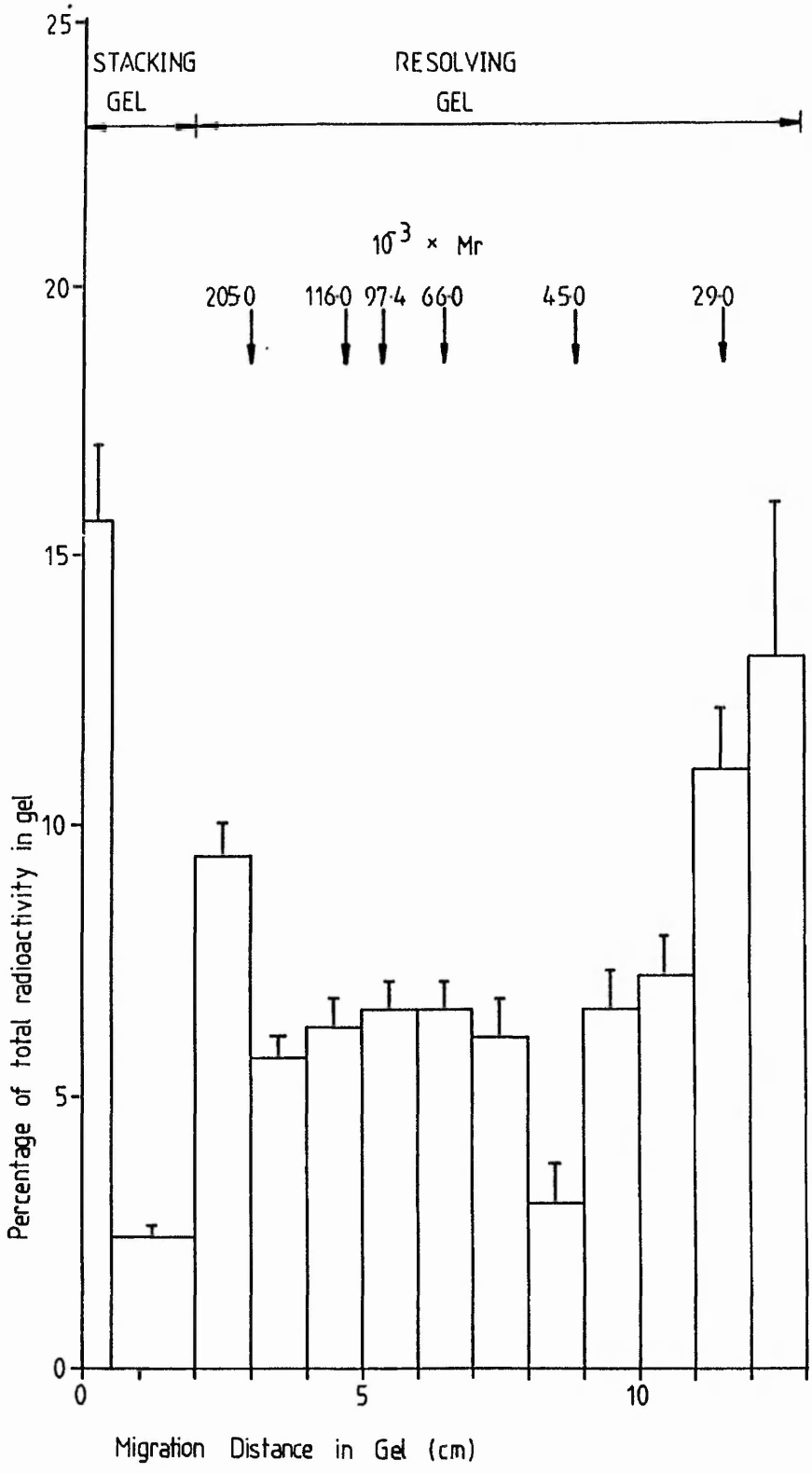

25
20
15
10
5
0
STACKING GEL
RESOLVING GEL
10^3 × Mr
205·0
116·0
97·4
66·0
45·0
29·0
Percentage of total radioactivity in gel
0
5
10
Migration Distance in Gel (cm)

Table 3.11.

Summary of the phosphorylation pattern data obtained with islet homogenates pretreated with RNAase and DNAase prior to electrophoresis

Gel Section		c.p.m. ± S.E.	% of total
first 0.5cm of stacking gel		1090 ± 99	15.6 ± 1.4
rest of stacking gel		166 ± 12	2.4 ± 0.2
resolving gel	1cm	658 ± 43	9.4 ± 0.6
	2cm	396 ± 28	5.7 ± 0.4
	3cm	471 ± 34	6.3 ± 0.5
	4cm	458 ± 38	6.6 ± 0.5
	5cm	463 ± 34	6.6 ± 0.5
	6cm	428 ± 47	6.1 ± 0.7
	7cm	213 ± 50	3.0 ± 0.7
	8cm	459 ± 47	6.6 ± 0.7
	9cm	502 ± 52	7.2 ± 0.7
	10cm	771 ± 80	11.0 ± 1.1
	11cm	914 ± 199	13.1 ± 2.8
total resolving gel counts		5733 ± 447	82.0 ± 6.4
total gel counts		6989 ± 479	100 ± 6.9

The heavy molecular weight phosphorylated component is further illustrated in Figure 3.29(2) which shows an autoradiogram of the separated islet proteins. The dense radiolabelling at the top of the stacking gel can be clearly visualised.

3.3.2. TREATMENT OF PHOSPHORYLATED ISLET PROTEIN HOMOGENATES WITH PROTEOLYTIC ENZYMES

In order to investigate the nature of the detected phosphopolymer, homogenates of islets that had been incubated with [^{32}P] under stimulatory levels of glucose (16.8mM) and subsequently treated with RNAase (50 µg/ml) and DNAase (50 µg/ml) were further treated with either of the proteolytic enzymes pronase (50 µg/ml) or trypsin (50 µg/ml). The homogenates were digested with the respective proteolytic enzyme for 30 min at 37^{o}C before preparation for electrophoresis. Controls were undertaken where no treatment with proteolytic enzymes was included. The results of these treatments are presented in Table 3.12.

Both pronase and trypsin treatment reduced the counts present at the top of the stacking gel by approximately 30%. These relatively small decreases in the counts present at the top of the stacking gel may be accounted for by the phosphopolymer being so extensively cross-linked that it is resistant to proteolytic breakdown under the short period of incubation allowed. The counts in the resolving gel were significantly reduced by both proteolytic enzymes (68-83%), indicating that the enzymes were active under the conditions of the assay.

Table 3.12.

Effect of proteolytic enzymes on the protein phosphorylation pattern of [^{32}P] - labelled islets

Homogenates of phosphorylated islets, incubated as described previously, were digested for 5 min at 37°C with RNAase (50 µg/ml) and DNAase (50 µg/ml) and subsequently further digested for 30 min at 37°C with pronase or trypsin each at a concentration of 50 µg/ml. Samples were then dissolved in electrophoresis sample buffer and electrophoresed. Control samples where proteolytic enzymes were omitted were used to calculate the % decrease obtained with the respective proteolytic enzymes. The counts present at the top of the stacking gel in control samples were 924 ± 71 and the total counts present in the resolving gel in control samples were 4860 ± 371.

	Treatment	
	Pronase	Trypsin
% decrease in counts at top of stacking gel	29.3 ± 3.2 ($p < 0.05$)	28.4 ± 9.2 ($p < 0.05$)
% decrease in counts in the resolving gel	68.3 ± 0.7 ($p < 0.01$)	83.1 ± 0.6 ($p < 0.001$)
	n = 4	n = 4

3.3.3. EXTRACTION OF PHOSPHORYLATED ISLET PROTEIN HOMOGENATES WITH ETHANOL-ETHER FOLLOWING T.C.A. PRECIPITATION AND SUBSEQUENT DIGESTION WITH PRONASE

It was possible that the detected phosphopolymer included in its composition some [^{32}P]-labelled phospholipid. In order to investigate this possibility homogenates of [^{32}P]-incubated pancreatic islets, obtained as previously described, were treated with T.C.A. to precipitate islet proteins. Control samples were then dissolved directly in electrophoresis sample buffer, whilst test samples were extracted with ethanol:ether (1:1 v/v), some of these test samples were then further digested with pronase overnight. Theoretically it should be possible to digest with pronase overnight since phosphatases that might otherwise have reduced the [^{32}P]-labelling were unlikely to be actively present in T.C.A. precipitated material. The data is presented in Table 3.13.

Although an 11.5% reduction in the counts present in the top of the stacking gel was observed after treatment with ethanol-ether, this decrease was not found to be statistically significant. Indicating that, using this method of analysis, there did not appear to be a significant amount of phosphorylated phospholipid present in the phosphopolymer. Similarly, a significant decrease in the counts present in the resolving gel was not observed. Treatment of T.C.A. precipitated homogenates with pronase after ethanol:ether (1:1 v/v) extraction did not result in any additional decrease in the counts present in the stacking gel. This result was surprising, particularly as the pronase digestion was undertaken overnight. The failure of pronase to breakdown the phosphopolymer may reflect the effect of T.C.A. precipitation and solvent extraction on the nature of the phosphopolymer, thus T.C.A. followed by ethanol and ether may have caused a conformational change in the polymeric

Table 3.13.

Effect of T.C.A. precipitation and ethanol-ether extraction, and subsequent pronase digestion on the phosphorylation pattern of islet proteins

Batches of 60 islets were incubated with [^{32}P] Pi and 16.8mM glucose as previously described. The islets were then homogenised in ice-cold 10% T.C.A. and the resultant protein pellet washed with ice-cold distilled water. Control samples were then dissolved in electrophoresis sample buffer, whilst test samples were extracted three times with water saturated ethanol/ether mixture 3:1 (v/v). Extracted samples were either dissolved in electrophoresis sample buffer or resuspended in homogenisation buffer excluding P.M.S.F. and digested with pronase at a concentration of 50 µg/ml overnight prior to dissolution in electrophoresis sample buffer. For the counts present in T.C.A. precipitated control samples see Figure 3.31.

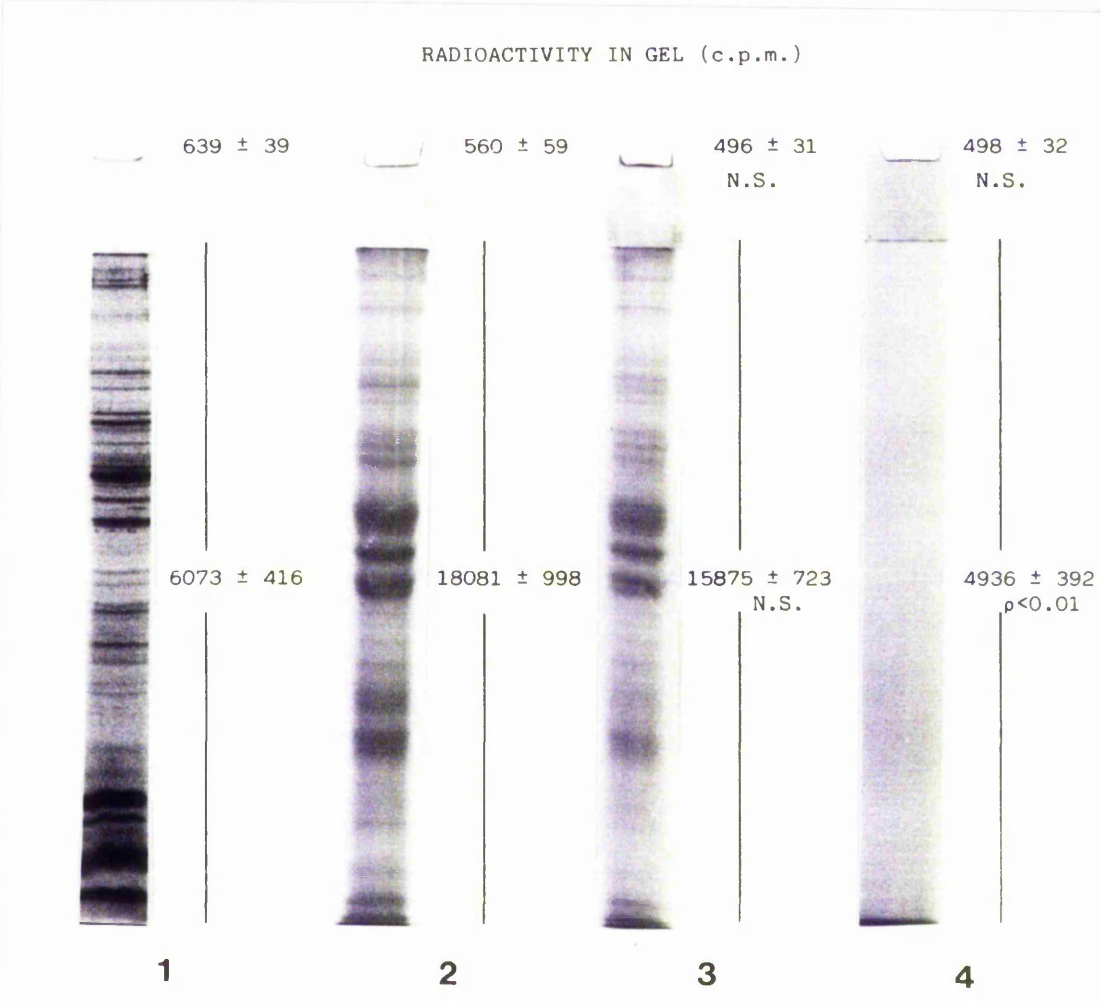
	% reduction in counts compared to T.C.A. precipitated control samples	
	Further Treatment	
	ethanol:ether extraction	ethanol:ether extraction and pronase digestion
Counts in top 0.5cm of stacking gel	11.5 ± 5.6 (n = 5) N.S.	11.0 ± 5.7 (n = 3) N.S.
Total counts in resolving gel	12.2 ± 4.0 (n = 5) N.S.	72.7 ± 7.7 (n = 3) $p < 0.01$

material resulting in its dehydration and thereby removing the sites of potential pronase attack since full rehydration of this polymeric material would be difficult to achieve. The pronase was again seen to be active under the conditions of incubation as determined by the significant decrease in the counts present in the resolving gel. This observation is illustrated in Figure 3.31 where treatment with pronase is shown to have removed the majority of the phosphorylated protein present in the resolving gel. Precipitation of islet homogenates with T.C.A. was found not to significantly alter the number of counts observed at the top of the stacking gel when compared with the control homogenates dissolved directly into electrophoresis sample buffer. However there was a very significant increase in the counts observed in the resolving gel. Homogenates dissolved straight into sample buffer had counts present in the resolving gel of 6073 ± 416 cpm while those samples treated with T.C.A. had counts present in the resolving gel of 18,081 ± 1040 cpm. Extraction of the T.C.A. precipitated homogenates with ethanol:ether did not appear to affect the general appearance of the protein stained electrophoretograms, although samples treated with T.C.A., in general, produced significantly less well defined bands as compared to homogenates dissolved straight into sample buffer.

Figure 3.31.

Effect of T.C.A. precipitation and ethanol-ether extraction, and subsequent pronase digestion on the phosphorylation pattern of islet proteins

Batches of 60 islets were treated as described in the legend to Table 3.13. Electrophoretogram 1 represents islets dissolved straight into electrophoresis sample buffer; electrophoretogram 2 represents islets after T.C.A. precipitation; electrophoretogram 3 represents islets after T.C.A. precipitation and ethanol:ether extraction; electrophoretogram 4 represents subsequent pronase digestion of islets following T.C.A. precipitation and ethanol:ether extraction. Significance tests were undertaken comparing the data from treatments 3 and 4 to that for treatment 2. (N.S. denotes no significant difference).


RADIOACTIVITY IN GEL (c.p.m.)
639 ± 39
560 ± 59
496 ± 31
N.S.
498 ± 32
N.S.
6073 ± 416
18081 ± 998
15875 ± 723
N.S.
4936 ± 392
p<0.01
1
2
3
4

3.3.4. DIGESTION OF PHOSPHORYLATED ISLET MEMBRANES WITH PHOSPHOLIPASE A_2/PHOSPHOLIPASE C AND PRONASE

As a result of the fractionation studies described in Section 3.3.7. which indicate the predominant membrane association of the phosphopolymer, it was decided to further investigate the nature of the polymer by its treatment with the two phospholipases A_2 and C, and by subsequent digestion of the remaining material with pronase. This investigation aimed to discover whether any of the ^{32}P detected was due to radiolabelled lipids or lipid associated with protein, and to investigate any association of the polymer with membrane components. Due to the requirement of phospholipase A_2 for Ca^{2+}, this investigation could not be undertaken on the islet homogenates as such an incubation would have resulted in activation of the islet transglutaminase. However, as the majority (86%) of the phosphopolymer was found in the islet membrane fractions, it was possible to undertake this investigation on the phosphorylated islet membranes since this fraction of the homogenates contained only a very small percentage of the islet transglutaminase. The phosphorylated islet membranes were obtained by a single centrifugation (80,000g av. for 45 min). The results of the phospholipase and pronase treatment on the phosphorylation pattern of these islet membranes is detailed in Table 3.14.

To minimise the activity of endogenous proteases and phosphatases in the homogenate, particularly as PMSF was omitted from the homogenisation medium (in order not to inhibit the activity of pronase) the total digestion time was limited to 30 min. The treatment of the phosphorylated islet membranes with phospholipase A_2/C (15 min) resulted in a percentage decrease in the counts present in the top of the stacking gel of 22.0 ± 9.5%, whilst the total counts present in the resolving gel decreased by 12.6 ± 2.4%.

Table 3.14.

Effect of phospholipase A_2/C and pronase on phosphorylated islet membranes

Following incubation with [^{32}P] and 16.8mM glucose, batches of 60 islets were pooled together and homogenised. The homogenate was then centrifuged at 80,000g av. for 45 min and the pellet resuspended in medium as described in section 2.9.10.2.3. Control samples were treated solely with RNAase (50 µg/ml) and DNAase (50 ug/ml), test samples were further treated with phospholipase C (5U) and phospholipase A_2 (5U) combined (15 min), some of these samples were then further digested with pronase (50 µg/ml) (15 min); all samples were dissolved in electrophoresis sample buffer, subsequently electrophoresed and the electrophoretograms sectioned and counted. The mean total counts present in the top of the stacking gel of control samples were 890 ± 92 and the mean total counts in the resolving gel were 4944 ± 501.

	% reduction in counts compared to untreated control samples	
	Treatment	
	phospholipase A_2/C (15 min)	phospholipase A_2/C (15 min) + pronase (15 min)
counts in top 0.5cm of stacking gel	22.0 ± 9.5 (n = 4) p <0.05	37.4 ± 3.5 (n = 4) p <0.05
total counts in resolving gel	12.6 ± 2.4 (n = 4) p <0.01	42.6 ± 0.4 (n = 4) p <0.001

Further treatment of the phosphorylated membranes with pronase (50 µg/ml for 15 min) resulted in a total decrease in the counts present in the top of the stacking gel of 37.4 ± 3.5% when compared with untreated controls and a total decrease in counts of 32.6 ± 0.4% in the resolving gel. Therefore during the 15 min pronase digestion the proteolytic enzyme had decreased the counts present in the top of the stacking gel by 15.4 ± 3.5% which is proportionally comparable to the 29.3 ± 3.2% decrease in the counts present in the top of the stacking gel when whole homogenates were digested with pronase for double the time (c.f. Table 3.12). Similarly the effect of pronase on the number of counts present in the resolving gel resulted in a 30.0 ± 0.4% reduction which is again proportionally comparable to the 68.3 ± 0.7% decrease that occurred for the same gel section when the whole homogenate was incubated with pronase for twice the time (c.f. Table 3.12).

3.3.5. INHIBITION OF GLUCOSE-STIMULATED FORMATION OF PHOSPHOPOLYMER IN INTACT ISLETS BY INHIBITORS OF TRANSGLUTAMINASE ACTIVITY

In order to test the hypothesis that transglutaminase was involved in the formation of the phosphorylated protein polymer in glucose-stimulated islets, intact islets were incubated with monodansylcadaverine (MDCD) or glycine methyl ester (GME) in the presence of stimulatory levels of glucose (16.8mM) and [^{32}P]Pi. GME is a further competitive inhibitor of transglutaminase activity which has been shown to specifically inhibit transglutaminase activity and thereby inhibit glucose-stimulated insulin release (Bersten et al., 1983). In addition, control experiments were performed using the methylated analogues of the two compounds, namely N-dimethylmonodansylcadaverine (dimethyl MDCD) and sarcosine methyl ester (SME) which lack the primary amine moiety essential for inhibition of transglutaminase activity. The results of these experiments are shown in Table 3.15.. MDCD (50μM) and GME (10mM) both inhibited the amount of phosphorylated material present at the top of the stacking gel by 25% and 37% respectively. In contrast, the control compounds dimethyl MDCD and SME did not significantly reduce the amount of phosphorylation in this component of the gel. When the total amount of phosphorylation present in the rest of the gel was taken into account, MDCD did not give rise to any significant inhibition of phosphorylation, whereas GME gave rise to a 57% stimulation. These results therefore suggest that the reduction in the amount of phosphopolymer present in the stimulated islet is due to its formation and not due to inhibition of islet protein phosphorylation. The comparable experiments undertaken to investigate the effects of MDCD and GME on glucose-stimulated insulin release indicated that

Table 3.15

Effects of transglutaminase inhibitors and their respective control compounds on glucose-stimulated insulin release and the protein phosphorylation pattern in glucose-stimulated intact islets

Batches of 60 islets were incubated in the presence of the test compound plus 2.8mM glucose for a preincubation period of 45 min, followed by a 90 min incubation in the presence of the test compound plus 16.8mM glucose and [^{32}P]Pi as described in Section 2.9.10.3. After incubation, islets were homogenised and immediately dissolved in electrophoresis sample buffer and electrophoresed as described in Section 2.4.3. The effects of test compounds on the phosphorylation pattern of islet proteins was carried out in paired experiments and expressed as a percentage (± S.E.M.) of the fraction of radioactivity found in controls. For insulin release measurements batches of 60 islets were taken through an identical incubation protocol excluding [^{32}P]Pi and the insulin content of the supernatant medium determined by radioimmunoassay. The inhibition of glucose--stimulated insulin release for each test compound was then expressed as a percentage (± S.E.M.) of the rate in control incubations, the number of experiments is shown in parentheses. Typical amounts of radioactivity found at the top of the stacking gel were 1099 ± 99 cpm and in the total resolving gel 5733 ± 447 cpm (n = 8). Typical rates of insulin release under the incubation conditions used were 3.4 ± 0.5 (n = 9) μUnits per 90 min per islet for islets incubated in 2.8mM glucose and 22.8 ± 2.9 μUnits per 90 min per islet for islets incubated in 16.8mM glucose.

N.S. denotes no significant difference from control values.

Table 3.15.

Test Compound	Conc (mM)	Radioactivity at the top of the stacking gel expressed as a percentage of the fraction found in controls	Radioactivity in the rest of the gel expressed as a percentage of the fraction found in controls	Inhibition of glucose-stimulated insulin release (%)
MDCD	0.05	74.7 ± 3.9 (8) p <0.01	91.8 ± 8.7 (8) N.S.	59.4 ± 2.4 (9) p <0.01
N-dimethyl-MDCD	0.1	101 ± 6.0 (7) N.S.	117.7 ± 11.2 (7) N.S.	-
GME	10	62.4 ± 5.3 (10) p <0.01	156.6 ± 15.5 (10) p <0.01	53.7 ± 4.1 (9) p <0.01
SME	10	122.6 ± 14.6 (4) N.S.	87.1 ± 6.9 (4) N.S.	-

MDCD (50μM) and GME (10mM) inhibited insulin release by 59% and 54% respectively. Earlier studies (Section 3.2.2.) indicated that N-dimethyl MDCD at a concentration of 400μM had no significant effect on glucose-stimulated insulin release, and previous evidence (Sener, et al., 1985) has shown that SME (10mM) likewise has no significant effect on glucose-stimulated insulin release.

3.3.6. THE CROSS-LINKING OF PHOSPHORYLATED ISLET PROTEINS INTO HIGH MOLECULAR WEIGHT PROTEIN AGGREGATES BY ISLET TRANSGLUTAMINASE

As a further means of investigating the involvement of islet transglutaminase in the formation of the phosphorylated polymer in the intact glucose-stimulated islet the ability of the enzyme to cross-link phosphorylated islet proteins to form high molecular weight aggregates was also assessed. Homogenates obtained from [^{32}P]-labelled islets were incubated in the presence and absence of Ca^{2+} for different time periods (0, 15, 30 and 45 min). The data obtained from these experiments is presented in Figure 3.32. The data shows a time dependent increase in the high molecular weight phosphorylated protein present in the top of the stacking gel over a thirty minute period in the presence of 5mM Ca^{2+}, no such significant increase occurred in the presence of 5mM EGTA, indicating that the phosphopolymer formation was likely to be the result of transglutaminase-catalysed cross-linking of phosphorylated proteins of lower molecular weight than the aggregate.

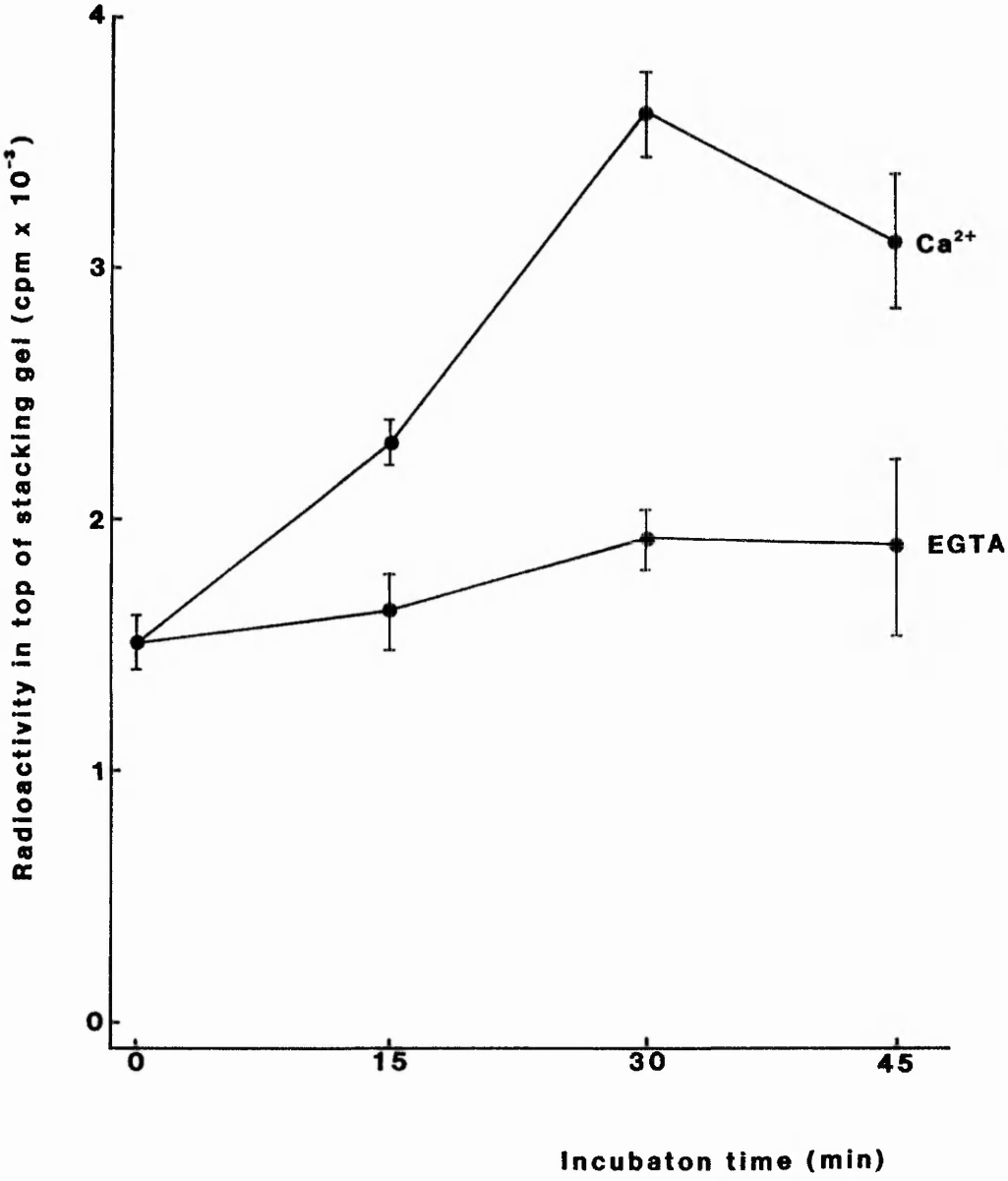
In separate experiments incorporating only a 30 min sample point, the Ca^{2+}-mediated time-dependent formation of the polymer was found to be totally inhibited by the addition of 1mM iodoacetamide to the incubation medium. Further indicating the involvement of transglutaminase in the formation of the phosphorylated polymer.

Figure 3.32.

The time dependent incorporation of phosphorylated islet proteins into high molecular weight protein aggregates

Homogenates of phosphorylated islets were incubated at 37°C in the presence or absence of either 5mM $CaCl_2$ or 5mM EGTA for 15, 30 and 45 min. Samples were digested with RNAase (50 µg/ml) and DNAase (50 µg/ml) for 5 min at 37°C prior to dissolution in electrophoresis sample buffer. Islet proteins were then separated by electrophoresis using the procedures previously described. The graph shows the amount of radioactivity present in the high molecular weight polymer at the top of the stacking gel at the different incubation times. The data are the mean c.p.m. (± S.E.M.) for three incubation samples at each time point.

Figure 3.32


Radioactivity in top of stacking gel (cpm x 10^{-3})
4
3
2
1
0
0
15
30
45
Incubaton time (min)
Ca^{2+}
EGTA

3.3.7. SUBCELLULAR DISTRIBUTION OF THE HIGH MOLECULAR WEIGHT PHOSPHOPOLYMER FOUND IN THE GLUCOSE-STIMULATED ISLET

Preliminary studies investigating the incorporation of [^{14}C]-methylamine into islet proteins have indicated that the islet proteins modified by islet transglutaminase are found in the membrane fraction of islet homogenates (Griffin et al., 1986). Therefore experiments were undertaken to determine whether the endogenous phosphorylated polymer present in glucose-stimulated islets was also membrane-associated.

Homogenates obtained from islets previously labelled with [^{32}P]Pi in the presence of 16.8mM glucose were fractionated into a low speed pellet, N (600g av.), a high speed pellet, P (80,000g av.), and a particle free supernatant, S. The resultant electrophoretic analysis of the radiolabel present in the fractions is presented in Table 3.16. Parallel experiments were undertaken in the absence of [^{32}P]Pi for which the transglutaminase and marker enzyme distribution between the fractions was determined. A histogram summarising the distribution of the radiolabel and the localisation of marker enzymes, transglutaminase and insulin is presented in Figure 3.33.

In further experiments homogenates incubated in the presence of either Ca^{2+} (5mM) or EGTA (5mM) for 30 min were also fractionated by differential centrifugation to investigate any possible changes that might occur in the subcellular distribution of the phosphopolymer following activation of the islet transglutaminase. The identity and purity of fractions was again assessed by measuring the levels of the markers 5'-nucleotidase, cytochrome C oxidase and lactate dehydrogenase in fractions obtained from Ca^{2+}-incubated homogenates. The resultant electrophoretic analysis of the radiolabel present in the different fractions is presented in

Table 3.16

Subcellular distribution of the high molecular weight phosphopolymer found in the glucose-stimulated islet

Islets (600-1,000 at a ratio of 2-3 islets per µl) previously labelled with [^{32}P]Pi in the presence of 16.8mM glucose (see Section 2.9.10.) were homogenised in medium comprising of 0.3M sucrose, 50mM sodium phosphate (pH 7.4), 1mM EGTA, 5mM NaF, 4mM ATP and 1mM P.M.S.F. The resultant homogenate was then fractionated into a 600g av. low speed pellet (N), a 80,000g av. high speed pellet (P) and a particle free supernatant (S). These fractions were then dissolved in electrophoresis sample buffer, the proteins separated by electrophoresis using the previously described procedures (Section 2.4.3.), and the radioactive counts present in the gel determined. The data is the mean ± S.E. for four separate experiments. The average total combined counts present in the top of the stacking gel for all three fractions was 1527 ± 126 per 100 islets, and the average total combined counts present in the whole gel for all three fractions was 9643 ± 228 per 100 islets.

	Subcellular Fraction		
	N	P	S
% distribution of total counts present in top of stacking gel	72.6 ± 5.1	13.3 ± 1.4	14.1 ± 3.9
% distribution of total counts found in whole gel	48.7 ± 5.0	19.4 ± 0.9	31.9 ± 4.5

Table 3.17., the data is also included in Figure 3.33 to allow comparison with the data obtained from homogenates where no incubation took place prior to fractionation. Apart from a change in the subcellular distribution of 5'nucleotidase in islet homogenates previously incubated with Ca^{2+} (as indicated in Fig. 3.33) no significant change in the distribution of the other markers occurred.

The distribution of transglutaminase activity was found to parallel that of the cytosolic enzyme lactate dehydrogenase, suggesting a cytosolic location for the islet enzyme. Whereas, in homogenates immediately fractionated, the distribution of the phosphopolymer was predominantly (73%) found in the low speed 600g av. pellet and showed no direct parallelism to any of the membrane markers. The distribution of the phosphopolymer obtained from homogenates incubated in the presence of EGTA was found to be comparable with the data obtained from islet homogenates which were fractionated without prior incubation. In this respect 73% of the phosphopolymer was present in the 600g av. pellet; 13% present in the high speed 80,000g av. pellet; and 14% present in the cytosol fraction. However, the distribution of the phosphopolymer obtained from homogenates incubated in the presence of Ca^{2+} indicated a statistically significant increase (12%, $p < 0.005$) in the amount of polymer found in the 600g av. pellet. There was also a statistically significant reduction (9%, $p < 0.05$) in the amount of phosphopolymer found in the cytosol fraction. Similarly when the percentage distribution of total radiolabel present in each of the subcellular fractions obtained from Ca^{2+}-incubated homogenates was compared to the EGTA incubated homogenates, the data indicated a significant

Table 3.17

Subcellular distribution of the high molecular weight phosphopolymer found in the glucose-stimulated islet after pre-incubation of islet homogenates in Ca^{2+}-containing medium

Following homogenisation of islets (600-1,000 at a ratio of 2-3 islets per µl) previously labelled with [^{32}P]Pi in the presence of 16.8mM glucose as previously described (Section 2.9.10.), the resultant homogenate was incubated for 30 min at 37°C in medium containing either

6mM $CaCl_2$ (net free Ca^{2+} concentration of 5mM) or 5mM EGTA. After this period the incubated homogenate was fractionated and processed, as described in the legend to Table 3.16. The data are the mean values obtained from four separate experiments. N.S. denotes no significant difference between the Ca^{2+}-incubated fraction and the corresponding fraction with no free Ca^{2+} present. The average total combined counts present in the top of the stacking gel for all three fractions was 1311 ± 89 per 100 islets, and the average total combined counts present in the whole gel for all three fractions was 8314 ± 163 per 100 islets.

