THE ROLE OF TRANSGLUTAMINASE IN THE PANCREATIC B-CELL

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ABSTRACT

The Role of Transglutaminase in the Pancreatic B-cell

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Electropermeabilised rat islets were employed to investigate a role for transglutaminase as a Ca^{2+} target during stimulus-secretion coupling. Incubation of electropermeabilised islets in the presence of the primary amine inhibitors of transglutaminase, methylamine, cystamine, glycine methyl ester and monodansylcadaverine inhibited Ca^{2+}-stimulated insulin release. In all cases except for monodansylcadaverine, which appeared to have caused maximum inhibition at the lowest concentration employed, the inhibition was dose-dependent. Incubation with the control compounds of monodansylcadaverine and glycine methyl ester, dimethyl-monodansyl-cadaverine and sarcosine methyl ester respectively, which lack the primary amine group necessary for inhibition, had no effect on Ca^{2+}-stimulated insulin release. The primary amines also failed to inhibit basal insulin release from electropermeabilised islets. These studies suggests a role for transglutaminase during Ca^{2+}-stimulated insulin release in electropermeabilised islets.

Incubation of electropermeabilised islets with monodansyl-cadaverine and glycine methyl ester failed to inhibit insulin release stimulated by either the protein kinase C activator, phorbol myristate acetate or cyclic AMP in the presence of the phosphodiesterase inhibitor, 3-iso-butyl-1-methylxanthine or arginine. These result suggest that transglutaminase acts at stage prior to these stimulators or that different insulin secretory mechanisms exist.

Using [^{14}C]-methylamine incorporation into islet proteins by transglutaminase as a probe, attempts were made to identify transglutaminase substrates using both homogenised and electropermeabilised islets. In homogenised islets the major substrate was confirmed as being a high molecular weight polymer which was unable to traverse a 3%(w/v) acrylamide gel following SDS-polyacrylamide gel electrophoresis. The polymer was predominantly associated with 71,000g av. particulate fraction upon subcellular fraction. Studies using electropermeabilised islets showed that three proteins of approximate molecular weights 78kDa, 34kDa and 32kDa were labelled at Ca^{2+} concentrations required for insulin release whilst the high molecular weight polymer was labelled at supra-physiological concentrations of Ca^{2+} (100μM and 1mM) which correlated with the loss of insulin release.

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 the assistance received.

Signed M A Lindsay .....

(Candidate)

Signed .....

(Director of Studies)

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DEDICATION

With love to Joanne Whetstone

PUBLICATIONS AND PRESENTATIONS

1. Griffin, M., Lindsay, M.A. and Bungay, P.J.
   Characterisation of the endogenous substrates of the pancreatic islet transglutaminase
   Diabetologia 31 (7) : 497A, 1988.

2. Lindsay, M.A., Bungay, P.J. and Griffin, M.
   Transglutaminase is important in the Ca^{2+}-induced events associated with the release of insulin from the pancreatic B-cell.
   Europ. J. Clin. Investigation. 19: 27, 1989.

3. Lindsay, M., Bungay, P.J. and Griffin, M.
   The importance of transglutaminase in Ca^{2+}-induced insulin release from electropermeabilised islets of Langerhans.
   Diabetologia 32 (7) : 510A-511A, 1989.

4. Lindsay, M., Bungay, P.J. and Griffin, M.
   Identification of the endogenous substrates of the pancreatic islet transglutaminase
   Biochem. Soc. Trans. 18 : 465-466, 1990.

5. Lindsay, M.A., Bungay, P.J. and Griffin, M.
   Transglutaminase involvement in the secretion of insulin from electropermeabilised rat islets of Langerhans.
   Bioscience Reports 10: 557-561, 1990.

ABBREVATIONS

ATP	Adenosine 5'-Trisphosphate
BSA	Bovine Serum Albumin
cAMP	Adenosine 3'-5'-cyclic monophosphate
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminetetra-acetic Acid
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic Acid
GME	Glycine Methyl Ester
IBMX	3-isobutyl-1-methylxanthine
IAB	Insulin Assay Buffer
MAP's	Microtubule Associated Proteins
MDCD	Monodansylcadaverine
Dimethyl-MDCD	Dimethylmonodansylcadaverine
NADH	Nicotinamide Adenine Dinucleotide (Reduced Form)
PAGE	Polyacrylamide Gel Electrophoresis
PFS	Particle Free Supernatant
PMA	Phorbol 12-Myristate 13-Acetate
PMSF	Phenylmethanesulfonyl Fluoride
SDS	Sodium Dodecyl Sulfate
SME	Sarcosine Methyl Ester
TCA	Trichloroacetic Acid
TEMED	N,N,N',N'-Tetramethylethylenediamine

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1 INTRODUCTION

1.1 INTRODUCTION TO THE TRANSGLUTAMINASES

1.1.1 Definition of Transglutaminases

A Ca^{2+}-dependent transamidating activity in guinea pig liver was first described by Sarker et al (1957). Since these early investigations a large volume of work has been published on these enzymes and they have been the subject of a number of reviews (Chung, 1972; 1975; Folk and Chung, 1973; Lorand and Stenberg, 1976; Folk and Finlayson, 1977; Folk, 1980; Williams-Ashman and Canellakis, 1980; Folk, 1983; Lorand and Conrad, 1984; Conrad, 1985).

At present, a transglutaminase is defined as a calcium dependent enzyme with an active site thiol which catalyses an acyl transfer reaction between peptide-bound glutamine residues and primary amine groups. This results in the post-translational modification of proteins either by the incorporation of amines or, if the amine is the ε-amino group of peptide bound lysine, the crosslinking of proteins via $\varepsilon(\gamma$-glutamyl)lysine bridges. Since it is peptide bound glutamine and not free glutamine which participates in the reaction, the name transglutaminase is a misnomer. Therefore, the enzyme commission recommends the systematic name R-glutaminyl-peptide:amine-γ-glutamyl transferase (E.C.2.3.2.13).

1.1.2 Classification of Transglutaminases

Transglutaminases have been reported both intracellularly and extracellularly in a wide variety of tissues (Folk, 1980; Conrad, 1985). It is now generally believed that the occurence of transglutaminases will prove to be ubiquitous in animal cells and tissues. At present these enzymes are classified according to their

localization and their physical, chemical, immunochemical and catalytic properties (Chung, 1975). On this basis the following groups of transglutaminases have been identified:

1) Factor XIII

This type of transglutaminase is involved in the blood clotting cascade. It has so far been identified in plasma (Lorand et al, 1968), placenta (Bohn & Schwick, 1971), platelets (Schwartz et al, 1973) and granulocytes, monocytes and macrophages (Berntorp et al, 1985).

2) Hair Follicle Transglutaminase

This type of transglutaminase has been identified in Mammalian hair follicles (Harding & Rogers, 1972; Chung & Folk, 1972), and been shown to be immunologically distinct from other forms of transglutaminases (Chung, 1975).

3) Epidermal and Keratinocyte Transglutaminase

To date four transglutaminases of molecular weights 54kDa (Goldsmith et al, 1974), 72kDa (Negi et al, 1985), 120kDa (Ando et al, 1989) and 92kDa (Thacher & Rice, 1985) have been identified in epidermal tissue. It is thought that the 92kDa or keratinocyte transglutaminase (Type I enzyme) plays an important role in cornified envelope formation during the terminal differentiation of keratinocytes (Eckert, 1989). The function of the other epidermal transglutaminases is uncertain although the 54kDa enzyme crossreacts immunologically with enzymes located in rabbit tracheal epithelium (Jetten & Shirley, 1983) and in the upper digestive tract and lower female genital tract of the rat (Parenteau et al, 1986). This implies that this transglutaminase may not be restricted simply to epidermal tissue and that it might play an important role in all epithelial cells.

4) Tissue (Type II) Transglutaminase

This family of transglutaminases has been identified in many tissues including liver, muscle, kidney, lung, adrenal gland, brain, testis, pancreas, erythrocytes and the uterus (Chung, 1972; Lorand and Stenberg, 1976; Conrad, 1985). It has been reported that this enzyme coexists in epidermal tissue (Lichti et al, 1985) with the Type I enzyme and in hair follicles (Chung & Folk, 1972) along with the hair follicle enzyme.

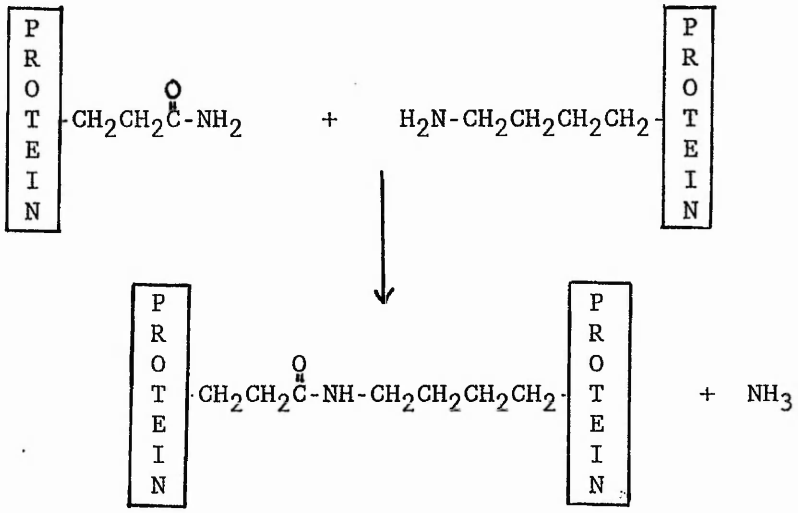
However, the classification of the transglutaminases in the above categories remains controversial. An example of this is the recent discovery of a particulate associated tissue transglutaminase in rat liver (Chang and Chung, 1986) and lung (Cocuzzi & Chung, 1986) which has not yet been tested for cross-reactivity with other particulate transglutaminases such as keratinocyte transglutaminase. Recently, Knight et al (1990a) have also demonstrated physical and immunological similarities between the cytosolic and particulate tissue transglutaminase.

1.1.3 Transglutaminase Catalysed Reactions

Although transglutaminases can catalyse a range of aminolytic and hydrolytic reactions, only three of these have been shown to occur in vivo and form the basis of the known physiological role of the enzyme (Figure 1.1). The three reactions involve the post-translational modification of proteins and result either in the crosslinking of proteins through the formation of ε-(γ-glutamyl) lysine bonds (Figure 1.1a) and the N,N-bis-(γ-glutamyl) polyamine derivative (Figure 1.1b), or the incorporation of primary amines into proteins (Figure 1.1c). The other reactions catalysed by transglutaminase

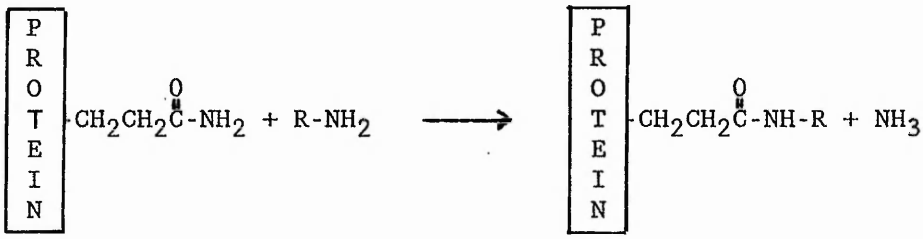
Figure 1.1 In Vivo Reactions Known to be Catalysed by Transglutaminase

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



(γ-glutamyl)lysine crosslink

B. Incorporation of primary amines into proteins :



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

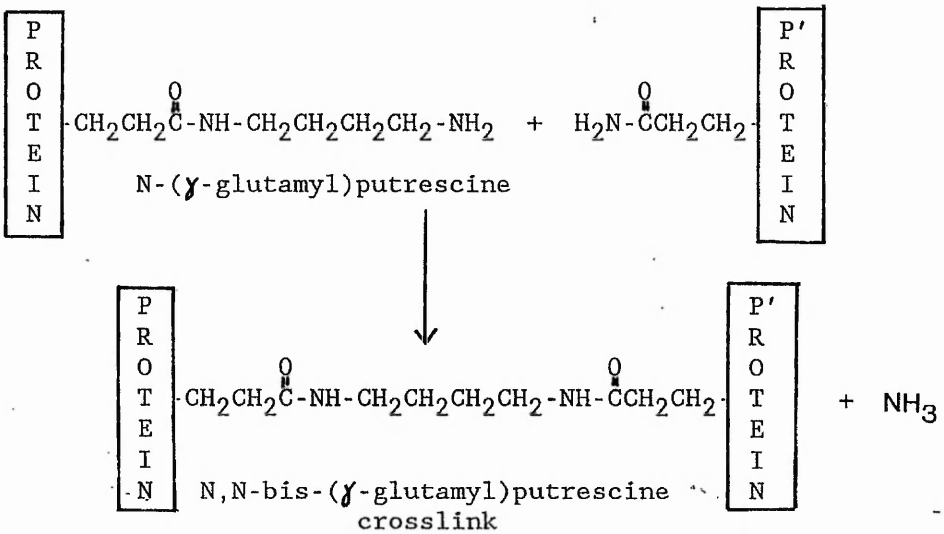


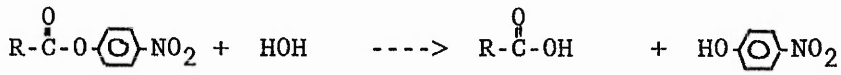
Table 1.1 Hydrolysis and Aminolysis Reactions Catalysed by Transglutaminase

1. Hydrolysis of peptide-bound glutamine

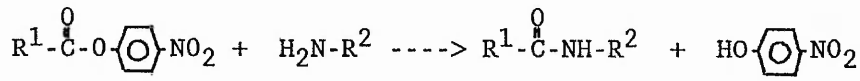
2. Hydrolysis of amides

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

involve either the aminolysis or hydrolysis of an amide or ester (Table 1.1).

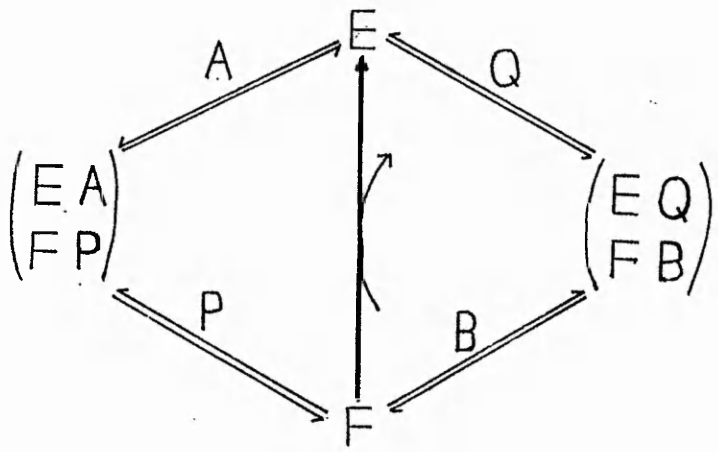
The incorporation of primary amines into proteins (Figure 1.1c) forms the basis of the most widely used assay of the different types of transglutaminases. This assay involves measuring the Ca^{2+}-dependent incorporation of radiolabelled primary amines (e.g. putrescine, methylamine or histamine) by transglutaminase into protein substrates (e.g. β-casein). This is done by TCA precipitation of proteins from the reaction mixture, followed by direct counting of protein bound radioactivity (Lorand et al, 1972). Blocking the ε-amino groups of lysine (by succinylation, methylation or acetylation) has been used recently since this prevents crosslinking of the protein substrates.

1.1.4 The Mechanism of Catalysis

The currently accepted model for the mechanism of catalysis by transglutaminases is a modified double-displacement (Figure 1.2). The evidence for this having been reviewed by Folk (1983). This mechanism envisages the binding of peptide bound glutamine to the enzyme active site cysteine residue to form a thioester intermediate, and the concomitant release of ammonia. This acyl-enzyme intermediate can then undergo nucleophilic attack either by water to form peptide bound glutamine, or by a primary amine such as putrescine, to form peptide-bound γ-glutamyl-putrescine.

This reaction is dependent on calcium ions, the binding of which is believed to result in small conformational changes in the enzyme struture which exposes the active site (Folk, 1983). The sequencing of Factor XIII (Ichinose & Davie, 1987; Putnam et al, 1987) has identified possible Ca^{2+} binding sites with homology to other Ca^{2+}-binding proteins such as calmodulin, although these sequences have not

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

been clearly identified in the sequence of tissue (type 2) transglutaminase (Ikura et al, 1987; 1988; Stein et al, 1987). Recently, Achyuthan & Greenberg (1987) have reported that tissue (type 2) transglutaminase binds to and is inhibited by GTP whilst Lee et al (1989) has shown that the enzyme possesses GTPase activity, suggesting that this type of transglutaminase may be regulated by both Ca^{2+} and GTP.

1.1.4.1 Substrate Specificity

To date substrate specificity studies have only been extensively performed on the guinea-pig liver transglutaminase and Factor XIII from plasma (Folk, 1983). However, these studies probably highlight some of the factors that are common to the biological function of all transglutaminases.

The substrate specificity has been investigated with respect to the following three factors:

i) **The Amide Site**

Waelsch and co-workers (1962) showed that only glutamine residues and not asparagine residues could act as substrates for transglutaminase. These conclusions were later confirmed by Chung et al (1970) whilst Gross & Folk (1973) and Chung (1975) have demonstrated that the enzyme exhibited stereospecificity for L-glutamine. Folk and Cole (1965; 1966) showed that only compounds blocked in the α-carbon NH_2 and COOH would act as substrates and that the enzyme preferred uncharged groups at or near the glutamine residue.

ii) **The Extended Glutamine Binding Site**

Chung et al (1970) demonstrated that substantial differences existed between the substrate specificity of liver transglutaminase and Factor XIII, the latter exhibiting a far greater specificity. Studies

by Gorman & Folk (1980, 1981) using peptide fragments of β-casein, showed that varying the amino-acid composition greatly affected the specificity of the enzyme. This work also suggested that the active site may interact with up to 10 amino acids and supported the idea that uncharged or hydrophobic amino-acids are important in substrate specificity.

iii) The Amine Site

Once the acyl-enzyme intermediate has been formed the thioester linkage may undergo hydrolysis by either water or a primary amine. Studies with a wide variety of primary amines showed lysine derivatives to be the most effective substrates for the enzyme (Gross et al, 1975; Lorand et al, 1979), and that the enzyme exhibited stereospecificity for the L-lysine derivative. Work using synthetic lysine containing peptides revealed that only those amino-acids adjacent to the lysine residue seemed to affect the interaction with enzyme (Gross et al, 1977).

Overall the studies using guinea-pig liver transglutaminase and Factor XIII suggest a common specificity of the enzymes for peptide bound glutamine (amide) and lysine (amine) but a difference in the specificity for the extented glutamine site, which may effect the biological function of the particular transglutaminase. However, the influence of substrate tertiary structure on catalysis, as well as the effect of quaternary structure, when both the enzyme and the two peptides (proteins) are bound, is completely unknown.

1.2 ROLE OF TRANSGLUTAMINASES

The biological role of some of the transglutaminases has been partially characterised and these are discussed in the first part of this section. However, the function of the tissue (type 2) transglutaminase is still uncertain. This enzyme has been proposed to have a variety of cellular functions and these are surveyed in the second part of the section.

1.2.1 Factor XIII

Activation of Factor XIII is known to be responsible for the stabilization of the fibrin clot during haemostasis. The inactive Factor XIII zymogen is composed of catalytic a-subunits of 75,000Da molecular weight and non-catalytic b-subunits of molecular weight 80,000Da, which associate to form an a_2b_2 structure (Siefring et al, 1978). The zymogen is activated by the calcium dependent protease thrombin, which catalyses the cleavage of a 4,000Da peptide from the a-subunit, to produce an $a_2'b_2$ structure (Takagi & Doolittle, 1974). This then separates in a Ca^{2+}-dependent manner (Lorand, 1974) to produce dimers of the a- and b-subunits (Chung et al, 1974). The limited proteolysis of the a-chain results in the unmasking of the active site cysteine, thereby permitting the a_2' dimer to act upon fibrinogen.

Fibrinogen, the substrate of Factor XIIIa, is composed of multiple polypeptides chains. Each of the polypeptides contains an α-β- and γ-subunit of molecular weights 70,000Da, 60,000Da and 50,000Da respectively, that are linked together by disulphide bonds (Mossesson & Finlayson, 1976). Thrombin dependent cleavage of the polypeptide results in the formation of an $(\alpha\beta\gamma')_2$ fibrin structures which assemble

in an uncatalysed reaction to form a gel. This fibrin gel is then crosslinked by Factor XIIIa to form a clot, initially through the rapid crosslinking of the γ-subunits, followed by the crosslinking of the α-subunits (Finlayson & Aronson, 1974). The β-subunits do not appear to be crosslinked.

Since the formation of the active enzyme Factor XIIIa and of its substrate fibrin, occurs mainly at the site of injury, this is thought to be one of the main mechanisms by which this process is regulated. However, fibrinogen has also been implicated in the regulatory process since it has been reported to increase the rate of cleavage by thrombin (Janus et al, 1983) and also lower the Ca^{2+} requirement for the dissociation of the modified Factor XIII zymogen ($a_2'b_2$) (Credo et al, 1978).

As well as in the plasma, Factor XIII has also been found in platelets, placenta, monocytes, macrophages and granulocytes (Conrad, 1985; Berntorp et al, 1985). Recently, Conkling et al (1989) showed that the enzyme is associated with the outer surface of the plasma membrane of human monocytes. This enzyme differs in that there appears to be no b-subunit present in the zymogen and in the case of the granulocyte enzyme, activation is not dependent upon thrombin (Berntorp et al, 1985). The function of these Factor XIII enzymes is uncertain although it is known that their release into plasma results in association with the b-subunit, thereby making them indistinguishable from the plasma enzyme (Folk & Finlayson, 1977). It has been proposed that these a_2 subunits either supplement the plasma enzyme or play some specialized role in wound healing.

As well as fibrinogen, several other plasma proteins including α_2-macroglobulin and fibronectin (Mosher, 1976), α_2-plasmin inhibitor (Sakata & Aoki, 1980; Carmassi & Chung, 1983), Factor V

(Francis et al, 1986), thrombosin (Bale & Mosher, 1986) and vitronectin (Sane et al, 1988), as well as apoliprotein B (Cocuzzi et al, 1990), have been reported as substrates of Factor XIIIa.

1.2.2 Hair Follicle Transglutaminase

Rogers & Springell (1959) first reported the presence of transamination and transpeptidation in wool which suggested that transglutaminase maybe found in the hair follicle. Chung & Folk (1972) eventually isolated two types of transglutaminases from homogenates of the inner sheath of the guinea-pig hair follicle. One of these was similar to the liver (type 2) transglutaminase. The other transglutaminase (hair follicle transglutaminase) which did not cross-react with antibodies to the liver enzyme, was found to have a molecular weight of 54kDa and to dissociate into two subunits of 27kDa molecular weight. Later studies have shown that the hair follicle transglutaminase is immunologically distinct from the epidermal transgutaminase (Buxman & Wuepper, 1976; 1978; Ogawa & Goldsmith, 1977).

The hair follicle transglutaminase is thought to crosslink precursor(s) into high molecular weight polymers within the inner root sheath and medulla of hair. A possible precursor is the citrulline-rich protein, trichohyalin. This is reported to have a high glutamic acid and low cysteine content, and to be crosslinked in vitro into a high molecular weight protein (Rogers et al, 1977). This proposal is supported by the earlier work of Harding & Rogers (1970; 1972) who demonstrated the presence of $\varepsilon(\gamma$-glutamyl)-lysine crosslinks in the citrulline-rich protein of the medulla and inner root sheaths of hair and reported enzyme activity in hair follicle homogenate which formed $\varepsilon(\gamma$-glutamyl)-lysine bonds.

1.2.3 Epidermal and Keratinocyte Transglutaminase

Following the discovery of the presence of transglutaminase in hair follicles, investigators began searching for similar activity in the epidermis itself. Goldsmith et al (1974) found transglutaminase activity in the epidermis associated with a protein of 54000Da molecular weight. This has since been confirmed from a variety of sources including bovine (Buxman & Wuepper, 1975), human (Ogawa & Goldsmith, 1975) and rat epidermis (Peterson & Buxman, 1981). This type of transglutaminase is reported to be immunologically distinct from Factor XIII, tissue transglutaminase and hair follicle transglutaminase (Ogawa & Goldsmith, 1977) and was named epidermal transglutaminase. Recently, several other transglutaminases with molecular weights 72kDa (Negi et al, 1985) and 120kDa (Ando et al, 1988) have been identified in the cytosolic fraction whilst a particulate transglutaminase of 92kDa has been isolated from cultured keratinocyte cells.

The keratinocyte transglutaminase has been shown to play an important role during the terminal differentiation of the keratinocytes of the epidermis (Eckert, 1989) and has been reported to be immunological distinct from other types of transglutaminases (Scott and Thacher, 1989). Thacher & Rice (1985) have shown that the enzyme exists both in a membrane associated and soluble form. However, the enzyme is thought to be primarily associated with the membrane where it is anchored by esterified fatty acids (Chakravarity & Rice, 1989). Recently, Rice et al (1990) have reported that the soluble enzyme, with a molecular weight of 80,000 Da, probably results from the proteolytic release of the particulate enzyme, which then associates to form dimers. These results have led to the suggestion that part of the

increase in activity seen during the terminal differentiation of the keratinocytes may result from proteolysis of the particulate keratinocyte enzyme. This process may also occur with the other epidermal transglutaminases. The activity of the 55kDa and 120kDa enzyme is increased 20 fold following proteolytic cleavage by calpain (Ando et al, 1988) whilst Negi et al (1985) has demonstrated a similar affect of trypsin on the 72kDa transglutaminase.

The terminal differentiation of the keratinocytes, which account for >90% of the cells of the epidermis, results in the formation of a layer of non-living, biochemically inert, barrier cells in which the organelles have been removed by digestive processes, and the remaining proteins stabilized by crosslinking reactions (Ecbert, 1989). The crosslinking of the major protein components of the cytosol, the keratins, is thought to result primarily from oxygen-mediated disulphide bonding following permeabilisation of the plasma membrane (Sun & Green, 1978). However, Rice and Green (1979) have reported that the transglutaminase catalyses the extensive crosslinking of proteins to form the characteristic dense accretion on the inner surface of the plasma membrane, called the marginal band (Farbman, 1966; Hasimoto, 1969). This polymer is thought to be made up from the crosslinking by keratinocyte transglutaminase of a variety of proteins including involucrin (Rice & Green, 1979), keratolinin (Zettergren et al, 1984), several membrane-bound proteins of high molecular weight (Simon & Green, 1984), trichohyalin (Rothnagel & Rogers, 1986), polyamines (Piacentini et al, 1988), a group of basic low molecular weight proteins (Phillips et al, 1990) and a variety of other unidentified polypeptides (Michel et al, 1987; Nagae et al, 1987).

1.2.4 Tissue Transglutaminase

It is now generally believed that tissue transglutaminases will be found to be ubiquitious in animal cells and tissue although the role of this transglutaminase still virtually unknown. Since the enzyme is so widely distributed, it seems likely that it plays some essential role in normal cell function. In recent years a considerable amount of work has accumulated to suggest a role for the enzyme during cellular growth, differentiation and death. In differentiated cells the enzyme has been implicated in the externally stimulated endocytotic and exocytotic events associated with the plasma membrane, as well as regulation of the cytoskeleton and the maintenance of cell contact.

1.2.4.1 Cellular Growth and Differentiation

Measurement of changes in the tissue transglutaminase activity during activation and differentiation of a range of cells has lead to the suggestion that the enzyme is important to these processes. Schroff *et al* (1981) demonstrated that the level of transglutaminase in macrophages was dependent upon the cell type and their state of differentiation. This has since been confirmed in a number of other cell types including platelets (Barnes *et al*, 1985), Chinese hamster ovary cells (Milhaud *et al*, 1980) and embryonic cells (Cariello *et al*, 1984; Maccioni & Arechaga, 1986; Uhl & Schindler, 1987).

Stimulation of growth and differentiation of a number of normal and leukaemic monocytic cells by retinoic acid derivatives has been shown to stimulate the rapid *de novo* synthesis of transglutaminase (Moore *et al*, 1984). Johnson & Davies (1986) have suggested that the action of the retinoids may be mediated by a membrane bound receptor

and G-protein since the retinoic acid induced expression of transglutaminase could be inhibited following pertussis toxin ribosylation of a 41,000kDa protein.

The role of tissue transglutaminase during growth and differentiation is still uncertain although Maccionini & Arechaga (1986) have studied the development of murine embryos and suggest that it may involve regulation of the cytoskeletal architecture possibly through stabilizing the microtubules (Maccioni & Seeds, 1986).

1.2.4.2 Tumours and Metastasis

If tissue transglutaminase is important in the regulation of the cellular life cycle it would be expected that abnormal cell growth would result in changes in the activity of the enzyme. Investigations appear to support this and have shown that reduction in transglutaminase activity occurs in rat liver tumours (Barnes et al, 1984, 1985), in cloned rat rhaddomyosarcoma cells (Delcros et al, 1986) and human colon tumours (Roch et al, 1987).

Further studies have suggested that the tissue transglutaminase may exists in both the well characterised cytosolic form as well as a predominantly membrane associated, particulate form (Chang & Chung, 1986) and that the induction of cancer in rats leads to both a reduction and redistribution of transglutaminase activity (Birckbichler et al, 1976; Barnes et al, 1985). Recent reports suggest that there is no change in the level of the particulate enzyme but that there is a 5 fold reduction in the expression of the cytosolic form of the transglutaminase (Hand et al, 1988). This reduction in cytosolic transglutaminase activity in metastatic tumours is associated with the build up of an inactive transglutaminase which can be proteolytically

cleaved to produce the cytosolic enzyme (Knight et al, 1990a).

Once again the way in which a reduction in transglutaminase activity may affect cell growth is uncertain. Haughland et al (1982) have demonstrated that inhibition of ε(γ-glutamyl)lysine formation using transglutaminase inhibitors results in increased membrane fluidity. Tyrrell et al (1988) identified the major substrate in liver cells as being a high molecular weight protein polymer which was associated with the plasma membrane and which maybe involved in regulating membrane fluidity and cell-cell adhesion. This report has since been confirmed by Hand et al (1990). However, the same authors found that the amount of the high molecular weight polymer was not reduced in chemically induced hepatocellular carcinomas even though there was a 3-fold reduction in the amount of ε-(γ-glutamyl)lysine crosslinks. They concluded that the cytosolic and particulate transglutaminases may be differentially activated and that they may act on different substrates within the cell.

1.2.4.3 Programmed Cell Death (Apoptosis)

Using morphological and biochemical studies it has been possible to identify two mechanisms of nucleated cell death, necrosis and apoptosis (Wyllie, 1980; Franceschi, 1989). Necrosis is characterised by loss in cell volume regulation, cessation of synthetic functions and then the influx of Ca^{2+} which results in the disruption of the cellular membranes. This commonly occurs in response to pathological events such as severe hypoxia, complement attack or exposure to toxins. Contrary to this mechanism, apoptosis or programmed cell death is dependent upon active metabolism and protein synthesis and occurs when death is apparently regulated as during embryogenesis, metamorphosis, hormone-dependent atrophy and in normal

cell turnover. The process is characterised by DNA fragmentation following the action of endogenous endonucleases (Duke et al, 1983) and the formation of detergent insoluble apoptotic envelope possibly by the action of transglutaminase (Fesus et al, 1989). A recent report (Knight et al, 1990c) using tumour cell lines of different metastatic potentials has shown a correlation between the activity of cytosolic transglutaminase and the formation of the highly crosslinked apoptotic bodies, the formation of which are inversely proportional to the metastatic potential of the cell line.

1.2.4.4 Receptor Mediated Endocytosis

Many investigators have demonstrated that competitive inhibitors of transglutaminase are able to inhibit receptor mediated endocytosis of hormones and proteins (Davies & Murtaugh, 1985). King et al (1980) and Yarden et al (1981) have questioned the conclusions of this work since they report that the primary amine inhibitors of transglutaminase are lysosomotrophic. They suggest that the inhibition of the internalization of the epidermal growth factor and α_2-macrophage receptor is a result of inhibition of their degradation. However, Van Leuven et al (1980) were able to dissociate the lysosomotrophic effect of the transglutaminase inhibitors, methylamine and monodansylcadaverine (MDCD) from the internalization of the a_2-macrophage and concluded that the decreased rate of uptake is due to inhibition of receptor processing rather than internalization.

1.2.4.5 Tissue Transglutaminase and the Cytoskeleton

A possible role for transglutaminase in the regulation of the cytoskeleton of eukaryotic cells was proposed following reports that tissue transglutaminase catalyses the in vitro incorporation of

polyamines into actin, α-actinin, desmin, tropomyosin and tubulin (Derrick & Lari, 1966; Iwanji, 1977; Gard & Lazarides, 1979; Cohen et al , 1979, 1980; Conrad, 1985).

Loewy et al (1981) demonstrated the presence of ε(γ-glutamyl)lysine crosslinks associated with the cytoskeleton of Physarium polycephalum that was decreased in the presence of Mg^{2+}-ATP and increased by Mg^{2+}-ATP plus Ca^{2+} and proposed that transglutaminase may play a dynamic role during cytoskeletal regulation. Indirect evidence to support transglutaminase regulation of the cytoskeleton has come from Alarcon et al (1985) who reported that primary amines inhibited proinsulin to insulin conversion, a process which occurs during the translocation of the proinsulin from the endoplasmic reticulum to the golgi. Several investigators have also implicated a role for transglutaminase during flagellar sliding in Arbacia sperm (Cariello & Nelson, 1985; Gagnon & Lamirande, 1986; Lamirande & Gagnon, 1989). They suggest that this may involve cycling of the ε(γ-glutamyl)lysine crosslink since 2-(3-diallylaminopropionyl) benzothiophene, a potent non-competitive inhibitor of transglutaminase, caused an increase in the forward swimming speed of spermatozoa.

Recent work has suggested that a calmodulin dependent transglutaminase is present in human platelets, red blood cells and chicken gizzard, that has a high affinity for the cytoskeletal proteins (Puszkin & Raghumaran, 1985; Puszkin & Billet, 1987).

1.2.4.6 Cell to Cell Adhesion

Studies on the hepatocytes of rat liver have suggested that membrane associated tissue transglutaminase may be involved in maintaining cell to cell contact. Slife et al (1985; 1986) reported that the membrane transglutaminase activity and a large molecular

weight substrate were associated with plasma membrane of rat liver hepatocytes. They found that the large molecular weight transglutaminase substrate which was unable to traverse a polyacrylamide stacking gel resembled a large molecular weight complex isolated from rat liver plasma membrane which was able to stimulate cell-cell adhesion. Tyrrell et al (1986) later demonstrated that both the enzyme and its substrate were specifically associated with the lateral plasma membrane which contained many filament bearing and junctional complexes. Recently, Tyrrell et al (1988a) has been able to isolate the large molecular weight substrates and has renamed them SITS (SDS-Insoluble Transglutaminase Substrates). Morphological studies have shown SITS to be irregularly shaped, flattened sheets ranging in size from 4-12μm. By raising polyclonal antibodies to the SITS, Tyrrell et al (1988b) has identified a major component as being fibronectin, which is known to be involved in variety of adhesion functions (Yamada and Olden, 1978; Hynes and Yamada, 1982). These anti-SITS antibodies also reacted with two proteins of molecular weights 32kDa and 35kDa present in the cytosol and proteins of 32kDa, 35kDa and 85kDa present in the plasma membrane fraction.

The fact that the SITS are so large has also led to the suggestion that they may serve as a scaffolding for the lateral plasma membrane, maintaining the proper orientation and distribution of the various components of this membrane domain.

1.2.4.7 Exocytosis

Several investigators have proposed a role for transglutaminase during stimulus-secretion coupling from islets of Langerhans. Transglutaminase activity in pancreatic islets was first measured by Bungay et al (1982) and later confirmed by Gomis et al

(1983). Since transglutaminase is a Ca^{2+}-responsive enzyme with a reported Km for Ca^{2+} of 3μM (Bungay et al, 1986) it was proposed that transglutaminase may be a target for the increase in the cytosolic concentration of Ca^{2+} which is thought to be the primary mediator of stimulus-secretion coupling during glucose-stimulated insulin release (Prentki & Matschinsky, 1987). Since several reports have shown that incubating islets with competitive substrate inhibitors of transglutaminase leads to inhibition of glucose stimulated insulin release (Bungay et al, 1984; Sener et al, 1985; Bungay et al, 1986), and that transglutaminase activity is increased in homogenates of glucose-stimulated islets (Gomis et al, 1986; 1989), this suggested that transglutaminase plays a role in stimulus-secretion coupling.