		Subcellular Fraction		
		N	P	S
% distribution of total counts present in top of stacking gel	Ca^{2+}	85.2±1.8 p <0.005	10.3±0.7 N.S.	5.0±1.5 p <0.05
	EGTA	73.4±1.1	13.2±1.1	13.5±1.1
% distribution of total counts found in whole gel	Ca^{2+}	61.1±1.2 p <0.05	18.7±1.3 N.S.	20.2±2.1 p <0.05
	EGTA	48.5±3.0	19.9±2.3	32.1±3.6

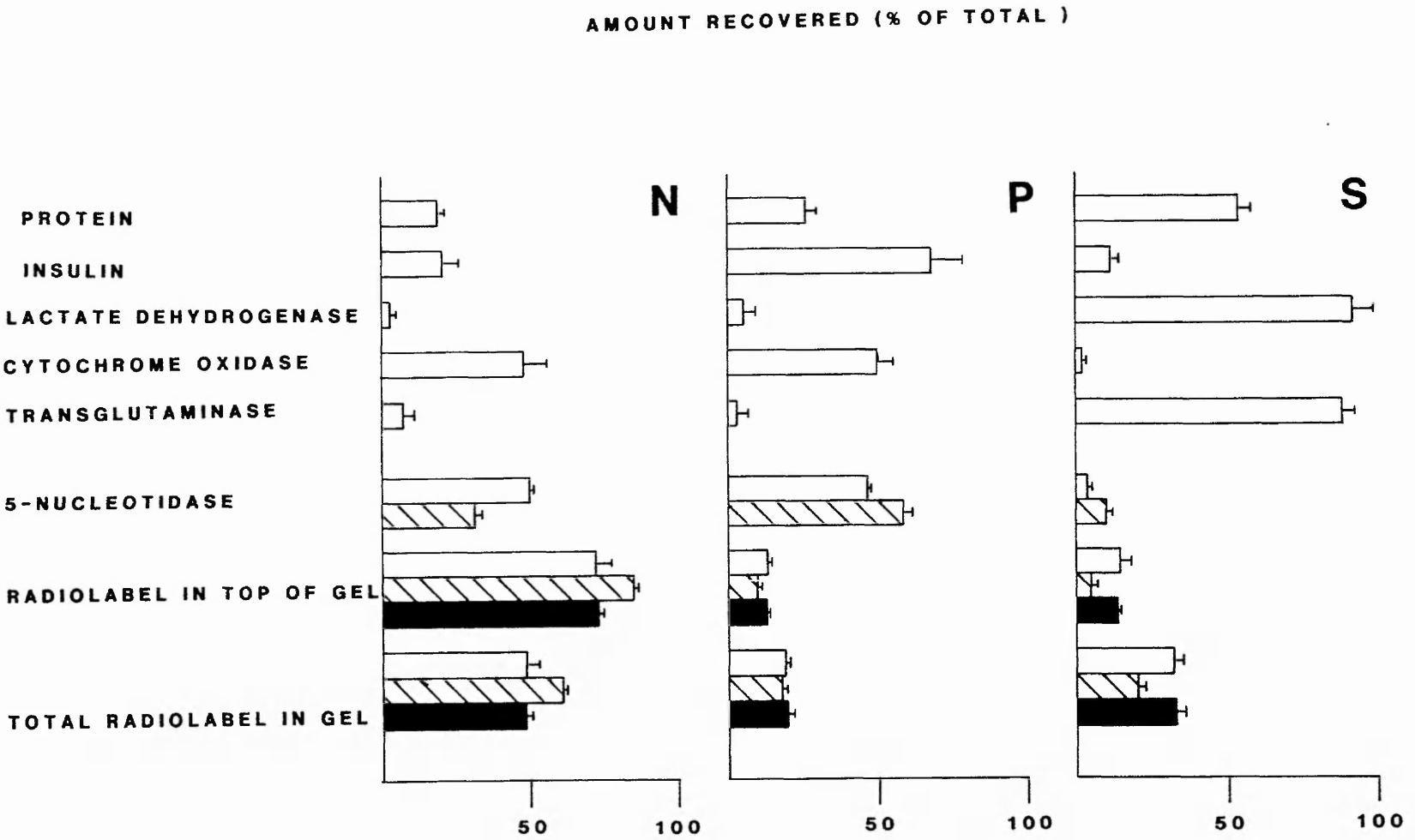
Figure 3.33

Distribution of phosphopolymer, marker enzymes and transglutaminase in islet subcellular fractions

Intact islets (600-1,000) previously incubated in the presence of [^{32}P]Pi and 16.8mM glucose were homogenised (2-3 islets per µl) as described previously (Section 2.9.10.) and then immediately fractionated by differential centrifugation into N (600g av.) and P (80,000g av.) particulate fractions and S, particle-free supernatant. The fractions were then dissolved in electrophoresis sample buffer and submitted to SDS-polyacrylamide gel electrophoresis and the radioactivity determined in the sections throughout the gel. Parallel experiments were undertaken in the absence of [^{32}P]Pi for which the transglutaminase and marker enzyme distribution between the fractions was detemined. The blank bars of the histogram show the amount of transglutaminase activity, marker enzymes, phosphopolymer and total radiolabel present in each fraction. Data for each fraction are expressed as a percentage of the total value for all fractions, and are given as means ±S.E. from three separate experiments. The percentage distribution of 5'-nucleotidase (±S.E., n=3), phosphopolymer and total radiolabel (± S.E., n=4) is also shown in each of the fractions following prior incubation of islet homogenates for 30 min in the presence of either 5mM Ca^{2+} (hatched bars) or 5mM EGTA (solid bars). No statistical significant difference was observed between the distribution of the other marker enzymes for the two differing treatments.

Typical combined values for all three fractions of the different components measured were:

	per 100 islets
Protein	58.7 ± 4.1 µg
Insulin	22.4 ± 7.3 mU
Lactate dehydrogenase	617 ± 186 µmol NADH oxidised per h
Cytochrome C oxidase	368.6 ± 56.1 µmol.cyt.C oxidised per h
Transglutaminase	14.4 ± 4.1 nmol.putrescine incorporated per h
5'-Nucleotidase	125.0 ± 10.5 nmol. AMP hydrolysed per h
Radiolabel in top of gel	1527 ± 126 c.p.m.
Total radiolabel in gel	9643 ± 228 c.p.m.


AMOUNT RECOVERED (% OF TOTAL)
N
P
S
PROTEIN
INSULIN
LACTATE DEHYDROGENASE
CYTOCHROME OXIDASE
TRANSGLUTAMINASE
5-NUCLEOTIDASE
RADIOLABEL IN TOP OF GEL
TOTAL RADIOLABEL IN GEL
50
100
50
100
50
100

increase (12%, $p < 0.05$) in the percentage radiolabel found in the 600g av. pellet. In parallel with this increase was a corresponding reduction in the amount of total radiolabel found in the cytosol fraction.

3.3.8. FURTHER EXPERIMENTS UNDERTAKEN ON THE CHARACTERISATION OF THE PHOSPHOPOLYMER

To ensure that the ^{32}P-radiolabel detected at the top of the stacking gel was a large molecular weight protein phosphopolymer and was not composed of aggregates of radiolabelled cellular debris either soluble or insoluble, a number of further experiments were undertaken. These investigations took three approaches, whereby the SDS content of the electrophoresis sample buffer was raised from 2% (w/v) to 4% (w/v) to ensure that all proteins were dissolved, the so prepared samples were centrifuged prior to electrophoresis to remove any possible insoluble cellular debris, and an agarose gel layer (0.8% w/v) was overlayed onto the stacking gel to investigate any loss of radiolabel at the top of the stacking gel. The effect of these treatments on the electrophoretic distribution of radiolabelled proteins in phosphorylated islet homogenates is given in Tables 3.18 and 3.19.

Raising the concentration of SDS in the sample buffer from 2% (w/v) to 4% (w/v), resulted in no significant change in the counts observed at the top of the stacking gel or the total counts found in the whole gel, indicating that in previous experiments the use of 2% (w/v) SDS had been satisfactory.

Similarly, incorporating a centrifugation step prior to electrophoresis did not significantly affect the counts in either the top of the stacking gel or the whole gel, indicating that no radiolabelled insoluble cellular debris was interfering with the results obtained previously.

Table 3.18

Effect of raising the SDS content of the electrophoresis sample buffer from 2% (w/v) to 4% (w/v) and of centrifugation of samples prior to electrophoresis on the protein phosphorylation pattern found in glucose-stimulated intact pancreatic islets

Homogenates (600-1000 islets at a ratio of 2-3 islets per µl) of previously phosphorylated islets (obtained as described in Section 2.9.10.) were digested for 5 min at 37°C with RNAase (50 µg/ml) and DNAase (50 µg/ml) prior to dissolution in electrophoresis sample buffer giving a final composition of 62.5mM Tris, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol, 0.01 mg/ml bromophenol blue, and either 2% (w/v) SDS or 4% (w/v) SDS. Some of the islet homogenates dissolved in electrophoresis sample buffer containing 2% (w/v) SDS were further centrifuged for 10 min at 11,600g max. and the supernatant electrophoresed. The phosphorylated proteins were then separated by electrophoresis using the previously described procedures, and the radioactive counts present in the gel determined.

N.S. denotes no significant difference in the samples when compared to the control values dissolved in electrophoresis sample buffer containing 2% (w/v) SDS and electrophoresed directly.

Table 3.18.

	Treatment prior to SDS-gel electrophoresis		
	2% (w/v) SDS	2% (w/v) SDS + centrifugation	4% (w/v) SDS
counts at top of stacking gel	416 ± 43	495 ± 52 N.S.	510 ± 51 N.S.
total counts in gel	4336 ± 376	5002 ± 411 N.S.	5542 ± 419 N.S.
stacking gel counts as a % of total gel counts.	9.6 ± 1.2	9.9 ± 0.6 N.S.	9.2 ± 0.2 N.S.
	n = 3	n = 3	n = 3

Table 3.19.

Effect of using a 0.8% agarose gel overlay on the protein phosphorylation pattern found in glucose-stimulated intact pancreatic islets

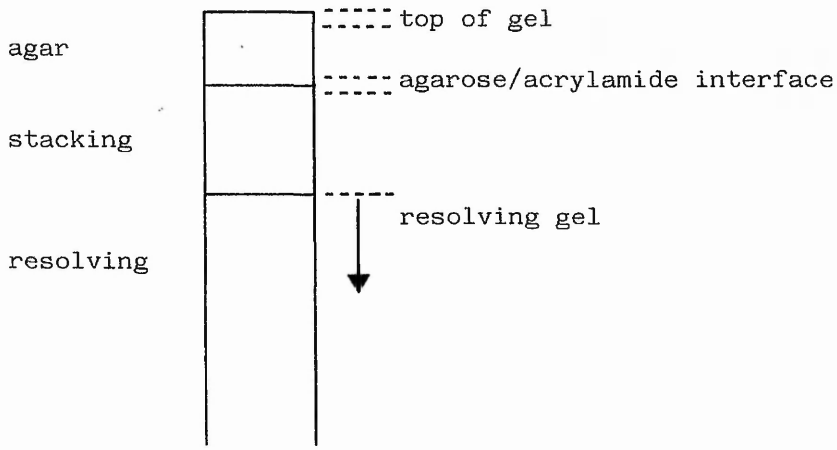
Homogenates (600-1000 islets at a ratio of 2-3 islets per µl) of previously phosphorylated islets treated with nucleases and dissolved in electrophoresis sample buffer (see Section 2.9.10.) were electrophoresed either on gels comprising of a 3% (w/v) acrylamide stacking gel on a 10% (w/v) acrylamide resolving gel, or on gels comprising of a 10% (w/v) resolving gel a 3% (w/v) stacking gel with a 0.8% (w/v) agarose gel. The radioactive counts present were then determined as previously described (see Section 2.3.2.).

N.S. denotes no significant difference.

Table 3.19

	Normal Gel	Agar Gel
counts at top of gel	416 ± 43	115 ± 11
(% of total gel counts)	(9.6 ± 1.2)	(2.1 ± 0.2)
counts at agarose/acrylamide gel interface	-	526 ± 21
(% of total gel counts)	-	(9.6 ± 0.4)
total gel counts	4336 ± 376	5482 ± 199 N.S.
	(n = 3)	(n = 3)

Schematic illustration of different gel components



The employment of a 0.8% (w/v) agarose gel accounted for only a small additional amount of radiolabelled high molecular weight polymer (2.1%). The percentage of the total gel counts found at the top of the 3% stacking gel did not significantly alter in the presence of the 0.8% (w/v) agarose gel. This data indicates that the phosphopolymer has a molecular weight in excess of 10^6 and since the phosphopolymer traversed a 0.8% (w/v) agar gel the material is unlikely to be insoluble or partly solubilised radiolabelled cellular debris.

3.3.9. DISCUSSION

The phosphorylation studies undertaken indicate that in intact islets incubated with [^{32}P]Pi under conditions of glucose-stimulation, a large portion of the total incorporated [^{32}P]-radiolabel was present in a high molecular weight component which could not traverse a 3% (w/v) stacking gel during electrophoresis of islet proteins. The amount of radioactivity present in this component was not altered by pre-digestion of islet homogenates with RNAase or DNAase thus indicating that it was unlikely to be radiolabelled nucleic acid or protein complexed to nucleic acid. However treatment of radiolabelled islet homogenates with RNAase and DNAase did result in a small reduction in the total amount of radiolabel present in the 10% (w/v) acrylamide resolving gel. Therefore it was necessary to undertake digestion of homogenates with RNAase and DNAase in all subsequent experiments.

The phosphopolymer was found to contain the largest single peak of [^{32}P]-radioactivity in the whole gel profile, this was particularly emphasised by autoradiography of electrophoretograms where the degree of labelling at the top of the stacking gel was very apparent. This observation suggests that this component is important in the system of phosphorylation reactions in the stimulated islets.

Treatment of the phosphopolymer with the proteolytic enzymes pronase and trypsin indicated that some reduction ($\simeq$ 30%) of the polymer occurred in 30 min. The finding that there was a smaller decrease in the radiolabel found in the top of the stacking gel compared with that found in the resolving gel following proteolytic digestion was not unexpected since a high molecular weight cross-linked matrix is likely to be much more resistant to proteolysis (Griffin and Wilson, 1984).

As a further means of demonstrating that the phosphopolymer was a high molecular weight phosphorylated protein, and not protein complexed with ^{32}P-labelled membrane lipids, treatments involving ethanol-ether extraction and phospholipase digestion were also undertaken.

Extraction of ^{32}P-labelled islet homogenates with ethanol-ether following precipitation of islet proteins with TCA did not result in a significant reduction of the [^{32}P]-labelling of the phosphopolymer, indicating there to be little if any [^{32}P]-labelled phospholipid associated with the phosphopolymer. However, subsequent investigations with phospholipase treatment of the particulate fraction (80,000g av. pellet) obtained from [^{32}P]-labelled homogenates resulted in a small but consistent 22 ± 9.5% (n = 4) reduction in the amount of radiolabel found in the top of the stacking gel. Thus, the two methods of analysis for characterisation of the phosphorylated phospholipid material present in the polymer did not accurately concur. However, the results from the digestion of the phosphopolymer with nucleases, proteases and phospholipases indicate that the phosphopolymer is predominantly composed of protein.

Further electrophoretic studies using an agarose gel (0.8% w/v) in combination with the 3% acrylamide stacking gel indicated that the phosphopolymer was small enough to traverse the agarose gel and that the majority of it (80-90%) entered but did not traverse the 3% (w/v) acrylamide stacking gel. These preliminary results suggest an approximate size for the phosphopolymer in excess of 10^6.

The amount of radiolabel present in the top of the stacking gel was not affected by prior centrifugation of the SDS

solubilised material nor by an increase in the SDS content of the electrophoresis sample buffer, thus indicating that the preparation of samples was scientifically sound. Additionally the phosphopolymer was able to traverse a 0.8% (w/v) agarose gel. The phosphopolymer detected at the top of the stacking gel is therefore unlikely to be due to the presence of radiolabelled cellular debris since such debris would be unlikely to traverse a 0.8% agarose gel.

Previous work in the laboratory (Bungay et al., 1986) had suggested that the cross-linking of membrane or membrane-associated proteins by the islet transglutaminase which can be demonstrated in Ca^{2+} incubated islet homogenates may represent the extreme of what may be a carefully regulated process in the intact cell. The finding of a protein phosphopolymer in the intact islet pointed to the intriguing possibility that this protein component was formed as a result of transglutaminase-catalysed protein cross-linking activity in the intact islet during glucose-stimulated insulin release. Inorder to investigate this possible relationship between islet transglutaminase activity and the formation of the phosphopolymer two further investigations were undertaken. The first consisted of looking at the effect of specific inhibitors of transglutaminase activity on the formation of the phosphopolymer. The second investigated whether the islet transgluaminase could increase the amount of phosphopolymer present in islet homogenates using incubation conditions which promoted activation of the islet enzyme.

Two specific inhibitors of transglutaminase activity were investigated for their effects on the formation of the polymer in glucose-stimulated intact islets. MDCD, which has been shown to be a specific inhibitor of transglutaminase activity in the previous section of this thesis (Section 3.2), in conjunction with the

dimethylated control compound dimethyl-MDCD. The second inhibitor employed was glycine methylester (GME), which has also been documented to be a specific inhibitor of transglutaminase activity (Bersten, et al., 1983) used in combination with its control analogue sarcosine methyl ester (SME).

These investigations indicated that those compounds capable specifically of inhibiting transglutaminase activity also inhibited the formation of the phosphopolymer, and did so at concentrations which gave rise to inhibition of glucose-stimulated insulin release.

These results strongly imply that transglutaminase is responsible for the formation of the endogenous phosphopolymer. Of particular importance to this suggestion is that the N-substituted analogues of MDCD and GME which acted as control compounds in the studies did not inhibit the formation of the phosphopolymer. A further important consideration is the finding that neither MDCD nor GME inhibited protein phosphorylation in the glucose-stimulated islet. Therefore the observed reduction in the amount of phosphopolymer in the presence of the inhibitors can only be due to inhibition of the formation of the phosphopolymer and not in the inhibition of the phosphorylation of its small molecular weight components.

Incubation of [^{32}P]-labelled islet homogenates under conditions of transglutaminase activation increased the amount of [^{32}P]-radiolabel recovered in the phosphopolymer. This apparent translocation of phosphorylated proteins to the phosphopolymer was inhibited by EGTA and by iodoacetamide indicating that transglutaminase-catalysed cross-linking was responsible for this process. The increase in phosphopolymer was found to be time

dependent over a 30 min period. Therefore, islet transglutaminase was capable of catalysing the formation of the phosphopolymer.

The observation that specific inhibitors of transglutaminase inhibit the formation of the phosphopolymer and that transglutaminase catalyses the formation of the phosphopolymer *in vitro* indicate the importance of transglutaminase activity in the formation of the phosphopolymer.

Preliminary attempts to characterise the subcellular niche of the islet phosphopolymer using differential centrifugation indicated that the phosphopolymer did not show any direct parallelism to the fractionation of either the plasma membrane marker 5'-nucleotidase or insulin. The majority of the phosphopolymer (73.4 ± 1.1%) was associated with the 600g particulate fraction, a fraction which in addition to containing islet nuclei (Christie *et al.*, 1985) contains approximately 50% of the islet plasma membrane and mitochondria.

Evidence to indicate that the islet transglutaminase is capable of catalysing, *in vitro*, the formation of phosphopolymer of similar nature to that found in the intact islet was additionally suggested by the fractionation studies. Fractionation of radiolabelled islets following incubation with Ca^{2+} indicated an increase in the amount of phosphopolymer which is associated with the 600g particulate fraction. Concomittant with this increase was a decrease in the percentage total radiolabel and radiolabelled phosphopolymer associated with the cytosol fraction.

4. GENERAL DISCUSSION

The overall aim of this thesis was to elucidate the role of the islet transglutaminase in the Ca^{2+}-mediated events leading to the secretion of insulin in the pancreatic β-cell. In pursuing this aim several relevant findings were made:

i) Islet transglutaminase was shown to be activated at Ca^{2+} concentrations likely to occur in the intact β-cell during glucose-stimulated insulin release.

ii) Using specific inhibitors of transglutaminase activity strong evidence was obtained to suggest that islet transglutaminase may be involved in the mechanism of glucose-stimulated insulin release from the pancreatic β-cell.

iii) Evidence was obtained to suggest the co-ordination of transglutaminase-catalysed cross-linking and phosphorylation of islet proteins in the process of glucose-stimulated insulin release.

The presence of transglutaminase activity in pancreatic islets had already been demonstrated prior to these studies (Bungay et al., 1982; 1984; Gomis et al., 1983). However in order to implicate a role for the enzyme in glucose-stimulated insulin release it was essential to indicate that the Ca^{2+}-activation of the islet enzyme would take place under the conditions found in the islet β-cell during glucose-stimulated insulin release. The finding of an apparent K_m for Ca^{2+} of 2-3 μM for the enzyme irrespective of the Mg^{2+} ion concentration was therefore an important result towards the aim of the thesis, since it is probable that localised concentrations of Ca^{2+} within the β-cell may be much higher than the overall quoted literature values of 0.1 - 0.2 μM approx. found during glucose-stimulated insulin release (cf. Section 3.1).

Therefore, in conjunction with other studies (Bungay et al., 1982; 1984; Gomis et al., 1983), it may be concluded that there is present in rat islets a transglutaminase enzyme which is activated by Ca^{2+} over a physiological range and which is thiol-dependent.

Definitive evidence for the involvement of this transglutaminase in glucose-stimulated insulin release is however still required. Such evidence would require the demonstration of an increase in the formation of γ-glutamyl conjugates, ε-(γ-glutamyl) lysine, γ-glutamyl polyamines, and bis-(γ-glutamyl) polyamines, in islet proteins as a consequence of glucose-stimulation. Such evidence represents one of the most important aspects of future work to be undertaken.

However, very strong evidence has been presented to imply a role for transglutaminase in glucose-stimulated insulin release. The use of the specific inhibitor of glucose-stimulated insulin release, monodansylcadaverine, indicated a paralleled inhibition of islet transglutaminase activity and glucose-stimulated insulin release. This evidence was enhanced by the lack of effect of the control compound dimethyl-monodansylcadaverine on both islet transglutaminase activity and glucose-stimulated insulin release. Similar evidence obtained by the use of a second specific inhibitor of transglutaminase, glycine methyl ester on the paralleled inhibition of islet transglutaminase and glucose-stimulated insulin release has also been obtained (Beresten et al., 1983). A lack of inhibition by the respective control compound, sarcosine methyl ester, on both islet transglutaminase activity and glucose-stimulated insulin release was also demonstrated by these authors. This strengthens the evidence presented in this thesis for the involvement of transglutaminase in the secretory process.

The observation that neither monodansylcadaverine or glycine methyl ester had any inhibitory effect on basal insulin release further implies that the expression of islet transglutaminase activity is integral to the process of glucose-stimulated insulin release.

These studies have therefore provided extensive evidence to suggest a role for the islet transglutaminase in glucose-stimulated insulin release. However the question still arises as to the nature of the substrates of the enzyme and its catalytic products. Furthermore, how do these products integrate into the secretory process?

These questions were addressed by investigating the labelling of islet proteins with [^{32}P]Pi. The outcome of these phosphorylation studies was the discovery of a transglutaminase-mediated high molecular weight phosphopolymer which was formed during glucose-stimulated insulin release, was composed predominantly of protein, and had a molecular weight of $<1 \times 10^6$.

Theoretically it would have been advantageous to label islet proteins with [^{32}P]Pi in the presence of low levels of glucose (2.8mM), and to subsequently stimulate the islets with high levels of glucose (16.8mM). Such experimentation would then directly address the question that formation of the phosphopolymer is related to glucose-stimulated insulin release. However, this type of experimentation was not feasible and accurate interpretation of the results would be difficult since formation of the [^{32}P]-labelled ATP pool is dependent on the rate of glucose utilisation and Pi uptake (Harrison & Ashcroft, 1984). At low glucose concentrations isotopic equilibrium is not reached between the γ-phosphate of intracellular ATP and ^{32}Pi even after 5 h incubation, a time period which is not fully satisfactory for the subsequent incubation of islets obtained

by collagenase digestion. Prior to attainment of equilibrium artefactual results concerning protein labelling are likely to occur if the specific activity of ATP changes on the addition of stimuli. Increasing the glucose concentration to stimulatory levels will lead to a change in ATP turnover and alteration in Pi uptake.

However the combined observations that : specific inhibitors of transglutaminase activity inhibit both glucose-stimulated insulin release and the formation of the phosphopolymer but do not affect phosphorylation per se in the cell; that transglutaminase can catalyse the formation of the phosphopolymer; and that transglutaminase activity is expressed during glucose-stimulated insulin release, provides very strong evidence for the transglutaminase-mediated formation of the phosphopolymer during glucose-stimulated insulin in the pancreatic β-cell.

Characterisation of the phosphopolymer with respect to identifying the constitutive proteins remains to be undertaken. Such characterisation could involve the generation of specific antibodies to the isolated phosphopolymer and subsequent search for major islet proteins with which these antibodies cross-react.

However, despite this current lack of information it is plausable to suggest that, in the islet, transglutaminase may be catalysing the cross-linking of cell membrane or cell membrane associated proteins. Other workers have suggested a role for transglutaminase in cell membrane-mediated events (cf. Section 1.4.). The importance of such membrane-mediated processes in the distal events associated with glucose-stimulated insulin release has been discussed in Section 1.6.6. Whilst it is difficult at this stage to ascribe any specific subcellular niche for the phosphopolymer from the studies undertaken, its association with the islet plasma

membrane or its underlying infrastructures present in the low speed (600g av.) particulate fraction is suggested by a number of factors. There is no evidence to suggest that the expression of transglutaminase is associated with either the cell nucleus or mitochondria, the other components present in the low speed (600g av.) particulate fraction. The presence of plasma membrane fragments in the low speed (600g av.) particulate fraction would tend to indicate that they are of greater density or size than those found in the high speed (80,000g av.) particulate fraction. The association of cross-linked cytoskeletal or microtubular elements (membrane infrastructures) with the plasma membrane would facilitate such a change in the biophysical character of the membrane. Evidence to support this comes from studies on the Ca^{2+}-loaded intact human erythrocyte (Griffin et al., 1980), whereby transglutaminase induced cross-linking of membrane and cytoskeletal proteins of the erythrocyte leads to an increase in its membrane density.

In later studies, Bjerrum et al. (1981), indicated that the preferred substrates of the red blood cell enzyme are band 4.1 (a synapsin like protein), spectrin (fodrin), and band 2.1 (ankyrin), these same membrane associated proteins are also good substrates for protein kinases (Home et al., 1985). Further studies with human erythrocytes (Lorand et al., 1987) indicated the transglutaminase-mediated formation of a high molecular weight ($>10^6$) membrane protein polymer. The polymer was formed from all of the band 4.1 (synapsin like) and band 2.1 (ankyrin), as well as some spectrin (fodrin), band 3 and haemoglobin. Band 3 has also been demonstrated to be the predominant substrate of tissue transglutaminase in studies involving the incorporation of monodansylcadaverine into inside-out vesicles from human erythrocytes (Lorand et al., 1986). Gomis et al. (1986) also indicated that the

endogenous substrates of the islet transglutaminase are predominantly particulate in origin. These authors also observed a transglutaminase-mediated polymeric product in the polyacrylamide stacking gel, and in addition a substrate protein of molecular weight 84,000. Interestingly the molecular weight of band 4.1, a synapsin like protein thought to be present in a number of different cell types is also 80,000 (Goodman et al., 1982; Leto & Marchesi, 1984)

Additionally, the observed increase in the amount of phosphopolymer which is associated with the 600g av. islet particulate fraction and the concomitant decrease in the percentage total radiolabelled phosphopolymer associated with the cytosol fraction following the incubation of radiolabelled islet homogenates with Ca^{2+}, indicated that cytosolic phosphorylated proteins may be coupled to particulate proteins by action of the islet transglutaminase. However it cannot be fully ruled out that part of the reduction seen in the cytosolic proteins is due to the action of phosphatases.

There are many interesting but diverse observations relating to structural membrane proteins, the majority of these observations do not relate to the β-cell per se but to other secretory cells. However many of the structural membrane proteins found in other secretory cell types have been detected in the β-cell (for review see Tyhurst & Howell, 1986).

Synapsin has been shown to cross-react with band 4.1 (a transglutaminase substrate in the erythrocyte, and a protein now found in many other cell types (Allen et al., 1987)), and has been demonstrated to bind spectrin (Baines & Bennett, 1985). In the erythrocyte band 4.1 (synapsin like) binds spectrin (fodrin) (Ungewickell, et al., 1979; Tyler, et al. 1980) and indirectly binds spectrin to actin by acting as a link between the two proteins

(Bennett et al., 1979; Ohanian et al., 1984; Cohen & Langley, 1984). Spectrin can additionally bind at least one transmembrane protein (Anderson & Lovrien, 1984; Mueller, 1984; Pasternak et al., 1985). In the brain synapsin is one of the major substrates for cAMP and Ca^{2+}-calmodulin-dependent kinases (Johnson et al., 1972; Sieghart et al., 1979). It has been suggested (Baines, 1987) that in the case of synaptic transmission, neurotransmitter-containing synaptic vesicles are restrained in a spectrin (fodrin)/actin network in the presynaptic terminal until nerve depolarisation elevates free Ca^{2+}. This activates a Ca^{2+}-calmodulin-dependent protein kinase that phosphorylates synapsin. On phosphorylation synapsin loses its ability to mediate the intereaction between spectrin (fodrin)/actin (Krebs et al. 1986) resulting in the dissolution of the network, thus freeing the vesicle for exocytosis at the presynaptic membrane. A similar process has been proposed in the process of chromatin granule secretion (Burgoyne & Cheek, 1987), indicating that reorganisation of actin filaments is an essential prelude to exocytosis. Trifaro et al. (1985) have also indicated the importance of the phosphorylation of an unidentified granule membrane protein or cytosolic component in the process, this component was subsequently identified as a synapsin-like protein (Burgoyne & Baines, 1987). Band 4.1 has also been identified as a phosphatidylserine binding protein in the human erythrocyte that may be involved in the molecular mechanisms that stabilise phosphatidylserine in the plasma membrane (Rybicki et al., 1988).

The involvement of spectrin in the secretory process from chromaffin cells has been further implicated by Perrin et al. (1987) who demonstrated that the stimulated secretion of adrenaline from chromaffin cells was inhibited by anti- fodrin, fodrin being a subunit of spectrin, which in turn is associated directly or

indirectly with actin.

Intermediate filaments, which may have a role in the effector system of glucose-stimulated insulin release (Tyhurst & Howell, 1986), have end domains which are sites for both phosphorylation and transglutaminase induced cross-linking (Fraser et al., 1987).

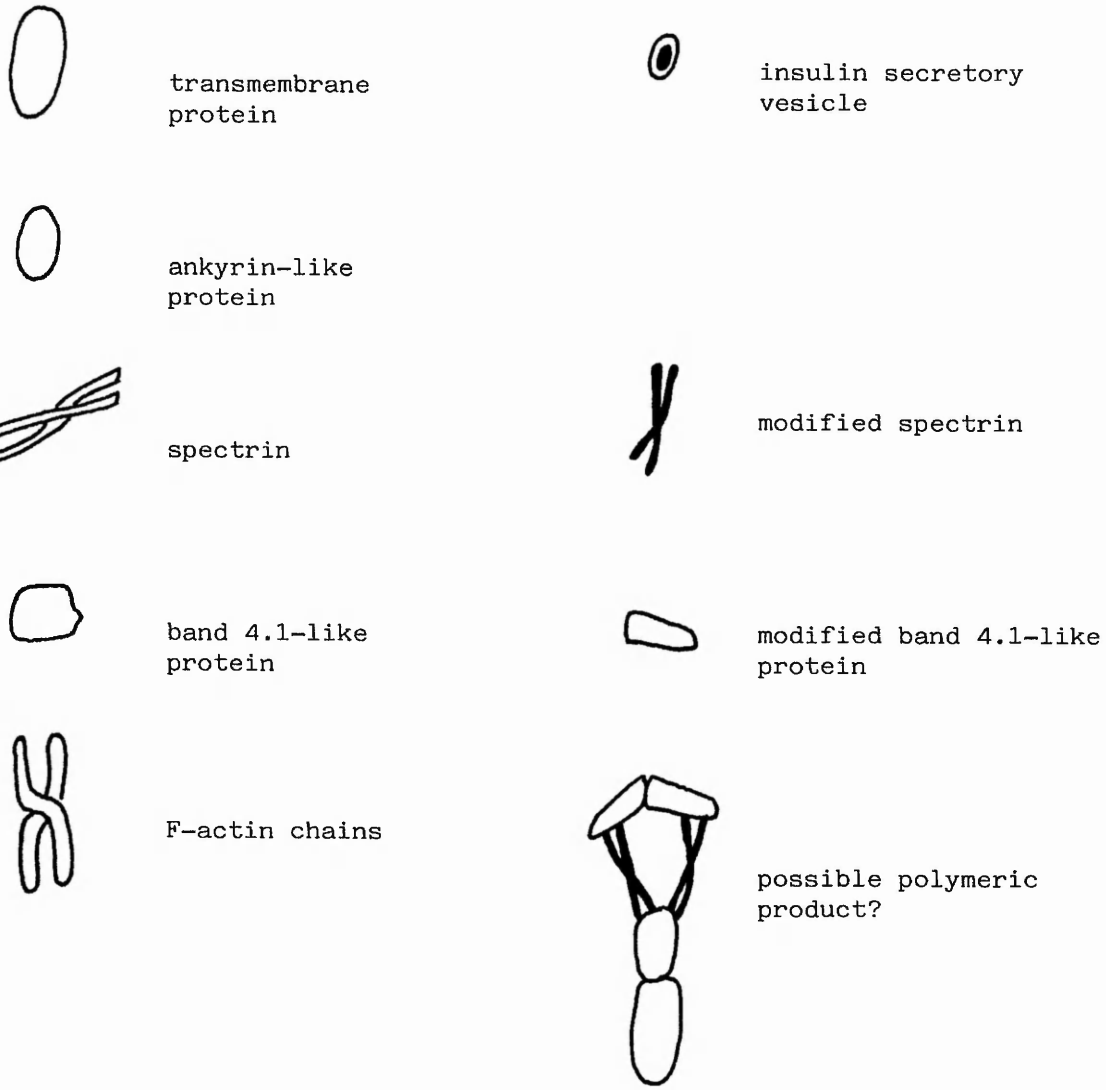
These observations represent only a portion of the wealth of information relating to the involvement of cytoskeletal components in the secretory apparatus. How therefore may the coordinated actions of protein kinases and transglutaminase be related to these phenomena? It is possible that the phosphorylation of specific membrane or membrane-associated proteins and the subsequent cross-linking of these proteins by transglutaminase could result in the release of secretory vesicles from the cytoskeletal network to facilitate exocytosis.

One potential candidate for such a mechanism is a band 4.1 like protein which may in its dephosphorylated state facilitate the immobilisation of secretory vesicles to the actin network (Bahler & Greengard, 1987). A consequence of the rise in Ca^{2+} concentration and other secondary messengers following stimulus response might be the phosphorylation of and subsequent cross-linking of band 4.1 to either itself or to other membrane or membrane associated proteins such that the secretory vesicles are released. This consideration could equally well apply to spectrin (fodrin), or possibly the two membrane associated proteins could function synergistically. A result of such concerted action of protein kinases and transglutaminase could be the localised disruption of the microfilaments thereby removing the barrier to exocytosis and the opening of routes to the plasma membrane. A possible model is illustrated in Fig. 4.1.

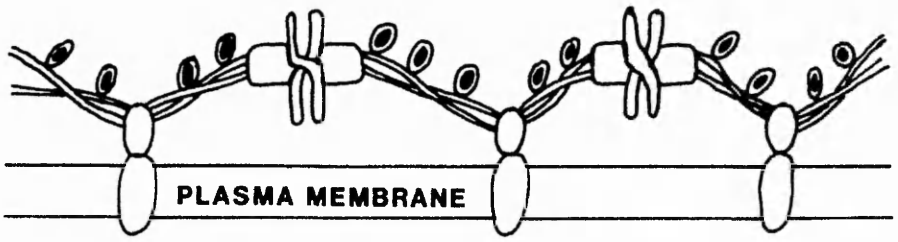
Figure 4.1.

Modification of the cytoskeletal network

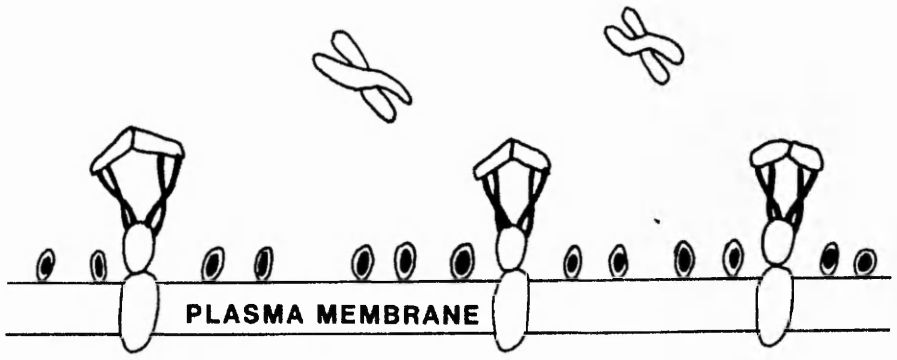
Under non-stimulatory conditions, insulin secretory vesicles may be prevented from gaining access to the plasma membrane. Upon stimulus perception the rise in Ca^{2+} and other secondary messengers may result in the modification of the cytoskeleton structure through the concerted action of transglutaminase and protein kinases, resulting in access of the insulin secretory vesicles to the plasma membrane and subsequent exocytosis.



Non-stimulatory - no access to plasma membrane



Stimulated cell - access to plasma membrane
- increase in Ca^{2+} and 2^{ndry} messengers
- concerted action of transglutaminase and protein kinases



An alternative consideration is that rather than resulting in a destabilisation of the cytoskeletal network, the co-ordinated action of protein kinases and transglutaminase may modify the polarity of the cell such that secretory granule migration is directed to specified areas of the β-cell plasma membrane. Recent evidence for polarisation of the β-cell (Lombardi et al., 1985) was obtained by using RNA viruses known to bud selectively from either the apical or basolateral domain in polarised epithelial cells (Rodriguez-Boulan et al., 1978); this evidence has been reviewed by Orci (1986). Therefore a possible model is that upon stimulation of the β-cell and the subsequent rise in Ca^{2+} and other secondary messengers, proteins become phosphorylated in areas away from the apical domain and are subsequently cross-linked by transglutaminase action. This enhanced cytoskeletal network could then physically direct the movement of secretory granules to the apical domain of the β-cell.

This model of a role for transglutaminase in enhancing the cytoskeletal network may also explain the consequences of reduced transglutaminase levels in tumour growth (c.f. Section 1.4.3.). The reduced levels of transglutaminase observed in neoplastic tissue may result in a disorganisation of the cytoskeleton which could explain the loss of cell shape and polarity, and impaired cell communication which are features of the neoplastic cell (Weinberg, 1983). The disorganisation of the cytoskeleton would also presumably lead to a decrease in cell-cell contact and hence facilitate metastasis.

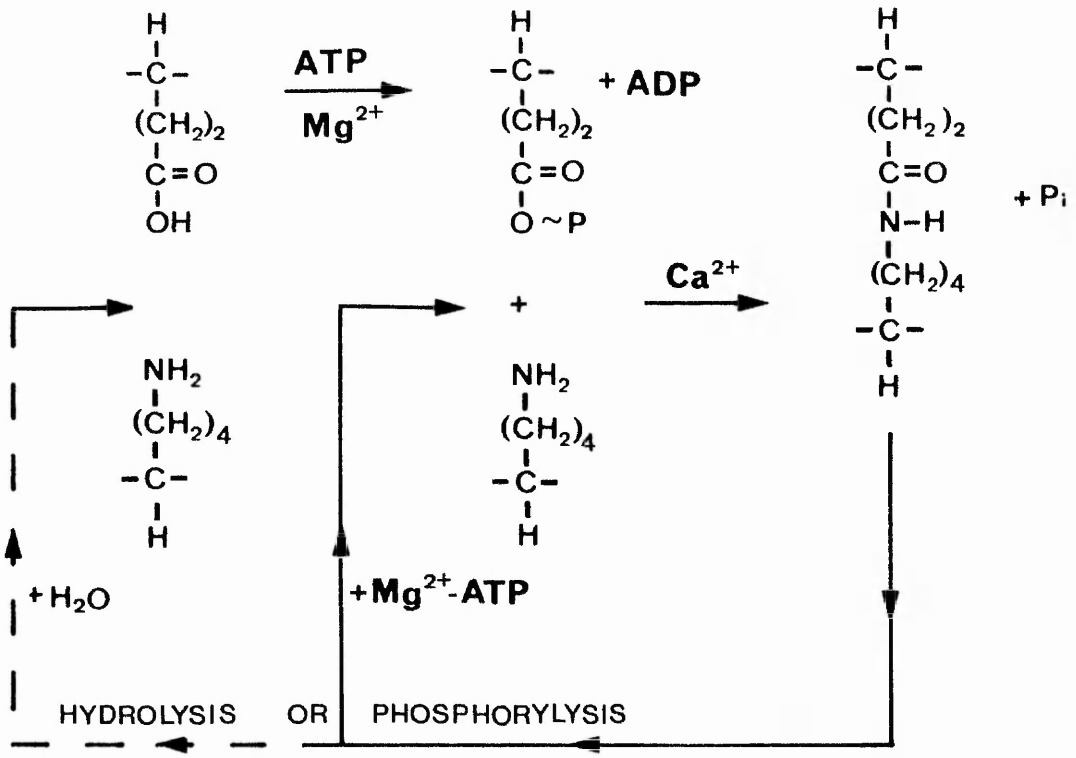
The exocytosis of insulin also requires the fusion of the secretory granules with the plasma membrane, resulting in the extrusion of the granule contents. This event is again a potential site for the involvement of transglutaminase in the secretory process. Of particular interest in this proposal is the

identification of a new family of Ca^{2+}-regulated-phospholipid binding proteins, the annexins (Geisow et al., 1987). These proteins potentiate the aggregation of phospholipid vesicles as well as natural membranes in the presence of elevated levels of Ca^{2+}. No evidence currently exists to indicate whether these proteins are substrates of either transglutaminase or protein kinases. As mentioned earlier, band 4.1 (synapsin like protein) is a phosphatidylserine binding protein which may be involved in the molecular mechanisms that stabilise phosphatidylserine in the cytoplasmic membrane of human erythrocyte plasma membrane (Rybicki et al., 1988). It is therefore possible that transglutaminase action may modify band 4.1 either by cross-linking of band 4.1 either to itself or to other membrane associated proteins. The result of such cross-linking may be the disassociation of band 4.1 to phosphatidylserine causing a destabilisation of the membrane and thereby allowing fusion between the secretory vesicle membrane and plasma membrane, and hence exocytosis.

The possibility that phosphorylation may be coupled to the formation of ε(γ-glutamyl) lysine cross-links in cellular systems has also been proposed by Loewy and Metacic (1981). These authors using studies performed in vitro demonstrated that the ε(γ-glutamyl) lysine cross-link of a variety of cell types could be modulated by ATP and ATP plus Ca^{2+}. They suggested that protein cross-linking by a transglutaminase-like enzyme may occur through prior phosphorylation of proteins at glutamine residues, the reaction proceeding via an activated acyl intermediate. Such a reaction mechanism where energy transfer accompanies alteration of protein structure may be applicable to the distal events of the secretory mechanism (Figure 4.2.).

Figure 4.2

A hypothetical cycle accounting for the making and breaking of ε(γ-glutamyl)lysine bonds



The models proposed within this discussion are highly speculative and are intended here only as areas of discussion, clearly much more specific information is required before any firm model can be constructed for the detailed role of transglutaminse in the distal events of insulin release.

Some of these answers may stem from the study of permeabilised islets. This technique circumvents many of the problems of access to the β-cell environment which were encountered through this and previous studies.

Further studies will also need to address the question of whether transglutaminase has a similar function in other secretory cell types within which it is found.

5. REFERENCES

Allan, D., Billah, M.M., Fineau, J.B., & Michell, R.H., (1976) Nature 261, 58-60.

Allen, D.P., Low, P.S., Dola, A., Maisel, H., (1987) Biochem. Biophys. Res. Commun. 149(1), 266-275.

Ambron, R.T., & Kremzner, L.T., (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 3442-3446.

Anderson, D.R., Davis D.L., & Carraway, K.L., (1977) J. Biol. Chem. 252(19), 6617-6623.

Anderson, R.A., & Lovrien, R.E., (1984) Nature 307, 655-658.

Ashcroft, F.M., Harrison, D.E., & Ashcroft, S.J.H., (1984) Nature 312, 446-448.

Ashcroft, S.J.H., (1980) Diabetologia 18, 5-15.

Axen, K.V., Schubart, K.K., Blake, A.D., & Fleischer, N., (1983) J. Clin. Invest. 72, 13-21.

Bachrach, U., Menasche, M., Faber, J., Desser, H., & Seiler, N., (1981) Adv. Polyamine Res. 3, 259-274.

Bahler, M., & Greengard, P., (1987) Nature 326, 704-707.

Baines, A.J., (1987) Nature 326, 646.

Baines, A.J., & Bennett, V., (1985) Nature 315, 410-413.

Baldwin, D., Prince, M., Marshall, S., Davies P., & Olefsky J.M., (1980) 77, 5975-5978.

Ball E.P., Guyre P.M., Shen L., Glynn J.M., Maliszewski P., Baker E., & Fanger M.W., (1984) J. Clin. Invest. 73, 1072-1077.

Barnes, R.N., Bungay, P.J., Elliot, B.M., Walton, P.L. & Griffin, M., (1984) Biochem. Soc. Trans. 12(2), 297-298.

Barnes, R.N., Bungay, P.J., Elliot, B.M., & Griffin, M., (1985) Carcinogenesis 6(3), 459-463.

Beattie, R.W., & Whitmore, F., (1933) J. Am. Chem. Soc. 55, 1546-1553.

Beniati, S., & Folk, J.E., (1987) Noble Conf. Cell. Mol. Biol.; Transglutaminase & Protein Cross-linking Reactions.

Beniati, S., Piacentini, M., Argento-Ceru, M.P., Russo-Caia, S., & Autuori, F., (1985) Biochim. Biophys. Acta. 841, 120-126.

Berbers, G.A.M., Feenstra, R.W., Bos, R.V.D., Hoekman, W.A., Bloemendal, H., & De Jong, W.W., (1983) Proc. Nat. Acad. Sci. U.S.A. 81, 7017-7020.

Berntorp, E., Sieving, B., & Stenberg, P., (1985) Scand. J. Haematol. 34, 71-77.

Berridge, M.J., & Irvine, R.F., (1984) Nature 312, 315-321.

Bersten, A.M., Ankong, Q.F., Hallinan, T., Nelson, S.J., & Lucy, J.A. (1983) Biochim. Biophys. Acta. 762, 429-436.

Best, L., Dunlop, M., & Malaisse, W.J., (1984) Experientia 40, 1085-1091.

Best, L., Lebrun, P., Saceda, M., Garcia-Morales, P., Hubinont, C., Juvent, M., Hurchuelez, A., Malaisse-Lagae, F., Valverde, I., & Malaisse. W.J., (1984) Biochem. Pharmacol. 33, 2033-2039.

Best, L., & Malaisse, W.J., (1983) Mol. Cell. Endocrinol. 32, 205-214.

Best, L., & Malaisse, W.J. (1984) Endocrinology 115, 1814-1820.

Biden, T.J., Prentki, M., Irvine, R.F., Berridge, M.J., & Wollheim, C.B., (1984) Biochem. J. 223, 467-473.

Birckbichler, P.J., Carter, H.A., Orr, G.R., Conway, E., & Patterson, Jr., M.K., (1978) Biochem. Biophys. Res. Commun. 84(1), 232-237.

Birckbichler, P.J., Orr, G.R., Conway, E., & Patterson, Jr., M.K., (1977) Cancer Res. 37, 1340-1344.

Birckbichler, P.J., Orr, G.R., & Patterson, Jr., M.K., (1976) Cancer Res. 36, 2911-2914.

Birckbichler, P.J., & Patterson, Jr., M.K., (1980) In: Tumour Cell Surfaces & Malignancy, A.R. Liss Inc., N.Y., U.S.A., pp845-855.

Bjerrum, O.J., Hawkins, M., Swanson, P., Griffin, M., & Lorand, L., (1981) J. Supramol. Struct. Cell. Biochem. 16, 289-301.

Blaustein, M.P., & Nelson, M.T., (1982) In: Membrane Transport of Calcium (Carafoli, E., ed.,) Academic Press, London, pp217-236.

Bohn, H., & Schwick, H.G., (1971) Arzneim. Forsch. 21, 1432-1439.

Bradshaw, B.S., & Wolfe, H.G., (1977) Biol. Reprod. 16, 292-297.

Brenner, S.C., & Wold, F., (1978) Biochim. Biophys. Acta. 522, 74-83.

Brisson G.R., & Malaisse, W.J., (1973) Metabolism 22, 455-465.

Brocklehurst, K.W., & Hutton, J.C., (1983) Biochem. J. 210, 533-539.

Bungay, P.J., Griffin, M., & Potter, J.M., (1982) Diabetologia 23, 159 (abstract).

Bungay P.J., Potter, J.M., & Griffin, M., (1984) Biochem. J. 219, 819-827.

Bungay, P.J., Owen, R.A., Coutts, I.C., & Griffin, M., (1986) Biochem. J. 235, 269-278.

Burgoyne, R.D., & Baines, A.J., (1987) 330, 115-116.

Burgoyne, R.D., & Cheek, T.R., (1987) Biosci. Rep. 7(4), 281-288.

Buxman, M.M., Kocarnick M.J., & Holbrook, K.A., (1979) Invest. Dermatol. 72, 171-177.

Buxman, M.M., Lobitz, C.J., & Weupper, K.D., (1980) J. Biol. Chem. 255(3), 1200-1203.

Buxman, M.M., & Weupper, K.D., (1974) Clin. Res. 22, 157 (abstract).

Buxman, M.M., & Weupper, K.D., (1975) J. Investig. Dermatol. 65(1), 107-112.

Buxman, M.M., & Weupper, K.D., (1976) Biochim. Biophys. Acta 452, 356-369.

Buxman, M.M., & Wuepper K.D., (1978) J. Histochem. Cytochem. 26, 340-348.

Campbell-Wilkes, L., (1973) Ph.D. Dissertation, Northwestern University, Evanston, Illinois, U.S.A. .

Campillo, J.E., & Ashcroft, S.J.H., (1982) FEBS Lett. 138(1), 71-75.

Cannelakis, Z., Lande, L.A., & Bandy, P.K., (1981) Biochem. Biophys. Res. Commun. 100(2), 675-680.

Cariello, L., Wilson, J., & Lorand, L., (1984) Biochemistry 23, 6843-6850.

Carpinelli, A.R., & Malaisse, W.J., (1980) Diabetologia 19, 458-464.

Casero, R.A., Bergeron, R.J., & Porter, P.W., (1984) J. Cell Physiol. 121, 476-482.

Chang, S.K., & Chung, S.I., (1986) J. Biol. Chem. 261(18), 8112-8121.

Chen, K.Y., (1983) Biochim. Biophys. Acta 756, 395-402.

Cheng, S.Y., Maxfield, F.R., Robbins, J., Willingham, M.C., & Pastan, I.H., (1980) Proc. Nat. Acad. Sci. U.S.A. 77(6), 3425-3429.

Christie, M.R., & Ashcroft, S.J.H., (1985) Biochem. J. 227, 727-736.

Chuang, D.M., (1981) J. Biol. Chem. 256(16), 8291-8293.

Chuang, D.M., (1984) Mol. Cell. Biochem. 58, 79-89.

Chung, S.I., (1972) Ann. N.Y. Acad. Sci. U.S.A. 202, 240-255.

Chung, S.I. (1975) In: Isoenzymes (Markert, C.L., ed.) Acad. Press, N.Y., Vol. 1, pp259-274.

Chung, S.I., & Folk, J.E., (1972) Proc. Nat. Acad. Sci. U.S.A. 69(2), 303-307.

Chung, S.I., Lewis, M.S., & Folk, J.E., (1974) J. Biol. Chem. 249, 940-950.

Chung, S.I., Schrager, R.I., & Lodish, H.F., (1970) J. Biol. Chem. 245(23), 6424-6435.

Clarke, D.D., Mycek, M.J., Neidle, A., & Waelsch, H., (1959) Arch. Biochem. Biophys. 79, 338-353.

Clements, R.S., & Rhoten, W.P., (1976) J. Clin. Invest. 57, 684-691.

Cocuzzi, E.T., & Chung, S.I., (1986) J. Biol. Chem. 261(18), 8122-8127.

Cohen, C.M., & Langley, C., (1984) Biochemistry 23, 4488-4495.

Colca, J.R., Brooks, C.L., Landt, M., & McDaniel, M.L., (1983) Biochem. J. 212, 819-827.

Cooke, R.D. & Holbrook, J.J., (1974) Biochem. J. 141, 71-78.

Cooper, H.L., Park, M.H., Folk, J.E. Safer, B., & Braverman, R., (1983) Proc. Nat. Acad. Sci. U.S.A. 80, 1854-1857.

Connelan, J.M., & Folk, J.E., (1969) J. Biol. Chem. 244(12), 3173-3181.

Conrad, S.M. (1985) In: Enzymol. Post-Transl. Mod. Prot. Vol.2, pp339-368.

Curtis, C.G., & Lorand, L., (1976) In: Methods in Enzymology (Lorand, L., ed.) Vol. 45, pp177-191.

Davidson, H.W., & Hutton, J.C., (1987) Biochem. J. 245, 575-582.

Davies, M.P., & Murtaugh, M.P. (1984) Mol. Cell. Biochem. 58, 69-77.

Davies, P.J.A., Cornwell, M.M., Johnson, B.S., Reggiani, A., Myers, M., & Murtaugh, M.P., (1984) Diabetes Care 7(1), 35-41.

Davies, P.J.A., Davies, P.R., Levitski, A., Maxfield, F.R., Milhaud, P., Willingham, M.C., & Pastan, I.H., (1980) Nature 283, 162-167.

Davies, P.J.A., Murtaugh, M.P., Moore, W.T., Jr., Johnson, G.S., & Lucas, D., (1985) J. Biol. Chem. 260, 5166-5174.

Davies, P.J.A., Podder, S., Basilion, J., Sabieski, M.M., Chiocca, E., & Stein, J.P., (1987) Noble Conf. Cell. Mol. Biol.; Transglutaminase & Protein Cross-Linking Reactions.

Deelers, M., Mahy, M., & Malaisse, W.J., (1985) Biochem. Int. 10(1), 97-103.

Deelers, M., Ruysschaert, J.M., & Malaisse, W.J., (1981) Biochem. Biophys. Res. Commun. 98, 255-260.

Delcros, J.G., Bard, S., Roch, A.M., Quash, G., Poupon, M.F., & Korach, S., (1986) FEBS Letts. 196(2), 325-330.

Dickson, R.B., Schlegel,R., Willingham, M.C., & Pastan, I.H., (1982) Exp. Cell. Res. 140, 215-225.

Dickson, R.B., Willingham, M.C., Gallo, M., & Pastan, I., (1981) FEBS Lett. 126, 265-268.

Dickson, R.B., Willingham, M.C., & Pastan, I., (1981) J. Biol. Chem. 256(7), 3454-3459.

Draznin, B., & Trowbridge, M. (1982) J. Biol. Chem. 257(20), 11988-11993.

Dunlop, M.E., & Larkins, R.G., (1984) Biochem. Biophys. Res. Commun. 118, 601-608.

Dunlop, M.E., Larkins, R.G., & Court J.M., (1982) FEBS Lett. 144, 259-263.

Fesus, L., & Arato, G., (1986) J. Immun. Methods 94, 131-136.

Fesus, L., Sandor, M., Horvath, L.I., Bagyinka, C., Erdei, A., & Gergely, J., (1981) Mol. Immunol. 18(7), 633-638.

Fex, G., & Lernmark, A., (1972) FEBS Lett. 25, 287-291.

Fillingame, R.H., & Morris, D.R., (1973) Biochemistry 12(22), 4479-4487.

Finlayson, J.S., & Aronson, D.L., (1974) Thromb. Diath. Haemorrh. 31, 435-438.

Fitzgerald, D., Morris, D., & Saelinger, C.B., (1980) Cell 21, 867-873.

Folk, J.E., (1980) Ann. Rev. Biochem. 49, 517-531.

Folk, J.E., (1983) Adv. Enzymol. 54, 1-56.

Folk, J.E., & Chung, S.I., (1973) Adv. Enzymol. 38, 109-191.

Folk, J.E., & Cole, P.W., (1965) J. Biol. Chem. 240(7), 2951-2960.

Folk, J.E., & Cole, P.W., (1966) Biochim. Biophys. Acta 122, 244-264.

Folk, J.E., & Finlayson, J.S., (1977) Adv. Prot. Chem. 31, 1-133.

Folk, J.E., Mullooly, J.P., & Cole, P.W., (1967) J. Biol. Chem. 242(8), 1838-1844.

Folk, J.E., Park, M.H., Chung, S.I., Schrode, J., Lester, E.P., & Cooper, H.L., (1980) J. Biol. Chem. 256(6), 3695-3700.

Fraser, R.B., Steinert, P.M., & Steven, A.C., (1987) TIBS 12, 43-45.

Frienkel, N., El Younsi, C., & Dawson, R.M.C., (1975) Eur. J. Biochem. 59, 245-252.

Geisow, M.J., Walker, J.H., Boustead, C., & Taylor, W., (1987) Biosci. Rep. 7(4), 289-298.

Gey, G.O., & Gey, M.K., (1936) Am. J. Cancer 27, 45-76.

Gilad, G.M., & Varon, L.E., (1985) J. Neurochem. 45(5), 1522-1526.

Goldman, R., (1987) Exp. Cell. Res. 168, 31-43.

Goldsmith, L.A., Baden, H.P., Roth, S.I., Colman, R., Lee, L., & Fleming, B., (1974) Biochim. Biophys. Acta 351, 113-125.

Goldsmith, L.A., & Martin, C.M., (1975) J. Invest. Dermatol. 64, 316-321.

Gomis, R., Casonovas, A., & Malaisse, W.J., (1986) Diabetologia 30, 574A.

Gomis, R., Sener, A., Malaisse-Lagae, F., & Malaisse, W.J., (1983) Biochim. Biophys. Acta 760, 384-388.

Goodman, S.R., Yu, J., Whitfield, C.F., Culp, E.N., Pasnack, E.J., (1982) J. Biol. Chem. 257, 4564-4569.

Gorman, J.J., & Folk, J.E., (1980) J. Biol. Chem. 255(3), 1175-1180.

Gorman, J.J., & Folk, J.E., (1981) J. Biol. Chem. 256(6), 2712-2715.

Gotterer, G., Ginsburg, D., Schulman, T., Banks, J., & Williams-Ashman, H.G., (1955) Nature 176, 1209-1211.

Gotterer, G., & Williams-Ashman, H.G., (1957) Proc. Soc. Exp. Biol. Med. 94, 60-64.

Goyns, M.H. (1982) J. Theor. Biol. 97, 577-589.

Griffin, M., Hussain, M., & Bungay, P.J., (1986) Diabetologia 29, 453.

Griffin, M., Swanson, P.E., Bjerrum, O.J., & Lorand, L., (1980) Biophys. J. 33, 177a.

Griffin, M., & Wilson, J., (1984) Mol. Cell. Biochem. 58, 37-49.

Griffith, L.M., & Pollard, T.D., (1978) J. Cell. Biol. 78, 958-965.

Grinnel, F., Feld, M., & Minter, D., (1980) Cell 19, 517-525.

Grodsky, G.M., Curry, D.L., Bennett, L.L., & Rodrigo, J.J., (1986) Acta Diabetol. Lat. 5(Suppl.1), 140-161.

Grodsky, G.M., Curry, D., Landhahl, M., & Bennett, L., (1969) Acta Diabetol. Lat. 6(Suppl.1), 544-579.

Gross, M., & Folk, J.E., (1973) J. Biol. Chem. 248, 1301-1306.

Gross, M., Whetzel, N.K., & Folk, J.E., (1975) J. Biol. Chem. 250(12), 4648-4655.

Gross, M., Whetzel, N.K., & Folk, J.E., (1977) J. Biol. Chem. 252(11), 3752-3759.

Grossie, V.B., Nishioka, K., Ota, D., & Martin, R.G., (1986) Cancer Res. 46, 3464-3468.

Grundmann, U., Amann, E., Zettlmeissl, G., & Kupper, H., (1987) Noble Conf. Cell. Mol. Biol.; Transglutaminase & Protein Cross-linking Reactions.

Gunzler, V., Schopf, R.E., Hanauske-Abel, H.M., & Schulte-Wisserman, H., (1982) FEBS Lett. 150(2), 390-396.

Guyre, P.M., Morganelli, P.M., & Miller, R., (1983) J. Clin. Invest. 72, 393-399.

Haddock-Russel, D.H., (1981) Biochem. Biophys. Res. Commun. 99(4), 1167-1172.

Haigler, H.T., Maxfield, F.R., Willingham, M.C., & Pastan, I.H., (1980) J. Biol. Chem. 255, 1239-1241.

Haigler, H.T., Willingham, M.C., & Pastan, I.C., (1980) Biochem. Biophys. Res. Commun. 94, 630-637.

Hales, C.N., & Randle, P.J. (1963) Biochem. J. 88, 137-142.

Hand, D., Bungay, P.J., Elliot, B.M., & Griffin, M., (1985) Biosci. Rep. 5(12), 1079-1086.

Hand, D., Elliot, B.M., & Griffin, M., (1987) Biochim. Biophys. Acta 930, 432-437.

Hanigan, H., & Goldsmith, L.A., (1978) Biochim. Biophys. Acta 522, 589-601.

Hanks, J.H., & Wallace, R.E., (1949) Proc. Soc. Exp. Biol. Med. 71, 196-200.

Harding, H.J.W., & Rogers, G.E., (1970) Biochim. Biophys. Acta 257, 37-39.

Harding, H.J.W. & Rogers, G.E. (1972) Biochemistry 11(15), 2858-2863.

Harrison, D.E., & Ashcroft, S.J.H., (1982) Diabetologia 23, 172-173.

Harrison, D.E., Ashcroft, S.J.H., Christie, M.R., & Lord, J.M., (1984) Experientia 40, 1075-1084.

Harrison, D.E., Poje, M., Rocic, B., & Ashcroft, S.J.H., (1986) Biochem. J. 237, 191-196.

Haughland, R.B., Lin, T.I., Dowben, R.M., & Birckbichler, P.J., (1982) Biophys. J. 37, 191-193.

Hawthorne, J.N., (1982) Nature 295, 281-282.

Hazum, E., Kwen-Jen, C., & Cuatrecasas, P., (1979) Nature 282, 626-628.

Heby, O., (1981) Differentiation 19, 1-20.

Hedeskov, C.J., (1980) Physiol. Rev. 60, 442-509.

Heding, L., (1972) Diabetologia 8, 260-266.

Hempel, J., & Klauke, E., (1971) Brit. Pat. 1258557(1970) Chem. Abs. 75, P19924d.

Henquin, J.C., & Meissner, H.P., (1984) Endocrinology 115, 1125-1134.

Hermansen, K., (1985) Endocrinolgy 116, 2251-2258.

Herzog, V., (1981) Trends Biochem. Sci. 6(12), 319-322.

Hidaka, H., & Tanaka, T., (1983) Methods Enzymol. 102, 185-194.

Higgins, S.J., Burchell, J.M. & Mainwaring, W.I.P., (1976) Biochem. J. 160, 43-48.

Higuchi, H., Takeyasu, K., Uchida, S., & Yoshida, H., (1982) Eur. J. Pharmacol. 79, 67-77.

Hii, C.S.T., Jones, P.M., Persaud, S.J., & Howell, S.L., (1987) Biochem. J. 246, 489-483.

Hii, C.S.T., Stutchfield, J., & Howell, S.L., (1986) Biochem. J. 233, 287-289.

Hodkin, M.R., & Hodkin, L.E., (1953) J. Biol. Chem. 203, 967-977.

Home, W.C., Leto, T.L., & Marchesi, V.T., (1985) J. Biol. Chem. 260, 9073-9076.

Howell, S.L., (1984) Diabetologia 26, 319-327.

Howell, S.L., & Tyhurst, M., (1979) Biochem. J. 178, 367-371.

Howell, S.L., & Tyhurst, M., (1982) Biochem. J. 204, 161-170.

Howell, S.L., & Tyhurst, M., (1984) Experientia 40, 1098-1105.

Howell, S.L., & Tyhurst, M., (1986) Diabetes Metab. Rev. 2(1&2), 107-123.

Hsu, L.K.H., Griffin, M., Wilson, J., & Lorand, L., (1981) Fed. Proc. 40(3), 781.

Hsu, L.K.H., & Friedman, H., (1983) J. Natl. Cancer Inst. 70(5), 965-969.

Hubinont, C., Best, L., Sener, A., & Malaisse, W.J., (1984) Febs Lett. 170, 247-253.

Hucho, F., & Bandini, G., (1986) FEBS Lett. 200(2), 279-282.

Ichinose, A., & Aoki, N., (1982) Biochim. Biophys. Acta 706, 158-164.

Ichinose, A., & Davie, E.W., (1987) Noble Conf. Cell. Mol. Biol.; Transglutaminase & Protein Cross-linking Reactions.

Iwanij, V., (1977) J. Biochem. 80, 359-368.

Janne, J., Poso, H., & Raina, A., (1978) Biochim. Biophys. Acta 473, 241-293.

Janne, J., Holtta, E., Kallio, A., & Kapyaho, K., (1983) In: Special Topics in Endocrinology & Metabolism. Vol. 5 (Cohen, M.P. & Foa, P.P., eds.,) A.R. Liss Inc., N.Y., U.S.A. pp227-293.

Jetten, A.M., & Shirley, J.E., (1983) J. Biol. Chem. 261(32), 15097-15101.

Johson, E.M., Ueda, T., Maeno, H., & Greengard, P., (1972) J. Biol. Chem. 257, 5650-5652.

Johnson, J.D., & Davies, P.J.A., (1986) J. Biol. Chem. 261(32), 14982-14986.

Julian, C., Speck, N.A., & Pierce, S.K., (1983) J. Immunol. 130(1), 91-96.

Juprelle-Soret, M., Wattiaux-De-Coninck, S., & Wattiaux, R., (1988) Biochem. J. 250, 421-427.

Kakiuchi, S., & Sobue, K., (1983) TIBS 59-62.

Kallio, A., Poso, H., Guha, S.K., & Janne, J. (1977) Biochem. J. 166, 89-94.

Kaminski, K., (1983) Adv. Polyamine Res. 4, 73-78.

Kamykowski, G.W., Mosher, D.F., Lorand, L., & Ferry, J.D., (1981) Biophys. Chem. 13, 25-28.

Kaplan, J., & Keogh, E.A., (1981) Cell 24, 925-932.

Kaplan, J., & Nielson, M.L., (1979) J. Biol. Chem. 254, 7323-7328.

Keusch, G.T., (1981) In: Receptor-Mediated Binding & Internalisation of Toxins & Hormones. (Middlebrook, J.L., & Kohn, L.D., eds.) Acad. Press, N.Y., pp95-112.

King, A.C., Hernaez-Davis, L., & Cuatraecasas, P., (1980) Proc. Nat. Acad. Sci. U.S.A. 77(6), 3283-3287.

Kojima, S., Hagiwara, H., Soga, W., Shimonaka, M., Saito, Y., & Inada, Y., (1987) Biomed. Res. 8(1), 25-29.

Knautila, S., & Pohjanpelto, P., (1983) Exp. Cell Res. 145, 222-226.

Krebs, H.A., & Hensleit, K., (1932) Hoppe Zyler's Physiol. Chem. 210, 33-66.

Krebs K.E., Zagon, I.S., & Goodman, S.R., (1986) J. Cell. Biol. 103, 2829a.

Kubilus, J., & Baden, H.P., (1984) Mol. Cell. Biochem. 58, 129-137.

Lacy, P.E., & Kostianovsky, M., (1967) Diabetes 16, 35-39.

Lacy, P.E., Walker, M.M., & Fink, C.J., (1972) Diabetes 21, 987-998.

Laemmli, U.K., (1970) Nature 227, 680-685.

Laki, K., Csako, G., Yancey, S.T., & Wilson, E.F., (1977) In: Search & Discovery (Kaminer B., ed.) Acad. Press, N.Y., pp303-312.

Landt, M., Mcdaniel, M.L., Bry, C.J., Kotagal, N., Colca, J.R., Lacy, P.E., & McDonald, J.M., (1982) Archs. Biochem. Biophys. 213, 148-154.

Laychock, S.G., (1983) Diabetes, 32, 6-13.

Leppla, S.H., Dorland, R.B., & Middlebrook, J.L., (1980) J. Biol. Chem. 255, 2247-2250.

Leto, T.L., & Marchesi, V.T., (1984) J. Biol. Chem. 259, 4603-4608.

Leu, R.W., Herriot, M.J., Moore, P.E., Orr, G.R., & Birchbickler, P.J., (1982) Exp. Cell Res. 141, 191-199.

Levitzki, A., Willingham, M.C., & Pastan, I.H., (1980) Proc. Nat. Acad. Sci. U.S.A. 77(5), 2706-2710.