Attempts to identify transglutaminase substrates in homogenized islets have suggested that the major substrate is a high molecular weight polymer which is unable to traverse a 3% (w/v) polyacrylamide gel (Bungay et al, 1986; Gomis et al, 1989). Owen et al (1988) have reported the formation of a high molecular weight phosphoprotein polymer during glucose stimulation which is inhibited by transglutaminase inhibitors. This may be the same as that found in islet homogenates although its subcellular distribution appears to be slightly different. The function of the high molecular weight polymer is uncertain but it has been proposed that it may be important to the membrane associated events of insulin release.

1.3 Aim of Thesis

The aim of this thesis was to investigate the function of transglutaminase in the B-cells of the rat pancreatic islets of Langerhans with a view to confirming the link between the Ca^{2+}-message, transglutaminase activity and insulin secretion. In an attempt to do this we :

1) Used electropermeabilised islets to investigated the role of transglutaminase during insulin secretion.

2) Used both homogenized and electropermeabilised islets to identify and characterise possible transglutaminase substrates.

Since much of this work involved study the role of transglutaminase during the process of stimulus-secretion coupling the second part of the introduction will briefly discuss what is known of this process.

1.4 MECHANISM OF INSULIN RELEASE

1.4.1 Insulin

Insulin, the polypeptide hormone secreted by the B-cells of the pancreatic islets of Langerhans is central to the control of glucose homeostasis in mammals. An increase in the blood concentration of insulin promotes uptake of both glucose and amino acids into most cells and particular those of muscle, liver and adipose tissue. Within many cells insulin also promotes glycogen, fat and protein synthesis. By stimulating this rapid uptake and utilization or storage of fuels, insulin is able to maintain the appropriate concentration of fuel molecules in the blood, most commonly in the form of glucose.

The release of insulin is regulated by a number of nutrient and neurohormonal factors. D-Glucose is the most important and is the only nutrient stimuli that promotes insulin release *in vitro*, at concentrations within the physiological range. However, other fuel molecules such as carbohydrates, amino acids, ketone bodies and fatty acids as well as neurohormonal factors, will affect insulin release. In circumstances where insulin production and release is either absent or reduced this leads to the clinical condition known as *diabetes mellitus*.

1.4.2 Cellular Pathway of Insulin Biosynthesis

Insulin is derived from a preproinsulin precursor which is subsequently proteolytically processed during its passage through the Golgi and coated secretory vesicles, to produce insulin. The preproinsulin is synthesized by polyribosomes on the rough endoplasmic reticulum with the concomitant removal of the pre-region to produce proinsulin (Patzelt *et al*, 1978). The removal of the pre-region allows

the protein to undergo rapid peptide chain folding. The proinsulin is then transported via microvesicles from the transitional endoplasmic reticulum to the cis-Golgi region (Orci, 1974; 1982; 1985) during which initial processing of the proinsulin is thought to occur (Steiner et al, 1974). The proinsulin is then packaged into clathrin coated vesicles with more than 100 different proteins (Hutton, 1984). Acidification of the coated vesicles following stimulation of an H^+-ATPase brings the intra-vesicular pH down to pH 5.0-5.5 which provides the optimum conditions for the proteolytic conversion of the proinsulin to insulin, with the release of the C-peptide (Orci, 1986). Following the proinsulin conversion, the immature granules with a low uniform density, change in morphology to non-coated mature granules with a dense inclusion of crystalline zinc-insulin. The cleaved C-peptide is thought to occupy the cytoplasm of the granule (Michael et al, 1987). The conversion of proinsulin to insulin is believed to be catalysed by a trypsin like and carboxypeptidase B like pair of enzymes which cleave the proinsulin, releasing the C-peptide. Recently, Davidson and Hutton (1987) reported that a carboxypeptidase H present in the pancreatic B-cell of rats was involved in proinsulin conversion.

1.4.3 Stimuli Leading to Insulin Release

The mechanism by which secretagogues are able to mobilize the secretory apparatus is still not understood in any great detail. The area has been the subject of intensive investigation since the development of methods for the isolation of intact functional islets of Langerhans. Islets are not a pure preparation of B-cells, however they do provide a suitable unit for in vitro experimental investigation of stimulus-secretion coupling. More recently the development of insulin-secreting cell lines and

fluorescent activated cell sorting for the preparation of near 100% B-cell suspensions has added to the experimental tools available.

An increasing number of fuel and non-fuel molecules are known to stimulate insulin release. However, under physiological conditions, only glucose is capable of initiating insulin secretion. Glucose is also required as a permissive factor in response to physiological modulators of insulin release. The potentiating action of amino acids, fatty acids, ketone bodies as well as other non-fuel secretagogues is closely linked to the ambient glucose concentration. However, under pharmacological conditions a variety of fuel molecules are able to act as secretagogues.

Secretagogues which stimulate insulin release can be generally classified as either primary stimuli which promote insulin release directly or secondary stimuli, which alter the response of the B-cell to a primary stimulus but do not directly affect secretion themselves. Additionally, there are inhibitors which inhibit the response to both primary and secondary stimuli.

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

Primary stimuli are in general fuel secretagogues which stimulate insulin release primarily as a result of the production of an intracellular Ca^{2+} signal, following depolarization of the plasma membrane, and the opening of Ca^{2+} channels. It is now well established that the metabolism of glucose and other fuel (nutrient) stimuli by the B-cell is a prerequisite for the secretory response (Ashcroft et al, 1980; Pralong et al, 1990). The currently accepted hypothesis is that a trigger substance(s) that stimulates insulin release is formed in

the B-cell during the metabolism of fuel molecules (the "Fuel Hypothesis", Malaisse et al, 1979)

Secondary stimuli are in general non-fuel secretagogues such as hormones and neurotransmitters which bind to receptors on the plasma membrane and affect the response of the primary stimuli by either modulating phospholipid and cAMP metabolism or by a direct affect on the ionic events at the plasma membrane.

1.4.4 Intracellular Messenger Systems and Stimulus-Secretion Coupling

Stimulus-secretion coupling from the pancreatic B-cell is believed to mediated by changes in the intracellular concentration of a number of secondary messengers. The most important of these are Ca^{2+}, cAMP and the products of phospholipid metabolism which include diacylglycerol (DAG) and D-myo-inositol 1,4,5-triphosphate (I-1,4,5-P_3) (Wollheim and Sharp, 1981; Malaisse and Malaisse-Lagae, 1984; Prentki and Matschinsky, 1987).

The mechanism by which nutrient stimuli and neurohormonal agonists activate the the B-cell messenger system is thought to be different (Prentki and Matschinsky, 1987). Fuel stimuli appear to initially stimulate an elevation in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ through the influx of extracellular Ca^{2+} and not through the early mobilization of stored Ca^{2+}. There then appears to be accelerated phospholipid metabolism which maybe Ca^{2+}-dependent. In contrast, neurohormonal agonists trigger a Ca^{2+}-independent increase in phospholipid metabolism followed by an elevation in $[Ca^{2+}]_i$ which is initially due to the mobilization of stored Ca^{2+}. These differences in the two systems probably result from the way in which the stimuli are recognised, since nutrient stimulation is mediated by the production of

metabolites whilst neurohormonal stimuli bind to plasma membrane receptors.

1.4.4.1 Cytosolic Ca^{2+} Metabolism

A rise in the cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ is thought to be of primary importance in mediating stimulus-secretion coupling from the pancreatic B-cell (Wollheim and Sharp, 1981; Rasmussen and Barrett, 1984; Prentki and Matschinsky, 1987).

The Ca^{2+} flux across the plasma membrane is regulated by three Ca^{2+}-transporting systems. Cytosolic Ca^{2+} is extruded from the cell by a ATP-dependent Ca^{2+} pump which is Ca^{2+}-calmodulin activated (Pershadsingh et al, 1980) and also through a Na^{+}-Ca^{2+} antiport system driven by the inward Na^{+} gradient (Blaustein and Nelson, 1982), whilst entry of extracellular Ca^{2+} occurs predominantly via voltage dependent Ca^{2+} channels and to a much lesser extent by diffusion along the inwardly directed electrochemical gradient.

The endoplasmic reticulum is known to play an important role in regulating $[Ca^{2+}]_i$. The uptake of Ca^{2+} from the cytosol is mediated by ATP dependent Ca^{2+} pumps (Colca et al, 1982; 1983) whilst efflux from the endoplasmic reticulum occurs via I-1,4,5-P_3 (Wolf et al, 1985) and arachidonic acid (Wolf et al, 1986) activated Ca^{2+} channels. Prentki et al, 1984 has shown that insulinoma microsomes possess a high capacity for Ca^{2+} accumulation and maintain microsomal Ca^{2+} concentration within the physiological range, suggesting that the endoplasmic reticulum is well suited to regulating $[Ca^{2+}]_i$.

Two other intracellular organelles have been implicated in regulating $[Ca^{2+}]_i$, the mitochondria and the secretory vesicles. Mitochondria from insulinoma cells have been shown to possess independent influx and efflux pathways for Ca^{2+} transport, and are able

to behave as efficient Ca^{2+} buffers of the surrounding medium, having been reported to lower ambient Ca^{2+} concentration in the micromolar range to a steady state of 0.5μM - 1μM (Prenkti et al, 1983). However, although secretory vesicles contain large amounts of stored Ca^{2+}, it seems unlikely that they play any role in Ca^{2+}-homeostasis under normal conditions (Prentki and Wollheim, 1984).

Studies with combinations of microsomes, mitochondria and secretory granules from rat insulinoma cells (Prentki and Wollheim, 1984) suggest that under non-stimulatory conditions the endoplasmic reticulum is the major Ca^{2+} buffering system. However, at the higher $[Ca^{2+}]_i$ resulting from stimulation, the mitochondria may play an important role in Ca^{2+} buffering by bringing the concentration down to the submicromolar range.

1.4.4.2 Effect of Secretatogues on Ca^{2+} Metabolism

The stimulus-secretion coupling of nutrient secretagogues is believed to be primarily mediated by an increase in the $[Ca^{2+}]_i$, following the influx of extracellular Ca^{2+}. This conclusion was drawn from experiments which showed that glucose stimulated insulin release required external Ca^{2+} (Grodsky and Bennett, 1966) and that $^{45}Ca^{2+}$ uptake was increased by a range of nutrient stimuli (Wollheim and Sharp, 1981). More recently it has been possible to directly measure an increase in $[Ca^{2+}]_i$ using quin 2 (Deleers et al, 1984; Rorsman et al, 1984) and fura 2 (Sussman et al, 1987). Evidence that the increase in cytosolic Ca^{2+} directly stimulates insulin release was obtained in permeabilised islets (Yaseen et al, 1982; Jones et al, 1985; Tamagawa et al, 1985) and in permeabilised RINm5F cells (Vallar et al, 1987) since insulin release could be induced by raising the Ca^{2+} concentration from the nM to the μM range.

The increased Ca^{2+} influx is thought to result from the activation of voltage-sensitive Ca^{2+} channels following membrane depolarization. Under resting conditions the membrane potential is determined by the ATP-sensitive K^+ channels which are open and result in a transmembrane potential close to the equilibrium potential for K^+. Closure of the ATP-sensitive K^+ channels produces rapid depolarization to a plateau potential, superimposed upon which are spikes. These spikes are thought to result from the opening of the voltage-sensitve Ca^{2+} channels. Unless B-cells are exposed to very high glucose concentrations, the spike activity eventually stops and the membrane potential repolarizes. The potential then begins to oscillate to produce regular slow waves with spike activity on the plateaus. At high glucose concentrations the membrane potential remains polarized (Henquin, 1987; Peterson and Findlay, 1987; Wang & McDaniel, 1990). Recently, Valdeolmillos et al (1989) has been able to measure oscillations in the $[Ca^{2+}]_i$ of the mouse pancreatic islets with a similar frequency to membrane depolarization. Since previous measurements on suspensions of single B-cells failed to show this (Grapengiesser et al, 1988) they believe that the extensive network of electrically coupled cells normally found in the islet is required to produce the $[Ca^{2+}]_i$ oscillations.

Although it has been shown using K^+-efflux (Henquin, 1978) and patch clamp (Ashcroft et al, 1984) studies, that glucose stimulation inhibits the plasma membrane K^+ permeability, the exact link between glucose metabolism and closure of the ATP-sensitive K^+ channels is uncertain. A major factor is thought to be the increase in the cytosolic concentration of ATP or in the ATP/ADP ratio (Dunne et al, 1988). However, other nucleotides including ADP (Dunne and Peterson, 1986a; Kakai et al, 1986), GDP and GTP (Dunne and Peterson,

1986b) and the pyridine based nucleotides NAD(P) and NAD(P)H (Dunne et al, 1988), as well as intracellular pH (Misler et al, 1989) and D-glyceraldehyde (Dunne et al, 1986), are known to gate the ATP-sensitive K^+ channels and could therefore be mediators, since their concentrations are altered during glucose stimulation.

A number of peptide hormones and neurotransmitters which influence insulin release are also known to modulate the ATP-sensitive K^+ channels. Galanin (DeWeille et al, 1988) and somatostatin (DeWeille et al, 1989) inhibit secretion by opening the ATP-sensitive K^+ channels whilst vasopressin, which stimulates secretion, inhibits channel opening (Li et al, 1988; Monaco et al, 1988). Dunne et al (1989) has since shown that the effect of galanin is mediated by a pertussis toxin sensitive G-protein coupled to the channel.

As well as the ATP-sensitive K^+ channel, a second high conductance Ca^{2+}-activated K^+ channel has been identified (Peterson and Findlay, 1987). Since this channel opens as the intracellular Ca^{2+} concentration rises, it has been speculated that this channel may play an important role in repolarizing the plasma membrane during electrical oscillation. However, Tabcharani and Misler (1989) have recently disputed this since the Ca^{2+}-sensitive K^+ channel does not appear to open under physiological conditions.

The spikes or action potentials measured during plasma membrane depolarization are thought to result from the opening of voltage-gated Ca^{2+} channels, which allow the passive entry of Ca^{2+} (Henquin, 1987). When voltage-gated Ca^{2+} channels are blocked, the oscillations in membrane potential rapidly stop, $^{45}Ca^{2+}$ influx decreases and insulin release is inhibited (Henquin and Lambert, 1975; Malaisse et al, 1976) whereas the dihydropyridine derivative, CGP 28392 which prolongs channel opening has the opposite effect (Henquin et al,

1985; Morgan et al, 1985). Recently, it has been possible to characterise the voltage-gated Ca^{2+} channel directly using patch clamping (Findlay and Dunne, 1985; Satin and Cook, 1985). This work has suggested that insulin secreting cells have two types of voltage-gated Ca^{2+} channels, a long lasting (L-type) and a transient (T-type) channel (Hiriart and Matteson, 1988; Satin and Cook, 1988; Ashcroft et al, 1989; Komatsu et al, 1989). However, only the dihydropyridine sensitive channel (L-type) appears to be responsible for spike activity and Ca^{2+} entry (Keahey et al, 1989; Plasman et al, 1990).

Recent work by Keahey et al (1989) suggests that catecholamines modulate the activity of the voltage gated Ca^{2+} channel via a pertussis toxin sensitive G-protein.

1.4.4.3 Cyclic AMP and Insulin Release

It is now generally accepted that cyclic AMP (cAMP) is not a major secondary messenger during stimulus-secretion coupling but that it sensitizes the B-cell response to nutrient stimuli (Malaisse and Malaisse-Lagae, 1984; Prentki and Matschinsky, 1987). This conclusion is based upon numerous experiments which show that cAMP or agents which stimulate a rise in cAMP (glucagon, B-adrenergic agents, cholera toxin and forskolin) have little or no affect on insulin release at low glucose, but enhance insulin release at high glucose. However, Zawalich et al (1990) has reported that prior stimulation of islet with cAMP suppresses insulin release probably by suppressing the activation of phospholipase C, and thereby preventing the generation of secondary messengers.

As well as playing a potentiating role, cAMP may also play a permissive role during nutrient stimulation. Pipeleers et al (1985) have shown that purified B-cells which contain 40% less cAMP than in

intact islets, have markedly impaired insulin secretion which could be restored following the addition of either dibutyryl-cAMP, glucagon or glucagon containing a-cells. It therefore appears that normal B-cell stimulation by fuels requires the presence of a-cells that release some glucagon to maintain B-cell cAMP content at a level necessary for fuels to be effective (Schuit and Pipeleers, 1985).

Previous reports have suggested that stimulatory concentrations of glucose result in a modest rise in the cytosolic cAMP concentration (Charles et al, 1973; Grill and Cerasi, 1974; Capito and Hedeskov, 1974). Since the adenylate cyclase of rat islets can be stimulated by Ca^{2+}-calmodulin it has been proposed that this may occur following the glucose stimulated rise in $[Ca^{2+}]_i$ and is supported by reports which show that the glucose stimulated rise in cAMP requires extracellular Ca^{2+} (Charles et al, 1975; Hellman, 1976). However, Ca^{2+}-calmodulin fails to affect mouse islet adenylate cyclase activity (Thams et al, 1982) and Thams et al (1988) have reported that the glucose stimulated accumulation of cAMP is mediated by activation of protein kinase C whose action may be mediated through phosphorylating the N_i and N_s coupling proteins of adenylate cyclase, as has been reported in other cell types (Katada et al, 1985; Bell et al, 1985).

The elevation in cytosolic cAMP is thought to potentiate nutrient induced insulin release either by changing the sensitivity of the secretory apparatus to Ca^{2+}, or by increasing the intracellular Ca^{2+} concentration. Both of the effects are probably mediated by the action of the cAMP-activated protein kinases (protein kinase A), as there are many reports of changes in the phosphorylation state of islet proteins induced by cAMP-raising agents in intact, homogenised and permeabilised islets. These reports do however show great variability in the molecular masses of the phosphorylated protein substrates

(Harrison et al, 1985; Prentki and Matchinsky, 1987; Jones et al, 1989).

Early work with $^{45}Ca^{2+}$ efflux studies suggested that cAMP may stimulate an increase in $[Ca^{2+}]_i$ as a result of Ca^{2+} flux from both intracellular stores (Brisson et al, 1972) and across the plasma membrane (Henquin and Messner, 1983). However, direct measurement of $[Ca^{2+}]_i$ using the fluorescent probe, quin-2, in both RIN5mF cells (Wollheim et al, 1984), HIT cells (Hill et al, 1987) and dispersed pancreatic islets cells (Rorsman and Abrahamsson, 1985) could not detect an increase in $[Ca^{2+}]_i$ following a rise in cAMP. Rajan et al (1989) has recently measured a rise in $[Ca^{2+}]_i$ using a second generation fluorescent probe, fura-2, and has suggested that the greater Ca^{2+} buffering capacity of quin-2 may have prevented detection of an increase in $[Ca^{2+}]_i$ in the past. However, this does imply that the increase in $[Ca^{2+}]_i$ is relatively small, and that sensitization of the secretory apparatus is the main mechanism by which elevated cAMP affects insulin secretion.

1.4.4.4 Phospholipid Metabolism and Insulin Release

Both nutrient secretagogues, hormones and neurotransmitters that use the Ca^{2+}-messenger system are known to stimulate the hydrolysis of inositol phospholipids to produce diacylglycerol (DAG), an activator of protein kinase C, and inositol 1,4,5-triphosphate (Ins-1,4,5-P_3) which triggers intracellular Ca^{2+} mobilization. The increased phospholipid metabolism also results in the production of arachidonic acid (AA) which serves as a precursor to produce eicosanoids (Best et al, 1984a; Prenkti and Matschinsky, 1987; Rana and Hokin, 1990).

Carbomylcholine binding to the muscarinic receptors of

insulin secreting cells activates phospholipase C which hydrolyses the polyphosphoinositols, phoshatidyl-D-myo-inositol 4,5-bisphosphate (PIP_2) and phosphatidyl 4-phosphate (PIP) (Best and Malaisse, 1983; 1984). Glucose stimulation of islets has been reported to activate phospholipase C to hydrolyse PIP_2 and possibly PIP and phosphatidylinositol (PI) (Laychock, 1983; Montague et al, 1985; Rana et al, 1985). Recently, Zawalich and Zawalich (1988) have been able to correlate glucose stimulated insulin release with phosphoinositide hydrolysis using intact islets.

The specific nature of the signal from glucose metabolism which promotes phosphatidylinositide hydrolysis has not yet been delineated. It is thought that the glucose stimulated hydrolysis could result from the rise in $[Ca^{2+}]_i$ which may activate phospholipase C. A possible link with NAD(P)H levels has also been suggested, since concentration response curves for NAD(P)H levels in response to glucose closely parallel those of ^{32}P labelling of phosphatidylinositol in response to glucose (Malaisse et al, 1979; Best and Malaisse, 1983). Recently, Wolf et al (1988) has reported that glucose is directly recognised by phospholipase C. In contrast to glucose stimulation, carbachol stimulated hydrolysis is not dependent on Ca^{2+} influx and elevation in $[Ca^{2+}]_i$ since it still occurs in the absence of external Ca^{2+} (Best and Malaisse, 1983) and even following depletion of the intracellular Ca^{2+} store (Wollheim and Biden, 1986). As with other cells, the stimulation of phospholipase C by carbachol is probably mediated by a G-protein (Dunlop and Larkin, 1986; Rana and Hokin, 1990).

The hydrolysis of inositol phospholipids following stimulation by both carbachol (Morgan et al, 1985) and glucose (Best and Malaisse, 1983; 1984; Montague et al, 1985) was concomitant with a rise in the concentration of inositol phosphates. Wollheim and Biden (1986) have reported that the concentration of the biologically active Ins-1,4,5-P_3 isomer increased following carbachol stimulation, as has Turk et al (1986) following glucose stimulation.

Hydrolysis of inositol phosholipids also results in the concomitant rise in the concentration of diacylglycerol following glucose stimulation of pancreatic islets (Montague and Parkin, 1980) and rat neonatal islets (Dunlop and Larkin, 1984) and following carbachol stimulation of pancreatic islet membranes (Dunlop and Malaisse, 1986). Wolf et al (1989) originally reported that the rise in the concentration of DAG produced by carbachol results from polyphosphoinositol hydrolysis but suggested that the increase following glucose stimulation is primarily as a result of de novo synthesis. This would mean that the islet DAG concentration could be regulated independently of phospholipid metabolism and inositol phosphate production. However, more recently they have shown the synthesis of DAG from glucose provides only a minor contribution to the overall increase in DAG concentration and is probably not important to the process of glucose stimulated insulin release (Wolf et al, 1990).

Since glucose and carbachol have been reported to exert additive effects on the formation of Ins-1,4,5-P_3 formation in isolated islets (Montague et al, 1985), it has been suggested that the fuel and neurotransmitter stimuli alter phosphoinositide metabolism by different mechanisms (Best and Malaisse, 1983). Since the action of carbachol is mediated through receptor-mediated phospholipid breakdown at the plasma membrane, whereas a change in the concentration of an intracellular

factor, possibly a rise in $[Ca^{2+}]_i$, mediates the action of glucose, they may activate different phospholipase C populations. In certain studies glucose (16.7mM) promoted a loss from PI and phosphatidylcholine only when islets were prelabelled at high (16.7mM) but not at low (3.3mM) glucose (Rana et al, 1985; Rana et al, 1986). This has led to the suggestion that islet cells may contain two pools of phosphoinositides, one that is labile and sensitive to fuel stimulation and a second that is more stable and insensitive to fuels. Identification of the subcellular location of the fuel sensitive phosphoinositide pool has produced conflicting reports. Clement et al (1977) found that glucose stimulation resulted in the hydrolysis and loss of phosphoinositides associated with the secretory vesicles whereas Rana et al (1986) reports that the loss is predominantly from the plasma membrane. Since glucose is also believed to stimulate de novo synthesis of phosphoinositides (Best et al, 1984; Farese et al, 1985), the concept of a fuel sensitive pool and the de novo synthesis of phosphoinositides may be an expression of the same process.

The increase in phospholipid metabolism stimulated by secretagogues also results in the release of arachidonic acid which can be subsequently metabolized by cyclooxygenases and lipooxygenases to produce eicosanoids (e.g. prostaglandins, leukotrienes and thromboxanes).

Arachidonic acid can be released from phospholipids either by the action of phospholipase C followed by DAG lipase (Dunlop and Larkin, 1984) or following deacylation by phospholipase A_2 (PLA_2) (Laychock, 1982; Dunlop and Larkin, 1984). Dunlop and Larkin (1984) report that the initial release of arachidonic acid from glucose stimulated islets is mediated by activation of PLA_2 which deacylates phosphatidylcholine and phosphatidic acid derived from phosphoinsitol,

whilst the later release of AA is mediated by the action of DAG lipase on diacylglycerol.

Previous reports have suggested that the production of arachidonic acid is important for glucose stimulated insulin release (Turk et al, 1984; Capito et al, 1989). Wolf et al (1986) has reported that arachidonic acid stimulated Ca^{2+} release from the endoplasmic reticulum and other membrane bound islet stores whilst more recently, Metz (1988) has reported that arachidonic acid stimulates Ca^{2+} efflux across the plasma membrane possibly by activation of protein kinase C. Fujimoto and Teague (1989) have reported that lysophospholipids, another product of the PLA_2-mediated phospholipid hydrolysis, may be important in the initiation of the biphasic pattern of glucose stimulated insulin release. This may at least in part result from the mobilization of a pool of Ca^{2+} bound to the plasma membrane and possibly other membranes (Metz, 1988). However, as with all these studies it is uncertain whether the secretory response results directly from the affect of liberated arachidonic acid, from lysophospholipids or from derivatives of arachidonic acid which are known to have a variety of affects on insulin release.

1.4.5 Mode of Action of Secondary Messengers on Insulin Release

The increase in the intracellular concentration of Ca^{2+}, cAMP and diacylglycerol are thought to be the link between stimulus perception and the activation of the secretory mechanism.

Many of the effects of Ca^{2+} are believed to be mediated by calmodulin (Tomlinson et al, 1984; Harrison et al, 1986). Ca^{2+}-calmodulin has been reported to increase the Ca^{2+}-ATPase activity of the islet plasma membrane (Pershadsingh et al, 1980). It may also influence cAMP metabolism since it has been shown to activate adenylate

cyclase (Valverde et al, 1979) and enhance cAMP phosphodiesterase activity (Sugden and Ashcroft, 1981). However, the primary means by which Ca^{2+}-calmodulin mediates insulin secretion is thought to be by altering the phosphorylation state of cellular proteins through activation of protein kinases. To date two Ca^{2+}-calmodulin dependent protein kinase enzymes, a phosphorylase kinase (Harrison et al, 1984) and a myosin light chain kinase (Harrison and Ashcroft, 1982; Landt et al, 1982; MacDonald and Kowluru, 1982; Penn et al, 1982) have been identified in insulin secretory cells. Protein phosphorylation studies have identified a Ca^{2+}-calmodulin dependent phosphorylation of proteins of molecular weight 57kDa and 53-54kDa which maybe the a- and b-subunits of tubulin (Colca et al, 1983). The stimulation of insulin release by an increase in $[Ca^{2+}]_i$ may also be mediated by Ca^{2+}-activated proteins such as the annexins (Burgoyne and Geisow, 1989), calpains (Suzuki et al, 1987) and many cytoskeletal-associated proteins, particularly those involved in the regulation of actin filaments such as gelsolin (Weeds, 1982).

It is now generally excepted that cAMP plays both a permissive and potentiating role during nutrient induced insulin release. It is thought that the effects of cAMP are also mediated through altering the phoshorylation state of islet proteins following activation of protein kinases. Montague and Howell (1972) have identified two cAMP-dependent protein kinase isoenzymes in rat islets.

A Ca^{2+}-phospholipid dependent protein kinase (protein kinase C) has been identified in insulin secreting cells (Tanigawa et al, 1982; Lord and Ashcroft, 1984) which is predominantly the type III isoenzyme (Onada et al. 1990). An increase in the concentration of diacylglycerol is thought to activate protein kinase C (PK-C) by increasing the affinity of the enzyme for Ca^{2+} and promoting its

movement from the cytosol to the membrane (Kaibachi et al, 1981). Synthetic diacylglycerols such as 1-oleayl-2-acetyl diacylglycerol (OAG) and the tumour promoting phorbol esters such as phorbol myristate acetate (PMA), which activate protein kinase C directly by substituting for diacylglycerol (Castagna et al, 1982; Kaibuchi et al, 1983; Nishizuka, 1984) have been shown to potentiate secretagogue induced insulin release from pancreatic islets in the absence or presence of basal glucose (2.8mM) (Malaisse et al, 1980; Malaisse et al, 1983; Zawalich et al, 1983). Eason et al (1989) has reported that exposure of islets to glucose or PMA results in the translocation of protein kinase C from the cytosol to the membrane and that this correlates with the onset of insulin release. The phorbol ester, phorbol myristate acetate, also stimulates insulin release from islets permeabilised by high voltage electrical discharge (Jones et al, 1985) and small amounts of digitonin (Tamagawa et al, 1985). Recently, stimulation of insulin secretory cell lines with PMA has been reported to result in plasma membrane depolarization following the closure of K^+ channels (Wollheim et al, 1988) and an increase in $[Ca^{2+}]_i$ (Yada et al, 1989). This has led to the proposal that protein kinase C may be involved in the critical initiating events of carbohydrate stimulated insulin release, although this conclusion has been questioned by Hii et al (1987). Overall it is thought that protein kinase C is an important regulator of insulin release from B-cells. However, it is still uncertain whether the effects of protein kinase C are due to the sensitization of the secretory apparatus to Ca^{2+} (Ca^{2+}-dependent) are a direct affect (Ca^{2+}-independent) or both. Jones et al (1989) has looked at the time course of PMA stimulated insulin release from electrically permeabilised islets and has reported that a Ca^{2+}-independent pathway may exist.

The action of protein kinase C is thought to be primarily mediated by protein phosphorylation. Studies using endogenous protein kinase C labelling and PMA stimulation in intact islets have identified a range of proteins phoshorylated by protein kinase C. Dunlop and Larkin (1986) have reported a 40kDa phosphoprotein in the particulate membrane fraction as a substrate for protein kinase C and Ca^{2+}-calmodulin, which is also phoshorylated in intact islets following exposure to glucose or PMA. They speculate that this phosphoprotein may be lipocortin. As with most aspects of stimulus-secretion coupling there is some controversy as to the importance of the respective secondary messenger systems in the secretory process. Several investigators have suggested that stimulation of the Ca^{2+}-calmodulin branch of the Ca^{2+}-messenger system is responsible for first phase insulin release, whereas protein kinase C activation is primarily responsible for the slowly developing second phase of release, following glucose stimulation (Zawalich et al, 1983; Rasmussen and Barrett, 1984; Zawalich et al, 1984) although Thams et al (1990) have recently found that PK-C depletion of islets inhibits phase 1 and stimulates phase 2 of both glucose and carbomylcholine induced insulin release. However, this is almost certainly an oversimplification of the process and more detailed study of the time course of the intracellular messenger system activation is required.

Although it is thought that secondary messengers stimulate insulin release, the question arises as to how this physically occurs. The release mechanism has two major components: i) the movement of insulin storage granules from the cytoplasm to the plasma membrane (margination), and ii) the subsequent release of the granule contents following the fusion of the granule membrane with the plasma membrane (exocytosis).

1.4.6 Margination

The intracellular movement of insulin secretory granules in the B-cell is believed to be associated with the network of microtubules and microfilaments (Howell, 1984; Howell and Tyhurst, 1984; 1986).

Microtubules were first implicated in the process of margination when it was found that colchicine, vinblastine and other drugs which disrupted microtubules, inhibited insulin release (Lacy et al, 1968; 1972; Howell et al, 1982; Yaseen et al, 1982). Since drugs that stabilize microtubule formation such as taxol, also inhibit insulin secretion, it is thought that a dynamic turnover of microtubules is required for margination. Microtubules are polymerized structures in a state of dynamic equilibrium with a pool of a-tubulin and b-tubulin subunits. In the unstimulated B-cell 30-40% of tubulin is polymerized into microtubules, whereas under conditions of glucose stimulated insulin release polymerization is increased (Montague et al, 1976). It is thought that the polymerization of microtubules is in part regulated by cAMP-dependent protein kinases, since Montague et al (1976) showed that an increase in cytosolic cAMP correlated with microtubule polymerization and insulin release. Colca et al (1983) have reported a Ca^{2+}-calmodulin dependent phosphorylation of two proteins with molecular weights 54 and 57 kDa in intact islets and microsomal fractions, as have Brocklehurst et al (1983) in rat insulinoma tissue. It has been suggested that these phosphoproteins may be the a- and b-tubulin subunits and that their phosphorylation may affect microtubule polymerization. Evidence of a role for microfilaments during margination has come from studies using cytochalasin B (Lacy et al, 1973) and phalloidin (Stutchfield and

Howell, 1984) which disrupt microfilament function and led to enhancement of insulin secretion. The constituent protein of microfilaments is thought to be actin which exists in a soluble, globular form (G-actin) and an insoluble filamentous form (F-actin). Glucose stimulation results in an increase in the proportion of filamentous actin in the islet cells from 40% to 70% (Blikstad et al, 1978; Howell and Tyhurst, 1980; Swanston-Flatt et al, 1980). This polymerization required ATP but not Ca^{2+}.

Myosin light and heavy chains as well as the enzyme myosin light chain kinase have been identified in rat islets of Langerhans (Ostlund et al, 1978). Since the interaction of actin and myosin to form actinomyosin is an important component in many contractile systems it may be involved in the granule movements of B-cells. Therefore, Ca^{2+} could regulate the movement of granules by stimulating Ca^{2+}-calmodulin dependent myosin light chain kinase to phosphorylate the myosin light chain and thus promoting interaction with actin, although it has not been possible to show the phosphorylation of myosin light chain in intact islets following glucose stimulation. However, Howell & Tyhurst (1982) have reported that secretory granules interact with actinomyosin.

Although no coherent overall model for vesicle movement has been formulated from the multitude of observations, it seems likely that the increase in $[Ca^{2+}]_i$ following islet stimulation plays an important role during margination. This could be mediated by alteration in the cytoskeletal structure or by altering the affinity of secretory granules to the cytoskeletal elements involved in the granule translocation. The phosphorylation of microtubule associated proteins (MAP's) in vivo by cAMP-and Ca2+-dependent kinases could result in the temporal dissociation of microtubules and microfilaments, which may in

turn allow the movement of secretory granules. Additionally, there are a range of calmodulin and Ca^{2+} binding proteins that can interact with components of the tubulin, actin and myosin cytoskeleton (Kakiuchi and Sabue, 1983) which may mediate vesicle movement.

1.4.7 Exocytosis

The final event of insulin release requires the fusion of the insulin storage granule with the plasma membrane. The major problem with this event is overcoming the repellent effect of the highly charged phospholipid head groups in the vesicle and plasma membranes.

Both Ca^{2+} and polyamines have been implicated in membrane fusion either by partially neutralizing these charges or by acting as bridges linking the phospholipids in the two membranes. It is however more probable that the loss of the polar head groups of phospholipids catalysed by phospholipase C plays an important role in facilitating membrane fusion. The phospholipase conversion results in the production of 1,2-diacylglycerol which promotes membrane fluidity.

A number of Ca^{2+} binding proteins, such as the annexins, have also been implicated in the process of membrane fusion in other secretory cells and are probably present and important to exocytosis in the islet B-cell.

2 MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 MATERIALS

The work for this thesis was undertaken in the laboratories of the faculty of Life Sciences, Nottingham Polytechnic (formerly Trent Polytechnic).

Chemicals used for electrophoresis were of Electran grade. All other chemicals were generally of Analar grade and some of general purpose grade.

The following indicates the source of the chemicals used.