Litchi, U., Ben, T., & Yuspa, S.H., (1985) J. Biol. Chem. 260(3), 1422-1426.

Lineweaver, H., & Burk, D., (1934) Biochem. J. 56, 658-666.

Loewy, A.G., & Metacic, S.S., (1981) Biochim. Biophys. Acta 668, 167-176.

Lombardi, T., Montesano, R., Wohlmend, A., Amherdt, M., Vassalli, J.D., & Orci, L., (1985) Nature 313, 694-696.

Lorand, L., Barnes, R.N., Bruner-Lorand, J.A., Hawkins, M., & Michalska, M., (1987) Biochemistry 26, 308-313.

Lorand, L., & Campbell-Wilkes, L.K., (1971) Anal. Biochem. 44, 207-220.

Lorand, L., Campbell-Wilkes, L.K., & Cooperstein, L., (1972) Anal. Biochem. 50, 623-631.

Lorand, L., & Conrad, S.M., (1984) Mol. Cell. Biochem. 58, 9-35.

Lorand, L., Downey, J., Gotoh, T., Jacobsen, A., & Tokura, S., (1968) Biochem. Biophys. Res. Commun. 31(2), 222-230.

Lorand, L., Murphy, S.N.P., Velasco, P.T., & Karush, F., (1986) Biochem. Biophys. Res. Commun. 134(2), 685-689.

Lorand, L., Paremeswaran, K.N., Stenberg, P., Tong, Y.S., Velasco, P.T., Jonsson, N.A., Mikiver, L., & Moses, P., (1979) Biochemistry 18(9), 1756-1765.

Lorand, L., Shishido, R., Paremeswaran, K.N., & Steck, T.L., (1975) Biochem. Biophys. Res. Commun. 67(3), 1158-1166.

Lorand, L., Siefring, Jr., G.E., & Lowe-Krentz, L., (1978) J. Supramol. Struct. 9, 427-440.

Lorand, L., Siefring, Jr., G.E., & Rafferty, N.S., (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 1356-1360.

Lorand, L., & Stenberg, P., (1976) In: Handbook of Biochem. & Mol. Biol. (Fasman, G.D., ed.,) C.R.C. Press, Cleveland, Vol 2, pp669-685.

Lorand, L., Wiessman, L.B., Ebel, D.L., & Bruner-Lorand, J., (1976) Proc. Nat. Acad. Sci. U.S.A. 73(12), 4479-4481.

Lord, J.M., & Ashcroft, S.J.H., (1984) Biochem. J., 219, 547-551.

Lott, W.A., & Krapcho, J., (1957) U.S. Patent 2,813,904.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., & Randall, R.J., (1951) J. Biol. Chem. 193, 265-275.

Maccioni, R.B., & Arechaga, J., (1986) Exp. Cell Res. 167(1), 266-270.

Maccioni, R.B., & Seeds, N.W., (1986) Mol. Cell Biol. 69(2), 161-168.

MacNeil, S., Walker, S.W., Senior, H.J., Bleehen, S.S., & Tomlinson, S., (1984) J. Invest. Dermatol. 83, 15-19.

Malaisse, W.J., Garcia-Morales, P., Dufrane, S.P., Sener, A., & Valverde, I., (1984) Experientia 40, 2015-2020.

Malaisse, W.J., & Malaisse-Lagae, F., (1984) Experientia 40, 1068-1065.

Malaisse, W.J., Malaisse-Lagae, F., & Sener, A., (1984) Experientia 40, 1035-1043.

Malaisse, W.J., Sener, A., Carpinelli, A.R., Poloczek, P., Winand, J., & Castagna, M., (1980) Cancer Res. 40, 3827-3831.

Malaisse, W.J., Sener, A., Herchuelz, A., & Hutton, J.C., (1979) Metabolism 28, 373-386.

Malaisse, W.J., Sener, A., Hurchuelz, A., & Hutton, J.C., (1979) Prog. Endo. Metab. 28(4), 373-386.

Malaisse, W.J., Sener, A., & Mahy, M., (1974) Eur. J. Biochem. 47, 365-370.

Mamont, P.S., Bey, P., Koch-Weser, J., (1980) In: Polyamines in Biomedical Res. (Gaugas, J., ed.,) John Wiley & Sons, London, pp147-166.

Mamont, P.S., Daznin, C., Wagner, J., Siat, M., Joder-Ohlenbusch, A.M., & Claverie, N., (1982) Eur. J. Biochem. 123, 499-504.

Mamont, P.S., Siat, M., Joder-Ohlenbusch, A.M., Bernhardt, A., & Casaro, P., (1984) Eur. J. Biochem. 142, 457-463.

Manjunath, R., Chung, S.I., & Mukharjee, A.B., (1984) Biochem. Biophys. Res. Commun. 121, 400-407.

Martelo, O.J., Woo, S.L.C., Reimann, E.M., & Davie, E.W., (1970) Biochemistry 9, 4807-4813.

Marton, L.J., Oredsson, S.M., Heng, D.T., & Deen, D.F., (1983) Adv. Polyamine Res. 4, 33-44.

Mato, J.M., Pencev, D., Vasanthakumar, G., Schiffman, E., & Pastan, I., (1983) Proc. Nat. Acad. Sci. U.S.A. 80, 1929-1932.

Maxfield, M.R., Davies, P.J.A., Klempner, L., Willingham, M.C., & Pastan, I.H., (1979) Proc. Nat. Acad. Sci. U.S.A. 76(11), 5731-5735.

Maxfield, M.R., Willingham, M.C., Davies, P.J.A., & Pastan, I.H., (1979) Nature 277, 661-663.

Maxfield, M.R., Willingham, M.C., Haigler, H.T., Dragsten, P., & Pastan, I.H., (1981) Biochemistry 20, 5353-5358.

McDonald, M.J., Kowluru, A., Chang, C.M., & Mertz, R.J., (1983) In: Biochemistry of Metabolic Processes (Lennon, D.L.F., Stratman, F.N., & Zahlten, R.N., Eds.,) Elsiever Science Publishing, Amsterdam, pp165-178.

McGovern, K.A., Clark, R.S., & Pegg, A.E., (1986) J. Cell Physiol. 12, 671-678.

McKenna, J.A., Haigler, H.T., & Cohen, S., (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 5689-5693.

Meglasson, M.D., & Matschinsky, F.M., (1986) Diabetes Metab. Rev. 2(3&4), 163-214.

Mehta, K., & Berestein, G., (1986) Cancer Res. 46(3), 1388-1394.

Mehta, K., Claringbold, P., & Lopez-Berestein, G., (1987a) J. Immunol. 138, 3902-3906.

Mehta, K., Lopez-Berestein, G., Moore, Jr., W.T., & Davies, P.J.A., (1985) J. Immunol. 134(4), 2053-2056.

Mehta, K., Turpin, J., & Lopez-Berestein, G., (1987b) J. Leukocyte Biol. 41, 341-348.

Metcalf, B.W., Bey, P., Danzin, C., Jung, M.J., Casara, P., & Vevert, J.B., (1978) J. Am. Chem. Soc. 100, 2551-2553.

Milhaud, P.G., Davies, P.J.A., Pastan, I.H., & Gottesman, M.M. (1980) Biochim. Biophys. Acta 630, 476-484.

Montague, W., & Howell, S.L., (1972) Biochem. J., 129, 551-560.

Montague, W., & Howell, S.L., (1976) In: Polypeptide Hormones : Molecular & Cellular Aspects (Porter & Fitzsimmons, Eds.,) C.I.B.A. Found. Symp. 41, 141-154.

Montague, W., Howell, S.L., & Green, S.I., (1976) Horm. Metab. Res. 8, 166-169.

Moore, Jr., W.T., Murtaugh, M.P., & Davies, P.J.A., (1984) J. Biol. Chem. 259, 12794-12802.

Morgan, M.G., Rumford, G.M., & Montague, W., (1985) Biochem. J. 228, 713-718.

Morgan, N.G., Rumford, G.M., & Montague, W., (1987) Biochem. J. 669-674.

Mosher, D.F., (1984) Moll. Cell Biochem. 58, 63-68.

Mossenson, M.W., Finlayson, J.S., (1976) Prog. Hemostasis Thromb. 3, 61-107.

Mueller, T., (1984) Fedn. Proc. 43, 1849a.

Mukherjee, D.C., Agrawal, A.K., Manjunath, R., & Mukherjee, A.B., (1983) Science 219, 989-991.

Murtaugh, M.P., Dennison, O., Stein, J.P., Davies, P.J.A., (1986) J. Exp. Med. 163, 1325-1330.

Murtaugh, M.P., Mehta, K., Johnson, J., Myers, M., Juliano, L., & Davies, P.J.A., (1983) J. Biol. Chem. 258, 11074-11081.

Mycek, M.J., & Waelsch, H., (1960) J. Biol. Chem. 235, 3513-3517.

Neidle, A., & Acs, G., (1961) Fed. Proc. 20, 234.

Nishida, E., Kuwaki, T., & Sakai, H., (1981) Biochem. J. 90, 575-578.

Nishizuka, Y., (1984) Nature 308, 693-698.

Noguchi, T., Kashiwaga, A., & Tanaka, T., (1976) J. Biochem. (Tokyo) 79, 451-454.

Notides, A.C., & Williams-Ashman, H.G., (1967) Proc. Nat. Acad. Sci. U.S.A. 58, 1991-1995.

Novogradsky, A., Quittner, S., Rubin, A.L., & Stengel, K.H., (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 1157-1161.

Ogawa, H., & Goldsmith, L.A., (1976) J. Biol. Chem. 251(23), 7281-7288.

Ogawa, H., & Goldsmith, L.A., (1977) J. Investig. Dermatol. 68(1), 32-35.

Ohanian, V., Wolfe, L.C., John, K.M., Pinder, J.C., & Lux, S.E., (1984) Biochemistry 23, 4416-4420.

Orci, L., (1974) Diabetologia 10, 163-187.

Orci, L., (1982) Diabetes 31, 538-565.

Orci, L., (1985) Diabetologia 28, 528-546.

Orci, L., (1986) Diabetes Metab. Rev. 2(1&2), 71-106.

Pabalan, S.S., (1975) Guinea-Pig Seminal Vesical Basic Secretory Proteins, Ph.D. Thesis, Northwestern University, Evanston, Illinois.

Paonessa, G., Metafora, S., Tajana, G., Abrescia, P., De Santis, A., Gentile, V., & Porta, R., (1984) Science 226, 852-855.

Parenteau, N.L., Pilato, A., & Rice, R.H., (1986) Differentiation 33, 130-141.

Park, M.H., Chung, S.I., Cooper, H.L., & Folk, F.E., (1984) J. Biol. Chem. 259, 4563-4565.

Pastan, I., & Willingham, M.C., (1981) Science 214, 504-509.

Pasternack, G., Anderson, R.A., & Marchesi, V.T., (1985) J. Biol. Chem. 260, 3676-3683.

Patterson, M.K., Maxwell, M.P., Birckbichler, P.J., Conway, E., & Carter, H.A., (1982) Cell Biol. Int. Reports 6(5), 461-470.

Patzelt, C., Labreque, A.P., Duguid, J.R., Carrol, R.J., Keim, P.S., Heinrikson, R.L., & Steiner, D.F., (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1260-1264.

Pegg, A.E., (1984) Biochem. J. 224, 29-38.

Pegg, A.E., (1986) Biochem. J. 234, 249-262.

Pegg, A.E., & Coward, J.K., (1985) Biochem. Biophys. Res. Commun. 133, 82-89.

Pegg, A.E., & McCann, P.P., (1982) Am. J. Physiol. 243, C212-C221.

Pegg, A.E., Seely, J.E., Poso, H., Della Ragionne, F., & Zagon, I.S., (1982a) Fed. Proc. Fed. Am. Exp. Biol. 41, 3065-3072.

Pegg, A.E., Tang, K.C., & Coward, J.K., (1982b) Biochemistry 21, 5082-5089.

Penn, E.J., Brocklehurst, K.W., Sopwith A.M., Hales, C.N., & Hutton, J.C., (1982) FEBS Lett. 130, 4-8.

Perrin, D., Langly, K., & Aunis, D., (1987) Nature 326, 498-501.

Perussia, B.P., Dayton, E.T., Faning, V., Thiangrajan, P., Hoxie, J., & Tinchieri, G., (1983) J. Exp. Med. 158, 2058-2067.

Piacentini, M., Sartori, C., & Ceru Argento M.P., (1987) Noble Conf. Cell. Mol. Biol.; Transglutaminase & Protein Cross-linking Reactions.

Pipeleers, D.G., Harnie, N., Haylen, L., & Wauters, G., (1979) In: Biochemistry & Biophysics of the Pancreatic Beta Cell (Malaisse, W.J., & Taljedal, I.B., eds.) European Workshop, Brussels. Georg Thieme Verlag, Stuttgart. pp163-167.

Pohjanpelto, P., Hollta, E., Janne, O.A., Knautila, S., & Alitalo,K., (1985) J. Biol. Chem. 260, 8532-8537.

Pohjanpelto, P., & Knautila, S., (1982) Exp. Cell Res. 141, 333-339.

Porta, R., Metafora, S., Esposito, C., Pucci, M., Malorni, A., & Marino, G., (1987) Noble Conf. Cell. Mol. Biol.; Transglutaminase & Protein Cross-linking Reactions.

Porta, R., Esposito, C., De Santis, A., Fusco, A., Iannone, M., & Metafora, S., (1986) Biol. Reprod. 35, 965-970.

Porter, C.W., Cavanaugh, P.F., Stolowich, N., Ganis, B., Kelly, E., & Bergeron, R.J., (1985) Cancer Res. 45, 2050-2057.

Prentki, M., Biden, T.J., Janjic, D., Irvine, R.F., Berridge, M.J., & Wollheim, C.B., (1984) Nature 309, 562-564.

Prentki, M., & Wollheim, C.B., (1984) Experientia 40, 1052-1060.

Puszkin, E.G., & Billett, H.H., (1987) Noble Conf. Cell. Mol. Biol.; Transglutaminase & Protein Cross-linking Reactions.

Puszkin, E.G., & Raghuraman, V., (1985) J. Biol. Chem. 260(29), 16012-16020.

Putnam, F.W., Takahashi, Y., & Takahashi, H., (1987) Noble Conf. Cell. Mol. Biol.; Transglutaminase & Protein Cross-linking Reactions.

Rana, R.S., Mertz, R.J., Kowluru, A., Dixon, J.F., Hokin, L.E., MacDonald, M.J., (1985) J. Biol. Chem. 260(13), 7861-7867.

Reimann, E.M., Walsh, D.A., & Krebs, E.G., (1971) J. Biol. Chem. 246, 1986-1995.

Rice, R.H., & Green, H., (1979) Cell 18, 681-694.

Robbins, K.C., (1944) Am. J. Physiol. 142, 581-588.

Robertson, R.P., (1986) Diabetes Metab. Rev. 2(3&4), 261-296.

Rodriguez-Boulan, E., & Sabatini, D.D., (1978) Proc. Natl. Acad. Sci. USA 75, 5071-5075.

Rogers, G.E., (1962) Nature 194, 1149-1151.

Rogers, G.E., Harding, H.W.J., & Llewellyn-Smith, I.J., (1977) Biochim. Biophys. Acta 495, 159-175.

Rogers, G.E., & Springell, P.H., (1959) Nature 183, 183-193.

Rudkin, B.B., Mamont, P.S., & Seiler, N., (1984) Biochem. J. 217, 731-741.

Russell, D.H., & Durie, B.G.M., (1978) Polyamines as Biochemical Markers of Normal & Malignant Growth, Raven Press, N.Y., U.S.A. .

Rybicki, A., Heath, R., Lubin, B., & Schwartz, R.S., (1988) J. Clin. Invest. 81, 255-260.
Saceda, M., Garcia-Morales, P., Valverde, I., & Malaisse, W.J., (1983) Diabetes 32(suppl.1), 39A.

Sakata, Y., & Aoki, N., (1982) J. Clin. Invest. 69, 536-542.

Sano, Y., Deen, F., Oredsson, S.M., & Marton, L.J., (1984) Cancer Res. 144, 577-581.

Sarkar, N.K., Clarke, D.D., & Waelsch, H., (1957) Biochim. Biophys. Acta 25, 451-452.

Schatzman, H.J., (1973) J. Physiol. 235, 551-569.

Schatzman, H.J., (1982) In: Membrane Transport of Calcium (Carafoli, E., ed.) Academic Press, London pp41-108.

Schrode, J., & Folk, J.E., (1978) J. Biol. Chem. 253, 4837-4840.

Schrode, J., & Folk, J.E., (1979) J. Biol. Chem. 254, 653-661.

Schroff, C., Nuemann, C., & Sorg, C., (1981) Eur. J. Immunol. 11, 637-642.

Schwartz, M.L., Pizzo, S.V., Hill, R.H., & McKee, P.A., (1971) J. Biol. Chem. 246, 5851-5856.

Schwartz, M.L., Pizzo, S.V., Hill, R.H., & McKee, P.A., (1973) J. Biol. Chem. 248, 1395-1407.

Seglen, P.O., & Gordon, P.B., (1980) Mol. Pharm. 18, 468-475.

Selkoe, D.J., Abraham, C., & Ihara, Y., (1982) Proc. Nat. Acad. Sci. U.S.A. 79, 6070-6074.

Sener, A., Dunlop, M.E., Gomis, R., Mathias, P.C.F., Malaisse-Lagae, F., & Malaisse, W.J., (1985) Endocrinology 117, 237-242.

Sener, A., Gomis, R., Lebrun, P., Hurchuuelz, A., Malaisse-Lagae, F., & Malisse, W.J., (1984) Mol. Cell. Endocrinol. 36, 175-180.

Sener, A., & Malaisse, W.J., (1984) Experientia 40, 1026-1035.

Sharp, G.W.G., (1979) Diabetologia 16, 287-296.

Shezen, E., & Goldman, R., (1985) Int. J. Cell Cloning 3, 65.

Sieghart, W., Forn, J., & Greengard, P., (1979) Proc. Natl. Acad. Sci. USA 76, 2475-2479.

Siefring, Jr., G.E., Apostol, A.B., Velasco, P.T., & Lorand, L., (1978) Biochemistry 17(13), 2598-2604.

Sjoerdsman, A., & Schechter, P.T., (1984) Clin. Pharmacol. Ther. 35, 287-300.

Slife, C.W., Dorsett, M.D., Bouquett, G.T., Register, A., Taylor, E., & Conroy, S., (1985) Arch. Biochem. Biophys. 241(2), 329-336.

Slife, C.W., Dorsett, M.D., & Tillotson, M.L., (1986) J. Biol. Chem. 261(7), 3451-3456.

Slife, C.W., Morris, G.S., & Snedeker, S.W., (1987) Arch. Biochem. Biophys. 257, 39-47.

Smith, B.D., LaCelle, P.L., Siefring, G.E., Lowe-Krentz, L., Lorand, L., (1981) J. Membr. Biol. 61, 75-80.

Steeg, P.S., Moore, R.N., Johnson, H.M., & Oppenheim, J.J., (1982) J. Exp. Med. 156, 1780-1793.

Stein, J.P., Chiocca, E., Saydak, M., & Davies, P.J.A., (1987) In: Noble Conf. Cell Mol. Biol.; Transglutaminase & Protein Cross-linking Reactions.

Steiner, D.F., Kemmler, W., Tager, H.S., & Peterson, J.D., (1974) Fed. Proc. 33, 2105-2115.

Steiner, D.F., Terris, S., Chan, S.J., & Rubenstein, A.H., (1976) In: Insulin (Luft, R., ed.) Nordisk Insulin Laboratorium, Genotte, Denmark pp53-103.

Streb, H., Irvine, R.F., Berridge, M.J., & Schulz, I., (1983) Nature 306, 67-69.

Stutchfield, J., & Howell, S.L., (1984) FEBS Lett. 175, 393-396.

Sugden, M.C., Ashcroft, S.J.H., & Sugden, P.H., (1979) Biochem. J. 180, 219-229.

Suprenant, K.A., & Dentler, W.L., (1982) J. Cell Biol. 93, 164-174.

Sztein, M.B., Steeg, P.S., Johnson, H.M., & Oppenheim, J.J., (1984) J. Clin. Invest. 73, 556-565.

Tabor, C.W., & Tabor, H., (1976) Ann. Rev. Biochem. 45, 285-306.

Tabor, C.W., & Tabor, H., (1984) Ann. Rev. Biochem. 53, 749-790.

Takagi, T., & Doolittle, R.F., (1974) Biochemistry 13, 750-756.

Takagi, T., & Konishi, K., (1972) Biochim. Biophys. Acta 271, 363-370.

Takermoto, T., Nagamatsu, Y., & Oka, T., (1983) Biochim. Biophys. Acta 740, 73-79.

Tamaki, T., & Aoki, N., (1981) Biochim. Biophys. Acta 661, 280-287.

Tanigawa, K., Kuzuya, H., Imura, H., Taniguchi, H., Baba, S., Takai, Y., & Nishizuka, Y., (1982) FEBS Lett. 138, 183-186.

Trifaro, J.M., Bader, M.F., & Doucet, J.P., (1985) Can. J. Biochem. Cell Biol. 63, 661-679.

Tomlinson, S., MacNeil, S., Walker, S.W., Ollis, C.A., Merritt, J.E., & Brown, B.L., (1984) Clin. Sci. 66, 497-508.

Tong, Y.S., (1980) Ph.D. Thesis, Northwestern University, Evanston, Illinois, U.S.A. .

Tyler, J., Reinhart, B., & Branton, D., (1980) J. Biol. Chem. 255, 7034-7039.

Tyrell, D.J., Sale, W.S., & Sliffe, C.W., (1986) J. Biol. Chem. 261(31), 14833-14836.

Tucciarone, L.M., & Lanclos, K.D., (1981) Biochem. Biophys. Res. Commun. 99, 221-227.

Uhl, L., & Schindler, J., (1987) Exp. Cell Biol. 55, 28-33.

Ungewickell, E., Bennett, P.M., Calvert, R., Ohanian, V., & Gratzer, W.B., (1979) Nature 280, 211-214.

Valverde, I., & Malaisse, W.J., (1984) Experientia 40, 1061-1068.

Vanella, A., Geremia, E., Pinturo, R., & Bernadini, R., (1983) Life Sciences 33, 555-559.

Van Leuven, F., Cassiman, J.J., & Van Den Berghe, H., (1980) Cell 20, 37-43.

Van Leuven, F., Marynen, P., Cassiman, J.J., & Van Den Berghe, H., (1981) FEBS Lett. 134, 83-87.

Van Obberghen, E., Somers, G., Devis, G., Vaughan, G.D., Malaisse-Lagae, F., Orci, L., & Malaisse, W.J., (1973) B. J. Clin. Invest. 32, 1041-1051.

Verelizier, J.L., Perez, N., Seisdedos, F.A., & Devos, R., (1984) Eur. J. Immunol. 14, 106-109.

Virji, M.A.G., Stefes, M.W., & Estensen, R.D., (1978) Endocrinology 102, 706-711.

Waelsch, H., (1962) In: Monoamines et Systeme Nerveux Central, Masson, Paris, pp93-104.

Wani, M.C., Campbell, H.F., Brine, G.A., Kepler, J.A., & Wall, A.E., (1972) J. Am. Chem. Soc. 94, 3631-3632.

Weinberg, R.A., (1983) In: Readings from Scientific American; Cancer Biology, pp77-87, W.H. Freeman & Company, N.Y., USA.

White, J.G., (1976) Sem. Hematol. 13, 121-132.

Wiedenkeller, D.E., & Sharp, G.W.G., (1983) Endocrinology 113, 2311-2313.

Williams-Ashman, A.G., Beil, R.E., Wilson, J., Hawkins, M., Grayhack, J., Zunamon, A., & Winstein, N.K., (1980) Adv. Enzyme Regul. 18, 239-258.

Williams-Ashman, H.G., & Cannelakis, Z.N., (1979) Physiol. Chem. Phys. 12, 457-472.

Williams-Ashman, H.G., & Cannelakis, Z.N., (1980) Perspect. Biol. Med. 22, 421-453.

Williams-Ashman, H.G., Notides, A.C., Pabalan, S.S., & Lorand, L., (1972) Proc. Nat. Acad. Sci. U.S.A. 69(8), 2322-2325.

Williams-Ashman, H.G., Wilson, J., Beil, R.E., & Lorand, L., (1977) Biochem. Biophys. Res. Commun. 79, 1192-1198.

Willingham, M.C., Maxfield, R., & Pastan, I.H., (1979) J. Cell Biol. 82, 614-625.

Willingham, M.C., & Pastan, I.H., (1981) Ann. Rev. Physiol. 43, 239-250.

Wing, D.A., (1977) Ph.D. Thesis, Northwestern University, Evanston, Illinois, U.S.A. .

Wise, B.C., Glass, D.B., Jen Chou, C.H., Raynor, R.L., Katoh, N., Schatzman, R.C., Turner, R.S., Kibler, R.F., & Kuo, J.F., (1982a) J. Biol. Chem. 257(14), 8489-8495.

Wise, C.B., Raynor, R.L., & Kuo, J.B., (1982b) J. Biol. Chem. 257(14), 8481-8488.

Wolf, B.A., Colca, J.R., Comens, P.G., Turk, J., & McDaniel, M.L., (1986) J. Biol. Chem. 261(35), 16284-16287.

Wollheim, C.B., & Biden, T.J., (1986) J. Biol. Chem. 261, 8314-8319.

Wollheim, C.B., & Pozzan, T., (1984) J. Biol. Chem. 259(4), 2262-2267.

Wollheim, C.B., & Sharp, G.W.G., (1981) Physiol. Rev. 61(4), 914-973.

Yamoto, S., Nakaki, T., Nakadate, T., & Kato, R., (1982) Eur. J. Pharmacol. 86, 121-124.

Yancey, S.T., & Laki, K., (1972) Ann. N.Y. Acad. Sci. 202, 344-348.

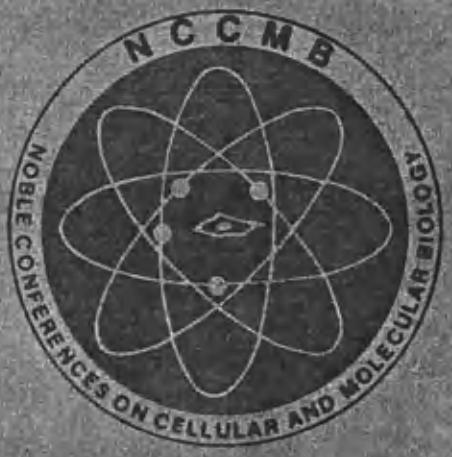
Yarden, Y., Gabbay, M., & Schlessinger, J., (1981) Biochim. Biophys. Acta 674, 188-203.

Yaseen, M.A., Pedley, K.C., & Howell, S.L., (1982) Biochem. J. 206, 81-87.

Zettergren. J.G., Peterson, L.L., & Wuepper, K.D., (1982) Clin. Res. 30(2), 616A.

NOBLE CONFERENCE
IN CELLULAR AND MOLECULAR BIOLOGY

TRANSGLUTAMINASE & PROTEIN CROSSLINKING REACTIONS



March 29 - April 1, 1987
James L. Knight International Center
Miami, Florida USA

Poster Board 22

Transglutaminase Catalysed Cross-linking of Islet Proteins may be Modulated by Protein Phosphorylation

R.A.Owen, M.Hussain, P.J.Bungay, and M.Griffin.
Dept. of Life Sciences, Trent Polytechnic,
Nottingham. NG11 8NS, England.

The islet transglutaminase may be a potential Ca^{2+}-target important to Ca^{2+}-mediated stimulus response coupling in the pancreatic B-cell. In addition to regulation by intracellular levels of Ca^{2+} our studies have indicated that expression of islet transglutaminase activity may also be modulated by phosphorylation of its substrate proteins during stimulus response. The possible relationship of protein phosphorylation in intact islets to transglutaminase activity was investigated by incubating islets with $^{32}PO_4$ in the presence of stimulatory levels (16.8mM) of glucose. Analysis of islet proteins by SDS-gel electrophoresis indicated the presence of radiolabelled polymeric material of high molecular weight at the top of the 3% acrylamide stacking gel. This phosphorylated polymer was associated with the 80,000g sedimented fraction of islet homogenates. Incubation of islets with monodansylcadaverine (50μM) or glycine methyl ester(10mM) prior to and during stimulation with 16.8mM glucose in the presence of $^{32}PO_4$ led to a significant reduction in the fraction of phosphorylated polymer present. Homogenates obtained from intact islets previously radiolabelled with $^{32}PO_4$ in the presence of 16.8mM glucose when incubated under conditions that activated transglutaminase led to an increase in the ^{32}P found in the phosphorylated polymer. These findings suggest that transamidation and phosphorylation reactions may be closely linked in the pancreatic B-cell.

Biochimica et Biophysica Acta 968 (1988) 220–230
Elsevier

BBA 12192

Transglutaminase-catalysed cross-linking of proteins phosphorylated in the intact glucose-stimulated pancreatic β-cell

Robin A. Owen, Peter J. Bungay *, Mashraf Hussain and Martin Griffin

Department of Life Sciences, Trent Polytechnic, Clifton, Nottingham (U.K.)

(Received 13 July 1987)
(Revised manuscript received 7 October 1987)

Key words: Transglutaminase; Protein cross-linking; Protein phosphorylation; Insulin release; (Pancreas β-cell)

Incubation of intact islets in the presence of [^{32}P]P_i and stimulatory levels of glucose followed by separation of phosphorylated islet proteins by SDS-polyacrylamide gel electrophoresis revealed the presence of a high molecular weight phosphopolymer which did not transverse a 3% (w/v) acrylamide gel. The majority of this phosphopolymer (approx. 70%) was present in the 600 × g sedimented fraction of islet homogenates. Islet homogenates obtained from intact islets previously incubated with [^{32}P]P_i and stimulatory levels of glucose when incubated under conditions that activated the islet transglutaminase resulted in an increase in the amount of phosphopolymer present in the 600 × g sedimented fraction. Inhibitors of transglutaminase activity which are known to inhibit glucose-stimulated insulin release led to a significant reduction in the fraction of phosphopolymer present in the glucose-stimulated intact islet. These findings suggest that protein cross-linking and phosphorylation reactions may be closely linked in the pancreatic β-cell.

Introduction

The stimulus-secretion coupling of glucose-stimulated insulin release from the pancreatic β-cell is thought to be mediated by a rise in the cytosolic concentration of Ca^{2+} [1,2]. Although the way in which Ca^{2+} mobilizes the secretory apparatus is not well understood, it is likely that a change in the activity of specific Ca^{2+}-binding proteins may be involved. A potential Ca^{2+}-responsive target in the pancreatic β-cell is the enzyme transglutaminase (EC 2.3.2.13) which catalyses an acyl-transfer reaction between peptide-bound glutamine and primary amine groups, resulting in the cross-linking of proteins with ε-(γ-glutamyl)lysine bridges [3,4]. Recent evidence based on the specific action of inhibitors of transglutaminase activity in pancreatic islet cells has indeed suggested that protein modification by this enzyme may be an essential activity in the insulin secretory mechanism [5–7]. Preliminary studies using homogenates of islets have demonstrated that endogenous transglutaminase activity is capable of cross-linking islet proteins into high molecular weight aggregates which are present in a particulate fraction of islet homogenates [6]. However, the significance of these observations to the function of the intact β-cell is not clear, since the substrate specificity of islet transglutaminase in the intact cell may not reflect that demonstrated in vitro, owing to differences in substrate availability.

Another mechanism whereby islet proteins may be modified post-translationally so that they affect

* Present address: Pfizer Central Research, Sandwich, Kent, U.K.

Correspondence: M. Griffin, Department of Life Sciences, Trent Polytechnic, Clifton, Nottingham, NG11 8NS, U.K.

0167-4889/88/$03.50 © 1988 Elsevier Science Publishers B.V. (Biomedical Division)

the secretory apparatus is through acting as substrates for specific protein kinases [8]. Thus, proteins involved in the secretory mechanism may be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinases, protein kinase C or cyclic nucleotide-dependent protein kinases during the process of stimulus secretion coupling. If protein kinase and transglutaminase activities play a role simultaneously in the intact β-cell during stimulus-secretion coupling, it is possible that they are closely coordinated. Indeed, it is possible that protein phosphorylation reactions may provide a further means of modulating transglutaminase activity through alteration of substrate availability.

This study has investigated the possible relationship between proteins which are phosphorylated and those which are modified by transglutaminase in the intact pancreatic β-cell during stimulus-secretion coupling. We report that the intact glucose-stimulated pancreatic β-cell contains a high molecular weight phosphorylated polymer which is associated with the particulate fraction of the cell. Evidence is presented which indicates that the formation of this phosphopolymer is mediated by the islet transglutaminase.

Materials and Methods

Materials. Collagenase used in the isolation of pancreatic islets was obtained from Serva Feinbiochimica, Heidelberg, F.R.G. and from Sigma (Collagenase type V) Chemical Co., Poole, Dorset, U.K., Glycine methyl ester was purchased from Aldrich Chemical Co Ltd., Gillingham, Dorset, U.K., Sarcosine methyl ester was purchased from Serva Feinbiochimica, Heidelberg, F.R.G. Monodansylcadaverine was purchased from Fluka A.G. Buchs, Switzerland. *N*,*N'*-dimethylmonodansylcadaverine, (*N*-(5-dimethylaminopentyl)-5-dimethyl amino-1-naphthalenesulphonamide) was synthesised by previously described methods [6]. [1,4-^{14}C]Putrescine, and [^{32}P]P_i (carrier free) were purchased from Amersham International, Amersham, Bucks, U.K.

Isolation of islets of Langerhans. Islets of Langerhans were isolated from the pancreas of 200–300 g Sprague-Dawley rats of both sexes using a modification of the collagenase digestion technique of Lacy and Kostianovsky [9]. Islets were individually harvested from the collagenase digestion using a finely drawn-out, siliconised glass pasteur pipette under a dissecting microscope. Only clean whole opaque islets were selected.

Measurement of insulin release. This was carried out as described previously [5]. Batches of 60 islets were incubated in 150 μl Gey and Gey medium [10] containing albumin (1 g/l) and additions and incubation times stated in the text and tables. Insulin released into the incubation medium was determined by radioimmunoassay [11] with rat insulin as standard.

Phosphorylation of peptides in intact islets. Batches of 60 islets were incubated for 90 min at 37 °C in 150 μl Gey and Gey medium containing albumin (1 g/l), 16.8 mM glucose and [^{32}P]P_i (1 mCi/ml). Following incubation, islets were quickly washed twice with medium containing 0.3 M sucrose, 50 mM sodium phosphate (pH 7.4), 5 mM sodium fluoride; 1 mM EGTA, 4 mM ATP and 1 mM phenylmethylsulphonyl fluoride prior to homogenisation in the same buffer. Homogenates were then used directly for electrophoresis by dissolution of proteins in 2 × strength electrophoresis sample buffer or used for further incubation studies.