Air Products Ltd., Moseley, Walton-on-Thames, U.K. ;

95% (v/v) O_2 in CO_2

Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K. ;

cystamine hydrochloride

glycine methyl ester

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

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

[^{125}I]-insulin

[^{14}C]-methylamine hydrochloride (100-200 mCi/mmol)

[^{14}C]-methylated molecular weight standards (Mr. 200,000-14,300)

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

Bio-Rad Laboratories Ltd., Watford, Hertfordshire, U.K. ;

ammonium persulphate

N,N,N',N'-tetramethylethylenediamine

British Drug House, Poole, Dorset, U.K. ;

acetic acid	acrylamide
N,N'-dimethylcasein	dimethylsulphoxide
E.D.T.A.	glycine
glycerol	L-glutamic acid

hydrogen peroxide 100 vols. (30% (w/v))

N,N'-methylenebisacrylamide PAGE blue 83

phenylmethanesulphonyl fluoride

sodium dodecyl sulphate

Fluka AG., Buchs, Switzerland ;

monodansylcadaverine

sarcosine methylester

IBF-Biotechnics, Villenuve la Garenne, France ;

trisacryl GF2000M

ICN Immunobiologicals, Washington, Tyne and Wear, U.K. ;

sac-cell donkey anti-guinea pig coated cellulose

L.K.B., Milton Keynes, Bucks, U.K. ;

optiphase safe and hi-safe liquid scintillation fluid

Novo, Bagsvaerd, Denmark ;

rat insulin

Sarstedt, Leicester, U.K. ;

microcentrifuge tubes, plastic scintillation-vial inserts

Serva Feinbiochemica, Heidleberg, W.Germany ;

collagenase from Clostridium histolyticum

Sigma Ltd., Poole, Dorset, U.K. ;

adenosine 3',5'-cyclic monophosphate

adenosine 5'-monophosphate bovine serum albumin (RIA grade)

collagenase from Clostridium histolyticum, type V & XI

cytochrome c (from bovine heart)

E.G.T.A. 2-mercaptoethanol

molecular weight standards (polyacrylamide gel electrophoresis)

molecular weight standards (gel chromotography)

phorbol 12-myristate 13-acetate

putrescine hydrochloride Sigmacote (siliconising fluid)

Trizma-base Tris.HCl

Wilfred Smith Ltd., Edgeware, Middlesex, U.K. ;

bovine serum albumin (Gey & Gey medium/protein assay)

Whatman Ltd., Maidstone, Kent, U.K. ;

3MM chromotography paper

The following equipment was used during experimentation :

Measurement of pH was undertaken using both a Corning pH meter 130 (Corning Ltd., U.S.A.) and a Pye Unicam (Phillips) model PW9409 pH meter.

Enzyme assays and kinetic analysis involving the absorbance changes were undertaken using a Beckman DU7 Spectrophotometer (Beckman Ltd., Warrington, U.K.)

Absorbance measurement during gel scanning was undertaken using a Pye Unicam SP1800 ultraviolet spectrophotometer (Pye Unicam Ltd., Cambridge, U.K.)

Sonication was performed with a M.S.E. Soniprep 150 sonicator (M.S.E. Scientific Instruments, Crawley, Sussex, U.K.).

The Gallenkamp Flask Shaker (Gallenkamp Biomedicals, Loughborough, U.K.) was used during the collagenase digestion of rat pancreati.

Electrophoretic separation of protein samples was undertaken with either a L.K.B. 2001 Vertical Electrophoresis Unit or a Bio-Rad Protean II Vertical Electrophoresis Unit.

Radioisotope emissions were detected using a Packard Auto-Gamma 500C with a 3 inch crystal (γ-emission) and a Packard 300 liquid scintillation counter (β-emission).

Electropermeabilisation was performed with the Bio-Rad Gene Pulser Unit.

Distilled water was de-ionised with the Milli-Q water purification system (Millipore Ltd., Harrow, U.K.).

Centrifugation was undertaken in the following centrifuges :

Centrifuge	Rotor	Relative Centrifugal Force (g av)
M.S.E. Superspeed 50	8x25ml angle, fitted with 3ml adaptors (r_{av}=6.22cm)	71,000
M.S.E. Chillspin	swing-out 4-place universal with multitube carrier (64 tubes)	2,000
M.S.E. MicroCentaur	12x1.5ml	11,600

2.2 ANIMALS

Pancreatic tissue for the isolation of islets of Langerhans was obtained from Sprague-Dawley rats of either sex weighing 250-400g.

2.3 ISOLATION OF ISLETS OF LANGERHANS

Islets of Langerhans were isolated from rat pancreati by digestion with collagenase. However, during the course of the project the method of digestion and the type of collagenase used was changed. Initially, a method based on that described by Lacy et al (1972) was used in which the pancreati were distended with physiological medium before being excised, chopped and then incubated in medium containing collagenase. In the later method the collenagenase was included in the

physiological medium used for the distension. This method had been developed from the method described by Howell & Taylor (1968). Comparison of the insulin secretion showed there to be no significant difference between the two methods but the new method permitted the isolationof larger numbers of islets.

2.3.1 Siliconisation of Glassware

To ensure that the islets would not adhere to the glass, all glassware was siliconised. This was performed by rinsing the glass surfaces with siliconising fluid (Sigmacote, Sigma). Before use all siliconised glassware was thoroughly washed with distilled water to remove any acidic traces resulting from the drying of the siliconising fluid.

2.3.2 Physiological Medium

Gey and Gey (1936) medium was used during both islet isolation procedures and in the subsequent separation of the islets from the digest.

The physiological medium contained :

NaCl	111.2mM
$NaHCO_3$	27mM
KCl	4.96mM
$MgCl_2.6H_2O$	0.98mM
$MgSO_4.7H_2O$	0.28mM
KH_2PO_4	0.22mM
$NaHPO_4$	0.80mM
$CaCl_2$	2.00mM

Gey and Gey medium was made up freshly each day from 100x strength stock solutions of the salts (10x strength for NaCl and

$NaHCO_3$). The relevant volumes of the concentrated stock solutions of each of the salts, except $CaCl_2.H_2O$ was pipetted into a 1l volumetric flask before addition of distilled water to give an approximate volume of 970ml. The solution was then gassed with 95% (v/v) O_2 in CO_2 for 15min at 37°C. Whilst the solution was gassed, $CaCl_2$ was added from the concentrated stock solution.

After gassing, glucose (2.8mM) and bovine albumin (0.5mg/ml) was added, before the solution was made up to volume. Finally, the solution was checked to ensure the pH was in the range 7.4 ± 0.2.

2.3.3 Excision of Pancreas

Sprague-Dawley rats of both sexes weighing between 250-400g were sacrificed by cervical fracture. The abdomen was opened and the common bile duct clamped at its point of entry into the duodenum. A small excision was made in the bile duct just prior to the bifurcation point of the duct into the liver, into which a cannula of 0.6mm diameter was inserted. The pancreas was then distended by injection of instillation physiological medium (Method 2.3.4/5).

Following removal of the clamp and cannula, the distended pancreas was excised by carefully cutting it away from its point of attachment to the duodenum, large intestine, stomach and liver. It was then lifted onto a petri-dish via the attached spleen before removal of the spleen and any excess fat-tissue.

2.3.4 Collagenase Digestion (Lacy et al. 1972)

Pairs of pancreati were distended by instillation with physiological medium from a reservoir via a peristaltic pump (3-4ml/min) until they appeared turgid (roughly 20ml of medium per pancreas), before excision.

Following excision, pairs of pancreati were vigorously chopped using a pair of fine pointed, long bladed scissors for 75sec. The chopped tissue was transferred to a plastic centrifuge tube (15ml) and rapidly accelerated to 1500rev/min (MSE Centaur bench centrifuge) before being immediately decelerated. The supernatant which included excess fat was discarded. This washing procedure was repeated once or twice until the supernatant appeared clear.

The washed tissue was then transferred into a 50ml glass conical flask and physiological medium was added to give a volume ratio of tissue to medium of 1:1. When the flask had been firmly attached to a shaker, a mixture of 10mg of Serva collagenase and 2mg of Sigma collagenase (Type V) was added and the flask stoppered with a suba-seal. The tissue was then shaken at a particular setting ($\sim$7.5 using a Gallenkamp flask shaker) and for a time duration ($\sim$8min) which by experimentation was found to be optimum for the particular batch of Serva collagenase. These parameters were determined by visually examining the digest until the loss of tissue strands was apparent.

After shaking, the digest was transferred with rinsing into a 15ml plastic tube. The tube was immediately centrifuged by rapid acceleration to 1500rev/min and then decelerated. The supernatant was discarded and the pellet containing the islets resuspended in fresh physiological medium using a pasteur pipette cut off at the point where the neck narrows. This washing procedure was repeated 4-6 times until the supernatant ceased to be cloudy. When this occurred the pellet was resuspended in fresh medium, overlaid with 95% (v/v) O_2 in CO_2 and the tube stoppered and left in a 37^{o}C waterbath ready for islet picking.

2.3.5 Collagenase Digestion (Howell & Taylor, 1968)

The pancreas was inflated (~4ml/min) from a 10ml syringe with 4ml of physiological medium at 4-8°C in which was dissolved 5mg of Sigma collagenase (Type XI). Following excision the pancreas was transferred into a 20ml glass scintillation vial stored on ice, and physiological medium added until the vial was approximately 1/3 filled. The process was repeated with a second rat. When both pancreati had been excised the scintillation vials were incubated at 37°C and shaken gently for 7-9min. The optimum incubation time for each batch of Sigma collagenase was determined by experimentation to obtain the maximum yield of islets. After incubation the collagenase digest was transferred with rinsing into a 20ml plastic centrifuge tube and immediately centrifuged by acceleration to 3000 rev/min (Gallenkamp Junior) followed by immediate deceleration. The supernatant was discarded and the pellet containing the islets resuspended in fresh physiological medium (37°C) using a pasteur pipette cut off at the point where the neck narrows. This washing procedure was repeated three more times. After the final wash, islets were resuspended in fresh physiological medium overlaid with 95% (v/v) O_2 in CO_2 and the tube stoppered and left in a 37°C waterbath ready for islet picking.

2.3.6 Picking of Islets

Following both methods of isolation, small volumes of digest (4-5ml) were transferred to black petri-dishes (90mm diameter) containing fresh physiological medium. The islets of Langerhans were picked individually under a binocular microscope, using a drawn out pasteur pipette. Only rounded and opaque islets were picked. Islets were transferred in the smallest possible volume to a single 50ml conical flask at 37°C containing fresh physiological medium overlaid

with 95% (v/v) O_2 in CO_2, until needed for experimentation.

2.4 ASSAYS

2.4.1 Radioimmunoassay of Insulin

The immunoassay was based on the method of Hales & Randall (1963) which separates antibody bound insulin from free insulin by a double antibody procedure.

The insulin assay buffer (IAB) which was used to dilute samples, standards, antibodies and radiolabelled insulin consisted of :

Na_2HPO_4 (pH 7.0)	40mM
KH_2PO_4	10mM
NaCl	154mM
EGTA	10mM
Bovine albumin (Fraction V; RIA Grade (Sigma))	1mg/ml
Sodium azide	1mg/ml

In each assay there were triplicates of zero's, totals, blanks and a set of standard insulins, as well as suitably diluted individual samples.

A range of insulin standards (0-10 ng insulin/ml) were prepared by suitable dilution of rat insulin (Novo). Samples taken from incubations of 3-5 electropermeabilised islets were diluted 1:2 whilst samples from incubations of 10 electropermeabilised islets were diluted 1:4.

Radio-iodinated insulin (Amersham 60-100 mCi/μg) was diluted to give 8000-10000cpm/100μl.

Having transferred 100μl of the appropriate samples or

insulin standard into plastic LP3 tubes (100µl of IAB was used for the blanks, zeros and totals), 100µl of anti-human insulin guinea pig antibody (ICN Biomedicals) which had been diluted x25,000 from the stock, was added with vortexing to all samples except the blanks. Following a 6h incubation at 4°C, 100µl of radio-iodinated insulin was added to all samples, which were then vortexed and left overnight at 4°C.

The antibody bound insulin was separated from free insulin by precipitation with anti-guinea pig cellulose labelled donkey antibody. To each sample 100µl of the second antibody was added before vortexing and incubation at room temperature for 20min. The precipitate containing antibody bound insulin was then separated from the unbound insulin by the careful but vigorous (sufficient to stir up the contents of the tube) addition of 1ml of IAB and subsequent centrifugation at 2000g for 15min. The supernatant from all samples except the totals was removed using a drawn out pasteur pipette attached to an aspirator. The tubes were then capped and the radioactivity of the pellets determined by gamma counting. Using the data obtained from the standard samples a graph of the log of the concentration of insulin versus the counts per minute was plotted to give the calibration graph, typically as shown in Figure 2.1.. Using this graph the values of unknown samples were determined by extrapolation back to the y-axis.

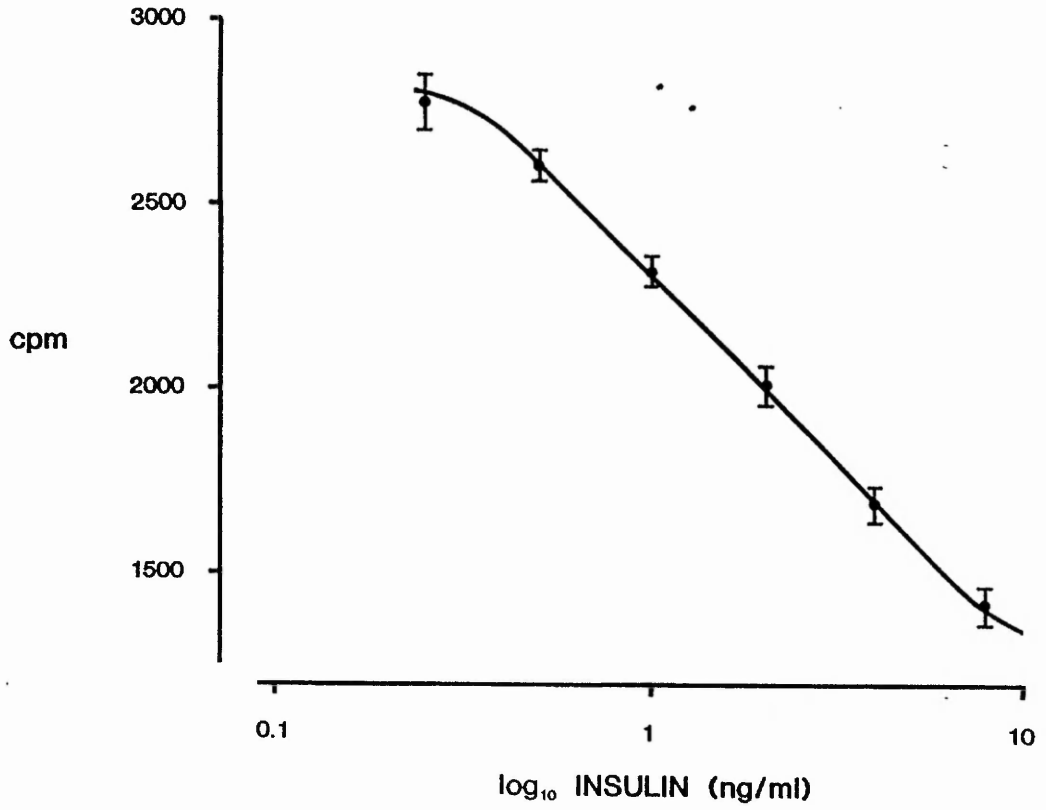
2.4.2 Protein Assay

Protein was estimated using a modification of the method of Lowry *et al* (1951).

Samples were diluted, if necessary, to bring them within

Figure 2.1

Insulin Standard Curve



range and 100µl of the sample solubilised in 100µl of 2% (w/v) SDS. To this was added 1ml of solution containing 49 parts of Folin A (0.4% (w/v) NaOH, 2% (w/v) $NaCO_3$, 0.02% (w/v) Na_2Tartarate) to 1 part of Folins B (0.5 (w/v) $CuSO_4.5H_2O$), and the mixture vortexed. Following 20min incubation, 100µl of a solution of freshly prepared Folin-Ciocalteau solution (1:1 distilled water) was added. The samples were again vortexed and then left for 20min before reading the absorbance at 750nm. Standard curves were prepared using bovine serum albumin (Fraction V; Wilfred Smith Ltd.) diluted with distilled water to give protein concentrations in the range 100µg/ml to 600µg/ml. Standards were taken through the same procedure.

2.4.3 Transglutaminase Assay

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

The standard incubation mixture following dilution with sample contained in a volume of 50µl :

27.50mM	Tris/HCl pH 7.4
3.85mM	dithiothreitol
2.50mM	$CaCl_2$ or
5.00mM	EDTA
1.20mM	[^{14}C]-putresine (3.96mCi/mmol)
5.0mg/ml	N-N'-dimethylcasein

Incubations were carried out in 0.8ml microcentrifuge tubes incubated in a waterbath at 37°C. The reaction was started by adding 22.5µl of sample (3 islets/ul) to 27.5µl of the reaction mixture. Following 30min incubation, 30µl samples were taken and spotted onto 2H pencil-labelled circles (2.2cm diameter) of Whatman 3MM filter paper

which had been pre-soaked in 100mM EDTA/1% (w/v) methylamine and then dried. These were immediately placed into a beaker of stirred ice-cold 10% (w/v) TCA for a minimum of 10min to precipitate the trichloroacetic acid-insoluble radioactivity. Any unbound radioactivity was removed from the filter papers by the following washing procedure :

4 x 10min wash in ice-cold 5% (w/v) TCA
and 1% (w/v) methylamine

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

1 x 5min wash in acetone

Washing was performed in a perforated beaker inserted within another beaker which contained a stirring magnetic flea. At least 25ml of TCA per paper circle per wash was maintained throughout.

Following washing the filter papers were allowed to dry overnight in air before being counting for radioactivity in 4ml of optiphase scintillation fluid. The Ca^{2+}-stimulated incorporation of [^{14}C]-putrescine into N-N'-dimethylcasein was calculated following the subtraction of EDTA containing incubations.

2.4.4 Lactate Dehydrogenase Assay

Lactate dehydrogenase activity was determined by following the decrease in the absorbance at 340nm of NADH during the conversion of pyruvate to lactate by lactate dehydrogenase.

The reaction mixture contained in a final volume of 1ml :

50mM potassium phoshate buffer, pH 7.4

1mM sodium pyruvate

0.1mM NADH

Reactions were started by addition of 50µl of sample to 0.95ml of reaction mixture. The decrease in absorbance at 340nm was

measured at 37°C for 3min in a DU-7 spectrophotometer (Beckman Instruments Ltd). Endogenous rates were measured by the substitution of phosphate buffer for pyruvate. Activity was calculated using a molar extinction coefficient for NADH of 3.22 x $10^{-3}M^{-1}cm^{-1}$ after subtraction of the endogenous rate.

2.4.5 5'-Nucleotidase Assay

The 5'-nucleotidase activity was measured using the method of Christie and Ashcroft (1985) which involves measuring the conversion of [^{3}H] 5'-AMP to [^{3}H]-adenosine by ion exchange chromotography.

The reaction mixture, following dilution with the sample contained in a final volume of 100µl :

50mM	Tris/HCl pH 8.0
3mM	$MgCl_2$
2mM	adenosine monophosphate
0.1µCi	2-[^{3}H]-adenosine monophosphate(19Ci/mmol)
0.1mg/ml	bovine serum albumin (Fraction V; Wilfred Smith Ltd)

Incubations were performed in 0.8ml microcentrifuge tubes and started by the addition of 50µl of sample (diluted by 2-5 times) to 50µl of the reaction mixture.

Duplicates of each sample were incubated for 1h at 30°C before the reaction was terminated by boiling for 5min. Following addition of 0.4ml of 0.1mM unlabelled adenosine, each sample was applied to a column containing 0.75ml of QEAE-A25 sephadex equilibrated 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 scintillation fluid. Controls for each sample in which the enzyme was inactivated by boiling for 2min

were taken through the same procedure. The radioactivity of these samples was subtracted as background during calculation of enzyme activity.

2.4.6 Cytochrome C Oxidase Assay

Cytochrome c oxidase activity was determined using the method of Cooperstein & Lazarow (1950), which follows the oxidation of reduced cytochrome c at 550nm.

The reaction mixture contained in a final volume of 650ul :

50mM potassium phosphate buffer, pH 7.4

1mM EDTA

41μM reduced cytochrome c

The reduced cytochrome c was prepared from freshly made cytochrome c solution, titrated with sodium dithionite (1.5mg/ml) to give an absorbance of approximately 0.8 at 550nm.

The reaction was started by addition of 50μl of sample to 600μl of the reaction mixture. The absorbance at 550nm was monitored for 15min at 37°C in a DU-7 spectrophotometer. Activity was calculated using a molar extinction coefficient for cytochrome c of $19.7 \times 10^3 M^{-1}{}_{cm}{}^{-1}$.

2.5 HOMOGENISED ISLET STUDIES

2.5.1 Homogenisation of Islets

Islets were homogenised at 4°C in the following medium :

0.3M	sucrose
50mM	Tris/HCl
1mM	EDTA
0.1mM	PMSF in DMSO (final concentration 0.1%(w/v))

Homogenisation was performed using a hand held 1ml glass/Teflon homogeniser (clearance 0.61mm). Islets of Langerhans were homogenised by 15 up and down passes of the Teflon plunger.

2.5.2 [^{14}C]-Methylamine Incorporation into Islet Homogenate

The reaction mixture following the addition of islet homogenate contained :

0.3M	sucrose
50mM	Tris/HCl pH 7.4
2mM	$MgCl_2$
3.85mM	dithiothreitol
1.78mM	[^{14}C]-methylamine (56mCi/mmol)
0.1mM	PMSF in DMSO (final concentration 0.1% (v/v))
2.5mM	$CaCl_2$ or
5.0mM	EGTA

Samples were incubated at 37°C for 30min before incorporation was stopped by cooling to 4°C.

2.5.3 Subcellular Fractionation of Islet Homogenate by Differential Centrifugation

Labelled and unlabelled homogenate was fractionated by two methods. In some cases homogenate was separated into high speed pellet (P) and a particle free supernatant(PFS) by centrifugation at 71,000g av for 45min at 4°C. The alternative was fractionation of the islet homogenate to produce a 600g av low speed pellet (N), an 71,000g av high speed pellet (P) and a particle free supernatant (PFS) using a modification of the method of Christie & Ashcroft (1985). All the pellets were resuspended by homogenisation in 200µl of homogenisation buffer (Methods 2.5.1).

Fractionation was performed in the 3ml adaptors to a 8 x 25ml rotor (MSE Superspeed 50), due to the small volume of homogenate.

All procedures were carried out at 4°C and fractions stored on ice.

2.6 SEPARATION AND DETECTION OF RADIOLABELLED PROTEINS

2.6.1 SDS-Polyacrylamide Gel Electrophoresis

The method employed was a modification of that described by Laemmli (1970) for use with a vertical slab gel apparatus. Two types of discontinuous gels were used to separate islet proteins. Both had a 3% (w/v) stacking gel but one had a 10% resolving gel whilst the other had a 5%-15% (w/v) gradient resolving gel.

2.6.1.1 Solutions

The solutions used were as follows :

Tris-glycine electrode buffer	15.2g	Tris base
(0.025M Tris, 192mM glycine)	72.0g	glycine
	5.0g	SDS
	pH 8.5 in 5000ml	
Tris-SDS stock solution, pH 6.8	3.03g	Tris base
(0.25M Tris)	0.2g	SDS
	pH 6.8 in 100ml	
Tris-SDS stock solution, pH 8.8	9.08g	Tris base
(0.75M Tris)	0.2g	SDS
	pH 8.8 in 100ml	
Acrylamide stock solution	30g	acrylamide
(30% (w/v))	0.8g	N-N'-methylene-bisacrylamide
		made up in 100ml
Ammonium persulphate	2% (w/v) in distilled water (freshly prepared)	

2.6.1.2 Sample Preparation

Islet homogenate samples were dissolved with a concentrated solution of the sample buffer to give the following concentrations (see legends for individual procedures):

62.5mM Tris/HCl pH 6.8

2% (w/v) SDS

5% (w/v) 2-mercaptoethanol

10% (w/v) glycerol

0.01mg/ml bromophenol blue

Following addition of the sample buffer the mixture was boiled for 5min and then stored at -70°C. Before electrophoresis the sample was boiled a second time for 5min to ensure the protein was fully dissolved, and then loaded onto the gels.

2.6.1.3 Procedure

The glass plates were cleaned by washing with detergent followed by rinsing with ethanol and distilled water. They were then assembled in the mould according to the manufacturers instructions to give gels with a width of 1.5mm.

The 10% (w/v) resolving gel solution, sufficient for 2 gels was made up of the following :

19.8ml acrylamide stock solution

30ml Tris-SDS stock solution pH 8.8

10.2ml distilled water

With the 5-15% gradient resolving gel the following two solution were made up :

Solution A	9.9ml	acrylamide stock solution
(5% (w/v) acrylamide)	30ml	Tris-SDS stock soln. pH 8.8
	20.1ml	distilled water

Solution B	29.7ml	acrylamide stock solution
(15% (w/v) acrylamide)	30ml	Tris-SDS stock soln. pH 8.8
	0.3ml	distilled water

The solutions were degassed for 15min using a vacuum pump. Immediately before pouring 1.5ml of a 1% (w/v) ammonium persulphate solution and 20µl TEMED (N,N,N',N'-tetramethylethylenediamine) was added to each. The gradient gel was poured whilst stirring, using a gradient former. Both types of resolving gels were poured to height of 12cm which would allow a stacking gel of a height 1.5cm. Once poured the resolving gels were overlaid with butan-2-ol to exclude air and to create a flat gel surface, and left to polymerize for a minimum of 60min. The butan-2-ol was then washed away and replaced with SDS-Tris stock solution pH 8.8/distilled water (1:2 (v/v)).

The stacking gel solution, sufficient for 2 gels was made up of the following :

2ml	acrylamide stock solution
10ml	Tris-SDS stock solution pH 6.8
8ml	distilled water

The solution was degassed for 10min. The diluted SDS-Tris stock, pH 8.8 was removed from the top of the resolving gel using a syringe/needle and the surface dried using filter paper. The stacking

gel solution was poured onto the resolving gel with a 10 well comb in place, after the addition of 1ml of 1% (w/v) ammonium persulphate solution and 12µl TEMED. The stacking gel was overlaid with butan-2-ol and left to polymerize for a least 60min. The combs were then removed, the butan-2-ol washed away and replaced with Tris-glycine buffer ready for the sample loading.

The gels prepared in this way had the following composition :

	Linear resolving gel	Gradient resolving gel	Stacking gel
acrylamide	10% (w/v)	5-15% (w/v)	3% (w/v)
N'-N-methylene-bisacrylamide	0.27% (w/v)	0.135%-0.405% (w/v)	0.08% (w/v)
SDS	0.1% (w/v)	0.1% (w/v)	0.1% (w/v)
Tris	0.375M	0.375M	0.125M

Samples were applied to the bottom of the wells using a Hamilton syringe. The rest of the apparatus was assembled according to the manufacturers instructions. Electrophoresis was carried out at 10-15^{o}C, at a constant current of 25mA/gel, whilst the samples were passing through the stacking gel and 35mA whilst samples were in the resolving gel.

Electrophoresis was continued for 4-6h until the bromophenol blue marker was within 1cm of the bottom of the resolving gel. Standards of proteins of known molecular weight were electrophoresised beside the samples on the gel, to allow calibration and the determination of protein molecular weight. For autoradiography, a set of [^{14}C]-methylamine labelled molecular weight standards were also electrophoresised.

The molecular weight standards were as follows : Unlabelled standards (Sigma)

Myosin	205,000 Da
β-Galactosidase	116,000 Da
Phoshorylase b	97,400 Da
Bovine serum albumin	66,000 Da
Egg Albumin	45,000 Da
Carbonic Anhydrase	29,000 Da

[^{14}C]-methylamine labelled standards (Amersham)

Myosin	200,000 Da
Phosphorylase b	97,400 Da
Bovine Serum Albumin	67,000 Da
Ovalbumin	47,000 Da
Carbonic Anhydrase	30,000 Da
Lysozyme	14,300 Da

2.6.1.4 Fixing, Staining and Destaining of Gels

Following electrophoresis the migration distance of the bromophenol blue front was noted and the plates removed from the mould. The gel was carefully removed from the plate and immersed overnight in 750ml of a fixing/staining solution consisting of :

0.125% (w/v)	PAGE 183
18% (v/v)	methanol
5% (v/v)	glacial acetic acid

The solution was freshly prepared by dissolving the PAGE 183 in methanol, and filtering. The glacial acetic acid was then added and the solution made up to volume with distilled water.

The gel was destained using :

5% (v/v)	methanol
7% (v/v)	glacial acetic acid

2.6.2 Scintillation Counting of Sectioned Gels

The lanes of the resolving gel were cut into 5mm sections, whilst the stacking gel was cut into 2 equal sections. Gel sections were placed into individual glass scintillation vials and dried under vacuum for 2-3h at 80°C. Whilst cooling to room temperature, 0.5ml of a solution of H_2O_2:NH_3 (100:3 (v/v)) was added to each vial which were immediately capped and the gel sections left to dissolve. The radioactivity of each vial was then determined by scintillation counting following the addition of 20ml of optiphase scintillation fluid.

2.6.3 Fluorography

Following destaining the gel was immersed for 15min in 200ml of Amplify (Amersham). The gel was then placed carefully on a sheet of Whatmann 3MM chromatography paper, covered with a sheet of cling-film and dried for 2h at 80°C on a gel dryer under vacuum (Bio-Rad Gel Dryer Model 543). The gel was then placed centrally within a film cassette holder and in the absence of light a sheet of X-ray (Fuji RX100 medical) was placed on the dried gel and the cassette sealed. The X-ray film was sensitized with a flash gun such that it had an absorbance of 0.15, following development, above that of unexposed film when measured at a wavelength of 540nm. The cassette was placed at -70°C for 60-90 days before the X-ray film was developed using Kodak LX-24 (90sec), fixed with Kodak FX-40 (120sec) and then washed.

The radioactive labelling of proteins was determined by scanning the individual gel lanes of the x-ray film, for their absorbance at 540nm (Pye Unicam SP1800 spectrophotomter). The molecular weight of proteins was determined by comparison to radiolabelled protein standards (see Methods 2.6.1.3.) which were run simultaneously on each gel.

2.6.4 Gel Filtration

Gel filtration was performed using either agarose A5M (range 10,000Da to 5 x 10^6Da) (Bio-Rad) and Trisacryl GF2000 (range 1.2 x 10^5Da to 15 x 10^6Da) (IPF Biotechnics) packed into a column of length 80mm and diameter 9mm. The column were pre-equilibriated with elution buffer (see below).

[^{14}C]-Methylamine labelled homogenate (100µl containing 100-200 islets) was dissolved in 20µl of 12% (w/v) SDS, 15% (v/v) 2-

mercaptoethanol and 50mM Tris/HCl, pH 7.4, boiled for 5min and then loaded onto the column. Sample was eluted from the column at a flow rate of 30-35µl/min using the following elution buffer :

1% (w/v)	SDS
50mM	Tris/HCl pH 7.4
1mM	dithiothreitol

Eluant was collected in 3min fractions (~100µl) using an LKB Redirac fraction collector.

The exclusion volume was determined with 25mM potassium dichromate (100µl) and the void volume with 100µl of blue dextran (5mg/ml). The column was calibrated with 100µl of bovine serum albumin (67,000 Da), apoferritin (444,000 Da) and thyroglobulin (669,000 Da) dissolved in the same buffer as the homogenate at a concentration of 5-10mg/ml and detected by protein assay (see Methods 2.4.2). The radioactivity of the fractions was determined by dissolving the 100µl samples in 4ml of optiphase, followed by scintillation counting.

2.7 ELECTROPERMEABILISED ISLET STUDIES

2.7.1 Preparation

Glassware used during experiments with electropermeabilised islets was siliconised (see Methods 2.3.1). All apparatus were acid washed with 20% (v/v) nitric acid and then rinsed with double de-ionised water (Millipore Milli-Q) to ensure the surfaces were clean. Double de-ionised water was used for all solutions and rinsing.

2.7.2 Electropermeabilisation Medium

For all experiments with electropermeabilised islets the medium developed by Jones et al (1985) was employed. This electropermeabilisation medium (permeation medium) contained :

140mM	K^+glutamate
15mM	HEPES pH 7.0
7mM	$MgSO_4$
5mM	ATP
5mM	glucose
1mM	EGTA
0.5mg/ml	bovine serum albumin (Fraction V; Wilfred Smith Ltd.)

Electropermeabilisation medium was made up fresh each day. The ATP, glucose, EGTA and bovine serum albumin were weighted out into a 100ml beaker and 10ml of concentrated (x10) stock solutions of K^+glutamate, HEPES and $MgSO_4$ pipetted out. The volume was made up to approximately 95ml, the pH at 37°C adjusted to 7.0 using HCl before the solution was made up to volume (100ml). A solution of $CaCl_2$ was added to give a free calcium concentration of 10nM. The concentration of free Ca^{2+} was controlled by the use of EGTA buffers (Portzehl et al, 1964) using the dissociation constants listed in Table 2.1., from the computer programme kindly donated by Professor S.L.Howell. The concentration of calcium required to give the relevant concentration of free Ca^{2+} are given in Table 2.2.

2.7.3 Electropermeabilisation of Islets

Islets were picked (see Methods 2.3.6) in the smallest possible volume and collected in a 1.5ml microcentrifuge tube, on ice.

Table 2.1

Dissociation Constants Used in the Calculation of Ca^{2+} Concentration in Electropermeabilisation Medium

The concentration of Ca^{2+} was controlled by the use of an EGTA buffer, the constants having been calculated from reported stability constants and pK values (Martell & Sillen, 1964; Grzybowski et al, 1970; Sillen et al, 1971), using the method described by Portzehl et al (1964). The dissociation constants (μM) were as follows:

Ligands	Bivalent Metal Ion	
	Ca^{2+}	Mg^{2+}
EGTA	0	24307
ATP	173	97
SO_4	5248	5370
Glutamate	15918	28307

Table 2.2

Determination of the Ca^{2+} Concentration in Electropermeabilisation Medium

The concentration of calcium (column 1) required to give the required concentration of Ca^{2+} (column 2) was calculated using the method of Portzehl et al (1964) (Table 2.1).

Concentration of Calcium (mM)	Calculated Ca^{2+} Concentration
0.005	10nM
0.01	13nM
0.1	140nM
0.5	1.2μM
0.9	5.7μM
1.15	10μM
1.25	20μM
2.50	100μM
13.0	1mM

Islets were pelleted by centrifugation at 13,000rpm for 15sec (MSE Micro Centaur) and the supernatant of physiological medium removed. Islets were resuspended in 1ml of permeation buffer using a Gilson pipette, pelleted and the supernatant removed. This washing procedure was repeated 4 times. After the final wash, the islets were resuspended in 0.8ml of permeation buffer and transferred using a Pasteur pipette into an electropermeabilisation cuvette (Bio-Rad cuvette for the Gene Pulser). Islets were permeabilised by 5 exposures to a uniform electric field of 3.0kV/cm from a capacitance of 3μF, using the Bio-Rad Gene Pulser. The optimum electric field and capacitance to give permeabilisation without excessive cell damage was determined by experimentation (Chapter 3). Each exposure was spaced by 15sec during which the islets were agitated with a Pasteur pipette to prevent them settling. Following electropermeabilisation, islets were washed 3 more times with permeation buffer. They were then transferred into a black petri-dish (4cm diameter)ready for picking. All the stages of electropermeabilisation were performed at 4°C.

2.7.4 Studies on Insulin Release from Electropermeabilised Islets

Groups of 3-10 electropermeabilised islets were picked out in the smallest possible volume (20-30μl) using a drawn out Pasteur pipette and transferred into a 10ml glass beaker. The beakers contained 1ml of permeation buffer and where necessary the insulin release stimulating agent (10mM arginine or 0.5μM phorbol 12-myristate 13-acetate (PMA) or 100μM cAMP/500μM 3-isobutyl-1-methylxanthine (IBMX)) and inhibitors of transglutaminase activity. Both PMA and IBMX were dissolved in DMSO which gave a final volume during incubations of 0.1% (v/v). Each batch of islets was pre-incubated at 4°C for 15min to allow equilibration of compounds. Incubations were started by sealing

the beakers with suba-seal stoppers and transferring to a 37°C waterbath. The starting times were staggered by 30sec to take account of the different starting times of the pre-incubations, which resulted from the islet picking process. In those experiments where the insulin release was stimulated by raising the concentration of Ca^{2+} from 10nM to 10μM, islets were pre-incubated in 0.9ml of permeation buffer and test compounds. Incubations were started following the addition of 0.1ml of permeation buffer with the relevant amount of $CaCl_2$.

After 30min incubation, 50μl samples were taken and stored in 0.8ml microcentrifuge tubes at -70°C awaiting assay for insulin. Samples were taken under a binocular microscope to ensure no islets or debris were collected. During each experiment controls were performed in which islet groups were incubated at 10nM Ca^{2+} and 10μM Ca^{2+} to ensure that islets were permeabilised and responding to Ca^{2+} stimulation.