Homogenisation of islets. Islets were homogenised with a glass-teflon homogeniser using an islet-to-medium ratio of 2–3 per μl, medium consisted of 0.3 M sucrose, 50 mM sodium phosphate, 1 mM EGTA, 5 mM sodium fluoride, 4 mM ATP and 1 mM phenylmethylsulphonyl fluoride (pH 7.4) (sucrose medium).

Incorporation of ^{32}P-labelled phosphorylated islet peptides into high molecular weight aggregates. Islet homogenates obtained from intact islets previously incubated with [^{32}P]P_i were prepared as described above. The incubation mixture for the cross-linking of phosphorylated islet proteins consisted of 0.3 M sucrose, 50 mM sodium phosphate (pH 7.4), 5 mM sodium fluoride, 4 mM ATP, 1 mM phenylmethylsulphonyl fluoride and 5 mM $CaCl_2$ or 5 mM EGTA in a final volume of 60 μl after the addition of 50 μl of islet homogenate. The mixture was incubated for 15, 30 and 60 min at 37 °C, after which time the reaction was stopped by the addition of 20 μl of 4 × strength electrophoresis sample buffer so that the components in the final incubation mixture consisted of 62.5 mM

Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol and 5% (w/v) 2-mercaptoethanol. The sample was then boiled for 5 min prior to electrophoresis.

Subcellular fractionation of islet homogenates. Islet homogenates were obtained from 500–800 islets which had been preincubated in the presence or absence of [^{32}P]P_i as described earlier. Homogenates were then fractionated using a scheme based on that described by Christie and Ashcroft [12]. Homogenates were first centrifuged at 600 × *g* av. for 10 min at 4°C, the supernatant was withdrawn and the pellet fraction was rehomogenised in 200 μl of sucrose medium (same composition as homogenisation medium) and centrifuged at 600 × *g* av. for a further 10 min at 4°C. The combined supernatants were then centrifuged at 80000 × *g* av. for 45 min at 4°C. Pellet fractions were resuspended in 150–200 μl of sucrose medium if used for enzyme assays or in electrophoresis sample buffer if used directly for electrophoresis. The supernatant or 'particle free' fraction was either used directly for enzyme assays or added to an equal volume of double strength electrophoresis sample buffer prior to electrophoresis.

Islet homogenates previously incubated in the presence of Ca^{2+} (5 mM) or EGTA (5 mM) as described earlier were fractionated in the same way. Following incubation, reactions were stopped by the addition of 10 μl of 26 mM EGTA and placed at 4°C prior to fractionation. Marker enzyme studies were carried out on fractions obtained from homogenates previously incubated in the presence of Ca^{2+}.

Enzyme assays. Transglutaminase activity was measured by [^{14}C]putrescine incorporation into *N*,*N'*-dimethylcasein using previously published methods [5]. Lactate dehydrogenase activity was measured spectrophotometrically by monitoring the rate of change of absorbance at 340 nm due to the pyruvate stimulated oxidation of NADH. Reaction mixtures contained in a volume of 1 ml, 40 mM potassium phosphate buffer (pH 7.4), 0.1 mM NADH, 0.5 mM sodium pyruvate and 150–250 μg of enzyme sample.

5'-Nucleotidase activity was measured by the method of Christie and Ashcroft [12] and cytochrome *c* was measured by the method of Cooperstein and Lazarow [13]. Protein was determined by the method of Lowry et al. [14].

SDS-polyacrylamide gel electrophoresis. Islet proteins were separated by discontinuous SDS-polyacrylamide gel electrophoresis as described by Laemmli [15]. The solubilised sample (approx. 50–75 μg of protein in 45–75 μl) was applied to the gels which consisted of a stacking gel of 3% (w/v) acrylamide, 0.125 M Tris-HCl (pH 6.8) and 0.1% (w/v) SDS and a resolving gel of 10% (w/v) acrylamide, 0.375 M Tris-HCl (pH 8.8) and 0.1% (w/v) SDS. Samples were run for 4–5 h together with the appropriate M_r standard proteins which were solubilised in the same buffer. After electrophoresis, gels were fixed in a solution of 10% (w/v) trichloroacetic acid and 3.4% (w/v) sulphosalicylic acid and then stained with a solution of 1.25 g Coomassie blue (PAGE 83; BDH)/l in 18% (v/v) methanol/5% (v/v) acetic acid. Gels were destained in 18% (v/v) methanol/5% (v/v) acetic acid containing 2 mM sodium phosphate. Stained gels were photographed, dried and then used for autoradiography by exposure to Fuji X-ray film. Alternatively, stained gels were sectioned every 1 cm apart from the stacking gel which was sectioned into two components, the first 5 mm and the rest of the stacking gel; radioactivity was then determined in each of the gel sections by placing in 10 ml water and radioactivity was determined by Cerenkov counting.

For some electrophoretic studies an additional agarose gel (3 cm in height) consisting of 0.8% (w/v) agarose, 0.12 M Tris-HCl (pH 6.8) and 0.1% (w/v) SDS was overlayed on top of the stacking gel.

Statistical analysis of data. Statistical test for significance were performed using a Students' *t*-test (two tailed) and values of $P < 0.05$ are taken as significant.

Results

Presence of a high molecular weight phosphorylated protein polymer in glucose-stimulated islets

To investigate a possible relationship between transglutaminase activity and protein phosphorylation in the glucose-stimulated islet involving the cross-linking of phosphorylated islet proteins, experiments were first undertaken to determine

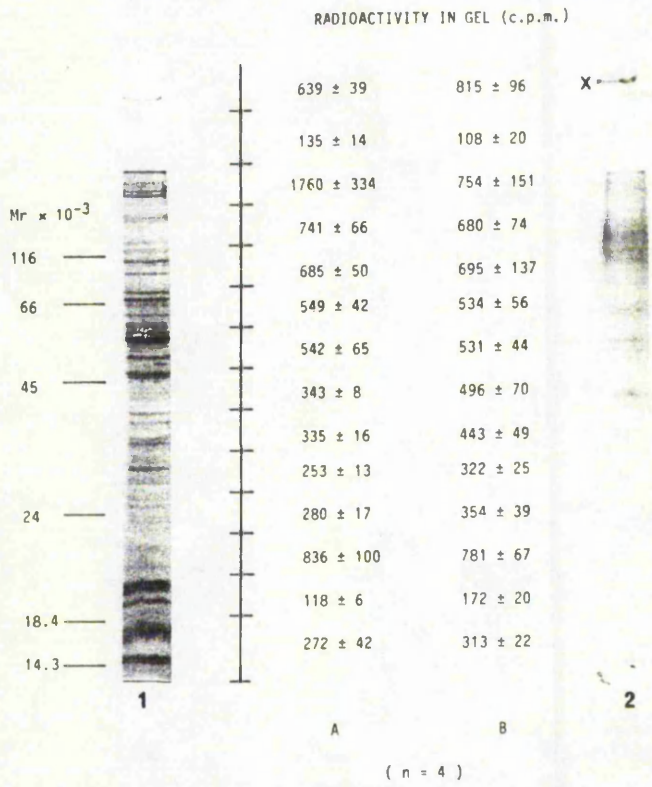


Fig. 1. Phosphorylation of islet peptides in glucose stimulated intact islets. 1 shows the electrophoretic separation of islet proteins in homogenates obtained from intact islets previously incubated with [^{32}P]P_i and stimulatory levels of glucose (16.8 mM). Column A shows the amount of radioactivity (cpm) at the points indicated obtained from untreated homogenates dissolved directly in electrophoresis sample buffer. Column B shows the radioactivity found in gels obtained from four paired experiments to those shown in column A where homogenates were treated with RNAase and DNAase (50 μg/ml) for 5 min prior to dissolution in electrophoresis sample buffer. 2 shows a representative autoradiogram from DNAase- and RNAase-treated homogenates with X indicating the presence of the radiolabelled phosphopolymer at the top of the 3% (w/v) acrylamide stacking gel.

whether a high molecular weight phosphorylated protein could be detected in the glucose-stimulated islet.

Batches of islets were incubated in the presence of [^{32}P]P_i and stimulatory levels of glucose (16.8 mM) to promote both maximum radiolabelling of ATP in the islet [17] and phosphorylation of islet peptides during β-cell stimulation. Islets labelled in this way were homogenised and the phosphorylated proteins were then analysed for radioactivity following their separation by SDS-gel electrophoresis. The phosphorylation pattern obtained for islet proteins using this procedure is illustrated in Fig. 1. To ascertain whether the radiolabelled proteins were in fact islet protein and not radiolabelled ribonucleic acid or islet proteins complexed with nucleic acids, islet homogenates were subjected to a number of different treatments prior to electrophoresis. The treatment, shown in Fig. 1, of DNAase and RNAase (50 μg/ml for 5 min) led to a small reduction in the total amount of radiolabel present in the gel, especially that found in the first 2 cm of the resolving gel. However, no significant reduction occurred in the radiolabelled phosphopolymer present at the top of the stacking gel. Following treatment of homogenates with RNAase and DNAase, homogenates were also incubated with pronase (50 μg/ml for 30 min). Comparison with control samples incubated without pronase indicated a 68% ± 0.7 ($n = 3$) reduction in the radiolabel present in the resolving gel and a 29 ± 3% ($n = 3$) reduction in the radiolabel present at the top of the stacking gel (data not shown). These data indicate that the radiolabelled phosphopolymer is not due to the presence of radiolabelled nucleic acids or radiolabelled nucleic acids complexed with islet protein. The decrease in radiolabel found with pronase digestion also indicates the phosphopolymer to be protein. The smaller decrease in radiolabel found in the phosphopolymer compared with that found in the resolving gel following pronase digestion is not unexpected, since a high molecular weight cross-linked matrix is likely to be much more resistant to proteolysis [16].

A summary of the phosphorylation pattern obtained from a number of experiments ($n = 18$) where islet homogenates were pretreated with RNAase and DNAase prior to electrophoresis is shown in Fig. 2. Of the material which incorporated ^{32}P, approx. 15% was present in the component at the top of the 3% (w/v) acrylamide stacking gel, making this the most heavily labelled component in the gel.

Additional experiments were undertaken on the phosphopolymer to give an approximate guide to its size and to ascertain whether any further phosphopolymer may be present in the stimulated islet which are not entering the standard 3% (w/v) acrylamide stacking gel. These experiments were performed by electrophoretic separation of islet

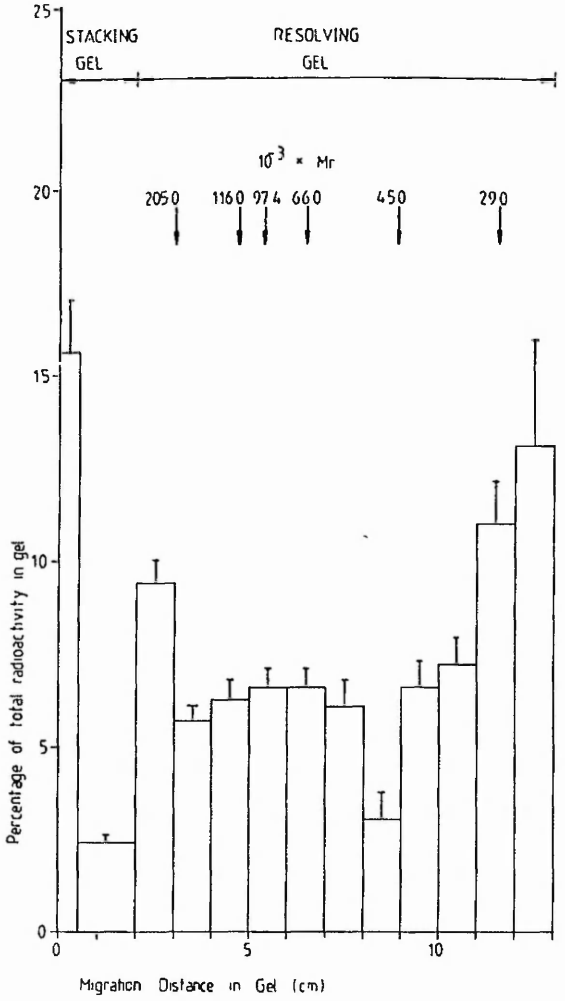


Fig. 2. Protein phosphorylation pattern in glucose stimulated intact islets of Langerhans. The histogram shows the protein phosphorylation pattern obtained from glucose stimulated islets following separation of islet proteins by electrophoresis. The bars of the histogram represent the mean ± S.E. percentage of the total radioactivity (cpm) found in the gel sections from 18 experiments. The mean total radioactivity present in the gels was 6989 ± 479 ($n = 18$). Islet homogenates were treated with DNAase and RNAase (50 μg/ml) for 5 min prior to electrophoresis.

proteins in a 0.7% agarose gel prior to their entry into the 3% (w/v) acrylamide stacking gel. If additional phosphopolymers of greater molecular weight were to be found in the stimulated islet which were unable to enter the 3% (w/v) acrylamide stacking gel, then these should be found in the agarose gel following electrophoretic separation of phosphorylated islet proteins. Using such a procedure it was found that only a small additional amount of radiolabel ranging from 1.6–2.7% ($n = 6$) of the total phosphorylated islet proteins was present in the agarose gel (data not shown). Our data, therefore, indicate that the majority of phosphopolymer which is present in the stimulated islet is, in fact, entering but not traversing the 3% (w/v) acrylamide. Its molecular size is therefore likely to be in excess of 10^6.

The cross-linking of phosphorylated islet proteins into high molecular weight protein aggregates by islet transglutaminase

As a means of investigating the involvement of islet transglutaminase in the formation of the phosphorylated polymer found in the intact glucose-stimulated islet, we assessed the ability of the enzyme to cross-link phosphorylated islet proteins to form high molecular weight aggregates. Homogenates obtained from ^{32}P-labelled intact islet were incubated for 30 min in the presence and absence of Ca^{2+}. Incubation mixtures contained 5 mM sodium fluoride to minimise the action of phosphatases during the incubation. The concentration of sodium fluoride in the incubations was limited to 5 mM, since higher concentrations were found to have a profound inhibitory effect on transglutaminase activity (data not shown). To prevent endogenous phosphorylation of proteins with phosphate derived from [γ-^{32}P]ATP present in the homogenates, unlabelled ATP (4 mM) was always present in the homogenisation and incubation mixtures.

Analysis of the radiolabelled proteins from these incubations following their separation by SDS-gel electrophoresis indicated that in the incubations containing Ca^{2+}, a marked increase occurred in the high molecular weight phosphorylated protein present in the top of the stacking gel. Small differences in the overall phosphorylation pattern obtained were observed between incubations containing Ca^{2+} and those in which Ca^{2+} was replaced with EGTA (data not shown). Whilst it is tempting to attribute differences in labelling pattern to the action of transglutaminase, it cannot be ruled out that phosphatase activity may account for at least some of the differences. In this respect the total amount of radiolabel found in gels obtained from incubations containing Ca^{2+} was generally 10–15% lower than that found in gels ob-

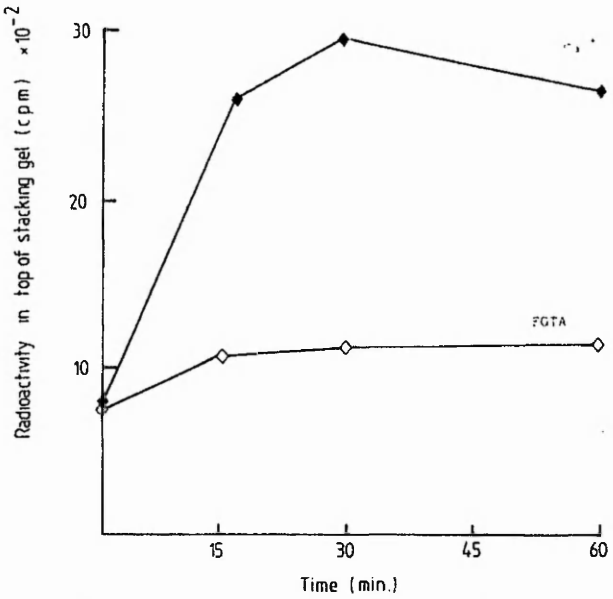


Fig. 3. The time-dependent incorporation of phosphorylated islet proteins into high molecular weight protein aggregates. Islet homogenates obtained from glucose stimulated intact islets previously incubated in the presence of [^{32}P]P_i were incubated at 37 °C in the presence of either 5 mM $CaCl_2$ or 5 mM EGTA for the times shown. The graph shows the amount of radioactivity present in the high molecular weight phosphopolymer at the top of the stacking gel at the different incubation times following electrophoretic separation of islet proteins. Data are the mean cpm obtained from two separate experiments each performed in duplicate.

tained from incubations containing EGTA. The formation of the Ca^{2+}-mediated phosphorylated polymer could also be inhibited by the addition of 1 mM iodoacetamide to the incubation mixtures (data not shown). Furthermore, when incubations were carried out in the presence of Ca^{2+} for different time periods (0–60 min), the Ca^{2+}-dependent formation of this polymer was found to be time dependent (Fig. 3). The formation of phosphorylated high molecular weight aggregates was therefore likely to be the result of transglutaminase-catalysed cross-linking of phosphorylated proteins of lower molecular weight than the aggregates.

Subcellular distribution of the high molecular weight phosphorylated polymer found in the glucose-stimulated islet

In order to identify which subcellular component contained the phosphopolymer in the glucose-stimulated intact islet, homogenates obtained from islets previously labelled with [^{32}P]P_i were fractionated by differential centrifugation prior to electrophoresis. In parallel experiments, the identity and purity of the fractions was assessed by measuring the levels of the markers 5′-nucleotidase, cytochrome-*c* oxidase, lactate dehydrogenase and insulin. Transglutaminase was also measured in each of the fractions. The results of this procedure are shown in Fig. 4. The distribution of transglutaminase activity paralleled that of the cytosolic enzyme lactate dehydrogenase, suggesting a cytosolic location for the enzyme. However, in contrast, the distribution of the phosphopolymer was predominantly (73%) found in the low-speed $600 \times g$ pellet and showed no direct parallelism to any of the membrane markers.

As a further means of demonstrating that the phosphopolymer was a high molecular weight phosphorylated protein and not protein associated with ^{32}P-labelled membrane phospholipids, the total membrane fraction obtained from ^{32}P-labelled islets (by centrifugation of homogenates at $80\,000 \times g$ av. for 45 min) was incubated with the phospholipases C and A_2 (5 U of each in the presence of 2 mM free Ca^{2+} and 100 μM Zn^{2+} for 15 min). Owing to the requirement of phospholipase A_2 for Ca^{2+} this incubation could not be performed with islet homogenates, since these conditions would also lead to the activation of the islet transglutaminase; in the membrane fraction only small amounts of enzyme activity could be detected (Fig. 3). Incubation of the total membrane fraction in this way led to a small but consistent 22% ($n = 4$) reduction in the amount of radiolabel found in the top of the stacking gel (data not shown), thus confirming that the phosphopolymer is in the main phosphorylated high molecular weight protein. The small amount of radiolabelled phospholipid which appears to be associated with the phosphopolymer does, however, suggest that it may be closely linked to membrane material.

In further experiments, homogenates incubated in the presence of either Ca^{2+} (5 mM) or EGTA (5 mM) for 30 min were also fractionated by differential centrifugation to investigate any possible changes that might occur in the subcellular distribution of the phosphopolymer following

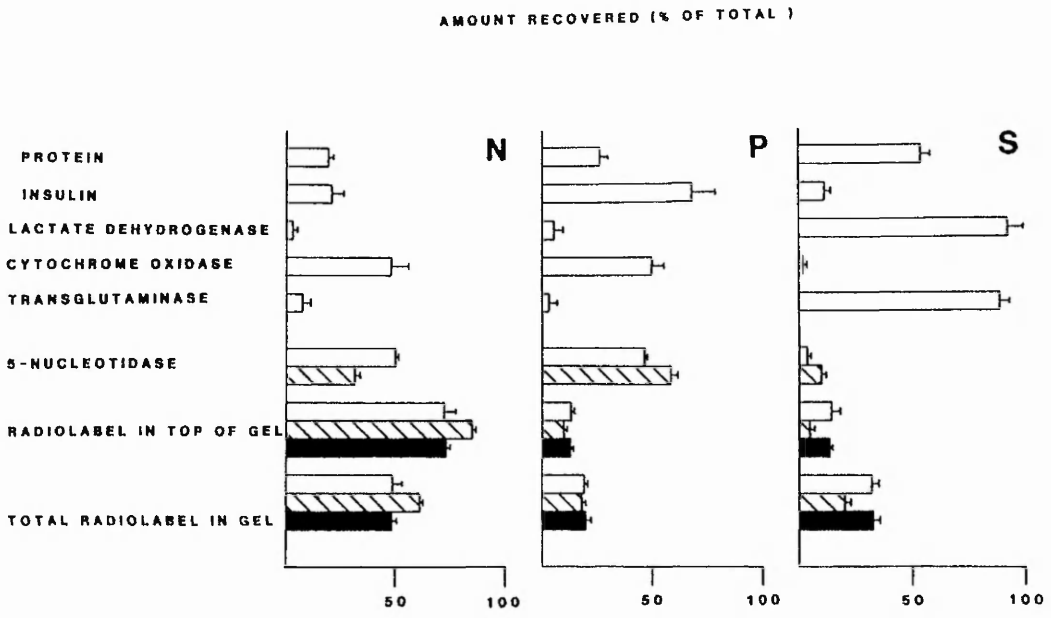


Fig. 4. Distribution of phosphopolymer, marker enzymes and transglutaminase in islet subcellular fractions. Intact islets previously incubated in the presence of [^{32}P]P_i and 16.8 mM glucose were homogenised and then fractionated by differential centrifugation into N,600 × *g* and P,80000 × *g* particulate fractions and S, particle-free supernatant. The blank bars of the histogram show the amount of transglutaminase activity, marker enzymes, phosphopolymer and total radiolabel present in each fraction. Data for each fraction are expressed as a percentage of the total value for all fractions, and are given as means ± S.E. from three separate experiments. The percentage distribution of 5′-nucleotidase ($n = 3 \pm$ S.E.), phosphopolymer and total radiolabel ($n = 4 \pm$ S.E.) is also shown in each of the fractions following prior incubation of islet homogenates for 30 min in the presence of either 5 mM Ca^{2+} (hatched bars) or 5 mM EGTA (solid bars).

activation of the islet transglutaminase. The identity and purity of fractions was again assessed by measuring the levels of the markers 5′-nucleotidase, cytochrome-*c* oxidase and lactate dehydrogenase in fractions obtained from Ca^{2+}-incubated homogenates. Apart from a small change in the subcellular distribution of 5′-nucleotidase (Fig. 4) the results indicated no significant changes in the distribution of the other markers used (data not shown). The distribution of the phosphopolymer obtained from homogenates incubated in the presence of EGTA was found to be comparable with the data obtained from islet homogenates which were fractionated without prior incubation, with 73% of the phosphopolymer present in the 600 × *g* av. pellet; 13% present in the high speed 80000 × *g* av. pellet; and 14% present in the cytosol fraction (Fig. 4). However, the distribution of the phosphopolymer obtained from homogenates incubated in the presence of Ca^{2+} indicated a statistically significant increase (12%, $P < 0.05$) in the amount of phosphopolymer found in the 600 × *g* pellet when compared to the EGTA incubated data; there was also a statistically significant reduction (9%, $P < 0.05$) in the amount of phosphopolymer found in the cytosol fraction. Similarly, when the percentage distribution of total radiolabel present in each of the subcellular fractions obtained from Ca^{2+}-incubated homogenates was compared to the EGTA-incubated homogenates, the data indicated an increase (12%, $P < 0.05$) in the percentage radiolabel found in the 600 × *g* av. pellet. In parallel with this increase, was a corresponding reduction in the amount of total radiolabel found in the cytosol fraction. Whilst it is once again tempting to attribute these differences in the distribution of radiolabel to the action of transglutaminase, it cannot be ruled out that phosphatase activity may account for part of the reduction seen in the amount of radiolabel present in the cytosolic proteins. However, the finding that an increase in the absolute amount of radiolabel occurs in the phosphopolymer (Fig. 3), when islet homogenates are incubated under conditions which activate trans-

glutaminase, and the fact that this increase occurs in the $600 \times g$ particulate fraction of the islet, without any marked change in the $80\,000 \times g$ particulate fraction, suggests that this translocation of phosphorylated proteins from the cytosol to the $600 \times g$ pellet is likely to be mediated through activation of the islet transglutaminase.

Inhibition of glucose-stimulated formation of phosphorylated polymer in intact islets by inhibitors of transglutaminase activity

To test directly the hypothesis that transglutaminase was involved in the formation of the phosphorylated protein polymer in glucose-stimulated islets, intact islets were incubated with monodansylcadaverine (MDCD) or glycine methyl ester, two competitive substrate inhibitors of transglutaminase activity [18,6] prior to electrophoretic analysis of phosphorylated islet proteins. In addition, control experiments were performed using the methylated analogues of the two compounds, namely *N*-dimethylmonodansylcadaverine (dimethylMDCD) and sarcosine methyl ester (SME) which lack the primary amine moiety essential for inhibition of transglutaminase activity. The results of these experiments are shown in Table I. MDCD (50 μM) and glycine methyl ester (10 mM) both inhibited the amount of phosphorylated material present at the top of the stacking gel by 25% and 37%, respectively. In contrast, the control compounds dimethylMDCD and sarcosine methyl ester did not significantly reduce the amount of phosphorylation in this component of the gel. When the total amount of phosphorylation present in the rest of the gel was taken into account, MDCD did not give rise to any significant inhibition of phosphorylation, whereas glycine methyl ester gave rise to a 57% stimulation. Our results therefore suggest that the reduction in the amount of phosphopolymer present in the stimulated islet is due to inhibition of its formation and not due to inhibition of islet protein phosphorylation. Comparable experiments undertaken to investigate the effects of MDCD and glycine methyl ester on glucose-stimulated insulin release indicated that MDCD (50 μM) and glycine methyl ester (10 mM) inhibited insulin release by 59% and 54% respectively. Previous evidence [5,6,19] has shown that the control compounds dimethylMDCD (100 μM) and sarcosine methyl ester (10 mM) have no significant effect on glucose-stimulated insulin release.

TABLE I

EFFECTS OF TRANSGLUTAMIANSE INHIBITORS AND CONTROL COMPOUNDS ON GLUCOSE-STIMULATED INSULIN RELEASE AND THE PROTEIN PHOSPHORYLATION PATTERN IN GLUCOSE-STIMULATED INTACT ISLETS

Incubation conditions were the same as those described in Fig. 1 except that test compounds were preincubated with islets for 45 min in Gey and Gey buffer containing 2.8 mM glucose prior to addition of [^{32}P]P_i and stimulatory levels of glucose (16.8 mM) after which islets were incubated for a further 90 min. Test compounds were kept at the concentration indicated throughout the incubation periods. The effects of test compounds on the phosphorylation pattern of islet proteins was carried out in paired experiments using the methodology described in Fig. 2 and the data are expressed as a percentage ($\pm$S.E.) of the fraction of radioactivity found incontrols. For insulin release, batches of 60 islets were preincubated with test compounds for 45 min in the presence of 2.8 mM glucose but in the absence of [^{32}P]P_i, followed by 90 min in 150 μl medium containing 16.8 mM glucose and test compound. The inhibition of glucose-stimulated insulin rleease for each test compound is expresed as a percentage ($\pm$S.E.) of the rate in control incubations, the number of experiments is shown in parenthesis, n.s. $P > 0.05$. Typical amounts of radioactivity found at the top of the stacking gel were 1099 ± 99 cpm and in the rest of the gel 5733 ± 447 cpm ($n = 8$). Typical rates of insulin release under the incubation conditions used were 3.4 ± 0.5 ($n = 9$) microunits per 90 min per islet for islets incubated in 2.8 mM glucose and 22.8 ± 2.9 microunits per 90 min per islet for islets incubated in 16.8 mM glucose.

Test compound	Concn. (mM)	Radioactivity at top of stacking gel (% fraction found in controls)	Radioactivity in rest of gel (% fraction found in controls)	Inhibition of glucose-stimulated insulin release (%)
Monodansylcadaverine	0.05	74.7 ± 3.9 (8) $P < 0.01$	91.8 ± 8.7 (8) n.s.	59.4 ± 2.4 (9) $P < 0.01$
N-Dimethylmonodansyl cadaverine	0.1	101 ± 6.0 (7) n.s.	117.7 ± 11.2 (7) n.s.	–
Glycinemethylester	10	62.4 ± 5.3 (10) $P < 0.01$	156.6 ± 15.5 (10) $P < 0.01$	53.7 ± 4.1 (9) $P < 0.01$
Sarcosinemethylester	10	122.6 ± 14.6 (4) n.s.	87.1 ± 6.9 (4) n.s.	–

Discussion

Previous work has suggested that during stimulus-secretion coupling in the pancreatic β-cell, islet transglutaminase may be activated by a rise in the cytosolic free Ca^{2+} concentration [6]. The result of activation of this enzyme may be post-translational modification of specific β-cell proteins which participate in the secretory mechanism. In the present investigation we have investigated the possibility that transglutaminase activity may be further modulated by a mechanism involving phosphorylation of its protein substrates. In islets labelled with [^{32}P]P_i under conditions of glucose-stimulation, we have demonstrated that a large proportion of the total incorporated ^{32}P radiolabel was present in a high molecular weight component which could not traverse 3% (w/v) acrylamide stacking gels during electrophoresis of islet proteins. The amount of radioactivity present in this component was not altered by digestion with RNAase or DNAase, but could be reduced by digestion with pronase, thus indicating that it was not radiolabelled nucleic acid or protein complexed to nucleic acid and, therefore, likely to be a protein phosphopolymer. This was further confirmed by phospholipase C and A_2 digestion of the particulate fraction of the islet which led to only a small reduction (approx. 20%) in the amount of phosphopolymer present in this fraction. Our electrophoretic studies using an agarose gel in addition to the 3% (w/v) acrylamide stacking gel indicated that this phosphopolymer is small enough to traverse the agarose gel and that the majority of it (80–90%) enters but does not traverse the 3% (w/v) acrylamide stacking gel. Although such studies only provide an approximate size for the phosphopolymer they do suggest that the phosphopolymer has a molecular weight in excess of 10^6. The fact that this phosphopolymer was found to contain the largest single peak of ^{32}P radioactivity in the whole gel profile further suggests that this component is important in the system of phosphorylation reactions in the stimulated islet.

We have previously suggested [16] that the cross-linking of membrane or membrane-associated proteins by the islet transglutaminase which can be demonstrated in vitro may represent the extreme of what may be a carefully regulated process in vivo. The present finding of a protein phosphopolymer in the intact islet also pointed to the intriguing possibility that this protein component was formed as a result of translutaminase-catalysed protein cross-linking activity in the intact islet during glucose-stimulated insulin release. Therefore, we carried out further experiments to determine the relationship between islet transglutaminase activity and the formation of the phosphopolymer. Firstly, incubation of ^{32}P-labelled islet homogenates under conditions of transglutaminase activation increased the amount of ^{32}P radiolabel recovered in the phosphopolymer. This apparent translocation of phosphorylated proteins to the phosphopolymer was inhibited by EGTA or iodoacetamide, suggesting that transglutaminase-catalysed cross-linking was responsible for this process. Therefore, islet transglutaminase was indeed capable of catalysing the formation of the phosphopolymer. Secondly, incubation of intact islets with specific inhibitors of transglutaminase activity [6,19] inhibited the formation of the endogenous phosphopolymer and did so at concentrations which gave rise to inhibition of glucose-stimulated insulin release. Of particular importance to the suggestion that transglutaminase activity is responsible for the formation of this phosphopolymer is the finding that the *N*-substituted analogues of glycine methyl ester and MDCD which acted as control compounds in these studies did not inhibit the formation of the phosphopolymer. Our results therefore indicate the importance of transglutaminase activity in polymer formation.

Preliminary attempts to characterise the subcellular niche of the islet phosphopolymer using differential centrifugation indicated that the phosphopolymer did not show any direct parallelism to the fractionation of either the plasma membrane marker 5′-nucleotidase or insulin. The majority of phosphopolymer (73%) was associated with the $600 \times g$ particulate fraction, a fraction which in addition to containing islet nuclei [12] contains approx. 50% of the islet plasma membrane and mitochondria. Although it is difficult at this stage to ascribe any specific subcellular niche to this phosphopolymer, its association with the islet plasma membrane or underlying infrastructures in

the 600 × *g* particulate fraction may be suggested by a number of factors. There is now considerable evidence to suggest that the expression of transglutaminase activity is concerned with cell membrane-mediated events [20–24] and not those concerned with either the cell nucleus or mitochondria. Further, the presence of plasma membrane fragments in this low-speed 600 × *g* av. fraction would tend to indicate that they are of greater density or size than those found in the high-speed 80 000 × *g* fraction. The association of cross-linked cytoskeletal or microtubular elements (membrane infrastructures) with the plasma membrane would facilitate such a change in the biophysical character of the membrane. Evidence to support this comes from studies on the Ca^{2+}-loaded intact human erythrocyte [25], whereby transglutaminase induced cross-linking of membrane and cytoskeletal proteins of the erythrocyte leads to an increase in membrane density. A further interesting analogy of our findings in the islet to those in the red blood cell is that the preferred cytoskeletal substrates of the red blood cell enzyme, band 4.1 (synapsin), spectrin (fodrin) and band 2.1 (ankyrin) are also substrates for protein kinases [26]. Evidence to indicate that the islet transglutaminase is capable of catalysing in vitro the formation of phosphopolymer of similar nature to that found in the intact islet was also suggested by these fractionation studies. Fractionation of radiolabelled islet homogenates following incubation with Ca^{2+} indicated an increase in the amount of phosphopolymer which is associated with the 600 × *g* particulate fraction. Concomitant with this increase was a decrease in the percentage total radiolabel and radiolabelled phosphopolymer associated with the cytosol fraction. Although we cannot rule out that part of the reduction seen in the cytosolic fraction is due to the action of phosphatases our data do indicate that cytosolic proteins may be coupled to particulate proteins by action of the islet transglutaminase. Such a mechanism is quite possible, considering the apparently soluble nature of the islet transglutaminase itself which we demonstrated in this study.

We have therefore provided evidence for the existence of transglutaminase-catalysed protein cross-linking in intact islet cells during glucose-stimulated insulin release. Combined with the evidence from inhibitor studies on glucose-stimulated insulin release, these results point to the participation of transglutaminase-catalysed cross-linking of phosphorylated proteins in the process of stimulus-secretion coupling. Furthermore, our observations imply that post-translational modifications catalysed by transglutaminase and protein kinases may be closely coordinated in the β-cell. Such coordination may represent a means of modulating transglutaminase activity by substrate availability in addition to direct control by cytosolic Ca^{2+} as previously suggested [27,6].

The possibility that phosphorylation reactions may be coupled to the formation of ε(γ-glutamyl)lysine cross-links in cellular systems has also been proposed by Loewy and Matacic [28]. These authors using studies performed in vitro demonstrated that the ε(γ-glutamyl)lysine cross-link content of a variety of cell types could be modulated by ATP and ATP plus Ca^{2+}. They suggested that protein crosslinking through a transglutaminase-like enzyme may occur through prior phosphorylation of proteins at glutamate residues, the reaction proceeding via an activated acyl intermediate. Such a reaction mechanism where energy transfer accompanies alteration of protein structure may be applicable to regulation of the distal events of the secretory mechanism. In keeping with our own studies, was their finding that these cross-links were present in high molecular weight proteins associated with both cytoskeletal and membrane components [29]. Furthermore, a functional relationship of the microfilament/microtubule system to the mechanism of cell secretion both in islets and other cells is now well documented [39,31].

Whilst the present work points to the importance of co-ordinated transglutaminase-catalysed cross-linking and phosphorylation of islet proteins, further work is required to identify the precise relationship of these two mechanisms of post-translational modification.

Acknowledgements

This work was funded by the Sir Halley Stewart Trust and the Wellcome Trust to whom we are extremely grateful.

References

1 Hedeskov, C.J. (1980) Physiol. Rev. 60, 442–509.
2 Wollheim, C.B. and Sharp, G.W.G. (1981) Physiol. Rev. 61, 914–973.
3 Folk, J.E. and Finlayson, J.S. (1977) Adv. Protein Chem. 31, 1–133.
4 Lorand, L. and Conrad, S.M. (1984) Mol. Cell. Biochem. 58, 9–35.
5 Bungay, P.J., Potter, J.M. and Griffin, M. (1984) Biochem. J. 219, 819–827.
6 Bungay, P.J., Owen, R.A., Coutts, I.C. and Griffin, M. (1986) Biochem. J. 235, 269–278.
7 Gomis, R., Sener, A., Malaisse-Lagae, F. and Malaisse, W.J. (1983) Biochim. Biophys. Acta 760, 384–388.
8 Harrison, D.E., Ashcroft, S.J.H., Christie, M.R. and Lord, J.E. (1984) Experientia 40, 1075–1084.
9 Lacy, P.E. and Kostianovsky, M. (1967) Diabetes 16, 35–39.
10 Gey, G.O. and Gey, M.K. (1936) Am. J. Cancer 27, 45–76.
11 Heding, L. (1972) Diabetologia 8, 260–266.
12 Christie, M.R. and Ashcroft, S.J.H. (1985) Biochem. J. 227, 727–736.
13 Cooperstein, S.J. and Lazarow, A. (1951) J. Biol. Chem. 189, 665–670.
14 Lowry, D.H., Rosebrough, N.J., Fass, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
15 Laemmli, V.K. (1970) Nature (London) 227, 680–685.
16 Griffin, M., Wilson, J. (1984) Mol. Cell. Biochem. 58, 37–49.
17 Christie, M.R. and Ashcroft, S.J.H. (1984) Biochem. J. 218, 87–99.
18 Bersten, A.M., Ahkong, Q.F., Hallinan, T., Nelson, S.J. and Lucy, J.A. (1983) Biochim. Biophys. Acta 762, 429–436.
19 Sener, A., Dunlop, M.E., Gomis, R., Mathias, P.C.F., Malaisse-Lage, F. and Malaisse, W.J. (1985) Endocrinology 117, 237–242.
20 Davies, P.J.A., Davies, D.R., Levitzki, A., Maxfield, R.R., Milhaud, P., Willingham, M.C. and Paston, I. (1980) Nature (London) 283, 162–167.
21 Fesus, L., Sandor, M., Horvarth, L.I., Bagymka, C., Erede, A. and Gergely, J. (1981) J. Mol. Immunol. 18, 633–638.
22 Griffin, M., Swanson, P.E., Bjerrum, O.J. and Lorand, L. (1980) Biophys. J. 33, 1771.
23 Maccioni, R.B. and Arechaga, J. (1986) Exp. Cell Res. 167, 266–270.
24 Tyrrell, D.J., Sale, W.S. and Slife, C.W. (1986) J. Biol. Chem. 261, 14833–14836.
25 Bjerrum, O.J., Hawkins, M., Swanson, P., Griffin, M. and Lorand, L. (1981) J. Supramol. Struct. 16, 289–301.
26 Home, W.C., Leto, T.L. and Marchesi, V.T. (1985) J. Biol. Chem. 260, 9073–9076.
27 Hand, D., Bungay, P.J., Elliott, B.M. and Griffin, M. (1985) Biosci. Rep. 5, 1079–1086.
28 Loewy, A.G. and Matacic, S.S. (1981) Biochim. Biophys. Acta 668, 167–176.
29 Loewy, A.G. and Matacic, S.S. (1981) Biochim. Biophys. Acta 668, 177–185.
30 Howell, S.L. and Tyhurst, M. (1984) Experientia 40, 1098–1104.
31 Perrin, D., Langly, K. and Aunis, D. (1987) Nature 326, 498–501.

Calmodulin antagonists of improved specificity and potency

SHEILA MACNEIL,* REBECCA A. DAWSON,* MARTIN GRIFFIN,† ROBIN OWEN,† A. MELVIN COOKE,‡ NANCY J. PETTETT‡ and G. MICHAEL BLACKBURN‡

**Department of medicine Clinical Sciences Centre, Northern General Hospital, Sheffield S5 7AU, U.K., †Department of Life Science, Trent Polytechnic, Clifton Lane, Nottingham NG11 8NS, U.K., and ‡Department of Chemistry, Sheffield University, Brook Hill, Sheffield S10 2TP, U.K.*

In the investigation of Ca^{2+}/calmodulin-mediated intracellular processes, a variety of drugs have been employed to inhibit calmodulin. However, it is recognized that many such agents (e.g. the phenothiazines) inhibit other non-calmodulin-dependent processes at only slightly higher concentrations. The best documented of the drugs currently available is *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7). A structure–activity study of napthalenesulphonamide derivatives by Hidaka *et al.* (1981) reported the affinity for calmodulin and potency as a calmodulin antagonist was dependent on the chlorination of position-5 of the naphthalene ring and the length of the hydrocarbon chain.

More recently Ito & Hidaka (1983*a*,*b*) have reported that W7, given either intraperitoneally or by ingestion to mice bearing tumours, is capable of significantly retarding solid tumour growth. This effect of W7 *in vivo* is obviously of great interest but unfortunately W7 also fails to be entirely specific for calmodulin: its IC_{50} against calmodulin-dependent phosphodiesterase is 25 μM and its IC_{50} against Ca^{2+}-dependent calmodulin-independent protein kinase C is reported to be of the order of 100 μM (Wise *et al.*, 1982) to 138 μM (Hidaka & Tanaka 1983).

In the current investigation we sought to extend the structure–activity study of Hidaka *et al.* (1981) in order to improve if possible both the calmodulin inhibitory potency of naphthalenesulphonamide derivatives and their specificity as estimated by their inhibition of two Ca^{2+}-dependent, calmodulin-independent enzymes, rat spleen protein kinase C and rat pancreatic islet transglutaminase.

Fourteen derivatives of W7 were synthesized differing from W7 in three respects, the halogen present on position-5 of the naphthalene ring, the position of the hydrocarbon chain and the length and composition of the hydrocarbon chain. The results, in terms of the ability of these drugs to inhibit Ca^{2+}/calmodulin-dependent beef heart phosphodiesterase activity (methodology as previously described, MacNeil *et al.*, 1984), are shown in Table 1.

From this study we conclude that iodine can effectively substitute for chlorine on position-5 of the naphthalene ring (CN and CF_3 are less effective than chlorine), that the sulphonamide substituent on position-2 can be almost as effective as on position-1, and that an optimum carbon chain length of C = 8–9 exists. (Additional carbons markedly decrease the water-solubility of the drug and slightly decrease its inhibitory potency also.)

Based on this series we find the 5-iodo-C_8 W7 analogue to be the most potent against calmodulin-dependent phosphodiesterase. Having improved the potency of W7 by a factor of almost 10-fold we examined the effect of this 'optimization' on the specificity of the drug. In our experiments the parent drug W7 had an IC_{50} of 29 μM against Ca^{2+} calmodulin-dependent phosphodiesterase, approximately 250 μM against Ca^{2+}-dependent protein kinase C activity (methodology adapted from Lord & Ashcroft, 1984) and 398 μM against Ca^{2+}-dependent transglutaminase (methodology as previously reported: Bungay *et al.*, 1984). This W7 analogue, 5-iodo-C_8, in contrast had an IC_{50} of 3 μM against kinase C and > 1000 μM against transglutaminase.

We conclude that optimization of the structure of the naphthalene sulphonamides for calmodulin-inhibitory potency has also resulted in an improvement in the specificity of drug action. We are currently investigating the use of this 5-iodo-C_8 W7 analogue in living cells.

We thank the Nuffield Foundation, The Yorkshire Cancer Research Campaign and the Sir Halley Trust for financial support for this work.

Abbreviation used: W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphanamide

Bungay, P. J., Potter, J. M. & Griffin, M. (1984) *Biochem. J.* **219**, 819–822

Hidaka, H. & Tanaka, T. (1983) *Methods Enzymol.* **102**, 185–194

Table 1. *Specifically synthesized W7 analogues*

Halogen at position-5	Position of side chain	Carbon length of side chain	Inhibition of phosphodiesterase activation by calmodulin (IC_{50}, μM)		
Cl	1	6	29 ± 2	(4)	5-Chloro-C_6 (W7)
I	1	4	503 ± 46	(3)	
I	1	6	29 ± 6	(6)	
I	1	7	6.5 ± 0.5	(2)	
I	1	8	3 ± 0.6	(4)	5-Iodo-C_8
I	1	9	3 ± 0.4	(3)	
I	1	10	5.3 ± 0.3	(2)	
I	1	12	11.2 ± 4.7	(2)	
I	2	8	7.7 ± 1.2	(2)	
CF_3	1	6	100 ± 10	(2)	
CN	1	8	17 ± 2	(2)	
CN	1	9	4.1 ± 1.9	(2)	
CN	1	10	8.5 ± 1.5	(2)	
I	1	2-0-2	26 ± 7	(4)	
Cl	1	2-0-2	116 ± 21	(4)	

Hidaka, H., Asano, M. & Tanaka, T. (1981) *Mol. Pharmacol.* **20**, 571–578
Ito, H. & Hidaka, H. (1983*a*) *Cancer Lett.* **19**, 215–220
Ito, H. Hidaka, H. (1983*b*) *Eur. J. Cancer Clin. Oncol.* **19**, 1183–1184
Lord, J. M. & Ashcroft, S. J. H. (1984) *Biochem. J.* **219**, 547–551
MacNeil, S., Walker, S. W., Senior, H. J., Bleehen, S. S. & Tomlinson, S. (1984) *J. Invest. Dermatol.* **83**, 15–19
Wise, B. C., Glass, D. B., Jenchou, C. H., Raynor, R. L., Katch, N., Schatzman, R. C., Turner, R. S., Kibler, R. F. & Kuo, J. F. (1982) *J. Biol. Chem.* **25**, 8489–8495

Biochemical Pharmacology, Vol. 37, No. 9, pp. 1717–1723, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
© 1988. Pergamon Press plc

CALMODULIN ANTAGONISTS OF IMPROVED POTENCY AND SPECIFICITY FOR USE IN THE STUDY OF CALMODULIN BIOCHEMISTRY

SHEILA MACNEIL,* MARTIN GRIFFIN,† A. MELVIN COOKE,‡ NANCY J. PETTETT,‡ REBECCA A. DAWSON,* ROBIN OWEN† and G. MICHAEL BLACKBURN‡

*Department of Medicine, Clinical Sciences Centre, Northern General Hospital, Sheffield S5 7AU,
†Department of Life Sciences, Trent Polytechnic, Clifton Lane, Nottingham, NG11 8NS, and
‡Department of Chemistry, The University, Sheffield S3 7HF, U.K.

(*Received* 26 *August* 1987; *accepted* 11 *November* 1987)

Abstract—Syntheses are described for a range of *N*-(ω-aminoalkyl)-5-iodo- and -5-cyanonaphthalene-1-sulphonamides. The selective activity of these compounds as inhibitors for calmodulin-dependent phosphodiesterase (EC 3.1.4.17) is compared with their activity for the calmodulin-independent but calcium-dependent enzymes protein kinase C and transglutaminase (EC 2.3.2.13). The results show a drastic improvement in the selectivity of effect for the 5-iodo-compounds compared with the widely-used drug, W7, *N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide.

Calmodulin mediates many of the diverse intracellular actions of calcium and roles for it have been suggested in cyclic nucleotide metabolism, hormone secretion, cell motility and contractility and cell division [1]. A range of chemically dissimilar drugs [2] will inhibit calmodulin activity and as a consequence have been employed as probes in the study of calmodulin-dependent processes in cells. However, many such drugs, for example the phenothiazines commonly used for this purpose [2], the substituted naphthalenesulphonamides, such as W7 [3], and others, such as R24571, "calmidazolium" which are particularly potent [4], will inhibit other non-calmodulin-dependent processes at concentrations only slightly higher than those required for calmodulin inhibition. In view of this, other important calcium-dependent but calmodulin-independent enzyme activities must also be considered in the current picture of intracellular regulation when using such probes. In particular, the calmodulin-independent but calcium-dependent enzyme protein kinase C, which is thought to be fundamental to many intracellular processes [5], is inhibited by a number of calmodulin antagonists over the concentration range used in studies involving intact cells [4]. For example, the most potent of the commonly available substituted naphthalenesulphonamides *N*-(4-aminohexyl)-5-chloronaphthalene-1-sulphonamide (W7) has been reported to have only a factor of five separating the potency of the drug against calmodulin-dependent phosphodiesterase activity from that against protein kinase C [6]. Similarly, the calcium-activated enzyme transglutaminase thought to be important in cellular processes, such as receptor mediated endocytosis [7] and stimulus secretion coupling [8, 9], is also reported to be inhibited by W7 [10] at concentrations similar to those required to bring about calmodulin antagonism.

Accordingly, the aim of this study was to develop calmodulin antagonists of improved specificity for use as pharmacological tools in the study of calmodulin biochemistry and also for evaluation as potential carcinostatic/antiproliferative agents. The approach we took was to extend the well-documented structure activity studies of Hidaka *et al.* [3] based on substituted naphthalenesulphonamides.

We report here that improvement in the potency of such drugs as calmodulin antagonists can be achieved with a very significant improvement in specificity.

MATERIALS AND METHODS

Materials

5-Aminonaphthalene-1-sulphonic acid, α,ω-diamines and *N*-dansylcadaverine were purchased from Fluka A.G. Chemische Fabrik and other chemicals from Aldrich Chemical Co. Ltd. Cyclic [^{3}H] AMP, [α^{32}P] ATPand [1,4-^{14}C]Putrescine were purchased from Amersham International plc. Calmodulin-dependent beef heart phosphodiesterase was obtained from Boehringer Mannheim Co. All other biochemicals were obtained from Sigma Chemical Co.

Methods

Confirmation of chemical structure. The purity and structure of each of the synthesised compounds was confirmed by determination of melting points and spectroscopic analysis using NMR and mass spectroscopy.

Proton NMR spectra were recorded using a Perkin-Elmer R34 instrument at 220 MHz with TMS as internal reference. ^{19}F NMR spectra were recorded at 75.2 MHz on a Bruker WP80 SY machine with $CFCl_3$ as external reference. Low resolution mass spectra were obtained with a Kratos MS25 machine and infrared spectra were recorded by Nujol mulls on a Perkin-Elmer 157G or a 457G instrument. Melting points were determined with a Kofler hot stage microscope and are otherwise uncorrected.

Synthesis of compounds

2,4,6-Isopropylbenzenesulphonamide was prepared as previously described [11].

5-Cyanonaphthalene-1-sulphonyl chloride was prepared from 5-amino-naphthalene-1-sulphonic acid as previously described [11], sodium 5-cyanonaphthalene-1-sulphonate being converted into the chloride by treatment with phosphoryl chloride to give brownish crystals from glacial acetic acid m.p. 144.5° (lit. [11] m.p. 140.3°).

5-Chloronaphthalene-1-sulphonyl chloride was prepared as previously described [12, 13] as dark brown crystals from glacial acetic acid, m.p. 940 (lit. [14] m.p. 95°).

5-Iodonaphthalene-1-sulphonyl chloride was prepared from 5-iodonaphthalene-1-sulphonic acid by reacting its anhydrous sodium salt with phosphoryl chloride at 80°. The product was crystallised from glacial acetic acid as yellow crystals, m.p. 115° (lit. [15] 114°. (Found: C, 34.1; H, 1.75; Cl, 9.65; I, 35.05; S, 9.8. Calc. for $C_{10}H_6ClIO_2S$: C, 34.07; H, 1.72; Cl, 10.06; I, 36.00; S, 9.10%).

5-Iodonaphthalene-2-sulphonyl chloride was prepared from sodium 5-iodonaphthalene-2-sulphonate as above, m.p. 97.9° (lit. [15] m.p. 102°).

1-Trifluoromethylnaphthalene was obtained from 1-iodonaphthalene by treatment with copper and iodotrifluoromethane in pyridine [16] as a pale yellow oil bp_4 70.2°. (Found: C, 67.3; H, 3.55. Calc. for $C_{11}H_7F_3$: C, 67.35; H, 3.60%).

5-Trifluoromethylnaphthalene-1-sulphonyl chloride was prepared by chlorosulphonylation [17] of 1-trifluoromethylnaphthalene [16] and the crude product crystallised from glacial acetic acid as cream coloured prisms m.p. 192.4°. (Found: C, 44.5; H, 2.55; Cl, 12.05; S, 11.05. Calc. for $C_{11}H_6ClF_3O_2S$: C, 44.97; H, 2.06; Cl, 11.76; S, 10.91%).

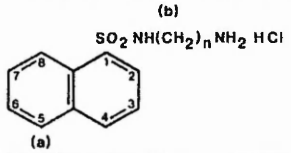
3-Trifluoromethylbenzenesulphonyl chloride was prepared [18] by the chlorosulphonylation of benzotrifluoride to give an oil, bp_{12} 103.8°. The product was further characterised by treatment with liquid ammonia to give 3-trifluoromethylbenzenesulphonamide m.p. 106.8° (lit. [19] m.p. 111.3°. (Found: C, 37.75; H, 2.8; N, 6.05; S, 14.45. Calc. for $C_7H_6F_3NO_2S$: C, 37.34; H, 2.69; N, 6.22; S, 14.24%).

General preparation of N-(ω-aminoalkyl)-arenesulphonamides. The ω-diaminoalkane (60 mmol) was dried by azeotropic distillation with benzene and dissolved in dry dioxane (80 ml). A solution of the arenesulphonyl chloride (10 mmol) in dry dioxane (40 ml) was added with vigorous stirring during 30 min at 10° then, after 1 hr, the solution was warmed to 80° for 30 min, cooled, and evaporated *in vacuo*. After removal of excess amine and salts by trituration with water, the solid residue was extracted with hot methanol (200 ml) and the solution acidified to pH 2 with HCl and evaporated to give the crude product which was crystallised from ethanol or aqueous methanol [20].

N-(t-butyl)-5-iodonaphthalene-1-sulphonamide. A solution of 5-iodonaphthalene-1-sulphonyl chloride (6.6 g, 20 mmol) in dichloromethane (80 ml) was added dropwise to a stirred solution of *t*-butylamine (2.72 g, 40 mmol) in the same solvent (50 ml) at 0°. After 48 hr the solution was evaporated *in vacuo* and the residue extracted with ether. The extract was washed with dilute HCl, water, dried and evaporated. The product was crystallised from toluene : hexane (1 : 1, v/v) as yellow needles (5.0 g, 68%) m.p. 238–241°. (Found: C, 42.9; H, 4.3; I, 32.4; N, 3.6; S, 8.2. $C_{14}H_{16}INO_2S$ requires C, 43.17; H, 4.44; I, 32.64; N, 3.60; S, 8.22%).

Calcium/calmodulin-dependent phosphodiesterase

Table 1. The effect of modifying (a) the choice of substituent at C_5 and (b) the composition and position of the sulphonamide side chain on calmodulin antagonist activity.



Compound	Name	(a)	(b)	IC_{50} $\bar{x} \pm$ SEM (N) (μM)
a	5-chloro-1-C_6 (W7)	Cl	$SO_2NH(CH_2)_6NH_2$	29 + 4(4)
b	5-iodo-1-C_4	I	$SO_2NH(CH_2)_4NH_2$	8 + 2(3)
c	5-iodo-1-C_6	I	$SO_2NH(CH_2)_6NH_2$	29 + 6(6)
d	5-iodo-1-C_7	I	$SO_2NH(CH_2)_7NH_2$	7 + 0.6(3)
e	5-iodo-1-C_8	I	$SO_2NH(CH_2)_8NH_2$	3 + 0.6(4)
f	5-iodo-2-C_8	I	$SO_2NH(CH_2)_8NH_2$	4 + 1.4(5)
g	5-iodo-1-C_9	I	$SO_2NH(CH_2)_9NH_2$	3 + 0.4(3)
h	5-iodo-1-C_{10}	I	$SO_2NH(CH_2)_{10}NH_2$	4 + 0.9(3)
i	5-iodo-1-C_{12}	I	$SO_2NH(CH_2)_{12}NH_2$	0.7 + 0.1(3)
j	5-CF_3-1-C_6	CF_3	$SO_2NH(CH_2)_6NH_2$	100 + 10(3)
k	5-cyano-1-C_6	CN	$SO_2NH(CH_2)_6NH_2$	59 + 10(5)
l	5-cyano-1-C_8	CN	$SO_2NH(CH_2)_8NH_2$	2 + 0.1(3)
m	5-cyano-1-C_9	CN	$SO_2NH(CH_2)_9NH_2$	3 + 0.7(4)
n	5-cyano-1-C_{10}	CN	$SO_2NH(CH_2)_{10}NH_2$	5 + 1.6(5)
o	5-iodo-1$^+$	I	$SO_2NHC(CH_3)_3$	20 + 2(3)
p	3-CF_3-1-C_6^{++}	CF_3	$SO_2NH(CH_2)_6NH_2$	250 + 62(3)

Compounds o and p differ from a–n above in that o^+ is a *t*-butyl derivative of b, p^{++} is 3-trifluoromethyl-benzene-sulphonamide, prepared as a molecule having a similar spatial relationship between the halogen and the sulphonamide group as that in W7 (see Table 2 for structure).

assay. The calmodulin inhibitory potency of the compounds prepared (Table 1) was determined with commercially-available (Boehringer-Mannheim) beef heart cyclic nucleotide phosphodiesterase (EC 3.1.4.17) using the methodology previously described [21]. Assays were performed in triplicate. Each drug was examined in not less than three separate assays.

Calcium-dependent transglutaminase assay. Particle free supernatant from extracts of rat liver [18] was used as a source of transglutaminase. Transglutaminase activity was measured using the procedure previously described [8]. Assays were performed in triplicate.

Calcium-dependent protein kinase C assay. The potency of compounds as inhibitors of protein kinase C was determined by measuring protein kinase C activity in extracts prepared from rat spleen. Rat spleen was homogenised in 3 vol. of Tris/HCl (20 mM, pH 7.4), EGTA (2 mM), 2-mercaptoethanol (50 mM) with a Potter-Elvehjem homogeniser. The homogenate was centrifuged at 71,000 *g* for 45 min at 4° and the supernatant retained. This preparation was diluted 1:10 with Tris/HCl (20 mM, pH 7.4) for use in the protein kinase C assays. The standard reaction mixture (100 μl) contained the following components: Tris/HCl (20 mM, pH 7.4), Mg $(OAc)_2$ (5 mM), histone H1 (200 μg ml^{-1}). [γ-^{32}P] ATP (10 μM, 0.6 Ci mmol^{-1}), $CaCl_2$ (98 μM), EGTA (48 μM), 2-mercaptoethanol (1.2 mM), phosphatidylserine (16 μg ml^{-1}), 1,2-diolein (2.4 μg ml^{-1}), and the appropriate concentration of test compound. Reactions were started by the addition of spleen extract (24 μl, approx 25 μg protein). The incorporation of ^{32}P from [γ-^{32}P] ATP into histone H1 was measured by the modified procedure of Lord and Ashcroft [22] whereby phosphorylated proteins were recovered by precipitation with trichloroacetic acid (5% w/v) containing osmium tungstate (0.25% w/v). Protein kinase C activity was calculated by subtracting the phosphorylation obtained in the presence of Ca^{2+} alone from that obtained in the presence of Ca^{2+}, 1,2-diolein, and phosphatidylserine over a period of 2 min incubation at 37°. Addition of phospholipid and diolein stimulated enzyme activity by approx 60%. Under these conditions, the incorporation of ^{32}P was linear with time, and the standard inhibitor of protein kinase C, trifluoperazine [6] was found to give 50% inhibition of enzyme activity at a concentration of 74 μM. Assays were performed in triplicate.

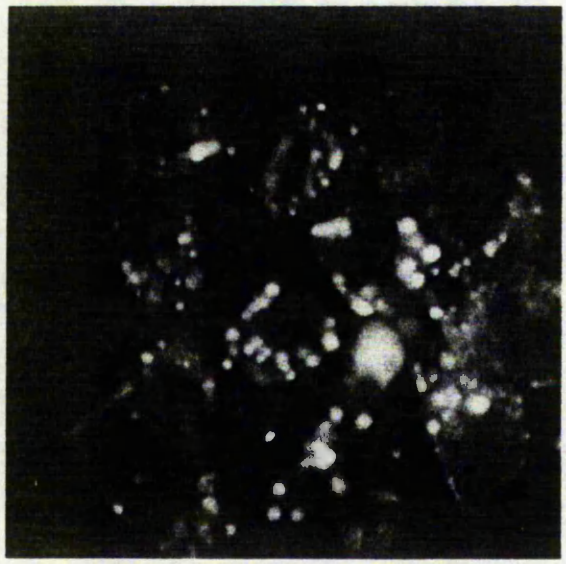


Fig. 1. Fluorescence of 5-iodo-1-C_8 within living cells. Human leukaemic (K562) lymphocytes were exposed to 100 μM 5-iodo-1-C_8 in phosphate-buffered saline. The picture, taken at 20 min, shows the maximum fluorescence obtained which developed over 15–20 min.

Addition of test compounds to enzyme assays. Many of the naphthalenesulphonamides were poorly water soluble and dimethylsulphoxide, DMSO, was used to dissolve these drugs. Appropriate dilutions of DMSO (up to 1% final) were added to all assays where such drugs were present. That DMSO itself did not affect the calmodulin-inhibitory potency of the drugs was determined from a comparison of some of the more soluble drugs which were assayed in the presence and in the absence of DMSO.

Fluorescence microscopy. Compounds were examined for fluorescence inside cultured cells (human K562 leukaemic lymphocytes) using an Orthomat microscope camera on an Orthoplan microscope (Ernst Leitz GMBA Wetzlar, F.R.G.). Cells were washed free of medium, resuspended in phosphate-buffered saline and compounds added to this suspension. A UV filter system and Fujichrome 400 ASA film were used to record this fluorescence as illustrated in Fig. 1.

RESULTS AND DISCUSSION

Physical nature of compounds synthesized

Fifteen naphthalenesulphonamides were prepared (see Table 1) in addition to W7. They were designed to explore three structural variations on *N*-(6-aminohexyl-5-chloronaphthalene-1-sulphonamide, W7. First, the choice of substituent at C-5, restricted by Hidaka to the 5-chloro- and 5-bromo-compounds, was expanded to include 5-iodo, 5-cyano, and 5-trifluoromethyl groups. Secondly, the length of the side chain was varied from four to twelve carbon atoms. Thirdly, the sulphonamide function was moved from the 1- to the 2-position of naphthalene to allow evaluation of any isomeric advantage. The 3-trifluoromethylbenzenesulphonamide was prepared as a molecule having a similar spatial relationship between the halogen function and the sulphonamide group as that in W7 while the *t*-butyl derivative was prepared for evaluation as a possibly inactive control compound.

The water solubility of the drugs was found to decrease with increasing length of the side chain (for the iodo- and chloro- series of compounds) and also to decrease in the series 5-chloro > 5-iodo > 5-cyanonaphthalenesulphonamides.

Experience with the crystallisation of the drug hydrochlorides indicated that there is a general lowering of alcohol solubility for the odd-numbered side chains compared to their even-numbered neighbours. The diminished water solubility of the longer chain compounds was circumvented for enzyme assay purposes by the use of DMSO (up to 1% final in assay) as co-solvent but this problem may possibly limit their potential as drugs for use *in vivo*.

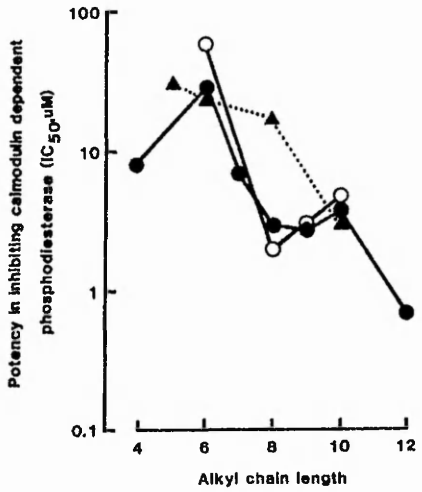


Fig. 2. The variation in potency of compounds as inhibitors of calmodulin-dependent phosphodiesterase as a function of the length of the alkyl chain: ●, 5-iodo-compounds; ○, 5-cyano-compounds; and – – ▲ – –, 5-chloro-compounds. Data for 5-chloro compounds were taken from Hidaka *et al.* [3].

Finally, it should be mentioned that all of the 5-iodonaphthalene-1-sulphonamides proved to have a marked fluorescence. This was found to show maximum emission at 345 nm and maximum excitation at 293 nm in water and is visible to the naked eye as a yellow fluorescence in daylight. This emission is, if anything, enhanced when such compounds enter cells (Fig. 1). Entry of 5-iodo-1-C_8 into cells (which had barely detectable autofluorescence) occurred within a few minutes as observed under a fluorescence microscope. Maximum fluorescence was obtained within 15–20 min and was at least 10 times greater than that attributable to the present compound W7. Figure 1 shows the fluorescence obtained after exposure of K562 human leukaemic lymphocytes to 100 μM 5-iodo-1-C_8 for 20 min. The non-fluorescent intracellular areas correspond to the position and size of the nuclei for these cells. The densely staining structures which appear to be located at or near the surface membrane have not yet been identified. This degree of fluorescence may prove to be useful for the future analysis of the location of calmodulin–drug interactions within living cells.

Ability of naphthalenesulphonamides to inhibit calmodulin. The calmodulin antagonist potency of these compounds was determined by their ability to inhibit calmodulin-dependent beef heart phosphodiesterase activity, which is one of the best established calmodulin-dependent enzyme processes [2]. The results are shown in Table 1. From these data, it is evident that the 5-iodo- and 5-cyano-substituents (c, k) can effectively replace the chlorine atom at C-5 in W7 (a) but that the trifluoromethyl group (j) is rather less active. There is no apparent inprovement in potency for the 5-iodonaphthalene-2-sulphonamide (f) relative to the 1,5-isomer (e)—a finding that contrasts with a report to the contrary for the 5-chloro-series of drugs [3]. Most significantly, the variation in calmodulin inhibition with variation in chain length (Fig. 2) (compounds b–i and k–n) is not as simple as that reported by Hidaka [3] for a limited number of compounds.

In particular, there seems to be a minimum activity for the C-6 chain and a sudden, fivefold increase in activity in going from C-10 to C-12 (h–i) which deserves to be further explored. However, this gain in activity with increasing chain length for the 5-iodo-series has to be offset against the diminished water

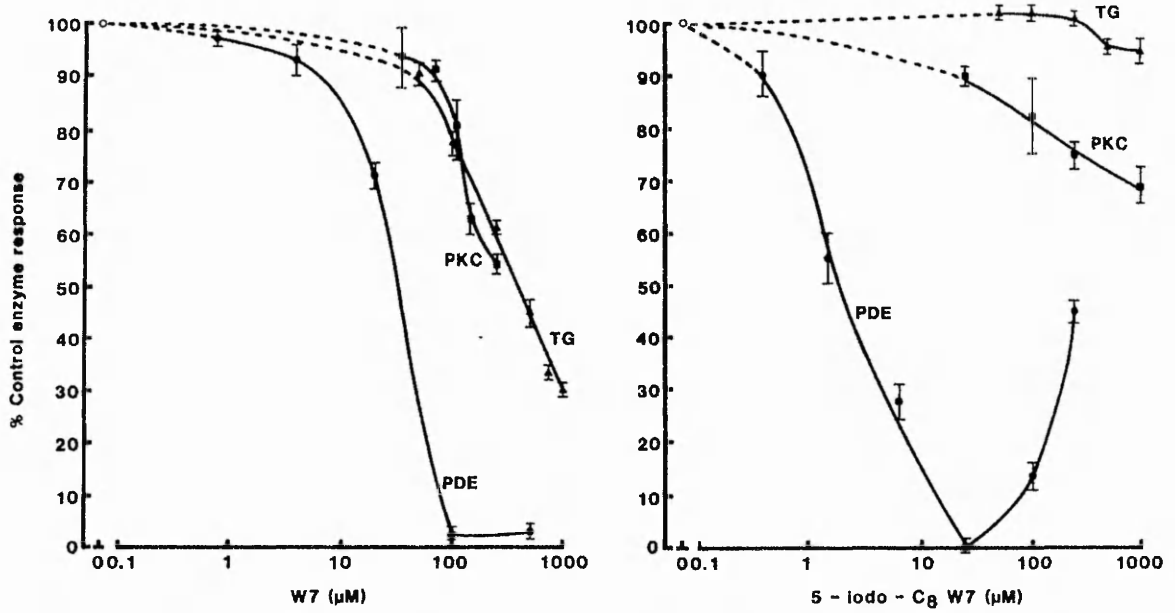
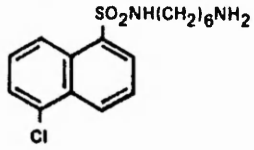
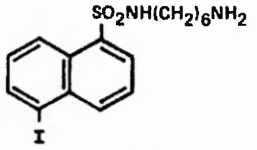
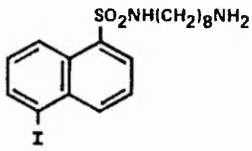
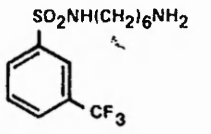
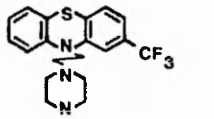
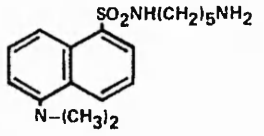
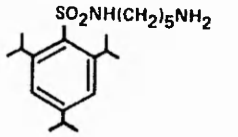
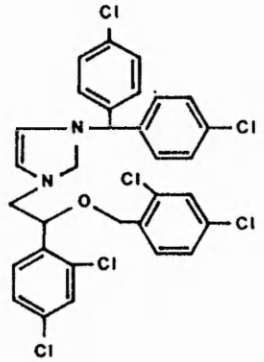


Fig. 3. A comparison of the specificity of action of W7 and 5-iodo-1-C_8 shown by their ability to inhibit calcium, calmodulin-dependent phosphodiesterase (PDE), calcium-dependent, calmodulin-independent rat liver transglutaminase (TG) and calcium-dependent, calmodulin-independent rat spleen protein kinase C (PKC). Results shown are means ± SEM of triplicate determinations of representative experiments. Results based on three such experiments are shown in Table 2.