2.7.5 Incorporation of [^{14}C]-Methylamine into Electropermeabilised Islets

Groups of 100 electropermeabilised islets were collected in a 1.5ml microcentrifuge tube on ice. The islets were pelleted by centrifugation at 13,000rpm for 5sec (MSE Micro Centaur) and the supernatant removed. Islets were then resuspended in 90μl of permeation buffer containing 4.4mM [^{14}C]-methylamine (56mCi/mmol). Where necessary inhibitors of transglutaminase activity, 0.5μM PMA or 500μM cAMP/100μM IBMX were also included. Both PMA and IBMX were dissolved in DMSO (final volume 0.1% (v/v)). Following pre-incubation at 4°C for 15min, incorporation was started by the addition of 10μl of $CaCl_2$ in permeation medium to give the relevant Ca^{2+} concentration, followed by incubation at 37°C for 30min. For each experiment controls

were performed, in which groups of 10 islets were incubated at 10nm Ca^{2+} and 10μM Ca^{2+} and the insulin release assayed.

2.7.5.1 TCA Percipitation of [^{14}C]-Methylamine Labelled Proteins

Following [^{14}C]-methylamine incorporation, electropermeabilised islets were pelleted by centrifugation at 13,000rpm (MSE Micro Centaur) for 15sec and the supernatant removed. Islet pellets were then immediately frozen in liquid nitrogen and stored at -70°C until processing.

When 10-15 samples had been collected, each pellet was defrosted and then disrupted and resuspended by ultrasonication in 35μl of homogenisation medium (see Methods 2.5.1) with 2 bursts of 5sec, each at 4μ peak to peak, with a 15sec interval between bursts. The temperature was maintained at 4°C throughout this process. Samples were immediately deposited onto 2.2cm circles of 3MM Whatmann filter paper which had been pre-soaked in 100mM EDTA/1% (w/v) methylamine, and immersed in stirred ice-cold 10% (w/v) TCA for a minimum of 10min. Unbound radioactivity was removed using the washing procedure described in Methods 2.4.3.. The radioactivity was determined in 4ml of optiphase scintillation fluid.

RESULTS

3 ELECTROPERMEABILISATION

All previous investigations into the role of transglutaminase in the B-cell of the islets of Langerhans have been performed in either intact islets or islet homogenate. Studies using intact islets have suggested a role for transglutaminase during stimulus-secretion coupling (Bungay et al, 1986) whilst homogenate work has identified possible transglutaminase substrates (Bungay et al, 1982; Gomis et al, 1989). However, both of these models have certain disadvantages when it comes to the further exploration of the function of transglutaminase. The studies concerned with the effect of transglutaminase inhibitors on glucose stimulated insulin release in intact islets, does not give any information as to where transglutaminase may be important in the process of stimulus-secretion coupling. Previous investigators have tentatively suggested that the enzyme is involved in the distal events associated with secretion, on the basis that transglutaminase is a Ca^{2+}-responsive enzyme and a rise in the intracellular Ca^{2+} concentration is thought to be the main mediator between stimulus perception and secretion (Bungay et al, 1986). However, the effects of transglutaminase inhibitors could be equally well explained if transglutaminase played a role during receptor mediated endocytosis (Introduction 1.2.4.4) or in the regulation of the cytoskeletal network (Introduction 1.2.4.5), as suggested in other cell types. Similarly, although it has been possible to identify possible transglutaminase substrates in islet homogenates using radiolabelled probes, these experiments have been performed at nonphysiological Ca^{2+} concentrations ranging from 0.5mM Ca^{2+} (Gomis et al, 1989) to 2.5mM Ca^{2+} (Bungay et al, 1986). Furthermore, since cellular integrity has been destroyed, it is

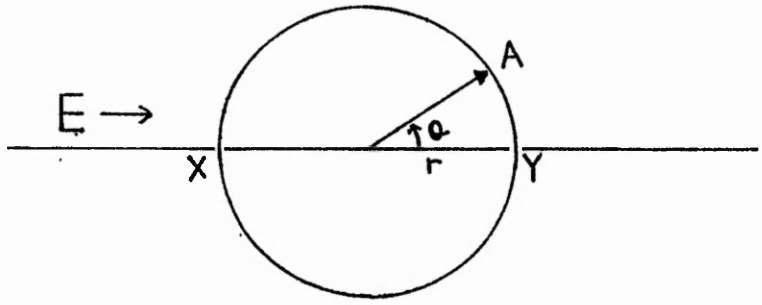
impossible to correlate the labelling of these substrates with insulin release. In order to overcome some of these problems it was decided to use islets in which the plasma membrane had been permeabilised by exposure to high voltage electrical discharge. This permits access of low molecular weight ions and molecules to the interior of the B-cell, thereby allowing the manipulation of the intracellular environment. Using this method membrane integrity is preserved and islets of Langerhans permeabilised have been reported to release insulin in response to a variety of stimuli including Ca^{2+} (Pace et al, 1980; Yaseen et al, 1982), cAMP (Jones et al, 1986), and the phorbol ester, PMA (Jones et al, 1985). The use of electropermeabilised islets would therefore allow investigations to be undertaken that would confirm or otherwise the link between the Ca^{2+}-message, transglutaminase activation and the mechanism of insulin release.

3.1 Theory of Electropermeabilisation

Although a number of methods have been used in an attempt to obtain selective breakdown of the plasma membrane of cells (permeabilisation), the most satisfactory of these for studies on intracellular signal transduction and secretion is electropermeabilisation, which has been applied to a variety of cell types (Knight and Scrutton, 1986). The technique involves exposing cells to the high voltage discharge of a capacitance, which above a threshold voltage results in the localized breakdown of the plasma membrane and the formation of stable pores.

To understand how this process occurs it is assumed that a cell in suspension undergoing electropermeabilisation, approximates to a hollow sphere. If this cell is subjected to a uniform electric field the potential difference at any point A (V_A) (Figure 4.1) will be given

by the equation:-



$$V_A = C\ r\ E\ \cos\theta \quad \text{volts}$$

where r = radius of the cell (cm)

E = field strength (V/cm)

C = a constant dependent on the relative conductivities of the extracellular fluid, the cytosol and the membrane

Since under most conditions the resistivity of the intracellular and extracellular medium is far less than that of the membrane, and the radius of the cell is much greater than that of the membrane, then under these conditions C approaches 1.5 (Knight et al, 1981). It can also be seen from the equation that the greatest potential difference, and therefore the points at which membrane breakdown is likely to occur first, is along the line of the electric field (points X,Y) where $\cos\theta = \pm 1$. This means that subjecting a cell to a electric pulse which generates a potential at the points X,Y just sufficient to cause membrane breakdown (V_{max}) should result in the formation of two pores.

The equation to describe the membrane potential at these points X,Y is:-

$$V_{max(x,y)} = 1.5\ r\ E$$

The potential difference V_{max} required to cause membrane breakdown will be dependent on a number of factors including membrane thickness, composition (Zimmermann et al, 1974; Benz et al, 1979) and temperature (Costa & Zimmermann, 1975). However, studies using suspensions of various cell types, artificial membranes and giant plants cells suggest that a membrane voltage of approximately 1.5V is required for membrane breakdown to occur (Knight, 1981). An important consequence of this relatively constant breakdown potential is that the electric field strength (E) required to obtain membrane breakdown is inversely proportional to the size of the sphere, so that the smaller the sphere the larger the field strength required. This means that it is possible to selectively permeabilise the plasma membrane without effecting the membranes of the subcellular organelles, such as mitochondria.

As well as requiring an electric field of sufficient magnitude to cause membrane breakdown, the duration of the electric field needs to be at least as long as the time taken for the membrane to charge up. If it is again assumed that the resistivity of the membrane is much greater than that of the intracellular and extracellular medium, then the time constant for the charging up of the

membrane, Tm is given by:-

$$T_m = r\ C\ (p_i + 0.5p_o) \quad \text{seconds}$$

where r = radius of the cell (cm)

C = capacitance of the membrane (F/cm^2)

$p_i + p_o$ = resistivity of the intracellular and extracellular fluids respectively

Under normal conditions Jeltsch & Zimmerwald (1979) have reported that the time required for the applied electric field to impose the voltage difference across the membrane is less than 1μs, whilst Riemann et al (1975) has reported that if the voltage difference is of sufficient strength, breakdown occurs within nanoseconds.

Applying this technique to the study of insulin release from the islet B-cell is complicated by the fact that cellular aggregates are being dealt with rather than single cells in suspension. Furthermore, although B-cells constitute from 60-80% of the cells of islets there are also a-cells and d-cells present. Initial studies were therefore directed towards optimising the conditions of the electropermeabilisation process to ensure that permeabilisation of the B-cells was occuring without excessive damage to the islet.

3.2 Electropermeabilisation of Rat Islets of Langerhans

Electropermeabilisation of islets was performed with the Bio-Rad Gene Pulser with which it is possible to vary the size of the capacitance (C) and the electric field strength (E). It was decided to base the method of electropermeabilisation on that described by Jones et al (1985), since rat islets permeabilised in this way had been shown

to release insulin in response to Ca^{2+} and activators of protein kinase A (Jones et al, 1985) and protein kinase C (Jones et al, 1986). In this method islets were washed 5 times in K^+glutamate permeabilising buffer, electropermeabilised by 5 exposures (15sec intervals) to an electric field of 3.4kV/cm from a 2μF capacitance and then washed 3 more times. All of these steps were performed at 4°C.

3.2.1 Optimisation of Electropermeabilisation

When developing the electropermeabilisation process a procedure similar to that described by Jones et al (1985) was initially adopted. The method only differed in the size of capacitance (3μF) used since the Bio-Rad Gene Pulser does not have a 2μF facility, which in turn affected the electric field strength.

3.2.1.1 Optimisation of Capacitance and Electric Field Strength for Electropermeabilisation

Using the K^+ glutamate medium, the discharging of a capacitance of 3μF was found to result in a half life decay ($t_{1/2}$) of 10-200μs, as registered on the Gene Pulser. Since a $t_{1/2}$ of at least 2μs was required to allow the development of a potential across the islet cell plasma membrane this appeared to be adequate. The electric field required for membrane breakdown was determined by exposing groups of 50 islets to 5 pulses (at 15sec intervals) to an electric field of increasing strength (2.2kV/cm to 3.4kV/cm). The amount of islet permeabilisation following 15min incubation in permeabilisation buffer was then determined using trypan blue, which is normally impermeable to intact islets (Figure 4.1). It was found that with electric fields of less than 2.6kV/cm there was only a low level of permeability to trypan blue. This probably results from damage to the islets during isolation

since it was comparable to the trypan blue permeability of intact islets incubated in permeabilisation buffer (8 $\pm$ 4 % (n=3)). With the electric field strength of 2.6kV/cm or above there was a significant increase in islet permeabilisation. At 3.0kV/cm this resulted in 92% permeabilisation as determined by trypan blue exclusion. Since electric field strengths greater than 3.0kV/cm failed to produce significantly greater permeabilisation it was decided to use this value during permeabilisation. When the extent of permeabilisation was tested after 45min incubation following exposure to an electric field strength of 3.0kV/cm, it was found that 94% of islets were still permeable to trypan blue, thereby suggesting that the islets had not resealed over this period.

Table 3.1

Effect of Electric Field Strength on the Trypan Blue Permeabilility of Electropermeabilised Islets

Groups of 50 islets were washed 5 times in 1ml of permeation buffer at 4°C containing 10nM Ca^{2+}. They were then electropermeabilised by 5 exposures (at 15sec intervals) from a 3μF capacitor at an electric strength which varied in size from 2.2kV/cm to 3.4kV/cm. Islet groups were then washed 3 more times in permeation buffer before incubation in 1ml of buffer for 15min at 4°C. They were then pelleted, resuspended in 100μl of permeation buffer containing 2mg/ml trypan blue in permeation buffer (10nM Ca^{2+}) and incubated at 4°C for a further 2min. The number of islets which appeared to be permeable to trypan blue was determined by examination under a binocular microscope on a glass microscope slide.

The results are expressed as the mean percentage of islets ± SEM permeable to trypan blue, determined from 3 separate experiments.

Electric Field Strength (kV/cm)	% of Islets Permeable to Trypan Blue
2.2	10 ± 4
2.6	58 ± 10
3.0	92 ± 8
3.4	94 ± 8

3.2.1.2 Lactate Dehydrogenase Release following Electropermeabilisation

To ensure that the parameters used during electropermeabilisation were not resulting in the destruction of the islets or producing pores of sufficient size to allow the loss of higher molecular weight components of the cytosol, the activity of the cytosolic marker, lactate dehydrogenase was measured in electropermeabilised and intact islets. Islets were electropermeabilised (Methods 2.7.3) and batches of 60 islets pre-incubated at 4°C for 15min and then incubated at 37°C for 30min in permeation buffer containing 10nM Ca^{2+}. Islets were then separated from the medium by centrifugation, resuspended in homogenising buffer and homogenised at 4°C (Methods 2.5.1). Lactate dehydrogenase activity (Methods 2.4.4) in the homogenate of electropermeabilised islets was found to be 3.58 ± 0.63 µmol NADH oxidised/islet/60min (n=4). This compares with the enzyme activity of 3.88 ± 0.93 µmol NADH oxidised/islet/60min present in islets which were not electropermeabilised but incubated at 37°C for 45min in Gey and Gey medium containing 2.8mM glucose. If it is assumed that the dry weight of an islet is 1.4µg (Beckmann et al, 1980) then this gives an enzyme activity of 2.76 mol/kg dry weight/60min in non-electropermeabilised islets. This lactate dehydrogenase activity is comparable with that reported in rat islets by Kissane & Brolin (1963) (2.97 mol/kg dry weight/60min), Hellman & Taljedal (1967) (4.65 mol/kg dry weight/60min) and Yaseen et al (1982) (1.5 mol/kg dry weight/60min).

This data therefore indicates that the parameters used during electropermeabilisation have not resulted in large scale damage to the islets and that the pores are small enough to prevent any significant loss of lactate dehydrogenase.

3.2.1.3 Transglutaminase Activity Following Electropermeabilisation

Since the aim of the project was to use electropermeabilised islets to investigate the role of transglutaminase, it was important to show that transglutaminase activity was present following permeabilisation. Groups of 200 electropermeabilised islets (Methods 2.7.3) were therefore pre-incubated for 15min at 4°C in 1ml of permeation buffer containing 10nm Ca^{2+} before being incubated at 37°C for 30min. Islet groups were then homogenised (Methods 2.5.1) and their transglutaminase activity determined (Methods 2.4.3). It was found that the transglutaminase activity in homogenates from electropermeabilised islets (1.74 ± 0.23 nmol putrescine incorporated/100 islets/30min (n=4)) was not significantly different from the activity in islets which had been incubated at 37°C for 45min in Gey and Gey medium containing 2.8mM glucose (1.63 ± 0.24 nmol putrescine incorporated/100 islets/30min (n=4)). The latter gives a transglutaminase activity of 1.3 nmol/40islets/60min which is comparable to that of 0.9 nmol putrescine incorporated/40 islets/60min measured by Bungay et al (1986). These results demonstrate that no significant loss of transglutaminase activity occurred during and upto 45min after the permeabilisation process.

3.2.2 Characterisation of Electropermeabilised Islets

The work so far has attempted to ascertain the optimum parameters required for the electropermeabilisation of rat islets of Langerhans. However, in order for these permeabilised islets to be useful as a model for studying a role for transglutaminase during Ca^{2+}-stimulated insulin release it was important to investigate whether they were able to secrete insulin in response to increased Ca^{2+} as was first

demonstrated by Pace et al (1980). Investigations were also undertaken on some of the physiological parameters that may influence this Ca^{2+}-stimulated release since previous work has demonstrated that this release is temperature (Jones et al, 1985) and ATP dependent (Pace et al, 1980; Jones et al, 1985) and glucose independent (Pace et al, 1980; Jones et al, 1988).

3.2.2.1 Ca^{2+}-Stimulated Insulin Release

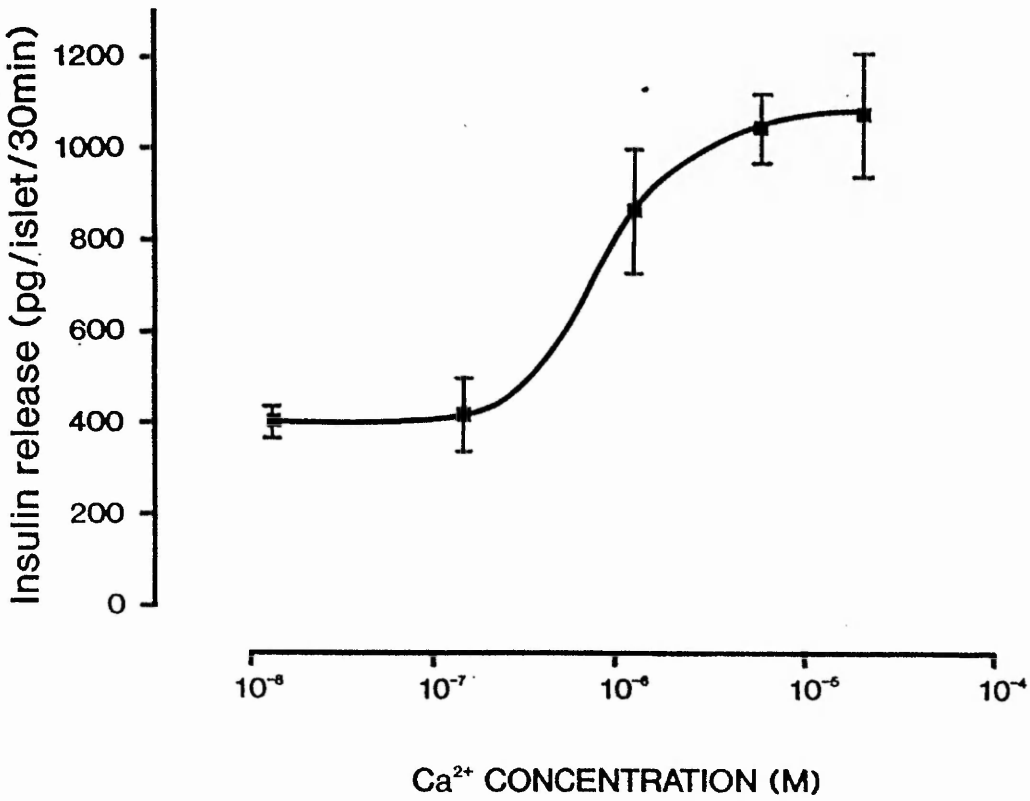
Incubation of electropermeabilised islets in permeation buffer containing increasing concentrations of Ca^{2+} (10nm to 20μM) produced a dose related increase in the insulin release, amounting to a 167% increase over this Ca^{2+}-concentration range (Figure 3.1). This Ca^{2+}-induced increase in insulin release produced a half maximal response at 0.8μM Ca^{2+}.

Figure 3.1

Ca^{2+}-Stimulated Insulin Release From Electropermeabilised Islets of Langerhans

Batches of 3-5 electropermeabilised islets (Methods 2.7.3) were pre-incubated for 15min at 4°C in 0.9ml of permeation buffer containing 10nM Ca^{2+}. The effect of Ca^{2+} on insulin release was then tested by adding 0.1ml of permeation buffer containing the relevant amount of $CaCl_2$ to give a range of Ca^{2+} concentrations (10nM to 20μM) (Methods 2.7.2) and incubated for 30min at 37°C. Following incubation, supernatant samples were taken and assayed for their insulin concentration by radioimmunoassay.

The results in Figure 3.1 give the mean insulin release ± SEM of 3-5 observations taken from a typical experiment.


Insulin release (pg/islet/30min)
1200
1000
800
600
400
200
0
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Ca²⁺ CONCENTRATION (M)

3.2.2.2 Effect of Temperature on Ca^{2+}-Stimulated Insulin Release

The effect of temperature on Ca^{2+}-stimulated insulin release from electropermeabilised islets of Langerhans was measured (Figure 3.2). At 37°C, increasing the Ca^{2+} concentration from 10nM to 10μM Ca^{2+} resulted in a 126% increase in the insulin release. However, when islets were incubated at 4°C, the rate of release was virtually eliminated having been reduced by 80% at 10nM Ca^{2+} and 91% at 10μM Ca^{2+}, to that measured at 37°C. The islets incubated at 4°C also failed to respond when the Ca^{2+} concentration was increased from 10nM to 10μM. This low level of insulin release at 4°C means that pre-incubating permeabilised islets at 4°C in the presence of compounds that we require to equilibrate with the intracellular space of the islet will only result in a minor contribution to the final measured level of insulin release. Since the process of Ca^{2+}-stimulated insulin release is temperature dependent this also implies that this mechanism is enzyme dependent and not a result of a non-physiological action of the Ca^{2+}.

Figure 3.2

Temperature Dependence of Ca^{2+}-Stimulated Insulin Release

Batches of 10 electropermeabilised islets (Methods 2.7.3) were pre-incubated for 15min at 4°C in permeation buffer containing 10nM Ca^{2+}. The effect of temperature on Ca^{2+}-stimulated insulin release was tested by incubating batches at either 4°C or 37°C for 30min with either 10nM Ca^{2+} or 10μM Ca^{2+}. Following incubation a supernatant sample was taken from each batch and assayed for insulin by radioimmunoassay.

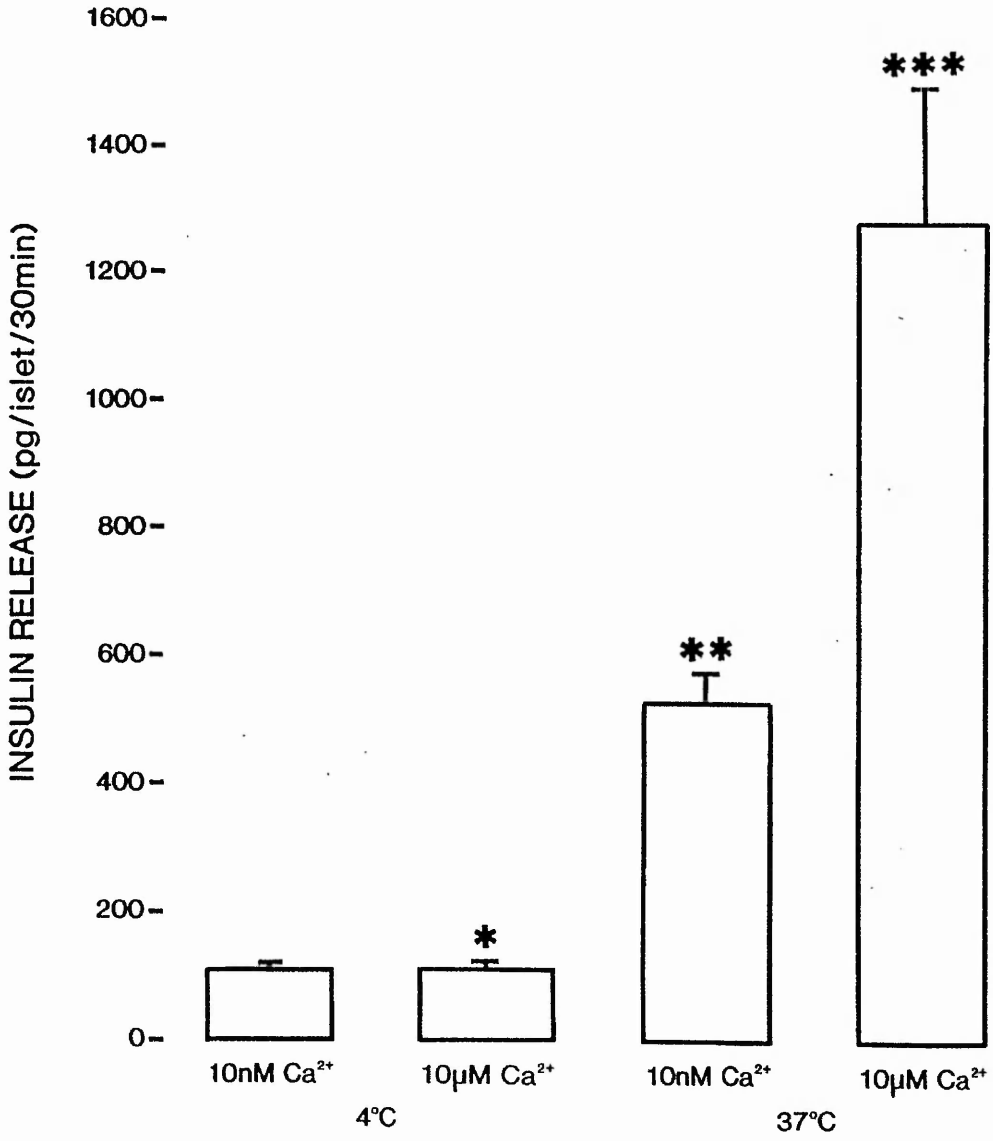
Data are the mean insulin release (pg/islet/30min) ± SEM taken from 3 separate experiments, each of 3-5 observations. The significance of the difference between conditions was determined using the Student paired t-test; N.S. denotes no significant difference

* P. N.S. versus 10nM Ca^{2+}/4°C

** P. < 0.001 versus 10nM Ca^{2+}/4°C

*** P. < 0.005 versus 10nM Ca^{2+}/37°C

< 0.0001 versus 10μM Ca^{2+}/4°C


INSULIN RELEASE (pg/islet/30min)
1600
1400
1200
1000
800
600
400
200
0
*
**
***
10nM Ca2+
10µM Ca2+
10nM Ca2+
10µM Ca2+
4°C
37°C

3.2.2.3 Effect of ATP on Ca^{2+}-Stimulated Insulin Release

The Ca^{2+}-stimulated insulin release from electropermeabilised islets was found to be dependent on the presence of MgATP (Figure 3.3). Islets incubated at 37°C in the presence of 5mM MgATP responded with a 144% increase in insulin release when the Ca^{2+} concentration was increased from 10nM Ca^{2+} to 10μM Ca^{2+}. When MgATP was absent from the external medium the rate of insulin release was reduced by 61% at 10nM Ca^{2+} and 80% at 10μM Ca^{2+} of that in the presence of 5mM MgATP. The islets without 5mM MgATP also failed to respond to an increase in the concentration of Ca^{2+} from 10nM to 10μM. These results demonstrate that MgATP is required for Ca^{2+}-stimulated insulin release from electropermeabilised islets. They provide further evidence to support the previous observation that the Ca^{2+}-stimulated insulin release is an enzymic reaction and an energy driven process.

Figure 3.3

ATP Dependence of Ca^{2+}-Stimulated Insulin Release

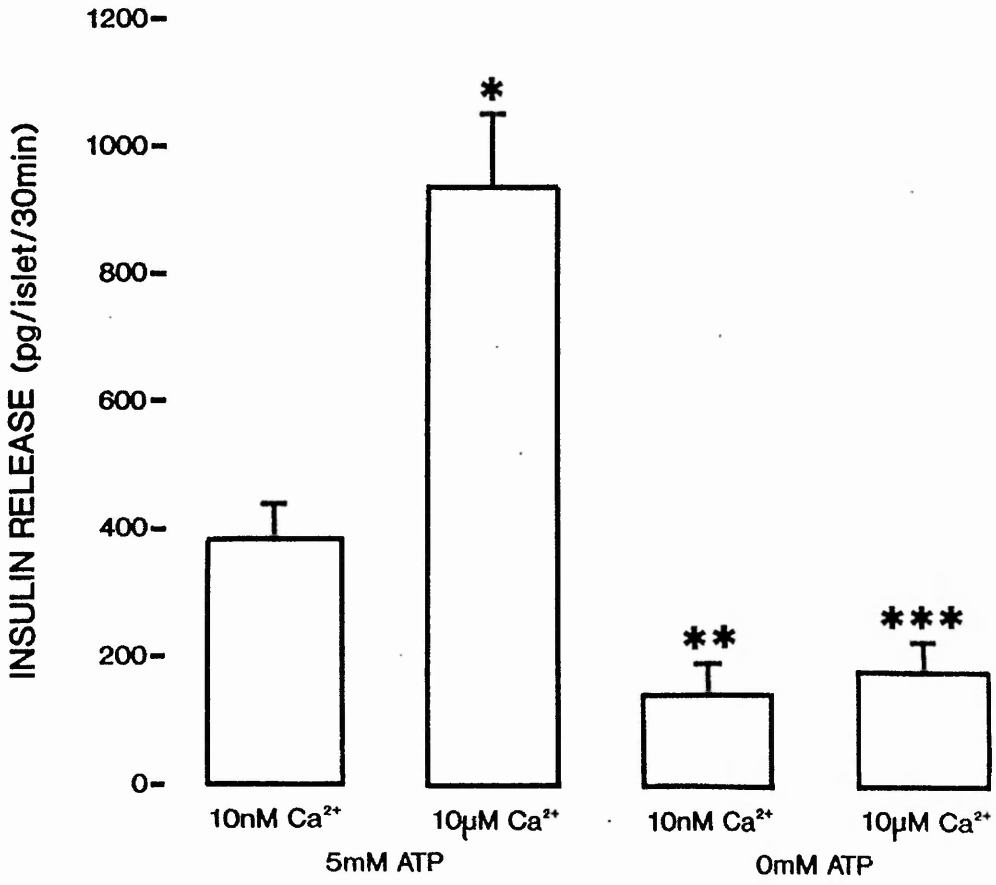
Batches of 10 electropermeabilised islets (Methods 2.7.3) were pre-incubated for 15min at 4°C in permeation buffer containing 10nM Ca^{2+} and with or without 5mM MgATP. The effect of MgATP on Ca^{2+}-stimulated insulin release was tested by incubating batches at 37°C for 30min with either 10nM Ca^{2+} or 10μM Ca^{2+}. Following incubation, supernatant samples were taken and assayed for insulin by radioimmunoassay.

Data are the mean insulin release (pg/islet/30min) ± SEM of 3 separate experiments, each of 3-5 observations. The significance of the difference between conditions was determined using the Student paired t-test; N.S. denotes no significant difference.

* P. < 0.005 versus 10nM Ca^{2+}/5mM MgATP

** P. < 0.01 versus 10nM Ca^{2+}/5mM MgATP

*** P. < 0.0005 versus 10μM Ca^{2+}/5mM MgATP


INSULIN RELEASE (pg/islet/30min)
1200
1000
800
600
400
200
0
*
**
***
10nM Ca2+
10µM Ca2+
10nM Ca2+
10µM Ca2+
5mM ATP
0mM ATP

3.2.2.4 Effect of Glucose on Ca^{2+}-Stimulated Insulin Release

Unlike intact islets, the Ca^{2+}-stimulated insulin release was found not to be dependent on the presence of glucose (Figure 3.4). The insulin release from electropermeabilised islets in the presence and absence of 5mM glucose was found to be increased by 144% and 142% respectively when the Ca^{2+} concentration was raised from 10nM Ca^{2+} to 10μM Ca^{2+}. Although the absolute levels of insulin release in the absence of 5mM glucose was slightly less than that in the presence of glucose this was not significant.

Figure 3.4

Effect of Glucose on Ca^{2+}-Stimulated Insulin Release

Batches of 10 islets electropermeabilised islets (Methods 2.7.3) were pre-incubated for 15min at 4°C in permeation buffer containing 10nM Ca^{2+} and with or without 5mM glucose. The effect of glucose on Ca^{2+}-stimulated insulin release was tested by incubating batches at 37°C for 30min with either 10nM Ca^{2+} or 10μM Ca^{2+}. Following incubation, supernatant samples were taken from each batch and assayed for insulin by radioimmunoassay.

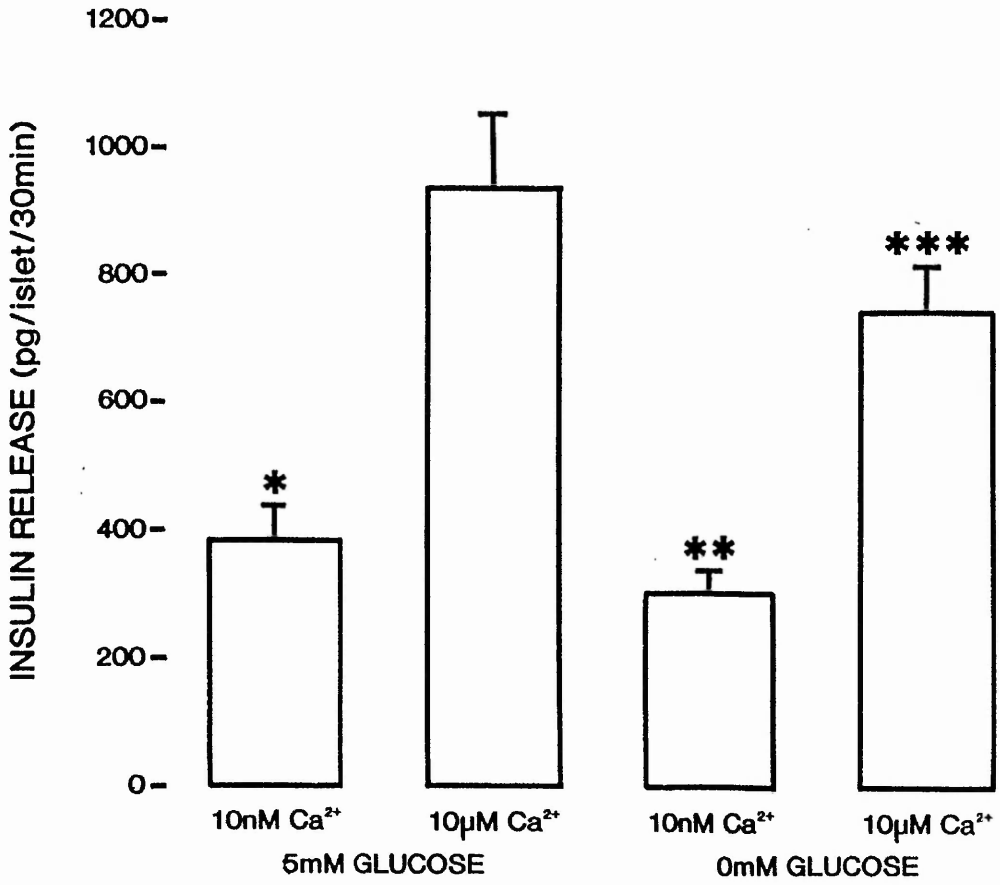
Data are the mean insulin release (pg/islet/30min) ± SEM taken from 3 experiments each of 3-5 observations. The significance of the difference between conditions was determined using the Student paired t-test; N.S. denotes no significant differences.

* P. < 0.005 versus 10μM Ca^{2+}/5mM Glucose

** P. N.S. versus 10nM Ca^{2+}/5mM Glucose

*** P. < 0.0005 versus 10nM Ca^{2+}/0mM Glucose

N.S. versus 10μM Ca^{2+}/5mM Glucose


INSULIN RELEASE (pg/islet/30min)
1200
1000
800
600
400
200
0
*
**
***
10nM Ca2+
10µM Ca2+
10nM Ca2+
10µM Ca2+
5mM GLUCOSE
0mM GLUCOSE

3.3 Discussion

In an attempt to overcome some of the problems of using either intact or homogenised islets to investigate the role of transglutaminase in the stimulus secretion mechanism of the B-cells of the rat islets of Langerhans, it was decided to use electropermeabilised islets. Previous work with electropermeabilised rat islets of Langerhans has demonstrated that insulin secretion can be stimulated when the Ca^{2+} concentration of the external medium is increased (Pace et al, 1980; Yaseen et al, 1982; Jones et al, 1985). Since transglutaminase has been implicated as a possible Ca^{2+} target (Bungay et al, 1986), this appeared to provide an ideal model in studying a role for the enzyme during this process.

Initial work involved optimising the electropermeabilisation method since it was required to permeabilise the islets to produce pores of sufficient size to allow the entry of small molecules and ions, but not large enough to result in the leaching of larger molecular weight components or the loss of membrane integrity. The method used was based on that developed by Yaseen et al (1982) and later refined by Jones et al (1985). Rat islets of Langerhans permeabilised by this procedure have also been demonstrated to secrete insulin in response to a variety of other intracellular messengers (Jones et al, 1985; Jones et al, 1986). The method involves exposing islets, suspended in a K^+ glutamate based medium to 5 exposures to a 2μF capacitance with an electric field strength of 3.4kV/cm. In order to follow this method as closely as possible a 3μF capacitance was decided upon. With the K^+ glutamate based medium this resulted in an electric field varying in duration from 10-200μs. Since the time required for the membrane to charge up and then breakdown is thought to take 1-2μs, then this appeared to be more than adequate. In order to

obtain the optimum electric field strength the trypan blue permeability of islets which had been electropermeabilised at field strengths varying in magnitude from 2.2kV/cm to 3.4kV/cm was examined. Using this procedure it was found that a field strength of 3.0kV/cm was the lowest value that gave a consistent, near 100% permeabilisation of islets. Islets were still found to be permeable to trypan blue even after 45min suggesting that they had not resealed in this period. This is in agreement with Yaseen et al (1982) who showed that the 80% of the islet space was still permeable after a 40min incubation but in disagreement with Pace et al (1980) who reported that resealing had occurred within 30min, since these islets were able to respond normally to stimulatory concentrations of glucose. However, the latter result may have been due to the lower voltage (2.5kV/cm) used by Pace et al (1980) to obtain permeabilised islets, since this would in theory have resulted in pores of smaller size (Knight, 1981), which may then have been repaired by the islet cells.

The work so far had demonstrated that islets subjected to 5 exposures from a 3μF capacitance at an electric field of 3.0kV/cm resulted in their permeability to the low molecular weight molecule, trypan blue. However, to rule out the possibility that this procedure had destroyed the islets or produced pores of sufficent size to allow the loss of larger cytosolic component, the activity of lactate dehydrogenase in intact islets was compared with that in islets that had been subjected to electropermeabilisation. It was found that there was no significant difference between the two treatments. The same result was found when the activity of the islet transglutaminase was measured, which ensured that the enzyme was still present following electropermeabilisation.

Having optimised the conditions for electropermeabilisation

further studies proceeded to assess whether membrane integrity of the B-cell had been maintained. This was undertaken by investigating whether the permeabilised islets were able to secrete insulin in response to an increase in the Ca^{2+} concentration of the external medium. These studies indicated there was a dose dependent (2-3 fold) increase in the release of insulin over the range 100nM Ca^{2+} to 20μM Ca^{2+}, with a half maximal response at 0.8μM Ca^{2+}. This is comparable to the dose-response curve measured by Jones et al (1985) and to the half maximal response of 0.6μM Ca^{2+} measured by Yaseen et al (1982) using electropermeabilised rat islets, as well as those measured in islets permeabilised with digitonin (Tamagawa et al, 1985; Best, 1986). The range of Ca^{2+} concentrations over which secretion occurs is also similar to that measured in other electropermeabilised cell preparations including catecholamine release from bovine adrenal medullary cells (Baker & Knight, 1981), cortical granule discharge from sea urchin eggs (Baker et al, 1980) as well as a host of other cell types (Knight & Scrutton, 1986). However, Yaseen et al (1982) found that electropermeabilised rat islets gave maximal insulin release at 1μM Ca^{2+} whilst Pace et al (1980) measured half maximal and maximal secretion at 50nM Ca^{2+} and 350nM Ca^{2+} respectively.

Measurement of the basal Ca^{2+} concentration in intact insulin secreting cell lines (Wollheim & Pozzan, 1984; Abrahamsson et al, 1985; Boyd et al, 1986) and B-cells (Rorsman et al, 1984; Deleers et al, 1985; Sussman et al, 1987) using the fluorescent dyes, quin-2 and fura-2 gave values in the range 50nM Ca^{2+}-200nM Ca^{2+} which are in agreement with the measured threshold Ca^{2+} concentration required for the stimulation of insulin release. However, stimulation of intact insulin secreting cells and B-cells is only thought to result in a 50-600% increase in the concentration of intracelluar Ca^{2+}, which is an order

of magnitude below that required to give maximal insulin release in electropermeabilised islets. However, this could be explained by the fact that measurement of cytosolic Ca^{2+} concentrations is not able to detect the localised development of high concentrations of Ca^{2+} possibly under the plasma membrane as a result of Ca^{2+} cycling across this membrane (Rasmussen, 1990).

To eliminate the possibility that the increase in insulin release from electropermeabilised islets resulted from a non-physiological action of the increase in Ca^{2+} concentration, possibly by acting on the plasma membrane, it was shown that both basal (at 10nM Ca^{2+}) and Ca^{2+}-stimulated (10μM Ca^{2+}) insulin release were temperature and MgATP dependent. These results are agreement with those of both Pace et al, (1980) and Jones et al (1985) and suggest that the both basal and Ca^{2+}-stimulated insulin release are enzymatic and energy driven processes. Since Pace et al (1980) have shown that intact islets only secrete insulin in response to changes in the Ca^{2+} concentration of the medium when these are in the millimolar range and only then in the presence of stimulatory concentrations of glucose, it was thought unlikely that insulin release resulted from a non-specific affect resulting from the increase in the Ca^{2+} concentration on islets that had not been permeabilised. This is partly supported by the results which demonstrated that insulin release was not stimulated in the absence of MgATP when the Ca^{2+} concentration was raised from 10nM to 10μM. This result as well as that demonstrating the requirement of permeabilised islets for MgATP to induce Ca^{2+}-stimulated insulin release implies that they are either unable to metabolise glucose, possibly as a result of the loss of an important co-factor through the plasma membrane pores or that the amount of ATP produced is insufficient.

Having developed a method for electropermeabilising rat islets of Langerhans further studies were then directed towards investigating a function for transglutaminase in the B-cells, since these cells make up the bulk of the cells present in these cellular aggregates.

4 THE ROLE OF TRANSGLUTAMINASE DURING INSULIN RELEASE FROM ELECTROPERMEABILISED ISLETS OF LANGERHANS

4 THE ROLE OF TRANSGLUTAMINASE DURING INSULIN RELEASE FROM ELECTROPERMEABILISED ISLETS OF LANGERHANS

4.1 The Importance of Transglutaminase in Ca^{2+}-, PMA- and cAMP-Stimulated Insulin Release

Several studies using transglutaminase substrate/inhibitors have reported that these compounds inhibit insulin release from intact islets, when evoked by both nutrient and non-nutrient secretagogues. The inhibition resulting from these compounds was found to be comparable to their measured inhibition on transglutaminase activity in vitro (Bungay et al, 1982; 1984; 1986; Gomis et al, 1983; 1984; Sener et al, 1985). Following reports that cytosolic transglutaminase is Ca^{2+}-dependent, with an apparent Km_{app} of approximately 3μM (Bungay et al, 1986), it was proposed that the enzyme may be an important target for the increase in the intracellular Ca^{2+} concentration thought to be an important mediator of stimulus-secretion coupling in the B-cell.

In an attempt to investigate this further it was decided to use electropermeabilised rat islets. The permeabilisation of the plasma membrane is thought to dissipate the electrical potential across this membrane. It is then possible to stimulate insulin release through manipulation of the intracellular Ca^{2+} concentration using a Ca^{2+}/EGTA buffer. Such a system would provide an ideal model system for confirming a role for transglutaminase as a Ca^{2+} target during insulin secretion.

As well as Ca^{2+}, increases in the concentration of other intracellular intermediates such as cAMP and diacylglycerol (DAG) whose effects are thought to be mediated by activation of protein kinase A and protein kinase C respectively, are also believed to be important in the distal events of secretion (Prentki & Matschinsky, 1987). It is

thought that these intermediates may act in part by a Ca^{2+}-dependent effect through altering the sensitivity of the secretory process to Ca^{2+} (Jones et al, 1985; Jones et al, 1986) and possibly by a Ca^{2+}-independent mechanism (Jones et al, 1989). Although the exact pathway for these different mechanisms is unknown it has been suggested that their effects are exerted by altering the phoshorylation state of B-cell protein following activation of protein kinases. Jones et al (1988) has demonstrated that protein phoshorylation occurs in electropermeabilised islets and that this pattern is altered following exposure to Ca^{2+}, cAMP and PMA, thereby suggesting that the various protein kinases systems are still at least partially functioning. Since Owen et al (1988) has reported that transglutaminase catalysed the formation of a high molecular weight phosphopolymer following glucose stimulation in intact rat islets, it was decided to investigate a role for transglutaminase during cAMP- and PMA-stimulated insulin release from permeabilised islets. This was on the basis that transglutaminase may affect insulin release by influencing the substrate availability to the various protein kinases or alternatively, the active role of the enzyme may be dependent on the phoshorylation of its protein substrates.

When investigating a role for transglutaminase during insulin release from electropermeabilised islets the alkylamines cystamine (Bungay et al, 1984), methylamine (Bungay et al, 1984; Gomis et al, 1984) and glycine methyl ester (Sener et al, 1985) and the naphthalenesulphonamide, monodansylcadaverive (Bungay et al 1984; 1986), were used. These competitive amine substrates of the enzyme have previously been shown to inhibit both transglutaminase activity and glucose stimulated insulin release from intact islets.

4.1.1 Effect of Transglutaminase Inhibitors on Ca^{2+}-Stimulated Insulin Release

In order to determine whether transglutaminase was important during Ca^{2+}-stimulated insulin release from electropermeabilised islets the effect of inhibitors of transglutaminase activity on this process was investigated. Incubation with cystamine, methylamine, glycine methylester and monodansylcadaverine resulted in the inhibition of Ca^{2+}-stimulated insulin release (Figures 4.1-4.4). In all cases except that found for monodansylcadaverine, the inhibition of Ca^{2+}-induced insulin release was shown to be dose dependent. The approximate concentration of the inhibitor required for 50% inhibition of Ca^{2+}-stimulated insulin release was 3.6mM cystamine, 7.6mM methylamine and 2mM glycine methyl ester. These values are comparable to those measured for inhibition of glucose stimulated insulin release from intact islets which were 0.1-0.5mM cystamine (Bungay et al, 1984), 1-2mM methylamine (Bungay et al, 1984; Gomis et al, 1984) and 5-10mM glycine methyl ester (Sener et al, 1985). With monodansylcadaverine, maximum inhibition appeared to be found at the lowest concentration of the inhibitor used (50μM). Since this inhibition of Ca^{2+}-stimulated insulin release is also comparable to the compounds measured inhibition of the activity of transglutaminase measured in vitro (Bungay et al, 1984; 1986; Gomis et al, 1984 ; Sener et al, 1985), it strongly suggests that they are acting on transglutaminase. However, to eliminate the possibility that these compounds may be acting by a non-specific action on another cellular target, the effect of sarcosine methyl ester and dimethylmonodansylcadaverine was also examined. These compounds are analogues of glycine methyl ester and monodansylcadaverine respectively and lack the primary amine group necessary for transglutaminase inhibition (Sener et al, 1985; Bungay et

al, 1986). Both of these compounds failed to inhibit Ca^{2+}-stimulated insulin release (Figure 4.5). In the case of dimethylmonodansylcadaverine which was dissolved in dimethylsulphoxide (DMSO), incubation of electropermeabilised islets in the same final concentration of 0.1%(v/v) DMSO, failed to cause significant inhibition of insulin release.

Figures 4.1 - 4.5

Effect of Transglutaminase Inhibitors on Ca^{2+}-Stimulated Insulin Release

Batches of 3-5 electropermeabilised islets were pre-incubated at 4°C for 15min in 0.9ml of permeation buffer containing 10nM Ca^{2+} and the relevant concentration of transglutaminase inhibitor. Following the addition of 0.1ml permeation buffer containing a concentrated Ca^{2+} solution that gave a final concentration of 10μM Ca^{2+}, islet batches were incubated at 37°C for 30min. Samples of supernatant were then taken and assayed for their insulin content by radioimmunoassay. Control experiments were undertaken in which islet batches were incubated in the presence of either 10nM Ca^{2+} or 10μM Ca^{2+}.

Figures 4.1 - 4.5 give the mean insulin release (pg/islet/30min) ± SEM of the number of experiments (n), each of 3-8 observations (group). The significance of the difference between the means of groups incubated in the presence 10μM Ca^{2+} and the means of groups incubated under identical conditions in the presence of either test compounds or at basal Ca^{2+} (10nM Ca^{2+}) was determined using the Student paired t-test.

N.S. denotes no significance.

GME = glycine methyl ester

SME = sarcosine methyl ester

MDCD = monodansylcadaverine

dimethyl-MDCD = dimethylmonodansylcadaverine

DMSO = dimethylsulphoxide

Figure 4.1

The Effect of Cystamine on Ca^{2+}-Stimulated Insulin Release

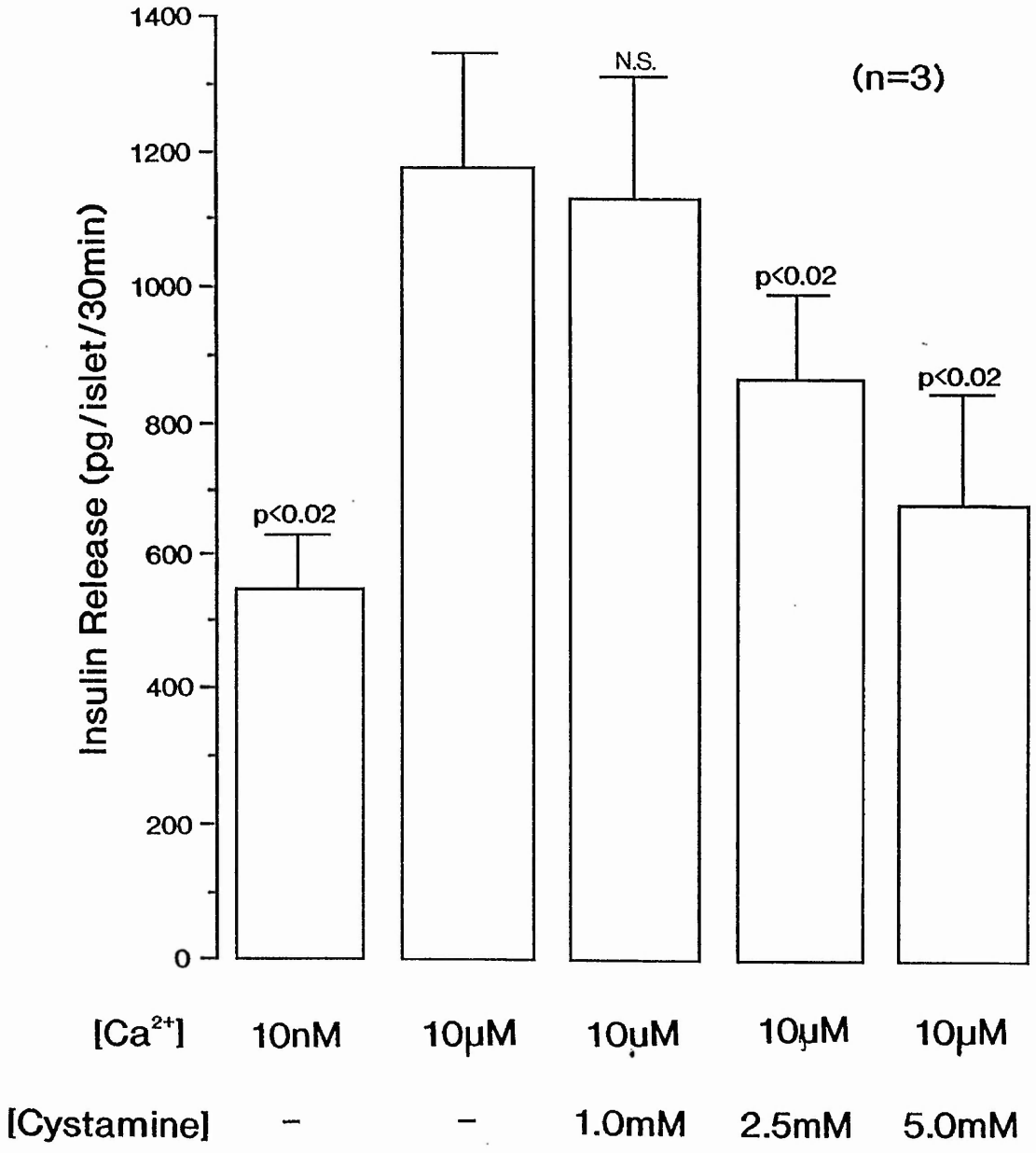


Figure 4.2

The Effect of Methylamine on Ca^{2+}–Stimulated Insulin Release

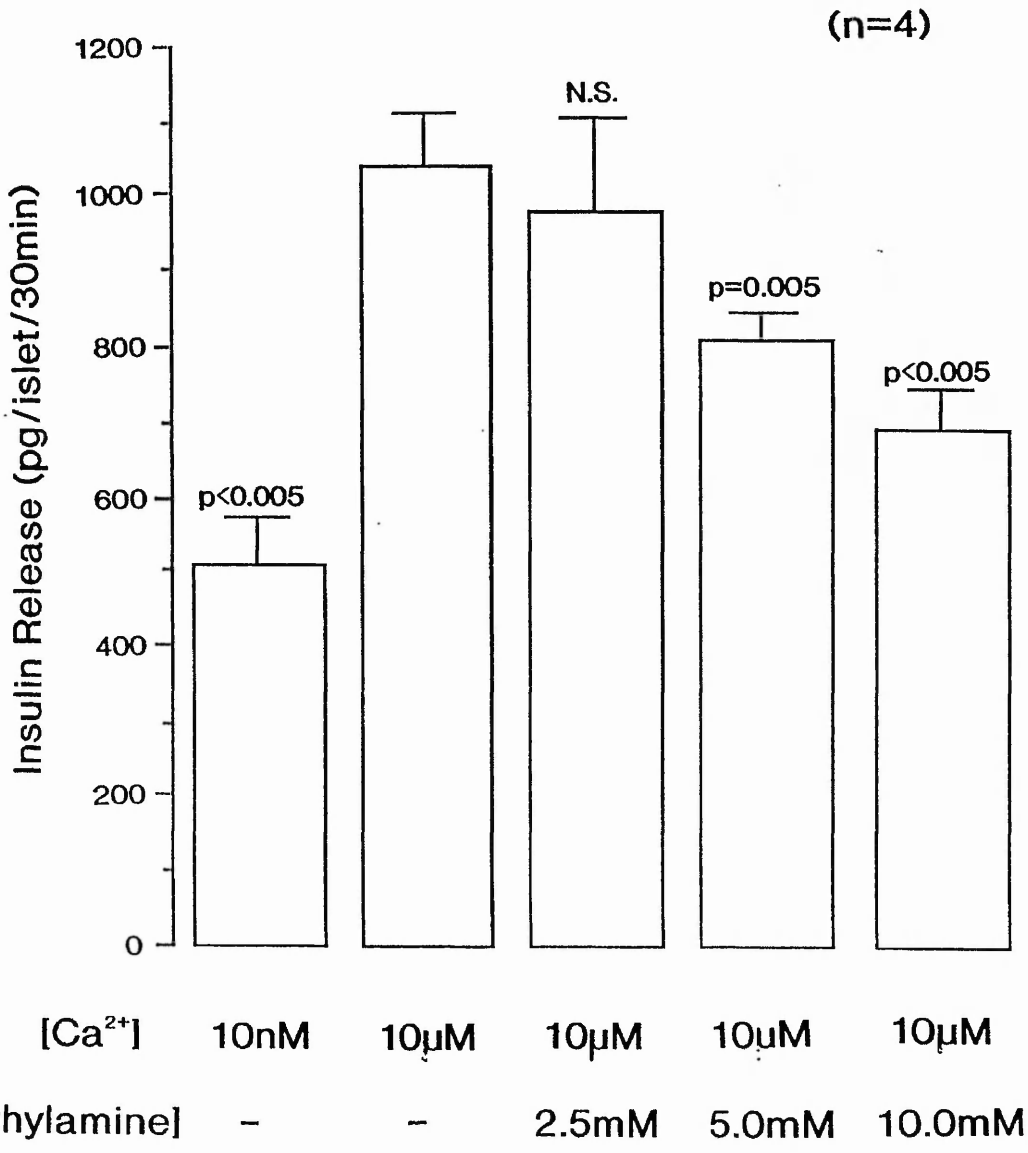


Figure 4.3

The Effect of Glycine Methyl Ester on Ca^{2+}-Stimulated Insulin Release

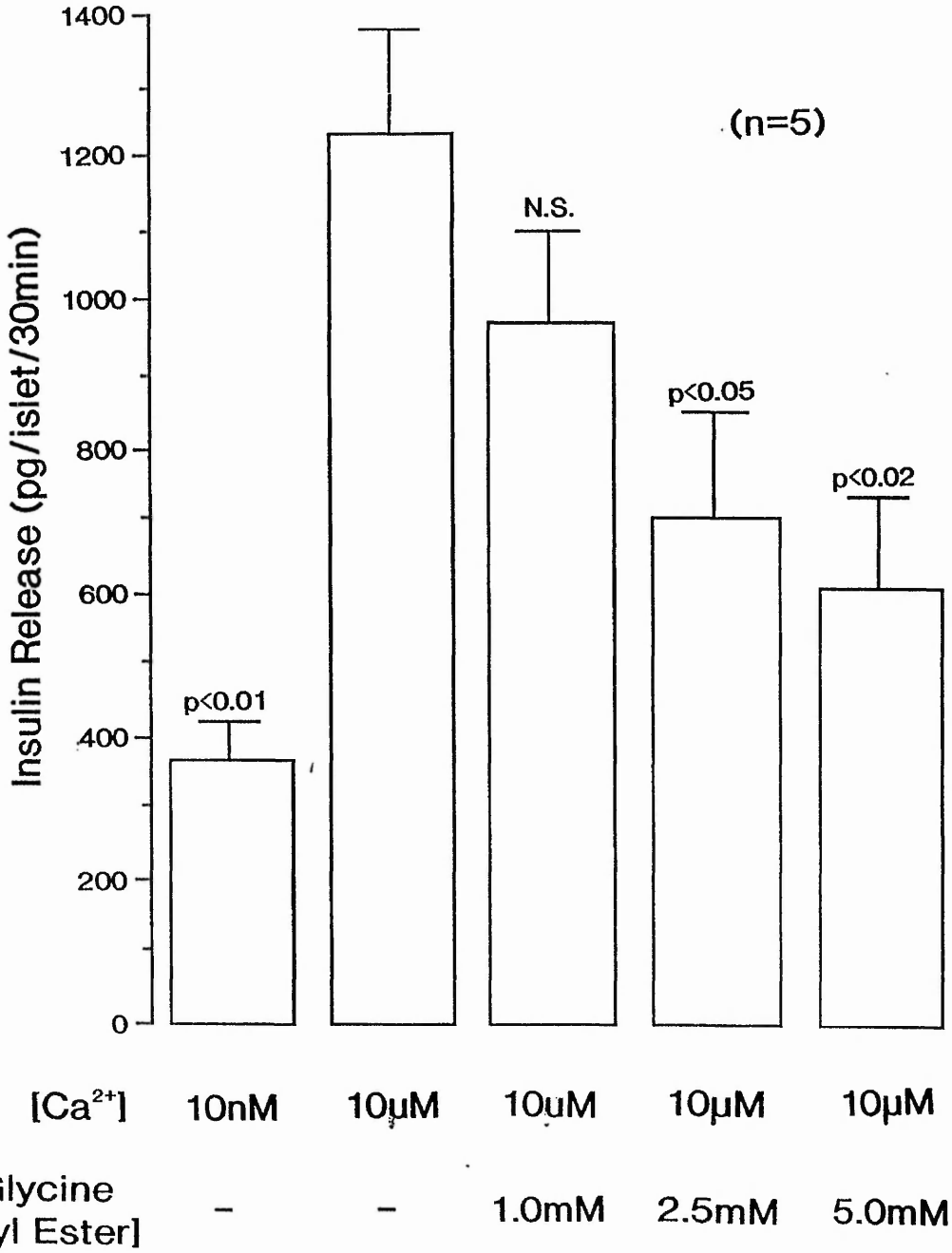


Figure 4.4

The Effect of Monodansylcadaverine on Ca^{2+}-Stimulated Insulin Release

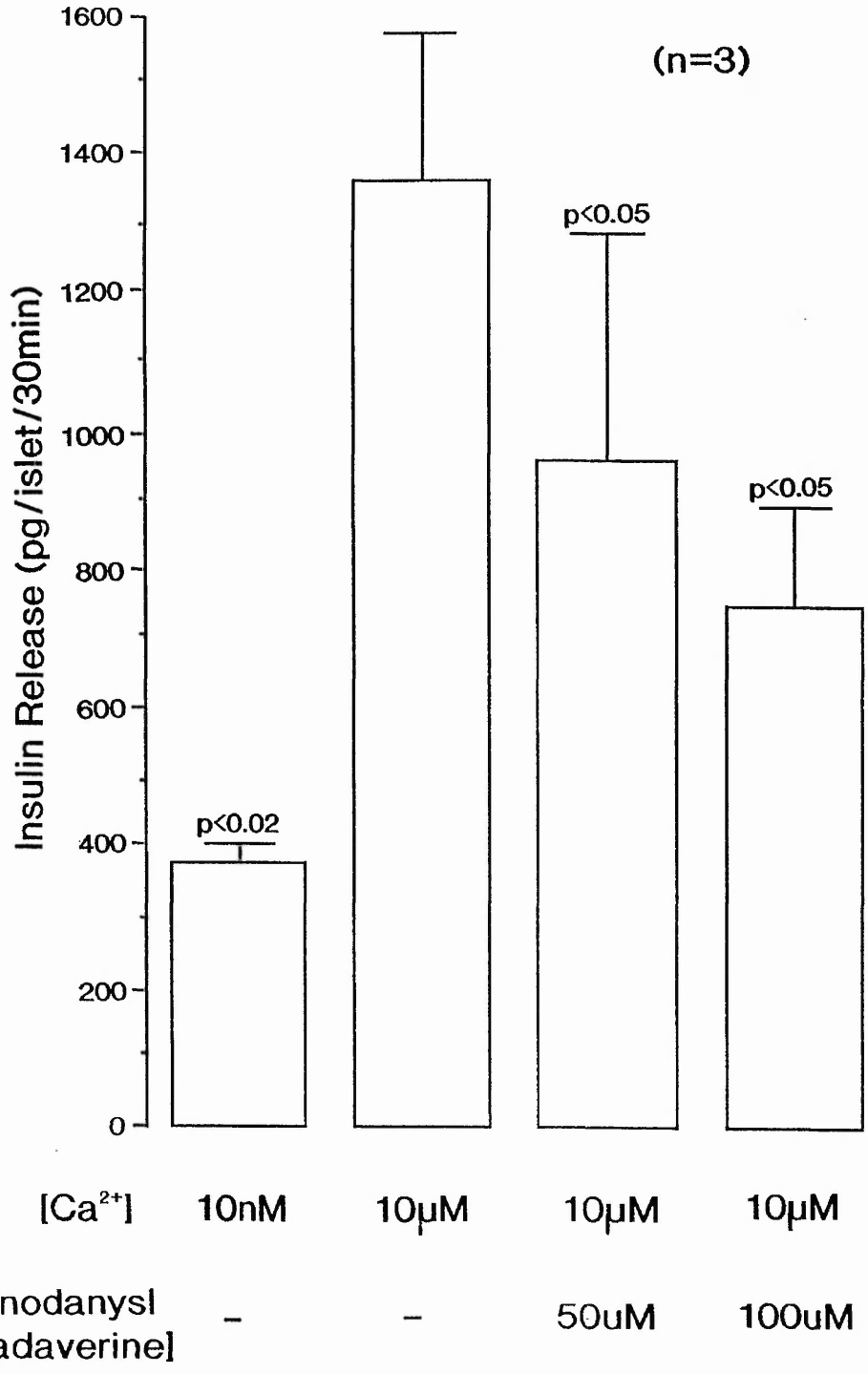


Figure 4.5

Effect of Non-Inhibitor Analogues of Transglutaminase Inhibitors on Ca^{2+}-Stimulated Insulin Release

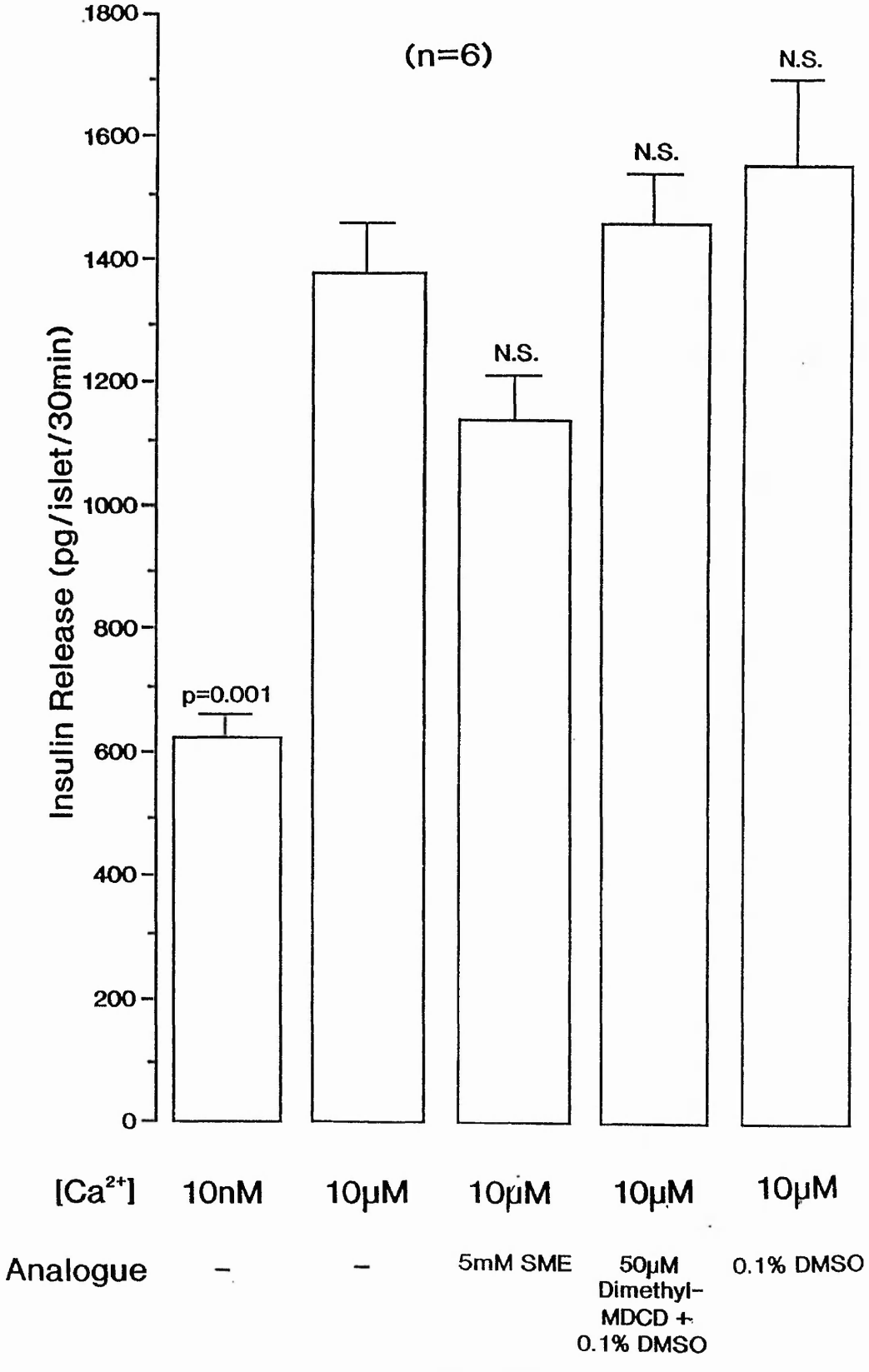


Table 4.1

Effect of Transglutaminase Inhibitors on Ca^{2+}-Stimulated Insulin Release

Table 4.1 is a summary of the results from Figures 4.1 to 4.4 expressed as the percentage of Ca^{2+}-stimulated insulin release.

Compound	Concentration (mM)	% of Ca^{2+}-Stimulated Insulin Release (±SEM)
Cystamine	1.0	92 ± 29
	2.5	50 ± 21
	5.0	9 ± 26
Methylamine	2.5	89 ± 15
	5.0	55 ± 7
	10.0	33 ± 9
Glycine Methyl Ester	1.0	77 ± 16
	2.5	42 ± 18
	5.0	30 ± 16
Monodansylcadaverine	0.05	59 ± 32
	0.10	37 ± 14

4.1.1.1 Effect of Transglutaminase Inhibitors on Basal Insulin Release

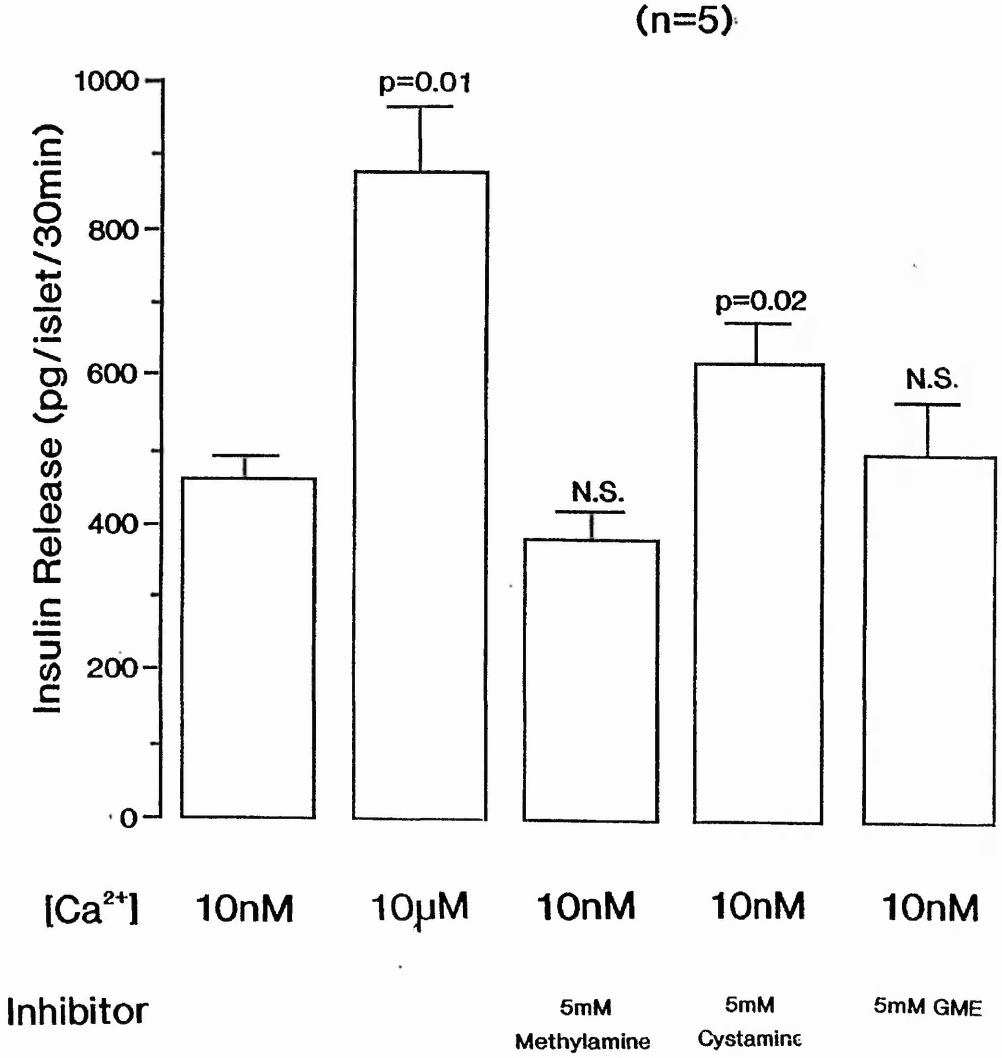
Since the observed effects of the transglutaminase inhibitors could also result from inhibition of basal insulin release the effect of test compounds on insulin release at 10nM Ca^{2+} was also examined. These results showed that at concentrations of the inhibitors that had previously been demonstrated to produce significant inhibition of Ca^{2+}-stimulated insulin release, that none of the compounds significantly inhibited basal insulin release from electropermeabilised islets (Figure 4.6). This is similar to the reported action of cystamine and monodansylcadaverine on basal insulin release from intact islet at 2.8mM glucose (Bungay et al, 1984). However, in the case of electropermeabilised islets incubated with cystamine (5mM) this led to a significant 34% rise in insulin release whilst with monodansylcadaverine, incubation at 10nM Ca^{2+} resulted in a massive release of insulin of a magnitude approaching 300-400% greater than that found in Ca^{2+}-stimulated islets. These results contradict those of Bungay et al (1984) who showed that methylamine stimulated a 60% increase in insulin release from intact islets incubated at 2.8mM but that cystamine (1mM) and monodansylcadaverine (100μM) had no affect under similar conditions. However, Gomis et al (1984) found that 2mM methylamine had no affect on intact islet at 5.6mM glucose.