Table 2. Inhibition of calmodulin dependent and independent enzymes by drugs

Name	Structure	IC_{50} (μm) (PDE)	(PKC)	(TG)
W7 (a)		29 ± 2	342 ± 47 [138 in (6)]	362 ± 93
5-iodo-1-C_6 (c)		29 ± 6	⩾1000	506 ± 59
5-iodo-1-C_8 (e)		3 ± 0.6	>1000	>1000
3-CF_3-1-C_6 (p)		250 ± 62	>1000	>500
Trifluoperazine		4 ± 2	74 ± 7	>1000
N-Dansylcadaverine		387 ± 9	⩾1000	43 ± 7
N-TIPS cadaverine		26 ± 15	200 (n = 2)	20 ± 2
Calmidazolium		0.03 ± 0.02 [0.1 in (4)]	[5.3 in (4)]	—

Phosphodiesterase (PDE), protein kinase C (PKC) and transglutaminase (TG) were assayed as described in Materials and Methods. Results shown are means of a minimum of 2, usually 3, experiments with each drug. Values shown in square brackets represent those obtained from the literature. *N*-TIPS is 2, 4, 6,-tri-isopropylbenzene sulphonamide. Values shown are means ± SEM based on 3 separate experiments for all except one case where N = 2 as indicated.

solubility for these compounds. It is important to discover whether this enhancement of activity stems from an improved hydrophobic interaction of the drug with calmodulin or from greater flexibility of the side chain.

One curious phenomenon was manifest by all of the iodo-compounds. While low concentrations ($\leq 50\ \mu M$) of these drugs (b–i) inhibit the calmodulin-dependent phosphodiesterase activity down to basal (i.e. calmodulin-independent) activity, at higher concentrations of the drugs ($\geq 50\ \mu M$) there is a restoration of enzyme activity approaching 50% of the maximum activity observed in the absence of drug (see Fig. 3). This effect appears (i) to be unrelated to the potency of the drug (b–i), (ii) to be unique in the whole range of compounds studied to the iodo-series, (iii) not to be an artefact of scintillation counting since the effect was not manifest in the presence of boiled, inactive phosphodiesterase—thereby excluding the possibility that fluorescence of the drugs affected the final scintillation counting in the assay, and (iv) did not occur in the absence of calmodulin. We have no explanation for this *in vitro* effect of these iodo-compounds at present and this is currently under investigation.

This anomalous behaviour for the 5-iodo-series of drugs seems unlikely to have any significance because we have been unable to elicit similar behaviour in experiments employing the iodo-series of drugs to inhibit DNA synthesis in cultured cells [23]. In this latter study [23] we found the 5-iodo-series of drugs to be more potent than the parent compound W7 in inhibiting proliferation of K562 tumour cells cultured in serum-free media. Closer examination of 5-iodo-1-C_8 (e) revealed a similar time course of inhibition to W7 and that, as with W7, growth was arrested by inhibiting entry of cells into S phase.

Specificity of action of naphthalenesulphonamides as calmodulin antagonists

Based on the potency data for this series of naphthalenesulphonamides, the C-6 and C-8 side chain 5-iodo-compounds, 5-iodo-1-C_6 (c) and 5-iodo-1-C_8 (e), were selected for further examination to compare their selectivity for non-calmodulin enzyme inhibition with that of W7. The question to be addressed was whether an improvement in calmodulin inhibitory potency was associated with any change in specificity of action of these drugs for calmodulin-dependent versus calmodulin-independent, but calcium-dependent, enzyme processes. Protein kinase C and transglutaminase (EC 2.3.2.13) were selected as good examples of calcium-dependent but calmodulin-independent enzymes [24, 25]. The results of this comparison are shown in Table 2 and Fig. 3.

5-Iodo-1-C_6 (c) is slightly more selective than W7 for calmodulin-dependent enzyme inhibition and 5-iodo-1-C_8 (e) is significantly more selective. Approximately 300 times more 5-iodo-1-C_8 is required for 50% inhibition of protein kinase C or transglutaminase than for inhibition of calmodulin-dependent phosphodiesterase. This compares to a selectivity factor in our hands of only 10-fold for W7 (Table 2 and Fig. 3). This clearly illustrates that improvements in potency of the naphthalenesulphonamides can be concomitant with improvements in specificity of action *in vitro*. Several other structurally different naphthalenesulphonamides and trifluoperazine are illustrated in Table 2 for comparison. *N*-Dansylcadaverine and *N*-TIPS cadaverine are compounds which show reasonable potency in inhibiting transglutaminase but the latter of these is, unfortunately, equipotent in inhibiting calmodulin-dependent phosphodiesterase. Trifluoperazine, like W7, shows only a factor of 10-fold separating its potency against protein kinase C [6] from that of its potency against phosphodiesterase. ^{3}CF-1-C_6 (p) shows little inhibitory effect on any enzyme activity. Comparison of the data in Table 2 indicate that the selectivity achieved for 5-iodo-1-C_8 is without parallel.

Despite its great importance, there are relatively few reports on the specificity of action of calmodulin antagonists. Some drugs, such as R24571 (calmidazolium), a derivative of the antimycotic agent miconazole, despite great potency as calmodulin antagonists (IC_{50} values of 0.03 μM in our own laboratory (see Table 2) and 0.1 μM elsewhere [4] for calmodulin-dependent phosphodiesterase), have been found to have relatively little specificity. Mazzei *et al.* [4] in a thorough study of calmidazolium found that it would also inhibit protein kinase C at relatively low concentrations (IC_{50} of 5.3 μM). The possibility also exists of course that any of the anti-calmodulin drugs may inhibit nonidentified calcium-independent enzymes *in vivo*.

In this study we have shown that it is possible, at least *in vitro*, to improve the potency at the same time as improving the selectivity of action of naphthalenesulphonamide drugs for the inhibition of calmodulin-dependent enzyme activity. The potential value of these new drugs as pharmacological agents for the study of calmodulin biochemistry and as possible clinical tools in carcinostasis and drug resistance is under investigation.

Acknowledgements—We thank the Yorkshire Cancer Research Campaign and the Sir Halley Stewart Trust for financial support of this work. S.M.N. is supported by a Wellcome Trust University Award.

REFERENCES

1. S. Tomlinson, S. MacNeil, S. W. Walker, C. A. Ollis, J. E. Merritt and B. L. Brown, *Clin. Sci.* **66**, 497 (1984).
2. W. C. Prozialeck and B. Weiss, *J. Pharmac. exp. Ther.* **222**, 509 (1982).
3. H. Hidaka, M. Asano and T. Tanaka, *Molec. Pharmac.* **20**, 571 (1981).
4. G. J. Mazzei, R. C. Schatzman, R. S. Turner, W. R. Vogler and J. F. Kuo, *Biochem. Pharmac.* **33**, 125 (1984).
5. Y. Nishizuka, *Science* **233**, 305 (1986).
6. B. C. Wise, D. B. Glass, C. H. Jen Chou, R. L. Raynor, N. Katoh, R. C. Schatzman, R. S. Turner, R. F. Kibler and J. F. Kuo, *J. biol. Chem.* **257**, 8489 (1982).
7. P. J. A. Dawes, D. R. Davies, A. Levitski, F. R. Maxfield, P. Malhaud, M. C. Willingham and I. M. Pasten, *Nature, Lond.* **283**, 162 (1980).
8. P. J. Bungay, J. M. Potter and M. Griffin, *Biochem. J.* **219**, 819 (1984).
9. P. J. Bungay, R. A. Owen, I. C. Coutts and M. Griffin, *Biochem. J.* **235**, 269 (1986).

10. M. M. Carnwell, R. L. Juliano and P. J. A. Davies, *Biochim. biophys. Acta* **762**, 414 (1983).
11. L. Lorand, K. N. Parameswaran, P. Stenberg, Y. S. Tong, P. T. Velasco, N. A. Jönsson, L. Mikiver and P. Moses, *Biochemistry* **9**, 1756 (1979).
12. R. W. Beattie and F. Whitmore, *J. Am. chem. Soc.* **55**, 1546 (1933).
13. P. Ferrero and G. Bollinger, *Helv. chim. Acta* **11**, 1144 (1928).
14. H. Hidaka, M. Asano, S. Iwadare, I. Matsumoto, I. Totsuka and N. Aoki, *J. Pharmac. exp. Ther.* **207**, 8 (1978).
15. R. Mercantou and H. Goldstein, *Helv. chim. Acta* **28**, 319 (1945).
16. K. Hosekawa and K. Inukai, *Nippon Kagaku Kaishi* 1791 (1976).
17. K. C. Joshi and S. Girl, *J. Ind. chem. Soc.* **38**, 117 (1961).
18. J. Hempel and E. Klauke, Brit. Pat. 1258557 (1970) *Chem. Abs.* **75**, P 19924d. (1971).
19. H. L. Yale and F. Sowinski, *J. org. Chem.* **25**, 1824 (1960).
20. A. V. Kirsanov and N. A. Kirsanova, *J. gen. Chem. USSR* **32**, 877 (1962).
21. S. MacNeil, S. W. Walker, H. J. Senior, S. S. Bleehen and S. Tomlinson, *J. Invest. Dermatol.* **83**, 15 (1984).
22. J. M. Lord and S. J. H. Ashcroft, *Biochem. J.* **219**, 547 (1984).
23. C. H. Barton, G. M. Blackburn, R. Rees, S. S. Bleehen, J. H. Senior, D. S. Munro and S. MacNeil, *Carcinogenesis* **8**, 919 (1987).
24. J. F. Kuo, R. G. G. Anderson, B. C. Wise, L. Mackerlova, I. Salomonsson, N. L. Brackett, N. Katoh, M. Shoji and R. W. Wrenn, *Proc. natn. Acad. Sci. U.S.A.* **77**, 7039 (1980).
25. P. J. Bungay, B. M. Elliott and M. Griffin, *Biosci. Rep.* **5**, 1079 (1985).

Biochem J. (1986) 235, 269–278 (Printed in Great Britain)

A role for transglutaminase in glucose-stimulated insulin release from the pancreatic β-cell

Peter J. BUNGAY,* Robin A. OWEN,* Ian C. COUTTS† and Martin GRIFFIN*‡
*Department of Life Sciences and †Department of Physical Sciences, Trent Polytechnic, Clifton, Nottingham NG11 8NS, U.K.

1. Preincubation of rat islets of Langerhans with the potent inhibitors of islet transglutaminase activity, monodansylcadaverine (30–100 μM) and *N*-(5-aminopentyl)-2-naphthalenesulphonamide (100–200 μM), led to significant inhibition of glucose-stimulated insulin release from islets. In contrast, the respective *N'*-dimethylated derivatives of these two compounds, which did not inhibit islet transglutaminase activity, were much less effective as inhibitors of glucose-stimulated insulin release. 2. None of the compounds inhibited rat spleen protein kinase C activity at concentrations which gave rise to inhibition of glucose-stimulated insulin release. When tested for their effects on calmodulin-stimulated bovine heart phosphodiesterase activity, of the compounds that inhibited insulin release, only monodansylcadaverine did not act as an effective antagonist of calmodulin at concentrations (up to 50 μM) that gave rise to significant inhibition of glucose-stimulated insulin release. Furthermore, at 50 μM, monodansylcadaverine did not inhibit methylation of islet lipids. The inhibition of glucose-stimulated insulin release by monodansylcadaverine is therefore likely to be attributable to its interference with islet transglutaminase activity. 3. The sensitivity of islet transglutaminase to activation by Ca^{2+} was investigated by using a modified assay incorporating dephosphorylated *NN'*-dimethylcasein as a substrate protein. The K_m for Ca^{2+} obtained (approx. 3 μM) was an order of magnitude lower than previously reported for the islet enzyme [Bungay, Potter & Griffin (1984) Biochem. J. **219**, 819–827]. Mg^{2+} (2 mM) was found to have little effect on the sensitivity of the enzyme to Ca^{2+}. 4. Investigation of the endogenous substrate proteins of islet transglutaminase by using the Ca^{2+}-dependent incorporation of [^{14}C]methylamine into proteins of islet homogenates demonstrated that most of the incorporated radiolabel was present in cross-linked polymeric aggregates which did not traverse 3% (w/v) acrylamide gels. The radiolabelled polymeric aggregates were present in 71000 *g*-sedimented material of homogenates, and their formation was transglutaminase-mediated. 5. These findings provide new evidence for the involvement of islet transglutaminase in the membrane-mediated events necessary for glucose-stimulated insulin release.

INTRODUCTION

The process of stimulus–secretion coupling in the pancreatic β-cell is mediated by the Ca^{2+}-messenger system, whereby stimulation of insulin release by glucose and other secretagogues is thought to involve a rise in the cytosolic concentration of Ca^{2+} (Hedeskov, 1980; Wollheim & Sharp, 1981; Prentki & Wollheim, 1984; Rasmussen & Barrett, 1984). The way in which Ca^{2+} elicits the release of insulin is not understood, but is likely to be the result of the binding of the cation to intracellular receptor proteins, whose activities may then increase rates of hormone secretion. One of these possible Ca^{2+} receptors is the Ca^{2+}-dependent enzyme transglutaminase (EC 2.3.2.13), which catalyses an acyl-transfer reaction between peptide-bound glutamine residues and primary amine groups. The result of this activity is the post-translational modification of proteins, either through incorporation of amines or through protein cross-linking (for reviews see Folk & Finlayson, 1977; Lorand & Conrad, 1984). Evidence has suggested that the transglutaminase activity present in isolated islets of Langerhans may be important in the pancreatic β-cell during glucose-stimulated insulin release (Bungay *et al.*, 198 1984*a*, *b*; Gomis *et al.*, 1983). Reactions catalysed transglutaminase have also been implicated in oth cellular processes regulated by Ca^{2+}, notably recepto mediated endocytosis (Davies *et al.*, 1980; Van Leuv *et al.*, 1980) and the activation of lymphocytes (Juli *et al.*, 1983) and macrophages (Fésüs *et al.*, 198 Günzler *et al.*, 1982).

Of importance to the assessment of a functional ro for the islet transglutaminase in the process stimulus–secretion coupling is its sensitivity to activati by Ca^{2+}. Earlier reports from our laboratory (Bung *et al.*, 1984*a*) and others (Gomis *et al.*, 1983) indicat that the islet transglutaminase operated at half-maxim velocity at concentrations of Ca^{2+} in the range 40–90 μ Since concentrations of Ca^{2+} as high as these are unlike to be found in the pancreatic β-cell during periods stimulated insulin release it is of great importance reassess the accuracy of these findings if a functional ro for this enzyme in stimulus–secretion coupling in t pancreatic β-cell is to be established.

Other previous evidence for the involvement transglutaminase in glucose-stimulated insulin relea

Abbreviations used: KRB, Krebs–Ringer bicarbonate medium (Krebs & Hensleit, 1932); dansyl, 5-dimethylaminonaphthalene-1-sulphon MDCD, monodansylcadaverine; 2-NSA, *N*-(5-aminopentyl)-2-naphthalenesulphonamide.

‡ To whom all correspondence should be addressed.

was based on the observation that compounds which inhibited transglutaminase activity *in vitro* also inhibited glucose-stimulated insulin release from islets. However, the difficulty with using this type of approach was that some of these 'probe' molecules may inhibit insulin release by interference with targets other than islet transglutaminase (Bungay *et al.*, 1984*a*; Sener *et al.*, 1984). For instance, alkylamines (which are normally used in millimolar concentrations) may exert their effects by disruption of lysosomal and endosomal function. The more potent inhibitors of transglutaminase activity, the naphthalenesulphonamides (Lorand *et al.*, 1979), may be antagonists of the Ca^{2+}-binding protein calmodulin (Hidaka & Tanaka, 1983) and inhibitors of the phospholipid- and Ca^{2+}-sensitive protein kinase (protein kinase C) (Wise *et al.*, 1982*b*). These two proteins have been implicated as important regulators of stimulus–secretion coupling in the pancreatic β-cell (Gagliardino *et al.*, 1980; Lord & Ashcroft, 1984). It is therefore important to establish the specificity of such 'probe' molecules if they are to be used to determine the involvement of transglutaminase in glucose-stimulated insulin release.

The present study was therefore undertaken in order both to reassess previous evidence and to provide further evidence of the involvement of transglutaminase in glucose-stimulated insulin release.

EXPERIMENTAL

Materials

Collagenase used in the isolation of pancreatic islets of Langerhans was obtained from Serva Feinbiochimica, Heidelberg, West Germany. Monodansylcadaverine was purchased from Fluka A. G., Buchs, Switzerland. Materials for the synthesis of other naphthalenesulphonamides (dansyl chloride, 2-naphthalenesulphonyl chloride and 5-chlorovaleronitrile) were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. [1,4-^{14}C]Putrescine, [^{14}C]methylamine, L-[*methyl*-^{3}H]-methionine, [U-^{14}C]glucose, cyclic [^{3}H]AMP and [γ^{32}P]ATP were all purchased from Amersham International, Amersham, Bucks., U.K., and ^{125}I-labelled insulin was obtained from Novo, Bagsvaerd, Denmark. Calmodulin from bovine brain and bovine heart phosphodiesterase were obtained from Boehringer Mannheim, London W.5, U.K., and phosphatidylserine and 1,2-diolein were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Standard rat insulin and anti-insulin antibody were obtained from Novo.

Methods

Synthesis of test compounds. The *NN'*-dimethylcadaverine sulphonamides *N*-(5-dimethylaminopentyl)-2-naphthalenesulphonamide and *N*-(5-dimethylaminopentyl)-5-dimethylamino-1-naphthalenesulphonamide (free bases) were made by reaction of the appropriate napthalenesulphonyl chloride in pyridine with *NN'*-dimethylcadaverine, which was prepared from 5-chlorovaleronitrile as detailed in Lott & Krapcho (1957). For the *N*-(5-aminopentyl)-2-naphthalenesulphonamide (hydrobromide salt), 6-(2-naphthalenesulphonamide)-hexanamide was treated with bromine in methanol as described by Lorand *et al.*, (1979) and the resulting carbamate was hydrolysed with a saturated solution of HBr in acetic acid (Wani *et al.*, 1972). Purity o compounds was assessed by t.l.c., mass spectrometry, i.r spectroscopy and n.m.r.

Isolation of islets of Langerhans. Islets of Langerhans were isolated from the pancreas of 200–300 *g* Sprague-Dawley rats of both sexes by a collagenase digestion technique similar to that of Lacy & Kostianovsky (1967). For studies with intact islets, islets were preincubated fo 5 min in KRB containing 16.8 mM-glucose, followed by 60 min in KRB containing 2.8 mM-glucose.

Homogenization of islets. Before homogenization, islets were washed in ice-cold 0.25 M-sucrose/1 mM-Tris/HCl/1 mM-EDTA or -EGTA, pH 7.4, and suspended in 150–300 μl of the same medium to give a final isle concentration of approx. 2 per μl. Homogenization was then carried out either by ultrasonication with 2 × 5 bursts at 3 μm amplitude with an MSE sonicator or b use of three strokes of a 5 ml Potter–Elvehjem homogenizer. In the assays for islet transglutaminase activity, a particle-free supernatant was used, which wa prepared by centrifugation of islet homogenates a 71000 $g_{av.}$ for 45 min.

Assay for transglutaminase activity. Transglutaminase activity was measured by [^{14}C]putrescine incorporation into *NN'*-dimethylcasein by using the method of Lorand *et al.* (1972). The incubation mixture, at 37 °C, consisted of 30 mM-Tris/HCl, pH 7.4, 3.85 mM-dithiothreitol, 2.5 mM-$CaCl_2$ or 5 mM-EDTA, 1.2 mM-[^{14}C]putrescine (3.96 Ci/mol), *NN'*-dimethylcasein (5 mg/ml) and 20 μ of islet preparation in a final volume of 50 μl. Samples (10 μl) were taken for the counting of trichloroacetic acid-insoluble radioactivity at appropriate time intervals and rates of putrescine incorporation were determined after subtraction of EDTA controls.

Measurement of the Ca^{2+}-sensitivity of transglutaminase. Islet supernatants were prepared as described above, except that 1 mM-EGTA was substituted for EDTA in the homogenization buffer. The islet supernatant extract was dialysed for at least 2 h at 4 °C against 50 mM-Tris/HCl (pH 7.4 at 37 °C)/1 mM-EGTA. Addition of 20 μl of islet preparation to the final assay volume of 50 μl then gave a final concentration of EGTA of 0.4 mM in 50 mM-Tris/HCl. Different amounts of $CaCl_2$ were then added to the assay medium such that the final desired concentration of free Ca^{2+} was obtained, ranging from 3.2 to 50.6 μM. For calculation of the free Ca^{2+} concentrations, an association constant at pH 7.4 of $10^{7.12}$ for [CaEGTA]/[Ca] [EGTA] was used (Schatzman, 1973). In these assays dephosphorylated *NN'*-dimethylcasein was used instead of native *NN'*-dimethylcasein. This protein was dephosphorylated by incubation with alkaline phosphatase as described by Cooke & Holbrook (1974).

Assay for protein kinase C activity. The potency of compounds as inhibitors of protein kinase C was determined by measuring protein kinase C activity in extracts obtained from rat spleen. With a Potter–Elvehjem homogenizer, rat spleen was homogenized in 3 vol of 20 mM-Tris/HCl (pH 7.4)/2 mM-EGTA/50 mM-2-mercaptoethanol. The homogenate was centrifuged at 71000 *g* for 45 min at 4 °C and the supernatant

retained. This preparation was diluted 1:10 with 20 mM-Tris/HCl, pH 7.4, for use in the protein kinase C assays. The standard reaction mixture (100 μl) contained the following final concentrations of components: 20 mM-Tris/HCl, pH 7.4, 5 mM-magnesium acetate, 200 μg of histone H1/ml, 10 μM-[γ-^{32}P]ATP (0.6 Ci/mmol), 98 μM-$CaCl_2$, 48 μM-EGTA, 1.2 mM-2-mercaptoethanol, 16 μg of phosphatidylserine/ml, 2.4 μg of 1,2-diolein/ml and the appropriate concentration of test compound. Reactions were started by the addition of 24 μl of spleen extract (approx. 25 μg of protein). The incorporation of ^{32}P from [γ-^{32}P]ATP into histone H1 was measured by the modified procedure of Lord & Ashcroft (1984), whereby phosphorylated proteins were recovered by precipitation with 5% (w/v) trichloroacetic acid containing 0.25% (w/v) sodium tungstate. Protein kinase C activity was calculated by subtracting the phosphorylation obtained in the presence of Ca^{2+} alone from that obtained in the presence of Ca^{2+}, 1,2-diolein and phosphatidylserine over a period of 2 min incubation at 37 °C. Addition of phospholipid and diolein stimulated enzyme activity by approx. 60%. Under these conditions, the incorporation of ^{32}P was linear with time, and the standard inhibitor of protein kinase C, trifluoperazine (Wise *et al.*, 1982*b*), was found to give 50% inhibition of enzyme activity at a concentration of 61 μM.

Assay for calmodulin-stimulated phosphodiesterase. The potency of compounds for their action as calmodulin antagonists was determined by using calmodulin-deficieint bovine heart phosphodiesterase, by the method of MacNeil *et al.* (1984). The reaction mixture at 37 °C contained, in a final volume of 400 μl: 40 mM-Tris/HCl, pH 7.0, 4 mM-2-mercaptoethanol, 5 mM-$MgCl_2$, cyclic [^{3}H]AMP (2×10^5 c.p.m./tube), 100 μM-cyclic AMP, 25 μM-$CaCl_2$, 2 munits of enzyme/tube and 12.5 ng of calmodulin. The amount of calmodulin in the assay was sufficient to produce approx. 80% of maximal activation of the phosphodiesterase enzyme. All assays were carried out at 37 °C for 15 min.

Incubation of islets for measurement of insulin release. This was done as described previously (Bungay *et al.*, 1984*a*). Insulin released into the incubation medium was determined by radioimmunoassay (Heding, 1972), with rat insulin as standard.

Measurement of glucose oxidation. Glucose oxidation by islets was measured by determination of the amount of $^{14}CO_2$ release from [U-^{14}C]glucose as described previously (Bungay *et al.*, 1984*a*).

Measurement of islet lipid methylation. Total islet lipid methylation was determined by measuring the incorporation of L-[*methyl*-^{3}H]methionine into chloroform/diethyl ether-extractable material, essentially as described by Best *et al.* (1984). Groups of 80–120 islets were preincubated for 45 min at 37 °C in 0.5 ml of KRB containing 2.8 mM-glucose with or without 50 μM-MDCD. After this preincubation, a further 0.5 ml of KRB containing 30.5 mM-glucose was added together with 10 μCi of L-[*methyl*-^{3}H]methionine (74 Ci/mol) to give final concentrations of 16.8 mM-glucose and 0.135 μM-methionine. After a further incubation period of 60 min, medium was removed from the islets, to which was added 1.0 ml of ice-cold 10% (w/v) trichloroacetic acid. The resulting precipitate was washed twice with 10% trichloroacetic acid and then transferred to a 15 ml gla centrifuge tube. To the precipitate was added 1.0 ml distilled water, and the aqueous mixture was extracte with 1.0 ml of chloroform/methanol/11 M-HC (200:100:1, by vol.). The lower organic layer wa removed to a scintillation vial and the organic solve evaporated with a stream of N_2. The residue was the counted for radioactivity. Counting efficiency wa determined by using the Quench Indicating Parameter Packard Instruments.

Incorporation of [^{14}C]methylamine into islet homoge ates. Homogenates of islets were prepared in 1 m Tris/HCl (pH 7.4)/0.25M-sucrose/1 mM-EGTA with Potter–Elvehjem homogenizer as described above. Th incubation mixture for incorporation of [^{14}C]methylamin consisted of 20 mM-Tris/HCl, pH 7.4, 3.85 mM-dithi threitol, 1.8 mM-[^{14}C]methylamine (56 Ci/mol), 0.1 m phenylmethanesulphonyl fluoride and 2.5 mM-$CaCl_2$ 0.625 mM-$CaCl_2$ or 5 mM-EGTA, in a final volume 100 μl after the addition of 60 μl of islet homogenat Under these conditions, the addition of 2.5 mM-CaC and 0.625 mM-$CaCl_2$ gave concentrations of free Ca^{2+} the incubation of 1.9 mM and 26.7 μM respectively. Th mixture was incubated at 37 °C for 15, 30 or 120 mi after which was added 100 μl of double-strengt electrophoresis sample buffer so that the final concentra tions of components in the homogenate sample we 62.5 mM-Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/ glycerol and 5% (v/v) 2-mercaptoethanol. The sample fo electrophoresis was boiled for 5 min and stored at 4 ° until required.

SDS/polyacrylamide-gel electrophoresis. Islet homo genate proteins were separated by discontinuous SDS polyacrylamide-gel electrophoresis as described b Laemmli (1970). The solubilized sample (approx. 50 μ of protein in 45–75 μl) was applied to the gels, whic consisted of a stacking gel of 3% (w/v) acrylamid 0.125 M-Tris/HCl, pH 6.8, and 0.1% SDS, and resolving gel of 10% acrylamide, 0.375 M-Tris/HC pH 8.8, and 0.1% SDS. Samples were run for 4–5 together with the M_r standard proteins β-galactosidas (116000), bovine serum albumin (66000), ovalbumi (45000), trypsinogen (24000), β-lactoglobulin (18400 and lysosyme (14300), which were solubilized in the sam buffer. After electrophoresis, the gels were fixed in solution of 10% trichloroacetic acid and 3.4% (w/ sulphosalicylic acid and then stained with a solution 1.25 g Coomasie Blue (PAGE 83; BDH):/l in 18% (v/ methanol/5% (v/v) acetic acid. After destaining i 18% methanol/5% acetic acid containing 2 m unlabelled methylamine, gels were photographed an resolving gels sectioned with a razor blade every 2 mm The whole stacking gel above the respective protein lane was taken as one section. The sections were thoroughl dried in scintillation vials, dissolved in H_2O_2 (30% w/v)/conc. NH_3 (sp. gr. 0.88) (19:1, v/v) and counted fo radioactivity in 10 ml of Instagel (Packard).

Addition of test compounds to enzyme assays an incubation media. Compounds were initially dissolved i 100 μl of dimethyl sulphoxide, except for MDCD, whic was initially dissolved in 100 μl of dilute HCl. From stock 10 mM solution in 10% (v/v) dimethyl sulphoxide

the compounds were added to the assay or incubation medium after serial dilution to give the desired final concentration. Control incubations contained the same final concentration of dimethyl sulphoxide as the incubations containing test compounds. For hydrobromide salts of test compounds, NaBr was added to control incubations so that the same final concentration of Br^- was present in control and test incubations.

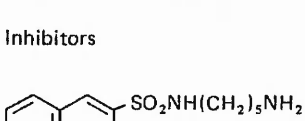
RESULTS

Effects of naphthalenesulphonamides on insulin release

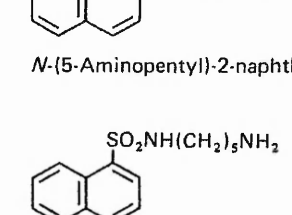
In order to investigate the involvement of islet transglutaminase in the mechanism of insulin release from the pancreatic β-cell, isolated islets of Langerhans were incubated with two known potent inhibitors (competitive substrates) (Lorand *et al.*, 1979) of the enzyme activity, monodansylcadaverine (MDCD) and *N*-(5-aminopentyl)-2-naphthalenesulphonamide (2-NSA). In addition, the *N*-dimethylated derivatives of these compounds, *N*-dimethylmonodansylcadaverine (dimethyl-MDCD) and *N*-(5-dimethylaminopentyl)-2-naphthalenesulphonamide (dimethyl-2-NSA), were tested for their effects on insulin release, since these act as control compounds in that they lack the primary amine group which is crucial to inhibition of transglutaminase activity (Fig. 1). The effects on insulin release of these compounds and their *N*-dimethylated derivatives are shown in Table 1. These chemically related compounds displayed varied effects on glucose-stimulated insulin release from islets. The most potent inhibitor was MDCD, which was effective over the concentration range 30–100 μM. 2-NSA was also an effective inhibitor of glucose-stimulated insulin release, but not as potent as MDCD, concentrations of 100–200 μM being required for effective inhibition.

In contrast with these primary amines, much higher concentrations of the *N*-dimethylated derivatives of these naphthalenesulphonamides were required for any effects on insulin release. Dimethyl-2-NSA did not inhibit glucose-stimulated insulin release at either 100 or 200 μM, but was able to cause 76% inhibition at 400 μM. Dimethyl-MDCD did not inhibit glucose-stimulated insulin release at either 200 or 400 μM. Therefore inhibition of glucose-stimulated insulin release by the range of compounds used appeared to depend on the possession of a primary amine moiety, since the tertiary-amine analogues were not as potent inhibitors as their primary-amine counterparts.

(*a*) Inhibitors

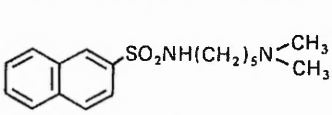


N-(5-Aminopentyl)-2-naphthalenesulphonamide (2-NSA)

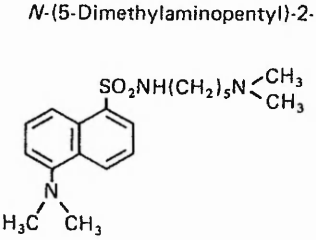


Monodansylcadaverine (MDCD)

(*b*) Control compounds for inhibitors



N-(5-Dimethylaminopentyl)-2-naphthalenesulphonamide (dimethyl-2-NSA)



N-Dimethylmonodansylcadaverine (dimethyl-MDCD)

Fig. 1. Inhibitors of transglutaminase activity (*a*) and control compounds for the inhibitors (*b*)

Effects of naphthalenesulphonamides on glucose-stimulated glucose oxidation

In view of the importance of glucose metabolism to stimulus–secretion coupling in the pancreatic β-cell, we compared the compounds used above for their effects on glucose oxidation by islets. The release of $^{14}CO_2$ from [U-^{14}C]glucose was measured over the same time-course as that used in the studies on insulin release. The results of these experiments are shown in Table 2. Of the compounds used that were shown to inhibit insulin release, 2-NSA significantly inhibited glucose oxidation when incubated with islets at a concentration of 200 μM, but not at a concentration of 100 μM. Dimethyl-2-NSA (400 μM) was also tested and caused no significant inhibition of glucose-stimulated glucose oxidation. In our previous experiments with MDCD, we demonstrated that it was non-toxic to islets at concentrations up to 100 μM (Bungay *et al.*, 1984*a*). Therefore, inhibition of glucose-stimulated insulin release by concentrations of MDCD below 100 μM, of 2-NSA below 100 μM and of dimethyl-2-NSA below 400 μM was independent of any effects of these compounds on islet glucose oxidation.

Effects of compounds on activities of transglutaminase, calmodulin-stimulated phosphodiesterase and protein kinase C

Since the β-cell contains a number of possible targets for the action of probe compounds such as the naphthalenesulphonamides used in the present work, it was crucial to determine the specificity of the compounds for intracellular targets whose inhibition could lead to inhibition of glucose-stimulated insulin release. The compounds were therefore screened for their possible action as inhibitors of islet transglutaminase and rat spleen phospholipid- and Ca^{2+}-dependent protein kinase (protein kinase C). In addition, the inhibition of the activation of bovine heart phosphodiesterase by calmodulin was used to measure the potency of the compounds as calmodulin antagonists (MacNeil *et al.*, 1984).

By comparison with control rates of enzyme activity obtained in the absence of the test compounds, the percentage inhibition of enzyme activity was calculated (Figs. 2 and 3). From these data, the concentration of compound that gave 50% inhibition of enzyme activity (IC_{50}) for each enzyme system was determined (Table 3).

Table 1. Effects of naphthalenesulphonamides on glucose-stimulated insulin release

Batches of five islets were incubated in KRB medium in the presence or absence of the concentrations of compounds stated as described in the Experimental section. Compounds were preincubated with islets for 45 min in KRB containing 2.8 mM-glucose, followed by 60 min in KRB containing 16.8 mM-glucose. The insulin content of the medium was determined by radioimmunoassay. Stimulation of insulin release over the 60 min period was determined by subtracting basal rates of insulin release from islets maintained in KRB containing 2.8 mM-glucose incubated simultaneously over the same total time period of 45+60 min. The inhibition of glucose-stimulated insulin release for each compound tested was then expressed as a percentage (±S.E.M.) of the rate in control incubations for the numbers of determinations shown in parentheses. The significance of difference from controls was determined by Student's *t* test: N.S., no significant difference. Typical rates of insulin release were 6.7±0.9 ($n = 33$) μunits/h per islet for islets incubated in 2.8 mM-glucose and 94.0±6.4 μunits/h per islet ($n = 48$) for islets incubated in 16.8 mM-glucose.