Figure 4.6

Effect of Transglutaminase Inhibitors on Basal Insulin Release

Batches of 3-5 electropermeabilised islets were pre-incubated at 4°C for 15min in 1ml of permeation buffer containing 10nM Ca^{2+} and the relevant transglutaminase inhibitor. Insulin release was stimulated by incubation at 37°C for 30min following which supernatant samples were taken and assayed for their insulin content by radioimmunoassay. Control experiments were performed in which islet batches were incubated in the presence of either 10nM Ca^{2+} or 10μM Ca^{2+}. Results are expressed as the mean insulin release (pg/islet/30min) ± SEM for the number of experiments (n), each of 3-8 observations (group). The significance of the difference between the means of groups incubated at 10nM Ca^{2+} and the means of groups incubated at 10nM Ca^{2+} in the presence of transglutaminase inhibitors or with 10μM Ca^{2+}, was assessed using the Student paired t-test. N.S. denotes no significant difference.

GME = glycine methyl ester


(n=5)
Insulin Release (pg/islet/30min)
1000
800
600
400
200
0
p=0.01
p=0.02
N.S.
N.S.
[Ca2+]
10nM
10µM
10nM
10nM
10nM
Inhibitor
5mM Methylamine
5mM Cystamine
5mM GME

4.1.2 Effect of Transglutaminase Inhibitors on PMA Stimulated Insulin Release

Previous work by Jones et al (1985) has demonstrated that exposure of electropermeabilised rat islets of Langerhans to the phorbol ester, phorbol 12-myristate 13-acetate (PMA) at both non-stimulatory (10nM) and stimulatory (10μM) concentrations of Ca^{2+} results in elevated insulin release. Other investigators have reported a comparable effect in digitonin permeabilised rat islets (Tamagawa et al, 1985), as well as electropermeabilised RINm5F cells (Vallar et al, 1987). It is thought that the action of PMA on insulin secretion is mediated by mimicking diacylglycerol which stimulates protein kinase C by increasing its affinity for Ca^{2+} and thereby stimulating the enzyme without elevating Ca^{2+} (Nishizuka et al, 1984). In order to investigate a possible interaction between transglutaminase and protein kinase C, the effect of transglutaminase inhibitors on PMA-stimulated insulin release from electropermeabilised islets incubated at basal Ca^{2+} concentrations was investigated.

Incubation of electropermeabilised islets with PMA resulted in a 130% increase in insulin release (Figure 4.7). This increase in insulin release was not due to stimulation by the PMA carrier DMSO since incubation in the same final concentration of DMSO (0.1% (v/v)) had no significant affect on basal insulin release (454 ± 65 pg/islet/30min n=3). When the transglutaminase inhibitors glycine methyl ester (5mM) and monodansylcadaverine (50μM) were included it was found that they had no significant affect on the PMA stimulated insulin release. However, incubation with the sarcosine methylester (5mM) the analogue of glycine methyl ester, which has been shown to have no inhibitory action on both Ca^{2+}-induced and glucose stimulated insulin release, resulted in a significant reduction in the rate of PMA induced

insulin release.

Figure 4.7

The Effect of Transglutaminase Inhibitors on PMA Stimulated Insulin Release

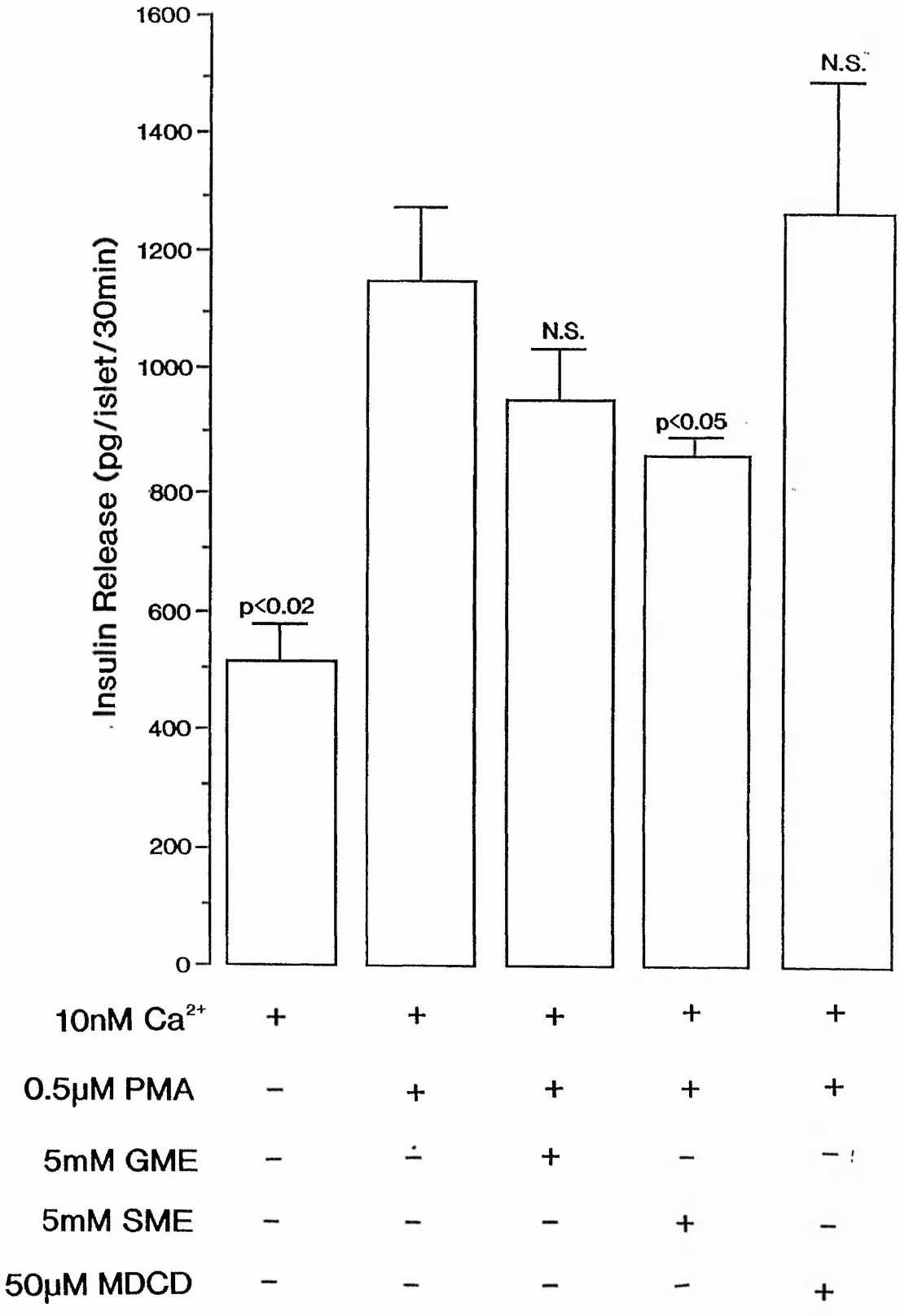
Batches of 10 electropermeabilised islets were pre-incubated at 4°C for 15min in 1ml of permeation buffer containing 10nM Ca^{2+} and 0.5μM PMA and the relevant test compound, where necessary. Insulin release was then stimulated by incubation at 37°C with supernatant samples being taken after 30min and the amount of insulin determined by radioimmunoassay. Results are expressed as the mean insulin release (pg/islet/30min) ± SEM for the number of experiments (n), each of 3-5 observations (group). The significance of the difference between the means of groups incubated in the presence of 0.5μM PMA and the means of groups incubated in the presence of 0.5μM PMA and the relevant test compounds was determined using the Student paired t-test. In control groups there was found to be a significant increase (p=0.05) in insulin release between electropermeabilised islets incubated at 10nM Ca^{2+} (510 ± 63 pg/islet/30min (n=3)) and those incubated at 10μM Ca^{2+} (987 ± 151 pg/islet/30min (n=3)).

GME = Glycine Methyl Ester

SME = Sarcosine Methyl Ester

MDCD = Monodansylcadaverine

PMA = Phorbol Myristate Acetate


Insulin Release (pg/islet/30min)
1600
1400
1200
1000
800
600
400
200
0
p<0.02
N.S.
p<0.05
N.S.
10nM Ca2+ + + + + +
0.5μM PMA − + + + +
5mM GME − ± + − −
5mM SME − − − + −
50μM MDCD − − − − +

4.1.3 The Effect of Transglutaminase Inhibitors on cAMP-Stimulated Insulin Release

Jones et al (1986) have reported that cAMP (10μM -100μM) and dibutyryl-cAMP (10μM - 100μM), as well as agents such as forskolin (0.2 -20μM) and 3-iso-butyl-1-methylxanthine (0.01mM - 5mM) which catalyse the formation and prevent the breakdown of cAMP respectively, stimulate insulin release from electropermeabilised rat islets, in a dose dependent manner, at both sub-stimulatory (50nM Ca^{2+}) and stimulatory (10μM Ca^{2+}) calcium concentrations. In order to investigate a possible role for transglutaminase during cAMP stimulated insulin release, the effect of transglutaminase inhibitors on insulin release stimulated by 100μM cAMP in the presence of the phosphodiesterase inhibitor, IBMX (500μM) was tested. From Figure 4.8 it can be seen that incubation of electropermeabilised rat islets with cAMP/IBMX resulted in a 125% increase in insulin release at sub-stimulatory calcium levels (10nM Ca^{2+}). As with PMA-stimulation, the transglutaminase inhibitors glycine methylester (5mM) and monodansylcadaverine (50μM) failed to significantly inhibit the cAMP-stimulated release of insulin. However, sarcosine methyl ester once again proved to have a significant inhibitory effect.

Figure 4.8

Effect of Transglutaminase Inhibitors on cAMP-Stimulated Insulin Release

Groups of 10 electropermeabilised islets were pre-incubated for 15min at 4°C in permeation buffer containing 10nM Ca^{2+}, 100μM cAMP/500μM IBMX and the test compounds where necessary. Insulin release was then started by incubation for 30min at 37°C after which time supernatant samples were taken and assayed for their insulin content by radioimmunoassay.

Results are expressed as the mean insulin release (pg/islet/30min) ± SEM for the number of experiments (n), each of 3 observations (group). The significance of the difference between the means of groups incubated with 100μM cAMP/500μM IBMX and those incubated under identical conditions in the presence of the relevant test compounds was assessed using the Student paired t-test. In control groups incubated in the presence of 10nM Ca^{2+} and 10μM Ca^{2+}, the insulin release was found to be 576 ± 43 pg/islet/30min (n=3) and 1142 ± 84 pg/islet/30min (n=3) respectively (p=0.02).

GME = Glycine Methyl Ester

SME = Sarcosine Methyl Ester

MDCD = Monodansylcadaverine

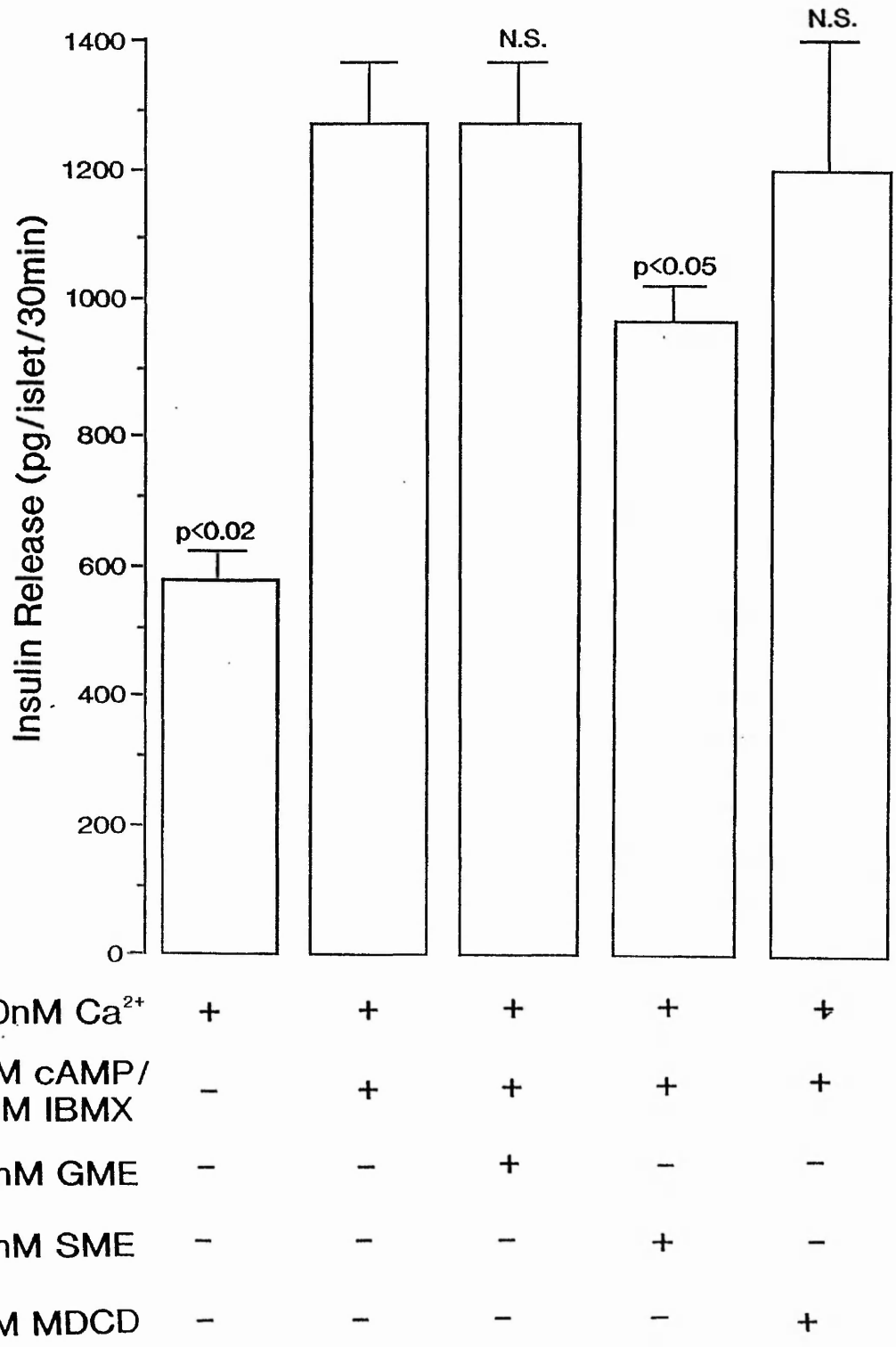

Insulin Release (pg/islet/30min)
1400
1200
1000
800
600
400
200
0
p<0.02
N.S.
p<0.05
N.S.
10nM Ca2+ + + + + +
100µM cAMP/ 500µM IBMX − + + + +
5mM GME − − + − −
5mM SME − − − + −
50µM MDCD − − − − +

Table 4.2

The Effect of Transglutaminase Inhibitors on PMA- and cAMP-Stimulated Insulin Release from Electropermeabilised Islets

Table 4.2 expresses the results from Figures 4.7 and 4.8 as the % of PMA- and cAMP-stimulated insulin release following incubation with transglutaminase inhibitors.

Compound	% of (±SEM) PMA-Stimulated Insulin Release	cAMP-Stimulated Insulin Release
5mM Glycine Methyl Ester	68 ± 12	101 ± 13
5mM Sarcosine Methyl Ester	54 ± 5	58 ± 7
50µM Monodanylcadaverine	117 ± 32	92 ± 26

4.1.4 Discussion

In order to investigate the role of transglutaminase during insulin release from electropermeabilised rat islets of Langerhans the primary amines cystamine (1mM - 5mM), methylamine (2mM -10mM) and glycine methylester (1mM - 5mM) were used, as well as the naphthalenesulphonamide, monodansylcadaverine (50μM - 100μM). These competitive amines are thought to inhibit the biological activity of transglutaminase by competing with the peptide bound ε-lysine residues during the crosslinking reaction, whilst cystamine may also act by binding to the active site of the enzyme under non-reducing conditions.

Using the transglutaminase substrate/inhibitors cystamine, methylamine and glycine methylester it was found that these compounds displayed a dose related inhibition of insulin release from electropermeabilised islets which had been stimulated by raising the concentration of Ca^{2+} from 10nM to 10μM. In the case of monodansylcadaverine maximum inhibition appeared to be achieved at the lowest concentration (50μM) of the inhibitor tested. The analogues of glycine methyl ester and monodansylcadaverine, sarcosine methyl ester and dimethylmonodansylcadaverine respectively, which lack the primary amine group necessary for inhibition of transglutaminase, were also tested and failed to inhibit Ca^{2+}-stimulated insulin release. These results therefore imply that the effects of the primary amines is a result of there specific action on transglutaminase, as opposed to some other cellular target. Since the effects of the competitive amines on Ca^{2+}-stimulated insulin release could also be explained by inhibition of basal insulin release from islets incubated at 10nM Ca^{2+}, we tested their affect on this process. It was found that none of the primary amines caused significant inhibition of basal insulin release. Paradoxically however, cystamine (5mM) resulted in a significant (36%)

stimulation of basal insulin release whilst incubation with monodansylcadaverine (50μM) lead to a massive release of insulin of a magnitude approaching 300-400% greater than that found in Ca^{2+}-stimulated islets. The latter result would seem to suggest that under basal conditions, monodansylcadaverine is toxic to the permeabilised B-cell.

Previous reports have shown that electropermeabilised rat islets, also secrete insulin at both non-stimulatory and stimulatory concentrations of Ca^{2+}, in response to the phorbol ester, phorbol myristate acetate (Jones et al, 1985) and to increases in the concentration of cAMP (Jones et al, 1986). Vallar et al (1987) found that PMA had the same effect in the insulin secreting cell line RIN5mF which had been electropermeabilised but that cAMP was only able to stimulate increased insulin release when stimulatory Ca^{2+} concentrations were used. The same result was reported by Tamagawa et al (1985) using digitonin permeabilised rat islets. Following electropermeabilisation it was shown that rat islets were able to stimulate insulin release at 10nM Ca^{2+} following exposure to either PMA (0.5μM) or cAMP (500μM) in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (100μM). Pre-incubation with the transglutaminase inhibitors monodanyslcadaverine (50μM) and glycine methylester (5mM) at concentrations which had been demonstrated to cause inhibition of Ca^{2+}-stimulated insulin release, produced no significant inhibition of PMA-or cAMP mediated insulin release. However, sarcosine methylester (5mM) resulted in significant inhibition under both of these conditions.

Overall, these results would imply that transglutaminase is important to the process of Ca^{2+}-stimulated insulin release from electropermeabilised islets. Earlier work on the effect of these

competitive amines on glucose stimulated insulin release from intact rat islets, had led to the suggestion that the enzyme may be an important target for the increase in intracellular Ca^{2+} that is thought to mediate stimulus secretion coupling from the B-cell. Although these investigations had demonstrated that the effects of methylamine (Sener et al, 1985), glycine methyl ester (Sener et al, 1985) and monodansylcadaverine (Bungay et al, 1984) did not result from their inhibition of glucose metabolism, it had not been possible to rule out the they may be acting on another metabolic process, such as the conversion of proinsulin to insulin (Alarcon et al, 1985). Furthermore, even if the effect of these compounds was mediated by their action on transglutaminase it was difficult to specify where in the stimulus response the enzyme acted. Therefore, by using electropermeabilised islets the distal events associated with insulin release can be isolated from the metabolic and membrane events that occur in intact islets. Indeed, these studies appear to have confirmed a possible role for transglutaminase as a Ca^{2+} target. This is supported by the fact that the observed potency of the transglutaminase inhibitors on Ca^{2+}-stimulated insulin release is comparable to their affects on glucose stimulated insulin release as well as their ability to inhibit islet transglutaminase activity as measured in islet homogenates. The inability of the transglutaminase inhibitors to affect PMA- and cAMP-stimulated insulin release at non-stimulatory Ca^{2+} concentrations implies that the effect of these primary amine inhibitors is not due to a non-specific action on either protein kinase C or cAMP-dependent protein kinases (Protein kinase A) or the overall secretory mechanism. Although we are unsure of why sarcosine methylester inhibits PMA-and cAMP-stimulated insulin release.

These result may also provides us with some information on

the interaction between the various secondary messenger systems that are thought to be important to the distal event involved in insulin secretion. Jones et al (1989) has reported that activation of protein kinase A and protein kinase C by cAMP and PMA respectively, may not only sensitize the response of permeabilised islets to an increase in Ca^{2+} but may also able to stimulate insulin release from B-cells by a Ca^{2+}-independent mechanism. This has also been suggested from work on permeabilised RIN5mF cells (Vallar et al, 1987) and with permeabilised neutrophils (Barrowman et al, 1986). Since the results show that transglutaminase is important to Ca^{2+}-stimulated insulin release but not PMA- or cAMP-stimulated insulin release this could be because these mechanisms are independent of one another in the permeabilised islet. Alternatively, they may indicate that transglutaminase acts at a stage prior to the sensitization by both PMA and cAMP.

4.2 Transglutaminase and Arginine Stimulated Insulin Release

Another compound that has been demonstrated to stimulate insulin release from electropermeabilised islets, in an ATP and temperature dependent manner, is the amino acid arginine (Bjaaland et al, 1989). Studies from intact islets have reported that arginine is a potent insulin secretagogue in the presence of threshold (7mmol/l) and stimulatory (10-15mmol/l) concentrations of glucose and that its action is exerted through depolarization of the plasma membrane (Hermans et al, 1987). Recent reports suggest that this depolarization results from the cellular accumulation of this positively charged amino acid, which has a direct biophysical effect at the membrane, rather than through its catabolism (Blanchier et al, 1989). However, this conclusion appears to be in contradiction to the results reported in electropermeabilised islets where the membrane potential is destroyed as a result of permeabilisation. We therefore decided to investigate a possible role for arginine through transglutaminase since it has been suggested that the catabolism of arginine may result in the production of polyamines and putrescine, which could in turn act as substrates for transglutaminase (Malaisse et al, 1989).

4.2.1 Effect of Transglutaminase Inhibitors on Arginine Stimulated Insulin Release

As was reported by Bjaaland et al (1989) incubation of electropermeabilised islets with 10mM arginine in the presence of sub-stimulatory Ca^{2+} concentrations (10nM) resulted in 120% increase in insulin release (Figure 4.9). Although incubation with the transglutaminase inhibitor glycine methylester (5mM) resulted in partial inhibition of arginine stimulated insulin release, this did not prove to be significant. Sarcosine methylester (5mM) had no

significant effect on arginine stimulated insulin release. These results appear to suggest that if arginine is metabolized by electropermeabilised islets to produce polyamines and putrescine then these compounds are not acting through transglutaminase. However, to provide further evidence to support this conclusion we examined whether putrescine could also stimulate insulin release directly from electropermeabilised islets (Figure 4.10). These experiments showed that putrescine (10mM) was unable to stimulate insulin release at basal Ca^{2+} (10nM). In fact at stimulatory Ca^{2+} levels (10μM), incubation with 10mM putrescine resulted in a 60% inhibition of Ca^{2+}-induced insulin release.

Figure 4.9

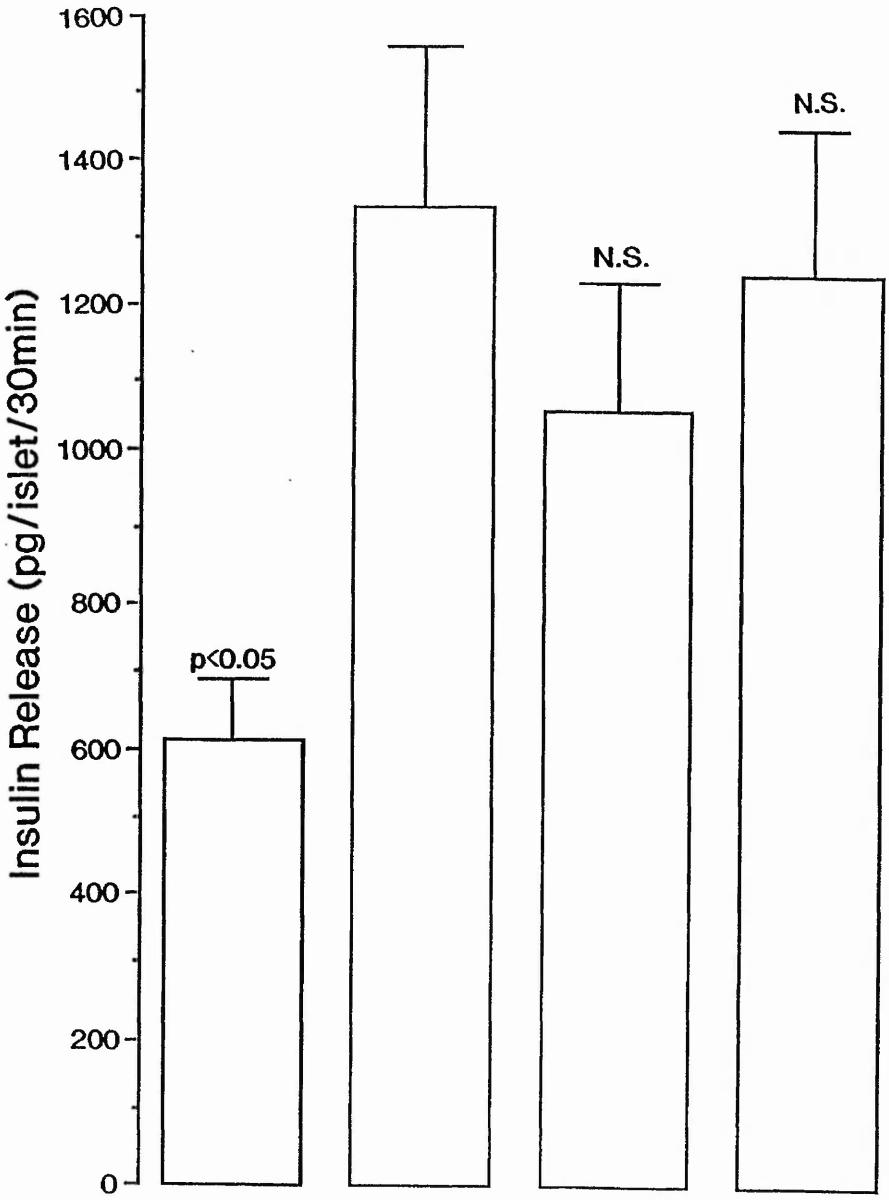
Effect of Transglutaminase Inhibitors on Arginine Stimulated Insulin Release

Batches of 10 electropermeabilised islets were pre-incubated at 4°C for 15min in 1ml of permeation buffer containing 10nM Ca^{2+}, 10mM arginine and the relevant test compound where required. Insulin release was then stimulated by incubation at 37°C for 30min after which supernatant samples were taken and assayed for insulin content by radioimmunoassay.

Results are given as the mean insulin release (pg/islet/30min) ± SEM taken from 3 separate experiments (n) each of 4 observations (group). The significance of the differences between the means of groups incubated in the presence of 10mM arginine/10nm Ca^{2+} and the means of groups incubated under identical conditions in the presence of test compounds was determined using the Student paired t-test. N.S. denotes no significant difference. The insulin release in control groups incubated at 10nM Ca^{2+} and 10μM Ca^{2+} were 610 ± 76 pg/islet/30min (n=3) and 1051 ± 146 pg/islet/30min (n=3) respectively (p=0.02).

GME = Glycine methyl ester

SME = Sarcosine methyl ester



10nM Ca^{2+}	+	+	+	+
10mM Arginine	–	+	+	+
5mM GME	–	–	+	–
5mM SME	–	–	–	+

Figure 4.10

Effect of Putrescine on Insulin Release from Electropermeabilised Islets

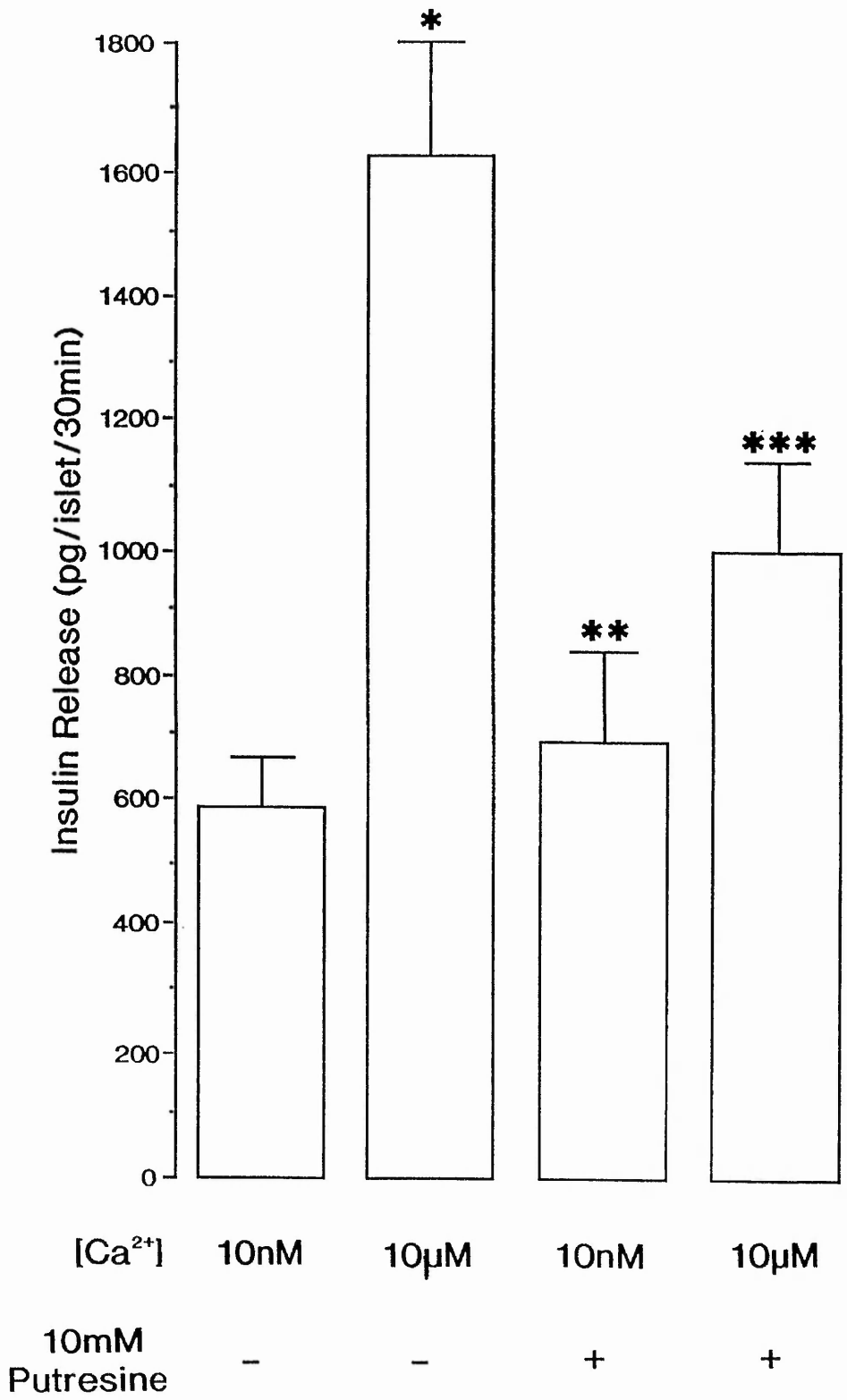
Batches of 10 electropermeabilised islets were pre-incubated at 4°C for 15min in 1ml of permeation buffer containing 10nM Ca^{2+}, 10mM putresine and the relevant concentration of test compound where required. Insulin release was stimulated by incubation at 37°C for 30min after which supernatant samples were taken and assayed for insulin content. In the experiments examining the effect of putresine on Ca^{2+}-stimulated insulin release, islet batches were pre-incubated in 0.9ml of permeation buffer and 0.1ml of permeation buffer containing the relevant amount of Ca^{2+} required to give 10μM Ca^{2+} added before incubation.

The results are given as the mean insulin release (pg/islet/30min) ± SEM taken from 3 experiment (n), each of 4 observations (group). The significance of the difference between means of groups was determined using the Student paired t-test. N.S. denotes no significant difference.

* P = 0.01 versus 10nM Ca^{2+}

** P = N.S. versus 10nM Ca^{2+}

*** P = 0.02 versus 10uM Ca^{2+}


Insulin Release (pg/islet/30min)
1800
1600
1400
1200
1000
800
600
400
200
0
*
**
***
[Ca2+]
10nM
10µM
10nM
10µM
10mM Putresine
–
–
+
+

4.2.2 Discussion

The cationic amino acid arginine has been shown to stimulate insulin release from both intact (Hermans et al, 1987) and electropermeabilised (Bjaaland et al, 1989) islets. Its action in intact islets is thought to be mediated by the depolarization of the plasma membrane following its accumulation within the B-cell. However, this cannot explain its ability to stimulate insulin release from electropermeabilised islets, since membrane potential is dissipated as a result of the permeabilisation process. Since it is known that arginine is metabolized to polyamines and putrescine which are known substrates of transglutaminase we investigated whether arginine stimulated insulin release was affected by transglutaminase inhibitors. This work showed that glycine methyl ester had no affect on arginine stimulated insulin release at basal Ca^{2+} levels. This suggested that either the arginine was not metabolized to polyamines and putresine in electropermeabilised islets or that if this did occur, then their metabolism by transglutaminase was not important to the process of insulin release. In order to test out the latter hypothesis, we examined whether putrescine could also stimulate insulin release. These results proved negative since putresine was unable to stimulate insulin release from electropermeabilised islets at basal Ca^{2+}. However, putrescine was able to inhibit Ca^{2+}-stimulated insulin release which is probably a result of putrescine acting as substrate/inhibitor of transglutaminase

Overall it appears that transglutaminase is not important to the process of arginine stimulated insulin release from electropermeabilised rat islets although this still leaves unanswered the question of mechanism by which arginine stimulates insulin release. A possible explanation is that arginine stimulates localized increases

in Ca^{2+} either via a cation-exchange reaction, resulting in the mobilization of Ca^{2+} from membranes or from intracellular organelles.

5 IDENTIFICATION AND CHARACTERISATION OF TRANSGLUTAMINASE SUBSTRATES IN ISLETS OF LANGERHANS

5 IDENTIFICATION AND CHARACTERISATION OF TRANSGLUTAMINASE SUBSTRATES IN RAT ISLETS OF LANGERHANS

5.1 Introduction

The work using electropermeabilised rat islets of Langerhans has suggested that transglutaminase is important to the process of Ca^{2+}-stimulated insulin release. However, it is still uncertain what the function of the enzyme is during this mechanism. Therefore to extend these studies on transglutaminase it was decided to attempt to identify and characterise possible transglutaminase substrates. Previous investigations have indicated a transglutaminase mediated incorporation of [^{14}C]-methylamine, [^{14}C]-putrescine and [2,5-^{3}H]-histamine into TCA precipitated proteins of both intact (Bungay et al, 1984; Gomis et al, 1984; Sener et al, 1985) and homogenised islets (Bungay et al, 1986; Gomis et al, 1989). Bungay et al (1986) reported that following incubation of islet homogenate with [^{14}C]-methylamine and Ca^{2+} for 120min, that >90% of the trichloroacetic acid insoluble radioactivity was present in the particulate fraction (71,000g.av). They then went on to demonstrate that the major protein labelled by transglutaminase was a high molecular weight polymer that was unable to traverse a 3%(w/v) polyacrylamide gel. Recently, Gomis et al (1989) has confirmed the existence of this high molecular weight polymer and has identified a further protein of molecular weight 84100Da as being a potential γ-glutamyl containing substrate of transglutaminase. Since it has been suggested that this polymer may be involved in the membrane events associated with secretion (Bungay et al, 1986) it was decided to characterise the polymer formed in homogenised islets further. The task of identifying and characterizing the lower molecular weight

protein substrates of transglutaminase, that may be crosslinked by the enzyme to produce the polymer was also undertaken.

In order to identify transglutaminase substrates [^{14}C]-methylamine was used as a probe. This method has been previously used by several investigators (Gomis et al, 1984; Bungay et al, 1984; 1986) since the enzyme catalyses the incorporation of this primary amine into proteins containing available γ-glutamyl residues.

5.2 HOMOGENIZED ISLET STUDIES

5.2.1 Subcellular Localization of the [^{14}C]-Methylamine Labelled High Molecular Weight Polymer

In order to determine the subcellular distribution of the radiolabelled polymer, islet homogenate was incubated in the presence of [^{14}C]-methylamine and 2.5mM Ca^{2+} and then fractionated to produce an N-pellet (600g.$_{av}$), a P-pellet (71,000g.$_{av}$) and particle free supernatant (PFS). The different fractions were separated by SDS-polyacrylamide gel electrophoresis (3%(w/v) stacking; 10%(w/v) resolving), the gels sliced and the radiolabelling profile determined (Figure 5.1). Parallel studies were undertaken in which [^{14}C]-methylamine was omitted and the distribution of the marker enzymes between the fractions determined (Figure 5.2).