Test compound	Conc. (μM)	Inhibition of glucose-stimulated insulin release (%)
Monodansylcadaverine	30	33±6 (5) $P \leqslant 0.05$
	50	65±11 (6) $P \leqslant 0.002$
	100	85±8 (5) $P \leqslant 0.001$
N-Dimethylmonodansylcadaverine	200	24±9 (6) N.S.
	400	9±7 (6) N.S.
N-(5-Aminopentyl)-2-naphthalenesulphonamide	100	55±9 (6) $P \leqslant 0.001$
	200	86±4 (6) $P \leqslant 0.01$
N-(5-Dimethylaminopentyl)-2-naphthalenesulphonamide	100	0 (7)
	200	0 (6)
	400	76±9 (7) $P \leqslant 0.01$

Table 2. Effects of naphthalenesulphonamides on islet glucose oxidation

Glucose oxidation was determined for groups of 10–20 islets by measuring $^{14}CO_2$ release from [U-^{14}C]glucose as described in the Experimental section. Incubations were carried out for 1 h after the addition of a stimulatory concentration (16.8 mM) of glucose. For each concentration of test compound, control rates were determined for untreated islets from the same isolated batch. The inhibition of glucose oxidation by test compounds was expressed as a mean percentage (±S.E.M.) relative to control incubations, with the numbers of observations shown in parentheses. The significance of difference from controls was determined by Student's *t* test. Typical rates of glucose-stimulated glucose oxidation were 344±35 pmol/h per 10 islets ($n = 17$).

Test compound	Concn. (μM)	Inhibition of glucose-stimulated glucose oxidation (%)
N-(5-Aminopentyl)-2-naphthalenesulphonamide	100	0 (4) N.S.
	200	40±7 (5) $P < 0.005$
N-(5-Dimethylaminopentyl)-2-naphthalenesulphonamide	400	18±3 (3) N.S.

Since the naphthalenesulphonamides used in these experiments are known to act as competitive substrates of transglutaminase (Lorand *et al.*, 1979), inhibition constants (K_i) were calculated (Table 3) using the data shown in Fig. 3.

The effects of the compounds on protein kinase C activity were determined over the concentration range 0.1–1.0 mM. Under the conditions of assay, inhibition of enzyme activity required concentrations of the compounds of at least 1.0 mM (Table 3). The compounds that inhibited protein kinase C activity (2-NSA and dimethyl-2-NSA) were tested for their effects on basal enzyme activity (measured in the absence of diolein and phospholipid) and were found not to inhibit this activity, indicating that their effects were directed against the phospholipid-stimulated protein kinase C activity.

When tested for their effects on the activation of phosphodiesterase by calmodulin, the naphthalenesulphonamides inhibited this enzyme more potently and to varying degrees. The most potent inhibitor wa 2-NSA ($IC_{50} = 105$ μM), with dimethyl-2-NS ($IC_{50} = 277$ μM) being approx. $\frac{1}{3}$ as potent as 2-NSA an MDCD ($IC_{50} = 387$ μM) being approx. $\frac{1}{4}$ as potent a 2-NSA (Fig. 2; Table 3). Dimethyl-MDCD was found t be the least effective inhibitor of calmodulin-stimulate phosphodiesterase activity ($IC_{50} > 500$ μM) when teste at concentrations up to 500 μM. The effects of thes compounds on basal phosphodiesterase activity measure in the absence of calmodulin were also determined. Bas activity was not significantly affected by concentratio of MDCD up to 500 μM, of 2-NSA up to 200 μM and dimethyl-MDCD and dimethyl-2-NSA up to 250 μM. Th two dimethylated analogues significantly stimulate basal phosphodiesterase activity between 2- and 4-fold a concentration of 500 μM. These results therefo indicate that the compounds inhibited calmoduli stimulated phosphodiesterase activity as a result calmodulin antagonism.

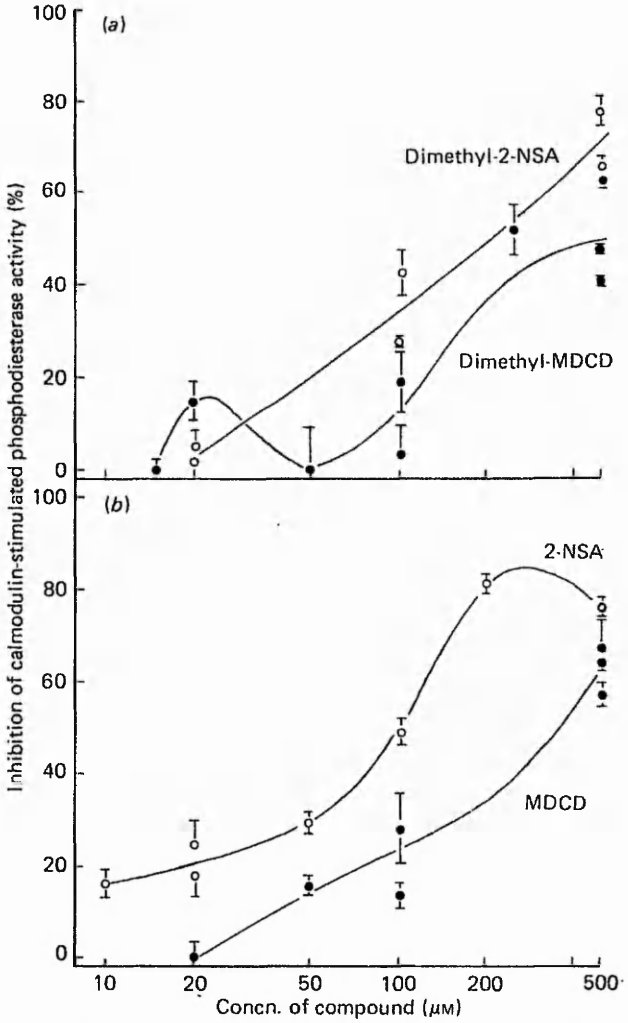


Fig. 2. Inhibition of calmodulin-stimulated phosphodiesterase activity by naphthalenesulphonamides

Calmodulin-stimulated phosphodiesterase activity was determined as described in the Experimental section in both the presence and the absence of the concentrations of compounds shown in the Figure. By reference to control rates of [^{3}H]adenosine production, results (obtained from three separate experiments) were expressed as a mean percentage (±S.E.M.) inhibition of enzyme activity, with each point the mean of three determinations. (*a*) ○, Dimethyl-2-NSA; ●, dimethyl-MDCD; (*b*) ●, MDCD; ○, 2-NSA. Typical control rates (±S.E.M.) representing 80% stimulation of maximal activity were 48.0±1.6 (n = 12) nmol of cyclic AMP hydrolysed/h.

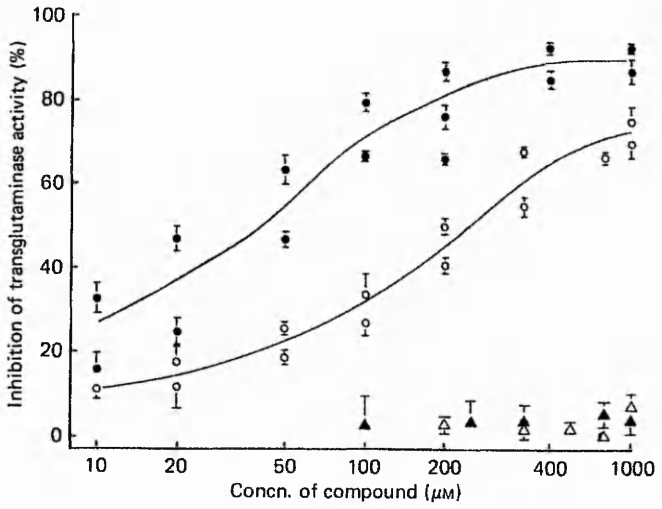


Fig. 3. Inhibition of islet transglutaminase activity by naphthalenesulphonamides

Transglutaminase activity in islet extracts was measured by [^{14}C]putrescine incorporation into *NN'*-dimethylcasein as described in the Experimental section in the presence and absence of the concentrations of MDCD (●), 2-NSA (○), dimethyl-MDCD (▲) and dimethyl-2-NSA (△) shown in the Figure. Rates of putrescine incorporation were calculated and, by reference to control values obtained in the same experiment, results were expressed as a mean (±S.E.M.) percentage inhibition of enzyme activity. All points are the mean of three determinations, and those for 2-NSA and MDCD represent data taken from three separate experiments. Typical control rates (±S.E.M.) for putrescine incorporation into *NN'*-dimethylcasein were 0.90±0.11 nmol of putrescine/h (n = 22).

When the same compounds were tested for their effects on islet transglutaminase activity, MDCD was found to be a potent inhibitor (IC_{50} = 43.3 μM; K_i = 9.8 μM) compared with 2-NSA (IC_{50} = 252 μM; K_i = 62.6 μM), a result comparable with those obtained by Lorand *et al.*, (1979), when these compounds were tested for their effects on guinea-pig liver transglutaminase. The two dimethylated analogues of these compounds did not significantly inhibit islet transglutaminase activity below concentrations of 1 mM (Fig. 3).

Reference to the data on insulin release (Table 1) reveals that the inhibition of transglutaminase activity by MDCD and 2-NSA and their respective dimethylated analogues correlated well with the potency with which they inhibited insulin release. For 2-NSA, the potency of the effect shown by this compound in its ability to inhibit the activation of phosphodiesterase by calmodulin suggests an overlap in the inhibitory action of the compound. However, such an overlap was not evident for MDCD, since the IC_{50} for calmodulin inhibition and the IC_{50} and K_i for transglutaminase inhibition were an order of magnitude apart. Furthermore, concentrations of MDCD could be defined (30–50 μM) that gave rise to significant inhibition of glucose-stimulated insulin release, but had little or no effect on the activation of phosphodiesterase by calmodulin.

Effect of MDCD on total islet methylation

It has been reported that MDCD incubated with neutrophils and fibroblasts caused an inhibition of phospholipid methylation, representing a non-specific action of the compound (Mato *et al.*, 1983). Phospholipid methylation has been shown to occur in islets, and its inhibition may lead to inhibition of glucose-stimulated insulin release (Best *et al.*, 1984). Therefore, as a further test for the specificity of MDCD as a probe for investigating transglutaminase involvement in glucose-stimulated insulin release, we investigated the effect of MDCD on total islet lipid methylation. Groups of 50–100 islets were preincubated for 45 min in the absence or presence of MDCD (50 μM), and the incorporation of methyl groups from [*methyl*-^{3}H]methionine into acidic

Table 3. Inhibition of transglutaminase, protein kinase C and calmodulin-stimulated phosphodiesterase activity by naphthalenesulphonamides

IC_{50} data represent the mean values calculated from three separate experiments by using the data shown in Figs. 2 and 3. The ranges of values obtained from these experiments are shown in parentheses. K_i values for inhibition of transglutaminase activity were calculated from the equation

$$K_i = \frac{[I]_{0.5}}{1+(s/K_m)}$$

by using a K_m of 0.59 mM for putrescine incorporation into *NN'*-dimethylcasein (Bungay *et al.*, 1984*a*). The $[I]_{0.5}$ values (concentration for half-maximal inhibition) used were the mean values calculated from the slopes of Eadie–Hofstee plots of fractional inhibition against fractional inhibition/inhibitor concentration, and obtained from three experiments by using the data shown in Fig. 3. $[I]_{0.5}$ (±S.E.M.) for MDCD = 29.9±11.1 μM and for 2-NSA = 190±20.6 μM. Numbers in square brackets show the percentage inhibition at the concentration shown.

Compound	IC_{50} (μM) or K_i* (μM)			
	Transglutaminase		Phosphodiesterase activation by calmodulin	Protein kinase C
Monodansylcadaverine	43.3 (34–56)	9.8*	387 (380–400)	> 1000 [0%]
N-Dimethylmonodansylcadaverine	> 1000 [6%]		> 500	> 1000 [2.1%]
N-(5-Aminopentyl)-2-naphthalenesulphonamide	252 (200–300)	62.5*	105 (90–110)	> 2500 [25%]
N-(5-Dimethylaminopentyl)-2-naphthalenesulphonamide	> 5000 [7%]		275 (210–370)	> 1000 [21%]

chloroform/methanol extracts of islets was determined after a further incubation for 60 min under glucose-stimulated conditions (16.8 mM-glucose), also in the absence or presence of MDCD. Comparison of the incorporation of radiolabel in control incubations of 29508±4118 d.p.m./h per 100 islets ($n = 5$) with the value obtained for islets incubated in the presence of 50 μM-MDCD, 34315±3321 d.p.m./h per 100 islets ($n = 5$) indicated that no significant inhibition of islet lipid methylation was caused by the compound (Student's *t* test). Therefore inhibition of glucose-stimulated insulin release by MDCD is unlikely to be the result of inhibition of islet phospholipid methylation.

Ca^{2+}-sensitivity of islet transglutaminase

Important to the evidence for the involvement of islet transglutaminase in insulin release is its sensitivity to activation by Ca^{2+}. Results from our laboratory (Hand *et al.*, 1985) have indicated that the use of dephosphorylated *NN'*-dimethylcasein in the enzyme assay is essential in order to obtain a true reflection of the activation of transglutaminase by Ca^{2+}, since significant amounts of Ca^{2+} can be bound by protein-bound phosphate groups. Therefore, in order to reassess the responsiveness of islet transglutaminase to Ca^{2+}, extracts of islets were incubated with dephosphorylated *NN'*-dimethylcasein in the presence of concentrations of free Ca^{2+} in the range 3.2–50.6 μM. Under these conditions, islet transglutaminase was found to be sensitive to concentrations of Ca^{2+} below 10 μM, and had a K_m for Ca^{2+} of 3.2 μM (Fig. 4). This value is a factor of 10 or so lower than values obtained previously (Bungay *et al.*, 1984*a*; Gomis *et al.*, 1983). This discrepancy probably reflects the use of a phosphorylated protein substrate in the enzyme assay before the present study.

Since Mg^{2+} is reported to be present in the cell at concentrations at least an order of magnitude higher than Ca^{2+} (Veloso *et al.*, 1973), it was feasible that this cation could affect the Ca^{2+}-sensitivity of islet transglutaminase. The use of dephosphorylated *NN'*-dimethylcasein allowed us to determine the effect of Mg^{2+} on the Ca^{2+}-sensitivity of the enzyme in the absence of any interference by Mg^{2+} through displacement of Ca^{2+} from protein-bound phosphate groups (Hand *et al.*, 1985). Measurement of the Ca^{2+}-sensitivity of islet transglutaminase in the presence of 2 mM-$MgCl_2$ revealed a K_m for Ca^{2+} of 2.2 μM (results not shown). Therefore Mg^{2+} appeared to have little effect on the Ca^{2+}-sensitivity of islet transglutaminase.

Identification of protein substrates for transglutaminase in islet homogenates

Experiments were carried out to determine which proteins in islets may be modified by transglutaminase action in the β-cell. Homogenates of islets were prepared and incubated with [^{14}C]methylamine in order to label proteins containing γ-glutamyl residues available for transglutaminase-catalysed reactions. Firstly, homogenates prepared by disruption of islets with a Potter–Elvehjem homogenizer were incubated with [^{14}C]methylamine in the presence of either 1.9 mM-Ca^{2+} or 5 mM-EGTA for 120 min. Homogenates labelled in this way with [^{14}C]methylamine were fractionated by centrifugation at 71000 g_{av} for 45 min, and the trichloroacetic acid-insoluble radioactivity in the pellet and supernatant was determined. Comparison of the Ca^{2+}-dependent labelling ($n = 3$) of the pellet (9371±373 c.p.m.) and supernatant (732±290 c.p.m.) fractions demonstrated that 93% of the label was present in the pellet fraction, indicating that the proteins labelled by the action of islet transglutaminase were probably membrane-associated.

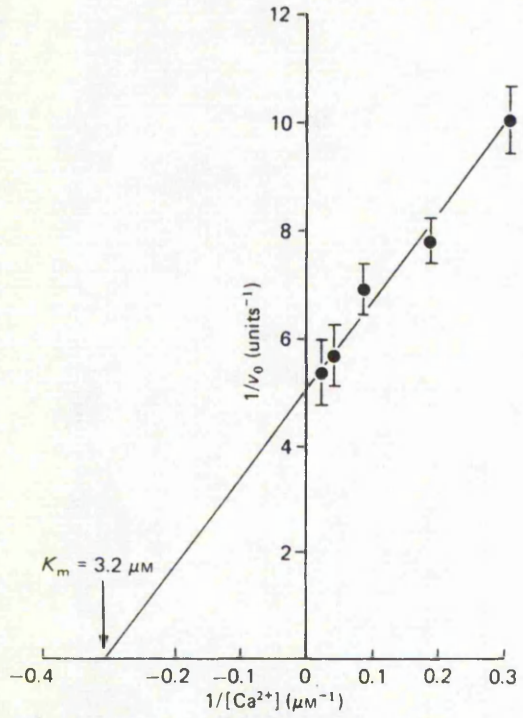


Fig. 4. Activation of islet transglutaminase by Ca^{2+}

Transglutaminase activity in islet extracts was measured by [^{14}C]putrescine incorporation into dephosphorylated *NN'*-dimethylcasein in the presence of 3.22, 5.45, 12.14, 26.1 and 50.6 μM free Ca^{2+} as described in the Experimental section. After calculation of rates of putrescine incorporation, data were treated to give a double-reciprocal plot. One unit of enzyme activity was equal to 1 nmol of putrescine incorporated/h. Points are means (±S.E.M.) for three determinations.

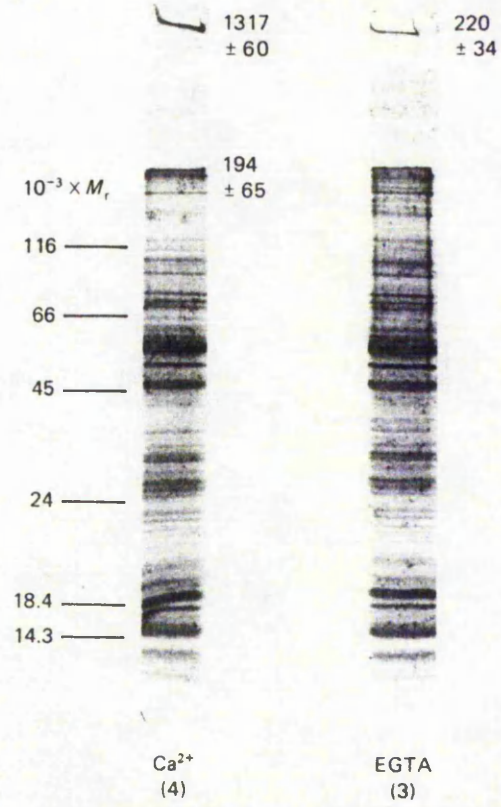


Fig. 5. Labelling of islet homogenate proteins wit [^{14}C]methylamine

Islet homogenates were incubated with [^{14}C]methylamine as described in the Experimental section. The samples were dissolved in electrophoresis sample buffer and electrophoresed by the method of Laemmli (1970). Stained gels were sectioned every 2 mm, apart from the stacking gel, which was treated as one unit, and the amount of radioactivity in the sections was determined. The photographs show examples of protein staining obtained from homogenates. The radioactivity (c.p.m.) incorporated was standardized per 50 μl incubation sample and is shown at the points indicated in the electrophoretograms. Amounts of radioactivity equal to or below control values are not shown. The bars indicate the mobility of standard M_r protein markers, which were electrophoresed under identical conditions. The number of experiments is shown in parentheses under each gel.

Further analysis of the labelled islet proteins was carried out by SDS/polyacrylamide-gel electrophoresis. Separation of islet proteins from homogenates incubated in the presence of [^{14}C]methylamine and 1.9 mM-Ca^{2+} for 120 min revealed that most of the radiolabel incorporated remained in the stacking gel, with a much smaller proportion remaining at the top of the resolving gel (Fig. 5). This labelling was inhibited by EGTA (5 mM) or by iodoacetamide (1.0 mM) in the incubation mixture. Furthermore, with homogenates incubated with 1.9 mM–Ca^{2+}, stained material of a very high M_r, presumably of polymeric nature, could be discerned at the top of the stacking gel. This material could not be discerned with homogenates incubated with either EGTA (5 mM) or iodoacetamide (results not shown). The patterns of bands observed after staining of the gels were otherwise similar regardless of the incubation conditions used. These results indicate that both the incorporation of [^{14}C]methylamine and the formation of the high-M_r material were a result of islet transglutaminase activity.

In an attempt to identify the individual proteins labelled by [^{14}C]methylamine in the absence of their cross-linking and incorporation into high-M_r material, various modifications were made to the incubation conditions. Firstly, the free Ca^{2+} concentration in the incubation mixture was decreased to 26.7 μM, which resulted in a decrease both in the incorporation of [^{14}C]methylamine and in the visibility of the high-M_r material in the stacking gel. However, all the radiolabe incorporated was found to be present in the stacking ge Secondly, a time-course experiment was undertake whereby incubations in the presence of 1.9 mM-Ca^{2+} wer carried out for 15 min or 30 min and compared wit incubations carried out for 120 min. Although decrease in the amount of labelling and polymer were apparent i the 15 min and 30 min samples, the label was detectabl mainly in the stacking gel and to a much lesser extent i the top of the resolving gel (results not shown).

DISCUSSION

The potency with which naphthalenesulphonamide inhibited glucose-stimulated insulin release appeared t depend on the possession by these molecules of a primar amine group. Thus MDCD was effective in the inhibitio

of insulin release (33–86% inhibition) in the concentration range 30–100 μM, whereas its dimethylated analogue failed to inhibit glucose-stimulated insulin release at concentrations as high as 400 μM. Comparison of the compounds possessing a primary amine group indicated that MDCD was a more effective inhibitor of glucose-stimulated insulin release than 2-NSA. At first glance, these findings correlate with the effects of these compounds on islet transglutaminase activity. However, further studies indicated that, with 2-NSA, more than one cellular process may be sensitive to this compound. Although islet glucose oxidation was not inhibited by 100 μM-NSA, the effects of the compound on the biological activity of calmodulin measured *in vitro* indicated that 2-NSA may not be specific in its action within islet cells. The sensitivity of calmodulin to antagonism by 2-NSA suggests that calmodulin may be the prime (though not the sole) target for this compound in the β-cell. Similarly, inhibition of insulin release by dimethyl-2-NSA at a concentration of 400 μM may be attributable to calmodulin antagonism. In this case, however, it is possible that dimethyl-2-NSA specifically inhibits calmodulin in the β-cell, since glucose oxidation, protein kinase C activity and transglutaminase activity were all unaffected by 400 μM-dimethyl-2-NSA.

In contrast with 2-NSA, MDCD gave significant inhibition of glucose-stimulated insulin release (33–65%) at low concentrations of the drug (30–50 μM) such that a specific mode of action for MDCD as an inhibitor of transglutaminase may be inferred. Thus we have previously shown that at 100 μM MDCD did not interfere with islet glucose oxidation (Bungay *et al.*, 1984*a*), and in addition the present findings suggest that MDCD (50 μM) did not significantly affect islet lipid methylation, nor did it affect the activity of protein kinase C measured *in vitro*. In common with its analogue, 2-NSA, MDCD was found to act as an antagonist of calmodulin, as measured in a standard assay using the calmodulin-sensitive phosphodiesterase. However, the potency of this effect was almost an order of magnitude lower than that measured for the effect of MDCD on islet transglutaminase. Thus concentrations of MDCD could be defined (30–50 μM) which gave rise to significant inhibition of glucose-stimulated insulin release, but which had little ($< 20\%$) or no effect on calmodulin-stimulated phosphodiesterase activity. The specificity of MDCD for islet transglutaminase thus demonstrated is therefore compatible with a role for this enzyme in the mechanism of glucose-stimulated insulin release. Furthermore, our results demonstrate that, within the naphthalenesulphonamide family of compounds, structural variations in the parent molecule can lead to substantial differences in their potency as inhibitors of various cellular targets which may lead to inhibition of glucose-stimulated insulin release.

A large body of evidence supports the view that a rise in the cytosolic concentration of Ca^{2+} is responsible for eliciting the secretory response of the β-cell to glucose (Prentki & Wollheim, 1984). The magnitude of this rise in intracellular [Ca^{2+}] has been estimated to be in the order of 0.1 μM, so that the concentration of free Ca^{2+} in the stimulated β-cell may reach approx. 0.2 μM (Wollheim & Pozzan, 1984; Deleers *et al.*, 1985). Therefore, for islet transglutaminase to act as a Ca^{2+}-receptor protein during β-cell activation, this enzyme must be sensitive to small changes in the free [Ca^{2+}] in the β-cell. Previous studies on the islet transglutaminase have suggested t half-maximal enzyme activity may occur between 40 a 90 μM-Ca^{2+} (Bungay *et al.*, 1984*a*; Gomis *et al.*, 198 However, these estimates were based on assays conduc with native *NN'*-dimethylcasein as the protein substra under which conditions the actual free [Ca^{2+}] may overestimated as a result of the binding of the cation protein-bound phosphate groups (Hand *et al.*, 198 Similarly, interference of this kind also provi difficulties in assessing the effects of other cellular cati (such as Mg^{2+}) on the sensitivity of transglutaminase Ca^{2+}. In the present paper we measured the sensitivity the islet transglutaminase to Ca^{2+}, using a dephospho lated *NN'*-dimethylcasein substrate. Under these m authentic conditions, the islet transglutaminase found to be at least an order of magnitude more sensit to Ca^{2+} than had been previously demonstrated, wit K_m for Ca^{2+} in the region of 2–3 μM. Furthermore, presence of Mg^{2+} (2 mM) appeared to have little effect the sensitivity of the enzyme to Ca^{2+}. Therefore Mg which is present in cells at millimolar concentrations unlikely to affect the sensitivity of islet transglutamin to activation by Ca^{2+}. The sensitivity of islet transgl aminase to Ca^{2+} is comparable with that measured the phospholipid- and Ca^{2+}-dependent protein kina (protein kinase C) obtained from islets, which posses K_m for Ca^{2+} of 3.9 μM (Lord & Ashcroft 1984), a from bovine heart, for which a K_m for Ca^{2+} of 5 was measured (Wise *et al.*, 1982*a*). Our results theref suggest that islet transglutaminase may be sufficien sensitive to activation by Ca^{2+} for this enzyme to direc act as a receptor for Ca^{2+} in the stimulated β-cell.

It is therefore proposed that, during stimulus–secret coupling in the pancreatic β-cell, islet transglutamin may be activated by a rise in the free [Ca^{2+}] and that result of the activation of this enzyme may be post-translational modification of specific β-cell prote which participate in the secretory mechanism. We h previously shown that primary amines such as meth amine and putrescine may be incorporated into i proteins both *in vitro* and in the intact islet (Bung *et al.*, 1984*b*). In the present work, we investigated th proteins further, using islet homogenates incubated w [^{14}C]methylamine. Crude subcellular fractionation these homogenates into a particle-free supernatant an 71 000 *g*-sedimentable particulate (membranous) fract indicated that most (93%) of the Ca^{2+}-depend incorporation of radiolabel occurred in the membr fraction. This finding is in keeping with other reports the specificity of the tissue transglutaminase in cross-linking of membrane or membrane-associa proteins (Bjerrum *et al.*, 1981; Pober & Strominger, 19 Birckbichler *et al.*, 1972).

Attempts to resolve the islet proteins labelled transglutaminase by using SDS/polyacrylamide-gel el trophoresis indicated that most of the incorporation [^{14}C]methylamine occurred in proteins which did traverse, but remained in, the stacking gel (3 acrylamide) during electrophoresis. These protei therefore possessed a very high M_r, probably in excess 10^6. In some incubations, a much smaller proportion radiolabel was detectable at the top of the running (10% acrylamide), suggesting an M_r for these protein excess of 250000, as was the case for incubations carr out with 1.9 mM-Ca^{2+} for 120 min. These experime also indicated that, the greater the Ca^{2+} concentration

the longer the incubation period, the greater was the amount of polymeric material and extent of radiolabelling of this material. Since this material was not detectable in homogenates of islets which were incubated in the absence of Ca^{2+}, it seems unlikely that large amounts of this material are present in the β-cell. However, these studies demonstrate the ability of the islet transglutaminase to cross-link islet proteins which appear to be membrane-associated. Furthermore, the absence of any incorporation of methylamine into individual islet proteins involved in the formation of the polymer may suggest that the islet transglutaminase possesses a high specificity for the cross-linking of these proteins. Alternatively, these substrate proteins may already be associated in a non-covalent matrix which may be cross-linked covalently by the action of transglutaminase. Such a mechanism is comparable with that found in the human erythrocyte, where both integral membrane proteins and the associated membrane infrastructures may become cross-linked into a high-M_r polymer under the action of erythrocyte transglutaminase (Bjerrum *et al.*, 1981). Although it seems unlikely that such a drastic modification to the β-cell membrane and associated proteins would play in glucose-stimulated insulin release, these observations *in vitro* may represent the extreme of what may be a carefully regulated process *in vivo*.

In conclusion, our results detailing (1) the specificity of MDCD as an inhibitor of transglutaminase in the β-cell, (2) the sensitivity of islet transglutaminase to activation by Ca^{2+} and (3) the substrate specificity of islet transglutaminase are consistent with a role for this enzyme in glucose-stimulated insulin release. Furthermore they add further evidence to our original proposal (Bungay *et al.*, 1984*a*) that the role of the islet transglutaminase in insulin secretion may be in the cross-linking of membrane proteins and/or membrane infrastructures, a process which may serve to selectively stabilize areas of membrane during membrane recycling.

This work was funded by the Wellcome Trust and by the Sir Halley Stewart Trust, to whom we are grateful. We are also grateful to Dr. S. MacNeil for helpful discussions and for assistance with the screening of test compounds for calmodulin antagonism.

REFERENCES

Best, L., Lebrun, P., Saceda, M., Garcia-Morales, P., Hubinont, C., Juvent, M., Herchuelz, A., Malaisse-Lagae, F., Valverde, I. & Malaisse, W. J. (1984) Biochem. Pharmacol. **33**, 2033–2039

Birckbichler, P. J., Dowben, R. M., Matacic, S. S. & Loewy, A. G. (1972) Biochim. Biophys. Acta. **291**, 149–155

Bjerrum, O. J., Hawkins, M., Swanson, P., Griffin, M. & Lorand, L. (1981) J. Supramol. Struct. **16**, 289–301

Bungay, P. J., Potter, J. M. & Griffin, M. (1982) Diabetologia **23**, 159

Bungay, P. J., Potter, J. M. & Griffin, M. (1984*a*) Biochem. J. **219**, 819–827

Bungay, P. J., Potter, J. M. & Griffin, M. (1984*b*) Biosci. Rep. **4**, 869–877

Cooke, R. D. & Holbrook, J. J. (1974) Biochem. J. **141**, 71–7

Davies, P. J. A., Davies, D. R., Levitzki, A., Maxfield, R. R Milhaud, P., Willingham, M. C. & Pastan, I. (1980) Natur (London) **283**, 162–167

Deleers, M., Mahy, M. & Malaisse, W. J. (1985) Biochem. In **10**, 97–103

Fésüs, L., Sándor, M., Horváth, L. I., Bagyinka, C., Erdei, A & Gergely, J. (1981) Mol. Immunol. **18**, 633–638

Folk, J. E. & Finlayson, J. S. (1977) Adv. Protein. Chem. **31** 1–133

Gagliardino, J. J., Harrison, D. E., Christie, M. R., Gagliardino E. E. & Ashcroft, S. J. H. (1980) Biochem. J. **192**, 919–927

Gomis, R., Sener, A., Malaisse-Lagae, F. & Malaisse, W. J (1983) Biochim. Biophys. Acta **760**, 384–388

Günzler, V., Schopf, R. E., Hanauske-Abel, H. M. & Schulte Wissermann, H. (1982) FEBS Lett. **150**, 390–396

Hand, D., Bungay, P. J., Elliott, B. M. & Griffin, M. (1985 Biosci. Rep. **5**, 1079–1086

Hedeskov, C. J. (1980) Physiol. Rev. **60**, 442–509

Heding, L. (1972) Diabetologia **8**, 260–266

Hidaka, H. & Tanaka, T. (1983) Methods Enzymol. **102** 185–194

Julian, C., Speck, N. A. & Pierce, S. K. (1983) J. Immunol. **130** 91–96

Krebs, H. A. & Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol Chem. **210**, 33–66

Lacy, P. E. & Kostianovsky, M. (1967) Diabetes **16**, 35–39

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lorand, L. & Conrad, S. M. (1984) Mol. Cell Biochem. **58**, 9–3

Lorand, L., Campbell-Wilkes, L. K. & Cooperstein, L. (1972 Anal. Biochem. **50**, 623–631

Lorand, L., Parameswaran, K. N., Stenberg, P., Tong, Y. S. Velasco, P. T., Jönsson, N. A., Mikiver, L. & Moses, P (1979) Biochemistry **18**, 1756–1765

Lord, J. M. & Ashcroft, S. J. H. (1984) Biochem. J. **219** 547–551

Lott, W. A. & Krapcho, J. (1957) U.S. Patent 2,813,904

MacNeil, S., Walker, S. W., Senior, H. J., Bleehen, S. S. & Tomlinson, S. (1984) J. Invest. Dermatol. **83**, 15–19

Mato, J. M., Pencev, D., Vasanthakumar, G., Schiffmann, E & Pastan, I. (1983) Proc. Natl. Acad. Sci. U.S.A. **80** 1929–1932

Pober, J. A. & Strominger, J. L. (1981) Nature (London) **289** 819–821

Prentki, M. & Wollheim, C. B. (1984) Experientia **40** 1052–1060

Rasmussen, H. & Barrett, P. O. (1984) Physiol. Rev. **64**, 938–984

Schatzman, J. H. (1973) J. Physiol. (London) **235**, 551–569

Sener, A., Gomis, R., Lebrun, P., Herchuelz, A., Malaisse Lagae, F. & Malaissee, W. J. (1984) Mol. Cell. Endocrinol **36**, 175–180

Van Leuven, F., Cassiman, J. J. & Van Den Berghe, H. (1980 Cell **20**, 37–43

Veloso, D., Guynn, R. W., Oskarsson, M. & Veech, R. L (1973) J. Biol. Chem. **248**, 4811–4819

Wani, M. C., Campbell, H. F., Brine, G. A., Kepler, J. A. & Wall, M. E. (1972) J. Am. Chem. Soc. **94**, 3631–3632

Wise, B. C., Raynor, R. L. & Kuo, J. F. (1982*a*) J. Biol. Chem **257**, 8481–8488

Wise, B. C., Glass, D. B., Jen Chou, C. H., Raynor, R. L. Katoh, N., Schatzman, R. C., Turner, R. S., Kibler, R. F. & Kuo, J. F. (1982*b*) J. Biol. Chem. **257**, 8489–8495

Wollheim, C. B. & Sharp, G. W. G. (1981) Physiol. Rev. **61** 914–973

Wollheim, C. B. & Pozzan, T. (1984) J. Biol. Chem. **259** 2262–2267

Received 21 August 1985/20 November 1985; accepted 5 December 1985