Over the three fractions, the major substrate labelled (>60%) was the high molecular weight polymer which was unable to traverse the 3%(w/v) polyacrylamide stacking gel (Figure 5.1). From Figure 5.2 it can be seen that the majority of this labelled high molecular weight polymer was present in the P-pellet and to a lesser extent in the N-pellet. However, the majority of the labelled protein in the resolving gel was found in the PFS. Calculation of the distribution of the total

counts in each fraction showed there to be 23 ± 8% (n=3) present in the N-pellet, 46 ± 12% (n=3) in the P-pellet and 31 ± 10% in the PFS. Enzyme marker studies (Figure 5.2) showed the distribution of labelled polymer to be similar to that of the plasma membrane marker, 5' nucleotidase and the mitochondrial marker, cytochrome oxidase. However under the experimental conditions employed for subcellular fractionation it is likely that the nuclear material would have fractionated with the mitochondria, and since there is no experimental evidence to suggest an association of transglutaminase with mitochondria this implies that the labelled substrates in this fraction may be associated with the nucleus.

Overall, these results therefore suggest that the high molecular weight polymer substrate of islet transglutaminase is particulate and predominantly associated with the plasma membrane and possibly with the nucleus. However, the lower molecular weight substrates that may be crosslinked by transglutaminase to form the polymer appear to be localized to the PFS.

Figure 5.1

[^{14}C]-Methylamine Labelling Profile of Islet Subcellular Fractions Following Separation of Proteins by SDS-PAGE

Islet homogenate (400 islets; 220µg protein) was incubated in the presence of 1.78mM [^{14}C]-methylamine (56mCi/mmol) and 2.5mM Ca^{2+} for 30min in a final volume of 200µl (Methods 2.5.2) and then immediately fractionated by differential centrifugation into N (600g.$_{av}$ for 10min) and P (71,000g.$_{av}$ for 45min) particulate fractions and particle free supernatant (PFS)(Methods 2.5.3). The particulate fractions were resuspended by homogenisation (Methods 2.5.1) in homogenisation buffer (100µl) and then boiled (5min) following the addition of sample buffer (x6 concentration) (Methods 2.6.1.2). The PFS protein was precipitated with 10% (w/v) TCA, pelleted and this procedure repeated twice. The precipitated protein was then dissolved by boiling (5min) in sample buffer (120µl). Fractions were separated by SDS-polyacrylamide gel electrophoresis through a 3%(w/v) stacking gel and a 10%(w/v) resolving gel (Method 2.6.1) and the radioactivity profile determined by gel sectioning (Method 2.6.2). Controls were performed in which subcellular fractions were incubated in the presence of 5mM EDTA rather than 2.5mM Ca^{2+}. Background levels were determined by counting the radioactivity in gel lanes in which no samples had been loaded. The arrows indicate the mobility of standard radiolabelled protein markers.

The counts given for each segment are the mean ± SEM from 3 experiments following the deduction of EDTA controls (71 ± 10 cpm). The radioactivity in the control samples segments (67 ± 11 cpm) was found not to be significantly different from background .

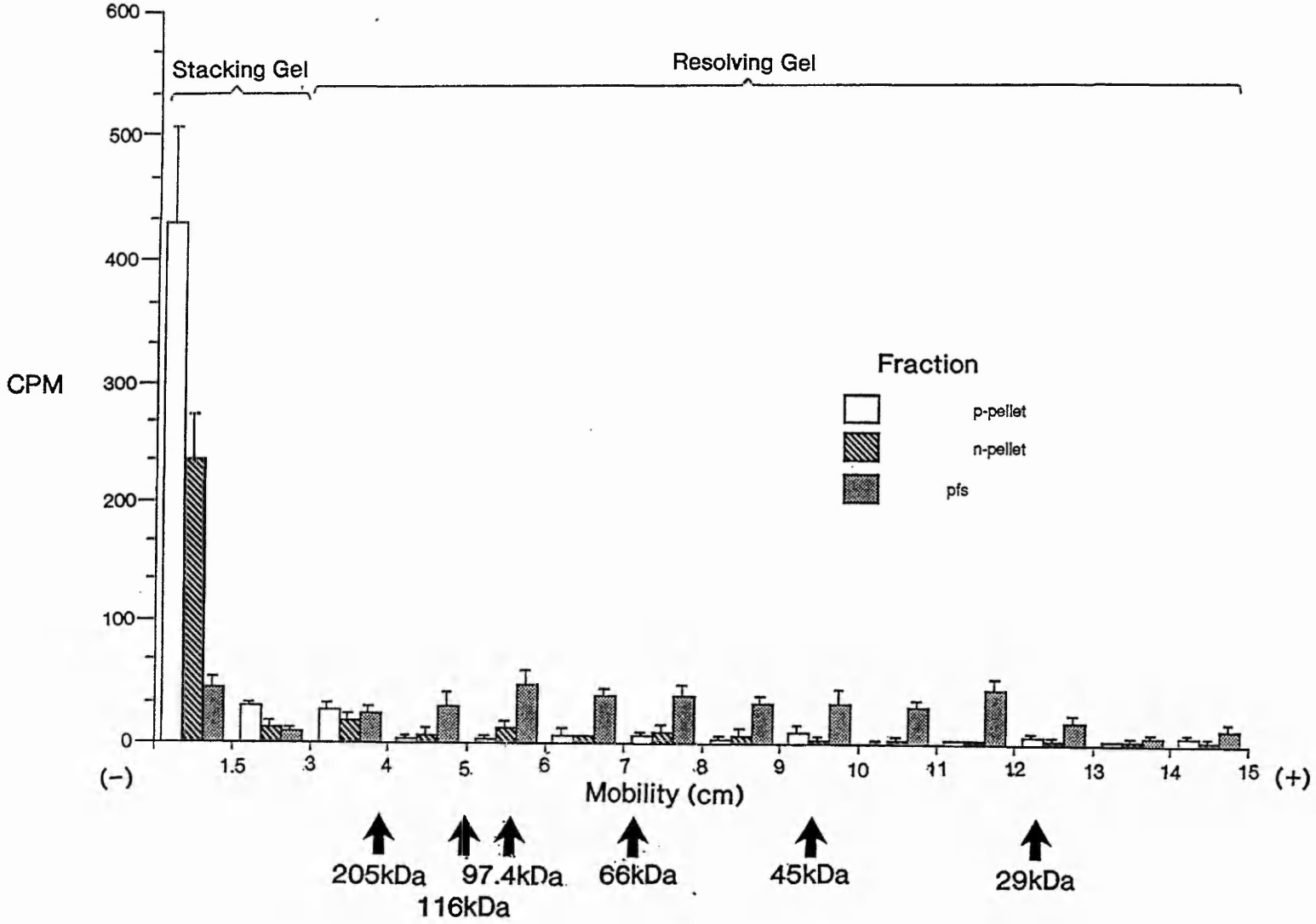

600
500
400
300
200
100
0
CPM
Stacking Gel
Resolving Gel
Fraction
p-pellet
n-pellet
pfs
(−)
1.5
3
4
5
6
7
8
9
10
11
12
13
14
15
(+)
Mobility (cm)
205kDa
116kDa
97.4kDa
66kDa
45kDa
29kDa

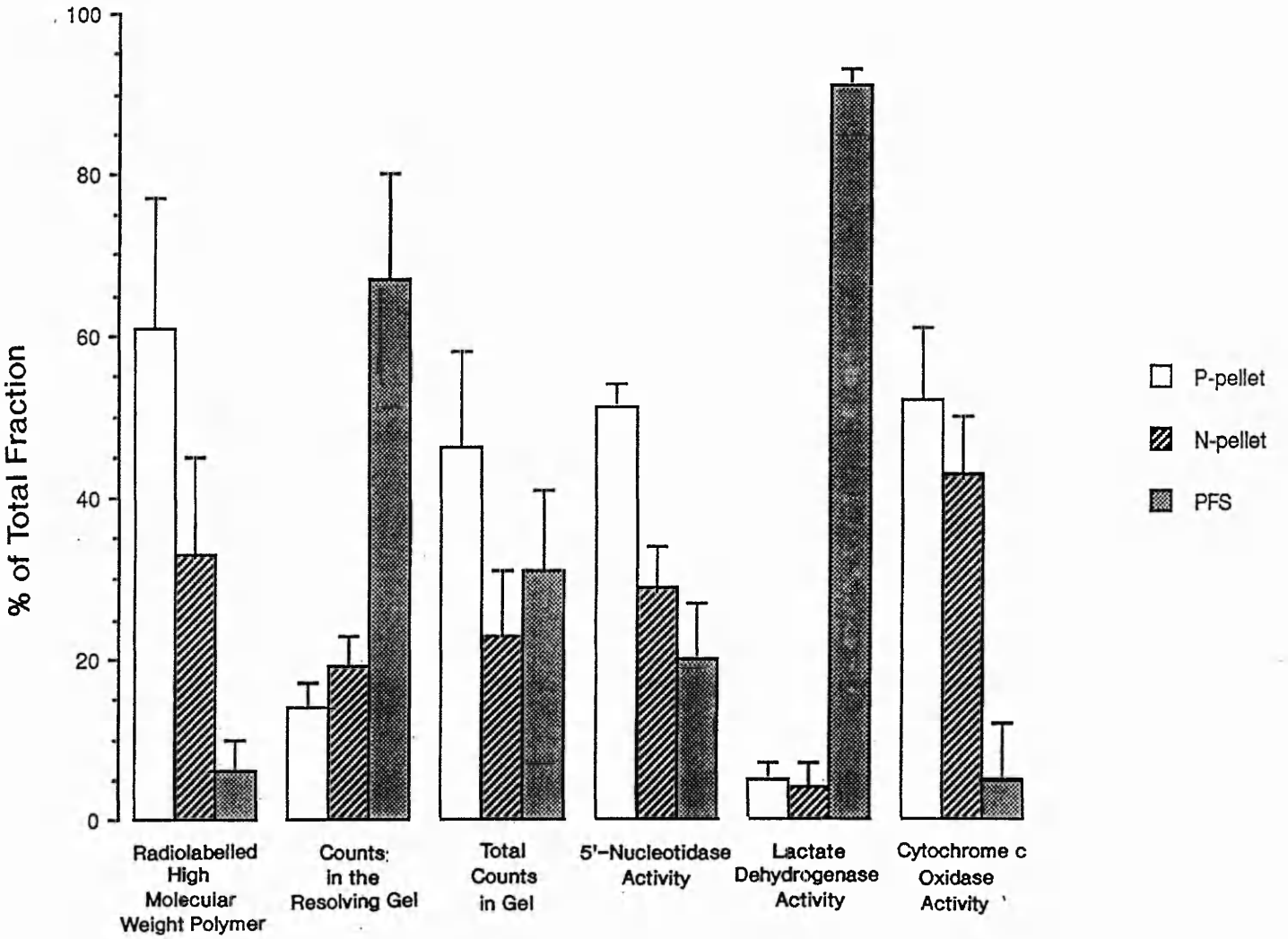
Figure 5.2

Distribution of the [^{14}]-Methylamine Labelling and Marker Enzymes in Islet Subcellular Fractions

The distribution of the [^{14}C]-methylamine labelling in the high molecular weight polymer at the top of the stacking gel and the lower molecular weight proteins of the resolving gel in each fraction was determined from Figure 5.1. Parallel experiments were undertaken to these in which islet homogenate (800 islets) was incubated in the presence of 2.5mM Ca^{2+} but in the absence of [^{14}C]-methylamine in a final volume of 400µl (Methods 2.5.2). Particulate fractions were resuspended by homogenisation (Methods 2.5.1) in homogenisation buffer (400µl). The activity of the marker enzymes 5'-nucleotidase (plasma membrane marker; Method 2.4.5), cytochrome c oxidase (mitochondrial marker; Method 2.4.6) and lactate dehydrogenase (cytosolic marker; Method 2.4.4) as well as the concentration of protein (Method 2.4.2) were then determined in the N-pellet and P-pellet and the PFS.

The activity of each fraction is expressed as a percentage of the total activity for all the fractions, and are given as mean ± SEM from 3 separate experiments. Typical combined values for all three fractions of the different enzyme markers measured were:

	per 100 islets
Protein	55 ± 8 µg
Lactate Dehydrogenase	410 ± 98 µmol NADH oxidised per h.
Cytochrome c Oxidase	322 ± 49 nmol.cyt.C oxidized per h.
5'-nucleotidase	112 ± 10 nmol. AMP hydrolysed per h.


% of Total Fraction
100
80
60
40
20
0
P-pellet
N-pellet
PFS
Radiolabelled High Molecular Weight Polymer
Counts in the Resolving Gel
Total Counts in Gel
5'-Nucleotidase Activity
Lactate Dehydrogenase Activity
Cytochrome c Oxidase Activity

5.2.2. Molecular Weight Estimation of [^{14}C]-Methylamine Labelled High Molecular Weight Polymer

Since the [^{14}C]-methylamine labelled polymer is unable to traverse a 3% (w/v) polyacrylamide gel, this suggests that it has a molecular weight equal to or greater than 10^6 Da.

To obtain a better estimation of the molecular weight of the labelled polymer, labelled homogenate was separated by gel filtration on columns containing agarose A5M (BioRad)(range 10,000Da - $5x10^6$Da) and Trisacryl GF2000 (range 120,000Da - $15x10^6$Da).

Using the BioRad A5M, the labelled polymer eluted with blue dextran at the void volume (Figure 5.3), which suggests that the polymer has a molecular weight of at least $5x10^6$Da. Elution of the labelled homogenate under identical conditions through GF2000 produced no distinct peak. This implies that the polymer has a molecular weight between $5x10^6$Da and $15x10^6$Da, the latter being the void volume of GF2000. Furthermore, since there are no distinct eluting peaks on the GF2000, it suggests that the polymer is heterogeneous in character.

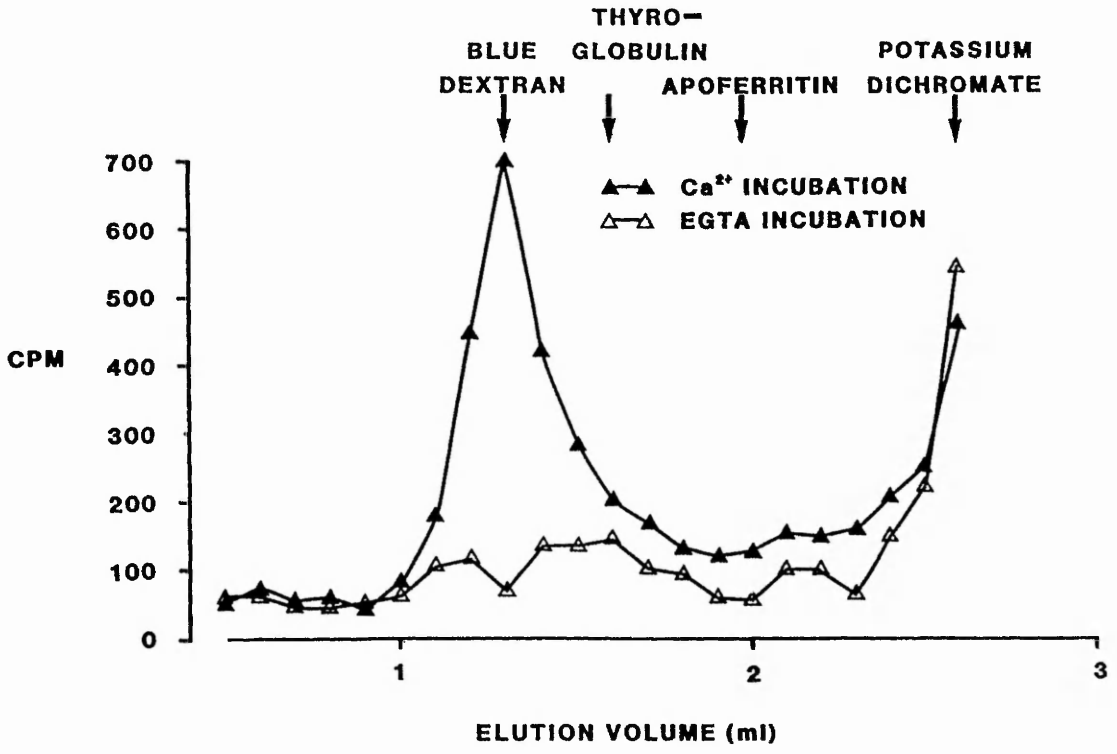
Figure 5.3

Characterisation of the [^{14}C]-Methylamine Labelled High Molecular Weight Polymer by Gel Filtration

Islet homogenate (100-200 islets) was labelled by incubation with 1.78mM [^{14}C]-methylamine (56mCi/mmol) and 2.5mM Ca^{2+} in a final volume of 100µl (Methods 2.5.2) for 30min and then immediately dissolved in sample buffer (20µl) to give a final concentration of 2%(w/v) SDS, 3%(v/v) 2-mercaptoethanol and 50mM Tris/HCl, pH 7.4 and loaded onto the column.

Labelled homogenate was eluted with 1%(w/v) SDS, 1mM dithiothreitol and 50mM Tris/HCl, pH 7.4 through pre-equilibrated columns (80mm x 9mm) containing either Bio-Rad agarose A5M or Trisacryl GF2000. Elution was performed at a rate of 30-35µl/min. Samples of 3min (~100µl) were collected and the radioactivity determined by scintillation counting. The columns were calibrated using protein standards of known molecular weights, whilst the void (v_o) and approximate total exclusion volume (v_1) were determined using blue dextran and K^+-dichromate (Methods 2.6.5).

Figure 5.3 gives a typical radioactivity profiles obtained from 3 separate experiments using Bio-Rad A5M. Control experiments were performed in which the homogenates were incubated in the presence of 5mM EDTA rather than 2.5mM Ca^{2+}.


THYRO–
BLUE
GLOBULIN
POTASSIUM
DEXTRAN
APOFERRITIN
DICHROMATE
Ca2+ INCUBATION
EGTA INCUBATION
CPM
700
600
500
400
300
200
100
0
1
2
3
ELUTION VOLUME (ml)

5.2.3 Formation of the [^{14}C]-Methylamine Labelled High Molecular Weight Polymer by Different Subcellular Fractions of Islet Homogenate

All previous work on the formation of the labelled polymer has been performed with whole homogenate. It was therefore decided to further characterise the dynamics of polymer formation/labelling by investigating whether the combined particulate fractions (N plus P pellets) or the particle free supernatant could be independently stimulated to produce the labelled polymer. Unlabeled islet homogenate was centrifuged to produce a total membrane fraction (71,000g.$_{av}$) and PFS. These were then incubated with [^{14}C]-methylamine and 2.5mM Ca^{2+} to stimulate labelled polymer formation, which was detected by gel filtration on agarose A5M (Bio-Rad). The radiolabelled profile of different fractions was compared with that found in whole homogenate simultaneously incubated with [^{14}C]-methylamine and 2.5mM Ca^{2+} (Figure 5.4).

In the PFS there was no detectable formation of the labelled polymer, whilst in the particulate fraction the incorporation of [^{14}C]-methylamine into the polymer was only 15 $\pm$ 6 % (n=4) of that found in whole homogenate (Table 5.1).

Figure 5.4

[^{14}C]-Methylamine Labelled High Molecular Weight Polymer Formation by Different Subcellular Fractions of Islet Homogenate

Islet homogenate (300 islets in 120µl homogenization buffer) was fractionated into a particulate (membrane) fraction (71,000g.$_{av}$ for 45min) and particle free supernatant (PFS). The particulate fraction was resuspended by homogenisation (Methods 2.5.1) in 120µl homogenisation buffer. Samples (60µl) from each of the fractions and whole homogenate (300 islets in 120µl homogenisation buffer), which had been stored on ice during fractionation, were incubated with 1.78mM [^{14}C]-methylamine (56mCi/mmol) and 2.5mM Ca^{2+} in a final volume of 100µl (Methods 2.5.2) for 30min, to stimulate the formation of the [^{14}C]-methylamine labelled high molecular weight polymer. Each sample was separated by gel filtration through an agarose A5M column (80mm x 9mm) and the radioactivity profile determined by scintillation counting. Figure 5.4 shows typical radioactivity profiles for particulate, PFS and whole homogenate taken from 4 separate experiments.

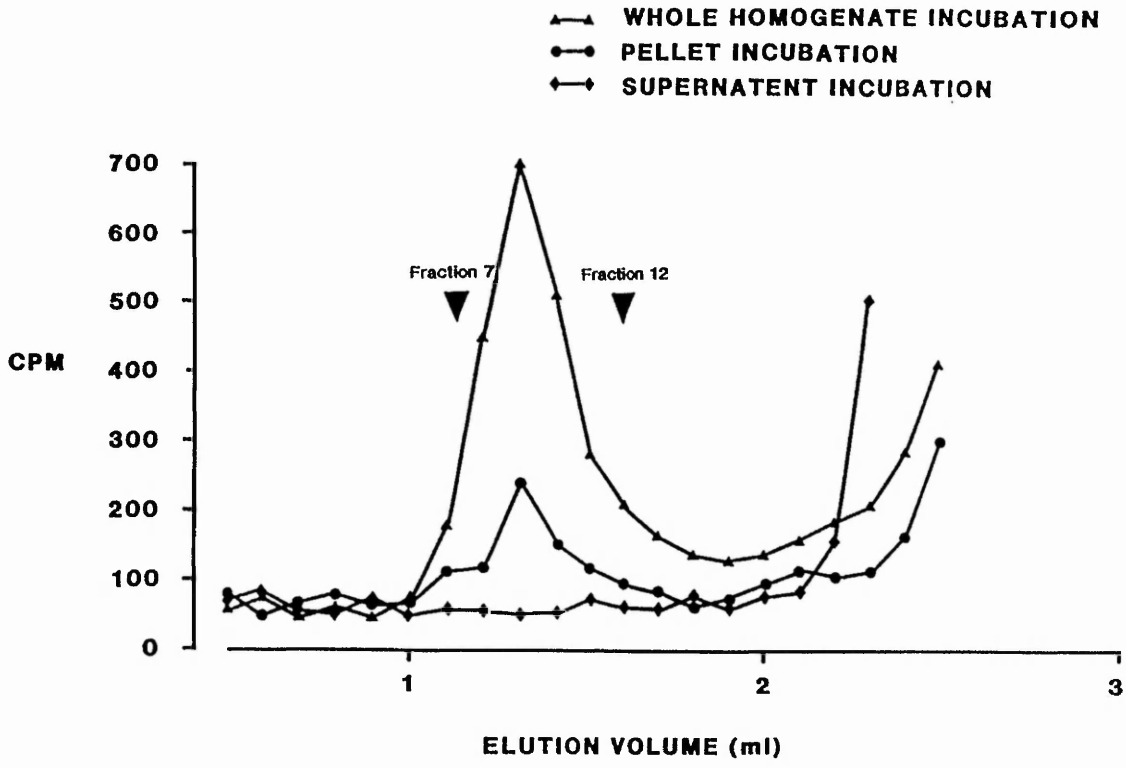

WHOLE HOMOGENATE INCUBATION
PELLET INCUBATION
SUPERNATENT INCUBATION
CPM
700
600
500
400
300
200
100
0
Fraction 7
Fraction 12
1
2
3
ELUTION VOLUME (ml)

Table 5.1

[^{14}C]-Methylamine Labelled High Molecular Weight Polymer Formation by Different Subcellular Fractions

Table 5.1 shows the amount of labelled polymer (cpm ± SEM) in the subcellular fractions and whole homogenate calculated from Figure 5.4. These were determined by totalling the radioactivity present in fractions 7 to 12 following deduction of EGTA controls (387 ± 15 cpm (n=12)). The second column expresses these as a percentage of those counts present in the whole homogenate.

Fraction	[^{14}C]-Methylamine Labelling	
	Total Counts (cpm)	% Whole Homogenate
Whole Homogenate	1952 ± 315	100 ± 17
Particulate Fraction	292 ± 117	15 ± 6
Supernatant Fraction	34 ± 20	2 ± 1

5.2.4 Identification and Subcellular Distribution of Lower Molecular Weight [^{14}C]-Methylamine Labelled Proteins

Although gel slicing allows the identification of a high molecular weight polymer as the major substrate labelled with [^{14}C]-methylamine by transglutaminase, the technique was not sensitive enough to identify and characterise less well labelled low molecular weight (LMW) substrates. To overcome this problem, [^{14}C]-methylamine labelled proteins were detected by fluorography. Labelled homogenate was fractionated to produce an N-pellet, a P-pellet and particle free supernatant (PFS), and the proteins in the fractions separated by SDS polyacrylamide gel electrophoresis. Following fluorography the labelled proteins were detected by densitometric scanning of the fluorographs (Figure 5.5).

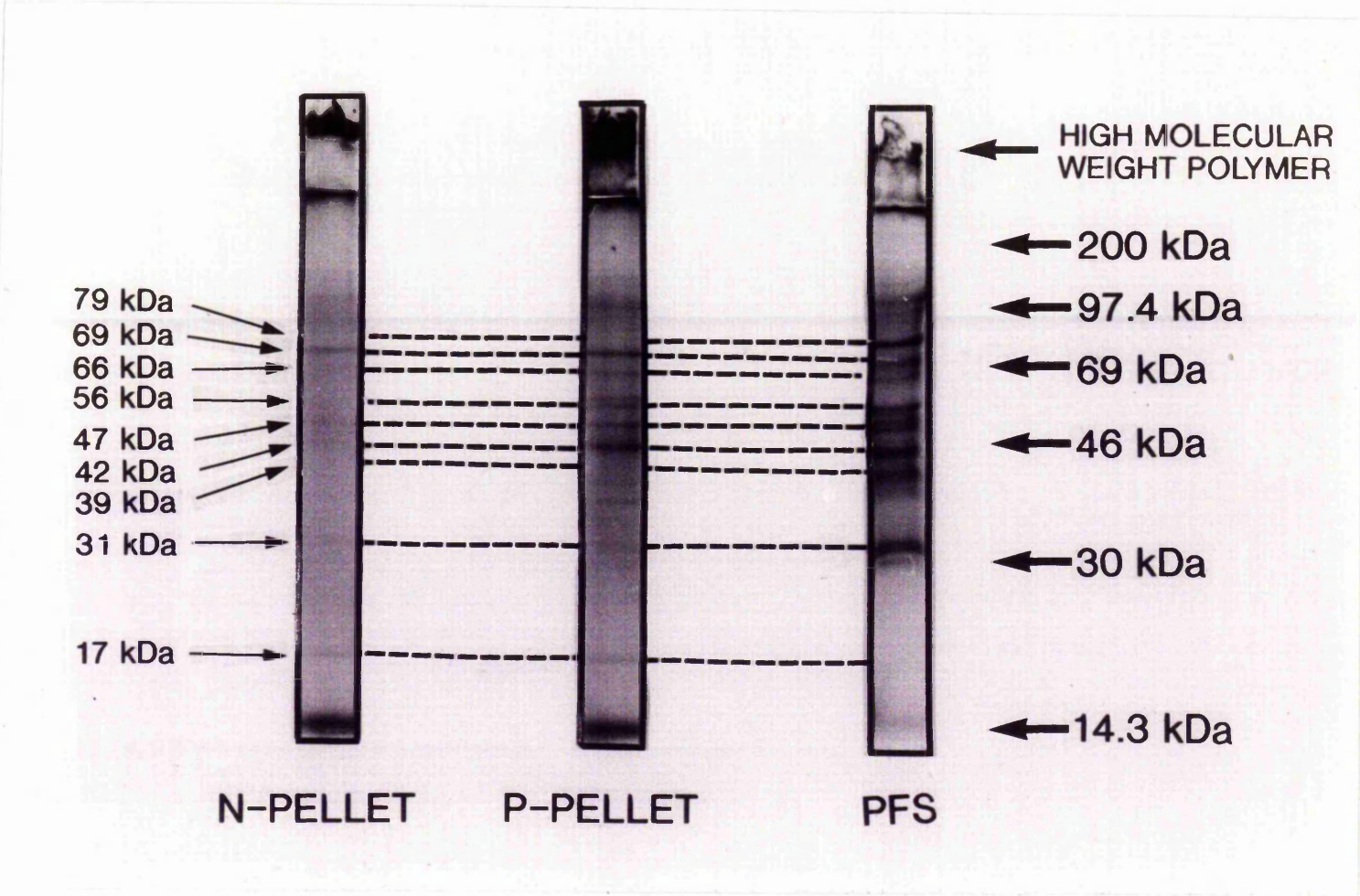
As expected the fluorographs showed considerable labelling of the polymer at the top of the stacking gel in both the N-pellet and P-pellet. Examination of the radiolabelling of lower molecular weight proteins in the resolving gel shows that a range of proteins had been labelled. The majority of these radiolabel proteins were found in the PFS fraction and had approximate molecular weights of 17kDa, 31kDa, 39kDa, 42kDa, 47kDa, 56kDa, 66kDa, 69kDa and 79kDa. Several of these labelled proteins were also found in the two membrane fractions. The P-pellet contained labelled proteins of approximate molecular weights 42kDa, 47kDa 56kDa, 66kDa and 69kDa whilst in the N-pellet only one band of approximate molecular weight 69kDa was discernible. In all the fractions, there was labelling of a protein of molecular weight >250kDa, that was unable to traverse the 10% (w/v) polyacrylamide stacking gel.

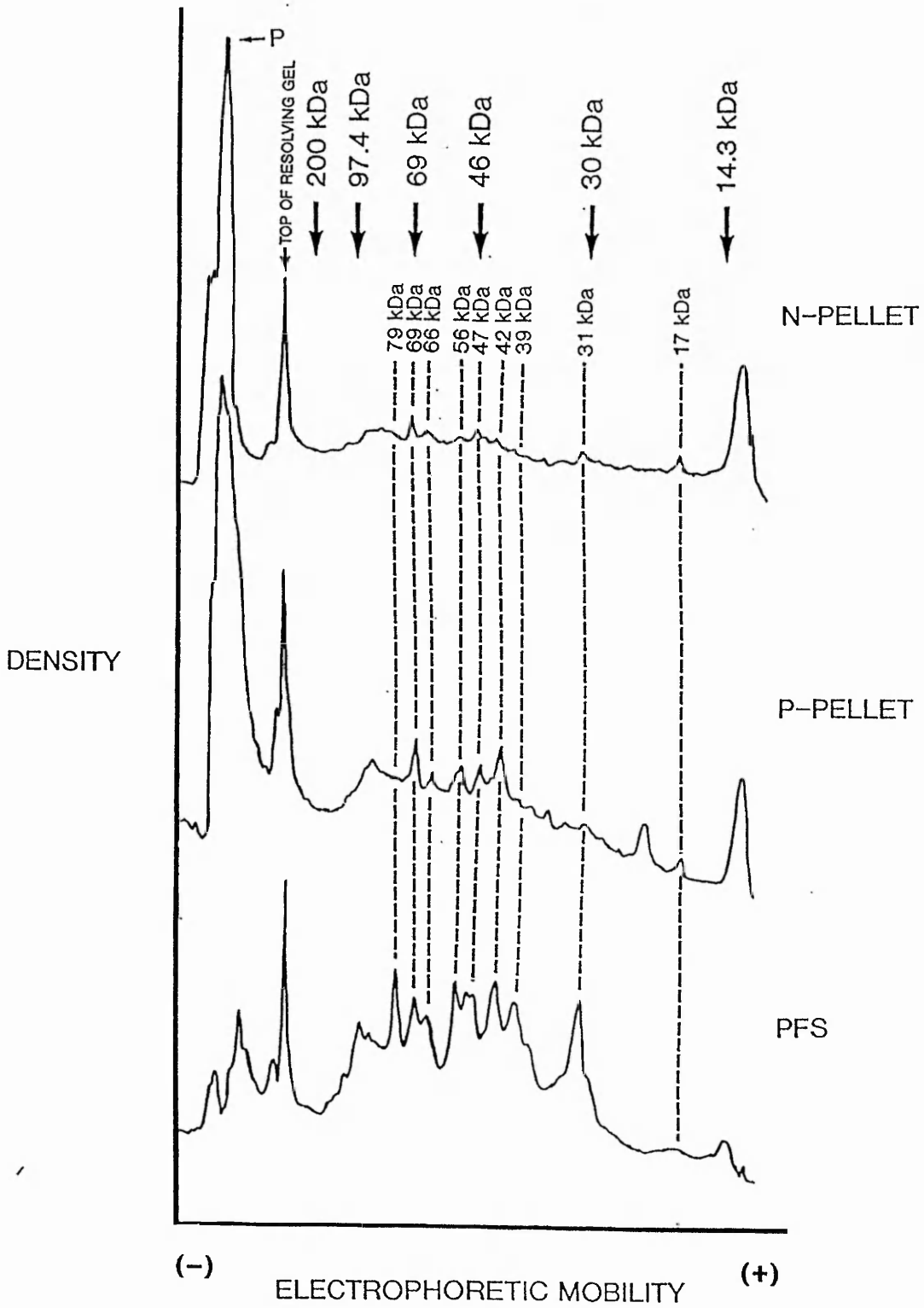
Figure 5.5 - 5.6

Densitometric Scans from Fluorographs of the Subcellular Fractions of [^{14}C]-Methylamine Labelled Islet Homogenate

Islet homogenate was labelled (Methods 2.5.2), fractionated (Methods 2.5.3) and the proteins seperated by SDS-polyacrylamide gel electrophoresis (Method 2.6.1) as described in the legend to Figure 5.1. The radiolabelling of proteins was detected by fluorography (Method 2.6.3). Gels were exposed for 60-90 days, developed and then photographed and densitometrically scanned.

The photographic image of a typical fluorograph and its densitometric scan, obtained from 3 separate experiments, is shown in Figure 5.5 and Figure 5.6 respectively. The large arrows indicate the mobility of standard protein markers. The small arrows show the approximate molecular weight of those proteins most highly labelled with [^{14}C]-methylamine. In control experiments where subcellular fractions were incubated with 5mM EDTA instead of 2.5mM Ca^{2+}, no protein labelling was detected following fluorography.


HIGH MOLECULAR WEIGHT POLYMER
200 kDa
97.4 kDa
69 kDa
46 kDa
30 kDa
14.3 kDa
79 kDa
69 kDa
66 kDa
56 kDa
47 kDa
42 kDa
39 kDa
31 kDa
17 kDa
N-PELLET
P-PELLET
PFS


P
TOP OF RESOLVING GEL
200 kDa
97.4 kDa
69 kDa
46 kDa
30 kDa
14.3 kDa
79 kDa
69 kDa
66 kDa
56 kDa
47 kDa
42 kDa
39 kDa
31 kDa
17 kDa
N-PELLET
P-PELLET
PFS
DENSITY
(-)
ELECTROPHORETIC MOBILITY
(+)

5.2.5 Discussion

Following incubation of islet homogenate with [^{14}C]-methylamine and 2.5mM Ca^{2+}, it was confirmed that the major substrate labelled by transglutaminase was a high molecular weight polymer (labelled polymer) that was unable to traverse a 3%(w/v) polyacrylamide gel during electrophoresis. Its inability to traverse the 3%(w/v) polyacrylamide gel suggests that the labelled polymer has a molecular mass of a least 10^6 Da. Further investigation into the molecular weight of the labelled polymer by gel filtration with agarose A5M (BioRad) and Trisacryl GF2000 implies that the polymer is heterogeneous in size and has a molecular weight in the range 5 x 10^6 Da to 15 x 10^6 Da.

To ascertain the subcellular localization of the labelled polymer, labelled homogenate was differentially centrifuged to produce an N-pellet ($600g_{av}$), a P-pellet ($71,000g_{av}$) and particle free supernatant (PFS). The labelled polymer was found predominantly in the P-pellet and to a lesser extent in the N-pellet. Parallel studies with marker enzymes suggested that the labelled polymer is predominantly associated with the plasma membrane since the distribution of the polymer is similar to plasma membrane marker 5'-nucleotidase. However, since 33% of labelled polymer was also found in the N-pellet which is likely to contain nuclear material, it is not possible to rule out an association of the polymer with the nucleus. This is supported by similar studies undertaken by Gomis et al (1989) in rat islet homogenate who have reported that 65% of radiolabel incorporation is found in the pellet following centrigation at 12,000g for 20min. However, the existence of a plasma membrane associated high molecular weight substrate for tissue transglutaminase has been suggested by several other investigators. Birchbichler et al (1973) reported that

the plasma membrane fractions of tissue cultured L-cells had a higher content of ε-(γ-glutamyl)lysine isopeptides than endoplasmic reticulum enriched fractions whilst Lorand et al, 1975 has reported the formation of a plasma membrane polymer following exposure of erythrocytes to Ca^{2+} in the presence of ionophore A23187. More recently Tyrrell et al (1988) has described an SDS-insoluble transglutaminase substrate in liver plasma membrane. Since there has been no reports of an association of transglutaminase or its substrates with the nucleus, it is likely that the polymer is predominating associated with the plasma membrane.

Since it is believed that the formation of the high molecular weight polymer results from the transglutaminase mediated crosslinking of lower molecular weight proteins (Bungay et al, 1986) it was attempted to identify these lower molecular weight proteins labelled with [^{14}C]-methylamine by fluorography. Examination of the fluorographs and their respective densitometric scans showed that a range of lower molecular weight proteins were radiolabelled. The majority of these proteins occured in the PFS where the major proteins labelled had approximate molecular weights of 17kDa, 31kDa, 39kDa, 42kDa, 47kDa, 56kDa, 66kDa, 69kDa and 79kDa, and to a lesser extent in the p-pellet. Five proteins of molecular weight 42kDa, 47kDa, 56kDa, 66kDa and 69kDa were found to be common to both the PFS and the P-pellet, whilst only one protein of approximate molecular weight 69kDa was found to be labelled in all 3 fractions. At present we have no information as to the identify of these labelled proteins. Gomis et al (1989) has reported that the major protein labelled with [2,5-^{3}H]-histamine by transglutaminase in homogenised rat islets has a molecular weight of 84kDa. Tyrrell et al (1988) has raised polyclonal antibodies to the large molecular weight substrate of transglutaminase in rat

hepatocyte and has found that these are also able to label fibronectin (230kDa) as well as two further proteins molecular weights 35kDa and 32kDa. Hand et al (1989) has confirmed the existence of these transglutaminase substrates in metastatic cells lines. Recent work by Ando et al (1989) has shown that transglutaminase is able to crosslink lipocortin I (calpactin II) of molecular weight 35kDa to produce higher molecular weight lipocortins of apparent molecular weights 75kDa and 160kDa.

In order to obtain a better understanding of the dynamics of formation of the polymer, experiments were undertaken in which islet homogenates was centrifuged to produce a particulate fraction ($71,000g_{av}$) and particle free supernatant and then incubated with Ca^{2+} and [^{14}C]-methylamine. Since these results showed that both the particulate and the PFS fractions were needed for polymer formation this means that the labelled polymer could be formed in several ways. Firstly, the transglutaminase responsible for the formation of the labelled polymer could be membrane bound and therefore does not have access to its substrates. However, this is contradicted by previous reports that have demonstrated that >90% of the transglutaminase activity is found within the PFS (Owen et al, 1988). Several investigators have also reported that the existence of a particulate associated transglutaminase activity is a result of a non-covalent interaction of transglutaminase with extracellular matrix proteins such as collagen (Juprelle-Soret et al, 1988) and fibronectin (Upchurch et al, 1987; Turner & Lorand, 1989) following homogenisation. Secondly, both the transglutaminase and its lower molecular weight substrates could be located in the PFS but they require some other component, possibly a template in the membrane, in order to catalyse the formation of the polymer. Finally, it also possible that the polymer is present

under physiological conditions and that stimulation of the cytosolic transglutaminase results in the incorporation of the radiolabelled amine into this structure.

As well as the problem of interpreting the dynamics of the high molecular weight labelled polymer formation it is also difficult to extrapolate from work in homogenised islets to its physiological role in intact islets. With both membrane and cytoskeletal integrity destroyed it is not possible to correlate the dynamics of polymer formation with physiological functions such as insulin release. For this reason it is difficult to make suggestions as to what role the polymer may have and therefore, what the function of transglutaminase might be. There is also the problem that unphysiologically high concentrations of Ca^{2+} are used in homogenised islets to obtain radiolabelling by the enzyme. This may explain why such large numbers of lower molecular weight proteins were labelled by the enzyme since the action of proteases may result in the exposure of γ-glutamyl groups into which radiolabelled methylamine can be incorporated. It has also been reported that transglutaminase catalyses the formation of apototic bodies during the final stages of programmed cell death. This process is characterised by an increase in the intracellular Ca^{2+} concentration. The polymer may therefore be the apototic body and have no role in insulin secretion.

Undertaking these same experiments in intact islets has to date proved impossible because of the difficulties of getting sufficient labelling of protein to be detectable. In an attempt to overcome these problems it was therefore decided to use electropermeabilised islets.

5.3 Electropermeabilised Islets Studies

The work so far reported has suggested that transglutaminase is important to the process of Ca^{2+}-stimulated insulin release from electropermeabilised rat islets of Langerhans. However, in order to confirm this theory it is necessary to demonstrate that the enzyme is activated during this process. [^{14}C]-methylamine was therefore used as a probe for transglutaminase activity during Ca^{2+}-stimulated insulin release from electropermeabilised islets. Since the electropermeabilised islets, unlike homogenised islets, still retain cellular integrity, it was hoped that the same method could be used to identify transglutaminase substrates under more physiological conditions and in this way extend previous studies undertaken in homogenised islets.

Transglutaminase has also been implicated in the process of programmed cell death, which is characterised during its later stages by an increase in the intracellular concentration of Ca^{2+} (Fesus et al, 1990; Knight et al, 1990). Since the intracellular Ca^{2+} concentration can be clamped in electropermeabilised islets it was decided to extend our studies by looking at the relationship between intracellular Ca^{2+} concentration, insulin release, transglutaminase activity and the labelling of substrate proteins containing available γ-glutamyl acceptor groups.

5.3.1 Relationship between Transglutaminase Activity, Intracellular Ca^{2+} Concentration and Insulin Release in Electropermeabilised Islets

Electropermeabilised islets were incubated in the presence of [^{14}C]-methylamine at increasing concentrations of Ca^{2+} (10nM Ca^{2+} - 1mM Ca^{2+}). In islets maintained at non-stimulatory levels (10nM Ca^{2+}) incorporation of methylamine into TCA precipitated proteins was easily detectable (Figure 5.7). Raising the Ca^{2+} concentration to 10μM did not increase the amount of methylamine incorporated. Similarly at 100μM Ca^{2+}, although a slight increase in incorporation of radiolabel could be detected, this increase was not statistically significant and it was only at 1mM Ca^{2+} that a significant increase in [^{14}C]-methylamine incorporation was observed. As an indication that the incorporation of radiolabelled methylamine was transglutaminase mediated, glycine methyl ester (5mM) was included during incubation (Figure 5.8). Inclusion of this competitive amine resulted in a significant ($p < 0.05$) decrease in radiolabel incorporation when tested at 10nM, 10μM and 1mM Ca^{2+}. In contrast, inclusion of the control compound sarcosine methyl ester, was found to have no significant affect on methylamine incorporation when tested at the same Ca^{2+} concentrations.

Insulin release was measured at identical Ca^{2+} concentrations to those used during determination of transglutaminase activity. As previously demonstrated, raising the Ca^{2+} concentration from 10nM Ca^{2+} to 10μM Ca^{2+} results in a 130% increase in insulin release (Figure 5.7). When the Ca^{2+} concentration is raised to 100μM, insulin secretion was found to decline significantly and at 1mM Ca^{2+} levels of secretion was grossly reduced and comparable to those at 10nM Ca^{2+}.

These results show that although transglutaminase is active,

there appears to be no stimulation in the activity of the enzyme during Ca^{2+}-stimulated insulin release. Only when the Ca^{2+} concentration is raised to 100μM and 1mM is there an increase in the activity of the enzyme and this correlates with a drop in insulin secretion (Figure 5.7).

paired Student t-test.

* denotes $p < 0.05$ otherwise there was no significant difference.

Figure 5.7

Relationship Between the Ca^{2+} Concentration, the Transglutaminase Mediated Incorporation of [^{14}C]-Methylamine into Protein and Insulin Release from Electropermeabilised Islets

For measurement of the incorporation of [^{14}C]-methylamine into islet, groups of 100-150 islets were pre-incubated at 4°C for 15min in 90µl of permeation buffer containing 10nM Ca^{2+} and 4.4mM [^{14}C]-methylamine (56mCi/mmol). Groups were then incubated at 37°C for 30min following the addition of 10µl of permeation buffer containing the relevant concentration of Ca^{2+} to give Ca^{2+} concentrations of 10nM, 10µM, 100µM and 1mM. The incorporation of radiolabelled methylamine was determined as described in Methods 2.7.5.1.

For measurement of insulin release, groups of 10 islets were preincubated at 4°C for 30min in 0.9ml of permeation buffer containing 10nM Ca^{2+}. Insulin release was then stimulated by incubation at 37°C for 30min following the addition of 0.1ml of permeation buffer containing the relevant amount of Ca^{2+} to give Ca^{2+} concentrations of either 10nM, 10µM, 100µM or 1mM. Following incubation samples of supernatant were taken and assayed for insulin by radioimmunoassay.

Radiolabel incorporation is expressed as the mean [^{14}C]-methylamine incorporation (cpm/100islets/30min) ± SEM taken from 4 separate experiments. The mean background value was 741 ± 52 (n=6). This was determined from electropermeabilised islet groups which were immediately TCA precipitated following pre-incubation in the presence of 10nM Ca^{2+} and radiolabelled methylamine. Insulin release is given as the mean insulin release (pg/islet/30min) ± SEM taken from 4 experiments, each of 4 observations. The significance of the difference in both methylamine incorporation and insulin release against those groups incubated at 10µM Ca^{2+} was determined using the

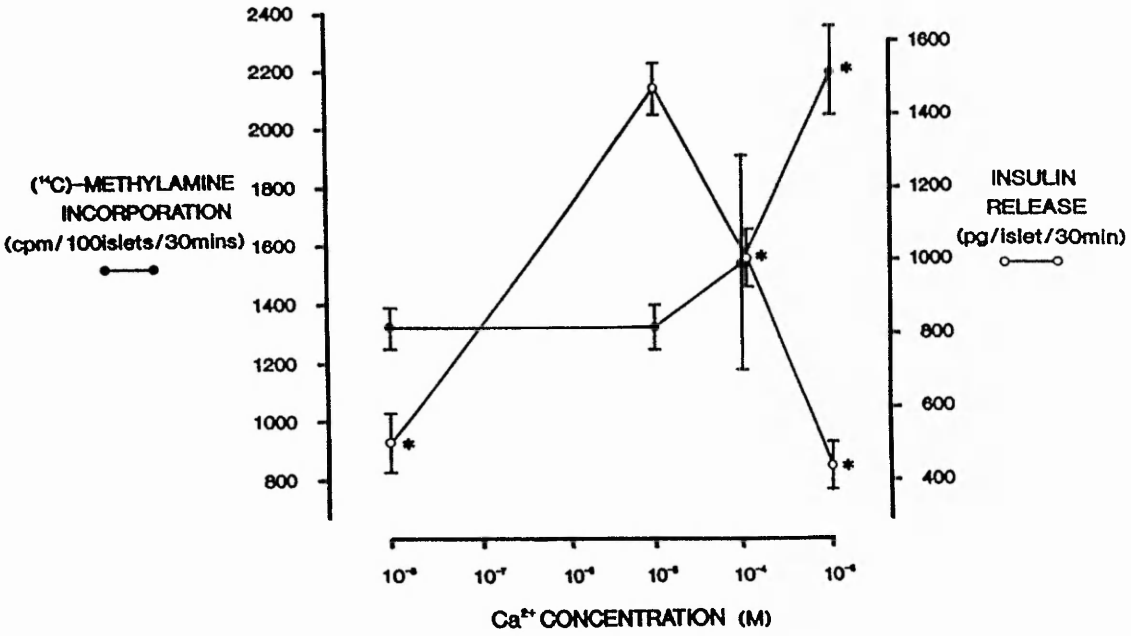

(¹⁴C)-METHYLAMINE INCORPORATION (cpm/100islets/30mins)
2400
2200
2000
1800
1600
1400
1200
1000
800
1600
1400
1200
1000
800
600
400
INSULIN RELEASE (pg/islet/30min)
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
Ca²⁺ CONCENTRATION (M)

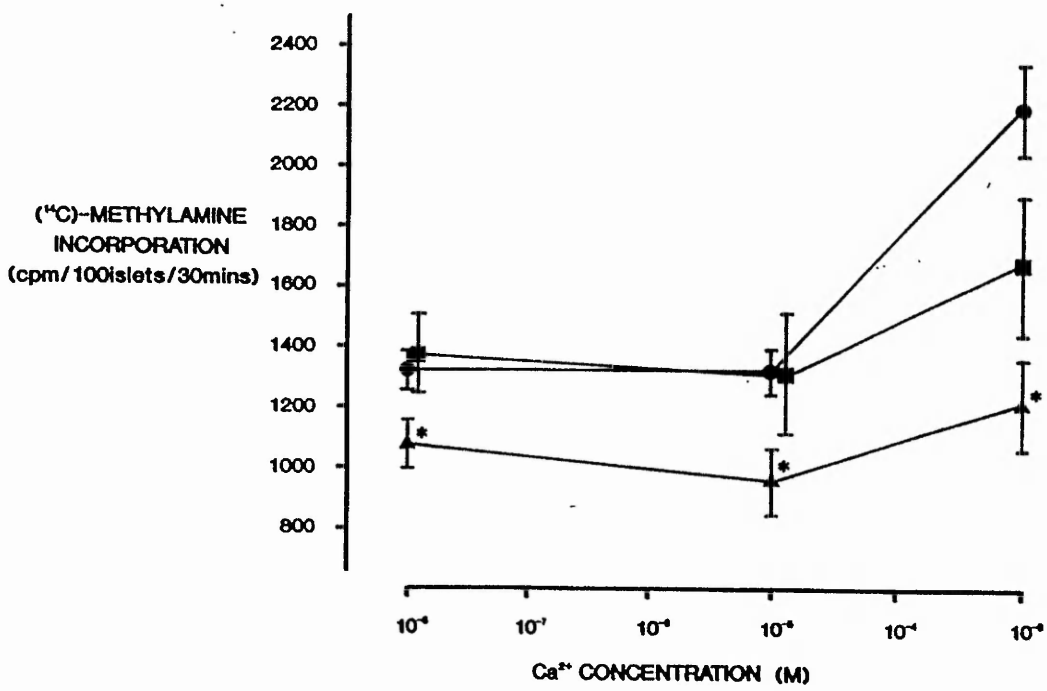
Figure 5.8

Effect of Glycine Methyl Ester and Sarcosine Methyl Ester on the Transglutaminase Mediated Incorporation of [^{14}C]-Methylamine into Electropermeabilised Islet Protein

Groups of 100-150 electropermeabilised islets were pre-incubated at 4^{o}C for 15min in 90µl of permeation buffer containing 10nM Ca^{2+}, 4.4mM [^{14}C]-methylamine (56mCi/mmol) (●——●) and either glycine methyl ester (5mM) (▲——▲) or 5mM sarcosine methyl ester (■——■), where required. Incorporation was started by incubation at 37^{o}C for 30min following the addition of 10µl of permeation buffer containing the relevant amount of Ca^{2+} to give Ca^{2+} concentrations of either 10nM, 10uM or 1mM. Following incubation the amount of radiolabel incorporated was determined as described in Methods 2.7.5.1.

Results are given as the mean incorporation (cpm/100islets/30min) ± SEM taken from 3 separate experiments. The mean background labelling was 790 ± 54 (n=5). The significance of the difference between the radiolabelled incorporation into islets incubated in the presence of glycine methyl ester (5mM) or sarcosine methyl ester (5mM) and those in permeation buffer alone, at any particular concentration of Ca^{2+} was assessed using the paired Student t-test.

* denotes $p < 0.05$ otherwise there was no significant difference.


(14C)-METHYLAMINE
INCORPORATION
(cpm/100islets/30mins)
2400
2200
2000
1800
1600
1400
1200
1000
800
*
*
*
10-8
10-7
10-6
10-5
10-4
10-3
Ca2+ CONCENTRATION (M)

5.3.2 Effect of PMA and cAMP on the Transglutaminase Mediated Incorporation of [^{14}C]-Methylamine into Electropermeabilised Islet Protein

Owen et al (1988) has demonstrated the phosphorylation of the high molecular weight substrate of transglutaminase following glucose stimulation of intact rat islets and has suggested the specificity of the enzyme towards its substrates may be regulated by phosphorylation. To investigate this further we examined the effect of including the protein kinase C activator, PMA and the protein kinase A activator, cAMP on the incorporation of radiolabelled methylamine into total islet protein by transglutaminase. Groups of 100-150 electropermeabilised islets were therefore pre-incubated at 4°C for 15min in 90µl permeation buffer containing 10nM Ca^{2+}, 0.5µM PMA and 100µM cAMP and 500µM of the phosphodiesterase inhibitor, IBMX. Following the addition of 10µl of permeation buffer to give a final concentration of 10µM Ca^{2+}, the islets were incubated at 37°C for 30min. The incorporation of [^{14}C]-methylamine into TCA precipitated islet protein of 1220 ± 32 cpm/100islets/30min (n=3) was found not to be significantly different from the radiolabel incorporation into islet groups that had been incubated under identical conditions but in the absence of PMA and cAMP/IBMX (1257 ± 52 cpm/100islets/30min (n=3)). This therefore suggests that the amount of incorporation of [^{14}C]-methylamine into transglutaminase substrates present in electropermeabilised islets is unaffected by protein kinase A and protein kinase C phoshorylation of the enzymes substrates.

5.3.3 Identification of Transglutaminase Substrates using Electropermeabilised Islets

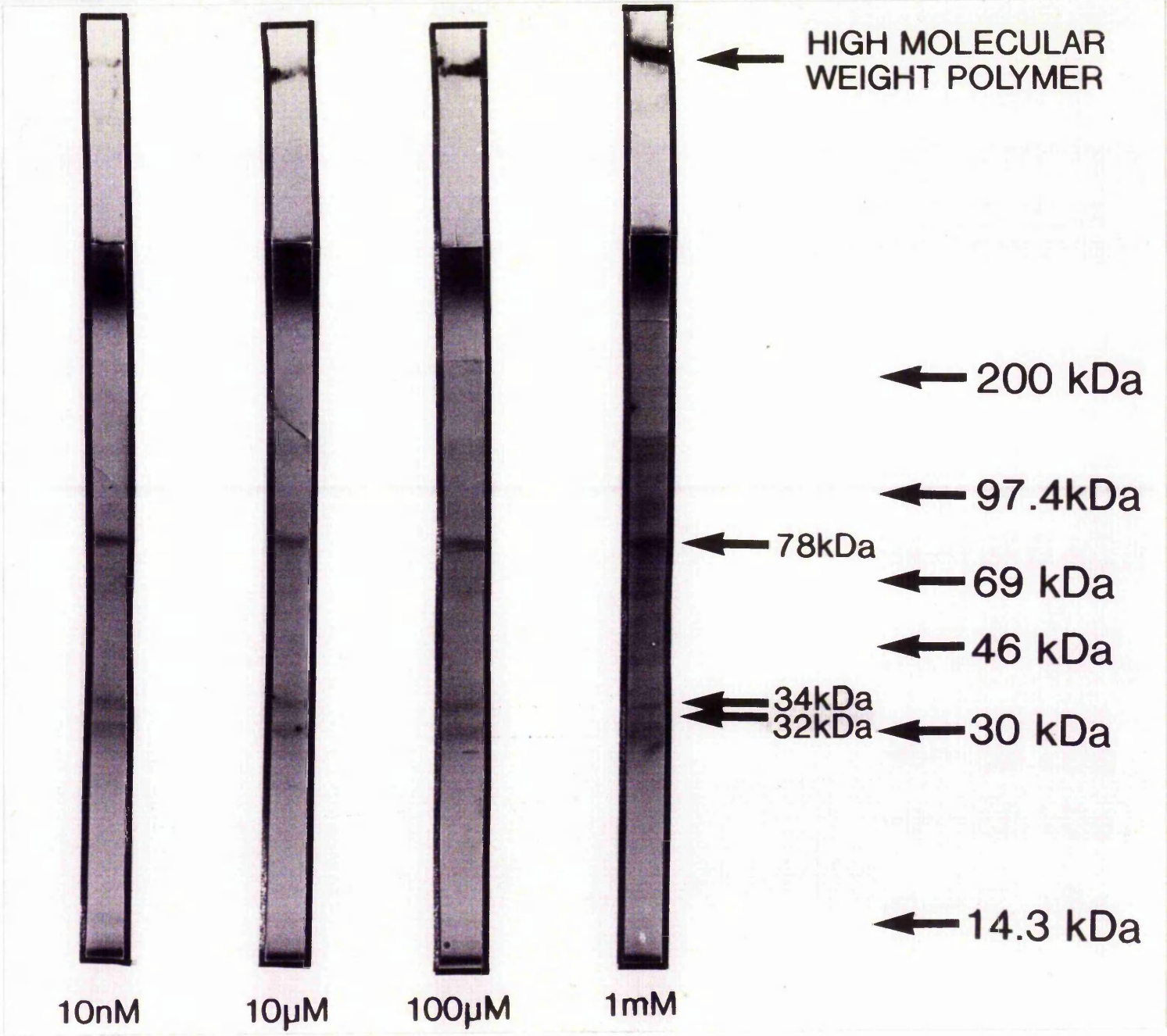
In order to identify other possible transglutaminase substrates labelled with [^{14}C]-methylamine by the enzyme and to examine if this changes at different concentrations of Ca^{2+}, electropermeabilised islet were incubated at 10nM Ca^{2+}, 10μM Ca^{2+}, 100μM Ca^{2+} and 1mM Ca^{2+} in the presence of [^{14}C]-methylamine. Labelled islet protein was then separated by SDS-polyacrylamide gel electrophoresis and detected using fluorography. Representative fluorographs of radiolabelled islet proteins and their densitometric scans are shown in Figures 5.9 and 5.10 respectively. At Ca^{2+} concentrations of 10nM and 10μM a major band of approximate molecular weight 78kDa is clearly visible with further proteins, less discernible, of approximate molecular weights 32kDa and 34kDa. This labelling pattern is also seen at 100μM Ca^{2+} and 1mM Ca^{2+} although at 1mM Ca^{2+} the labelling pattern is heavier and there is labelling of further small molecular weight proteins in the resolving gel. Examination of the stacking gel shows labelling of a high molecular weight polymer, which is unable to traverse the 3% (w/v) acrylamide stacking gel, at all Ca^{2+} concentrations. The amount of radiolabelling of this high molecular weight protein becomes more pronounced as the concentration of Ca^{2+} is increased and is particularly heavy at 1mM Ca^{2+}. From the densitometric scans it can be seen that at 1mM Ca^{2+}, the high molecular weight polymer is particularly prominent.

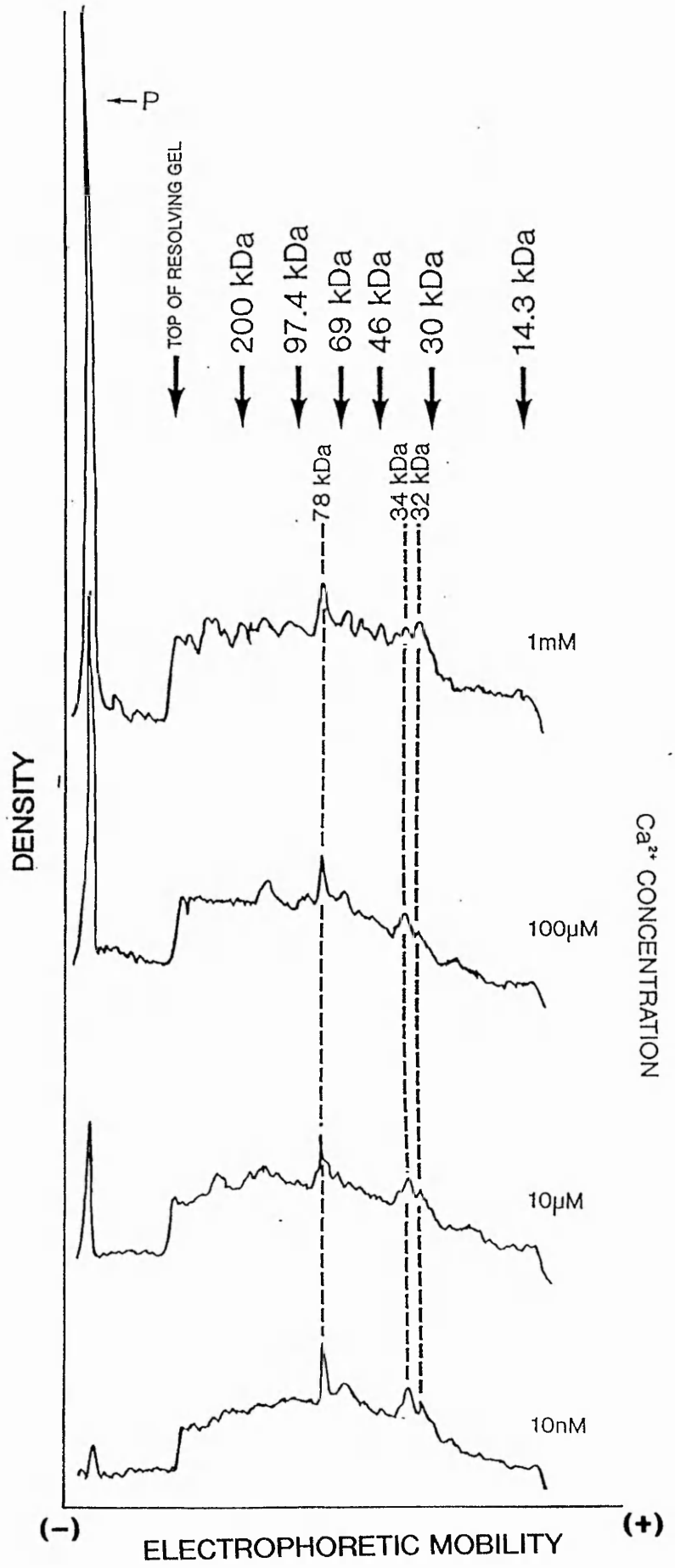
Figure 5.9 and 5.10

Densitometric Scans of Fluorographs obtained from Electropermeabilised Islet Proteins Labelled with [^{14}C]-Methylamine by Transglutaminase

Groups of 200 electropermeabilised islets were pre-incubated at 4°C for 15min in 90ul of permeation buffer containing 10nM Ca^{2+} and 4.4mM [^{14}C]-methylamine (56mCi/mmol). Following the addition of 10µM of permeation buffer containing the relevant amount of Ca^{2+} to give final Ca^{2+} concentrations of 10nM, 10µM, 100µM and 1mM, islet groups were incubated at 37°C for 30min. Following incubation, each group was dissolved in sample buffer and then separated by SDS-polyacrylamide gel electrophoresis (3%(w/v) stacking; 5-15%(w/v) resolving gel) (Methods 2.6.1). Labelled protein was then detected fluorographically (Methods 2.6.3). This involved exposing the dried gels to an pre-sensitised X-ray film for 60-90 days. The X-ray film was then developed before being photographed and densitometrically scanned.

The photographic image of a typical fluorograph and its respective densitometric scan, obtained from three separate experiments is shown in Figures 5.9 and Figures 5.10 respectively. The large arrows indicate the mobility of standard protein markers. The smaller arrows indicate the approximate molecular weights of proteins labelled with [^{14}C]-methylamine. The Ca^{2+} concentration at which the electropermeabilised islets were incubated is indicated in the figure.


HIGH MOLECULAR
WEIGHT POLYMER
200 kDa
97.4kDa
78kDa
69 kDa
46 kDa
34kDa
32kDa
30 kDa
14.3 kDa
10nM
10µM
100µM
1mM


P
TOP OF RESOLVING GEL
200 kDa
97.4 kDa
69 kDa
46 kDa
30 kDa
14.3 kDa
78 kDa
34 kDa
32 kDa
1mM
100μM
10μM
10nM
DENSITY
Ca2+ CONCENTRATION
(–)
(+)
ELECTROPHORETIC MOBILITY

5.4 Discussion

Previous work in this thesis has suggested a role for transglutaminase during Ca^{2+}-stimulated insulin release from electropermeabilised rat islets but the role the enzyme plays in this process is uncertain. Work with homogenised islets has suggested that the major substrate for transglutaminase is a high molecular weight polymer associated with the plasma membrane. However, in order to confirm a role for transglutaminase and the high molecular weight polymer it was decided to use electropermeabilised islets to correlate transglutaminase activity, polymer formation and insulin release.

The activity of transglutaminase was assessed by measuring the incorporation of [^{14}C]-methylamine into TCA-precipitated islet protein. Islets incubated at 10nM Ca^{2+} and 10μM Ca^{2+} were shown to incorporate radiolabel but there was no significant increase even though there was a 2-3 fold increase in insulin release. In fact, transglutaminase activity was only increased when the Ca^{2+} concentration was raised to 100μM and 1mM, which correlated with a loss in insulin release. Confirmation that this incorporation was transglutaminase mediated was demonstrated by showing it could be inhibited by another competitive amine substrate, glycine methyl ester but not by its control compound, sarcosine methyl ester. These results appear to suggest that the activity of islet transglutaminase is not increased during Ca^{2+}-stimulated insulin release, as would be expected if the enzyme was a Ca^{2+}-responsive target. This also contradicts the report by Bungay *et al* (1986) who determined the apparent Km of islet transglutaminase for Ca^{2+} as 3μM by measuring the incorporation of putrescine into dephosphorylated NN'-dimethylcasein. However, Gomis *et al* (1984) has reported that the incorporation of [^{14}C]-methylamine into endogenous islet protein is a saturable process and that incorporation

was not increased in intact islets incubated with stimulatory concentration of glucose (16.7mM). These results could therefore be explained by the increased availability of substrates, as the Ca^{2+} concentration is increased to 100μM and 1mM, possibly as a result of the activation of proteases and phosphatases. Alternatively, at 10nM Ca^{2+}, localised concentrations of Ca^{2+} are available to the enzyme or, conversely, it may have not been possible to clamp the Ca^{2+} concentration in the microenvironment. A further possibility could be the existence of two or more types of transglutaminase in rat islets each with different Km's for Ca^{2+}. Furthermore, Knight et al (1990) has reported that inactive transglutaminase exist in the plasma membrane which is activated following proteolytic cleavage.

When electropermeabilised islets were preincubated in the presence of activators of protein kinase A (cAMP/IBMX) and protein kinase C (PMA), this was found to have no affect on transglutaminase mediated incorportion of radiolabelled methylamine at 10nM Ca^{2+} and 10μM Ca^{2+}. This thereby implies that phoshorylation of transglutaminase substrates by these kinases does not increase their affinity for the enzyme, as proposed by Owen et al (1988). This also provides further evidence to suggest that a non-Ca^{2+} regulated mechanism of insulin release may exist.

In an attempt to identify possible transglutaminase substrates labelled with [^{14}C]-methylamine, electropermeabilised islets were incubated at increasing concentrations of Ca^{2+} (10nM, 10μM, 100μM and 1mM) in the presence of the radiolabelled probe, separated by SDS-PAGE and then subjected to fluorography. Unlike homogenised islets, only three proteins of approximate molecular weights 78kDa, 34kDa and 32kDa as well as the high molecular weight polymer, could be detected. At 1mM Ca^{2+}, many other protein became discernible on the resolving

gel. This may be the result of non-specific labelling by transglutaminase at this supra-physiological Ca^{2+} concentration, of proteins that are not its natural substrates and would explain the results obtained with homogenised islets where radiolabelling is performed at 2.5mM Ca^{2+}. This conclusion is supported by the work of Gomis et al (1989) using rat islet homogenate incubated at 0.5mM Ca2+, who were only able to identify one protein of molecular weight 84kDa labelled with [2,5-^{3}H]-histamine in the resolving gel as well as the high molecular weight polymer present in the stacking gel. Once again there is uncertainty as to the identify of these labelled lower molecular weight proteins although the 78kDa protein may be comparable to that identified by Gomis et al (1989).

Although the intensity of labelling of the lower molecular proteins remains constant as the concentration of Ca^{2+} is increased, the labelling of high molecular polymer is increased dramatically. At 10nM Ca^{2+} there is little labelling of the polymer which is only slightly increase at 10μM Ca^{2+}. When the Ca^{2+} was increased to 100μM and 1mM there was a dramatic increase in labelling which probably accounts for the measured increase in radiolabel incorporation into total islet protein. As stated early, this increase in labelled polymer is associated with a drop in insulin release to basal levels which suggests that polymer formation interfers with insulin release and may be consistent with a role for the enzyme during programmed cell death (apoptosis) where a high molecular weight polymer forms in dying cells (Fesus et al, 1989) in response to high levels of intracellular Ca^{2+} (Cohen and Duke, 1984). Alternatively, the polymer may be present under physiological conditions and increasing the concentration of Ca^{2+} to supraphysiological concentrations could be exposing possible transglutaminase labelling sites, as a result of the action of

proteases and phosphatases, which was previously discussed.

In conclusion, these result demonstrate that transglutaminase is active at concentrations of Ca^{2+} that stimulate insulin release. However, an increase in the activity of the enzyme during Ca^{2+}-stimulated insulin release has not been demonstrated possibly because labelling of substrates with the radiolabelled probe is a saturable process. At concentrations of Ca^{2+} that stimulate insulin release, three substrates labelled with [^{14}C]-methylamine by the enzyme have been identified, which may play a role during Ca^{2+}-stimulated insulin release. Studies of the dynamics of formation of the polymer suggest that it is not a substrate of transglutaminase at Ca^{2+} concentrations required for insulin release.

6 OVERALL DISCUSSION

The overall aim of this thesis was to elucidate a role for transglutaminase in the B-cell of the rat pancreatic islets of Langerhans. In pursuing this aim the following observations have been made:

1) Using competitive substrate inhibitors of transglutaminase it has been shown that the enzyme is important to the process of Ca^{2+}-stimulated insulin release but not PMA-, cAMP- or arginine stimulated insulin release from electropermeabilised islets.

2) The identification of three possible transglutaminase substrates that may be important to the mechanism of Ca^{2+}-stimulated insulin release from electropermeabilised islets.

3) That at supra-physiological concentrations (100μM-1mM) the major substrate of transglutaminase is a high molecular weight polymer. This polymer may be an apoptotic body which suggests a possible role for transglutaminase during the process of programmed cell death.

The presence of transglutaminase activity in rat pancreatic islets was first demonstrated by Bungay *et al* (1982) and later confirmed by Gomis *et al* (1983). Later studies on the effects of competitive substrate inhibitors of the enzyme showed that these were able to inhibit glucose-stimulated insulin release at concentrations comparable to those required to inhibit transglutaminase activity as measured *in vitro* (Bungay *et al*, 1984; Gomis *et al*, 1984; Sener *et al*, 1985; Bungay *et al*, 1986) . Since the Km for activation of the islet

enzyme by Ca^{2+} was reported to be 3μM (Bungay et al, 1986) it was proposed that transglutaminase may be a target for the increase in intracellular Ca^{2+} that is thought to be a primary mediator of stimulus-secretion coupling in the B-cell (Prentki & Matschinsky, 1987). In order to investigate a role for transglutaminase as a Ca^{2+} target it was decided to use rat islets that had been permeabilised by exposure to an electric field. This technique results in the formation of stable pores in the plasma membrane thereby permitting access and control of the intracellular environment whilst maintaining both the membrane and cytoskeletal integrity required for insulin release.

Islets electropermeabilised in this way have been shown to secrete insulin in response to a number of intracellular secondary messengers including Ca^{2+} (Pace et al, 1980; Yaseen et al, 1982; Jones et al, 1985), increases in cAMP (Jones et al, 1986) as well as activators of protein kinase C (Jones et al, 1985). Since the plasma membranes of electropermeabilised islets contains pores, this dissipates the membrane potential and probably results in the loss of cofactors required for metabolism. The electropermeabilised islets therefore provide an ideal model for investigating a role for transglutaminase during the distal events of insulin secretion.

Initial work involved development of the a method for electropermeabilising rat islets. Eventually this was optimized so that the electric field strength and capacitance chosen gave near 100% permeabilisation of the islets whilst maintaining islet membrane and cytoskeletal integrity. Islets permeabilised in this way were able to secrete insulin in response to an increase in Ca^{2+} from 10nM to 10μM, and following exposure at 10nM Ca^{2+} to either the protein kinase C activator, phorbol myristate acetate (PMA) or cAMP in the presence of the phosphodiesterase inhihibitor, IBMX or arginine. Since the Ca^{2+}-

stimulated insulin release was both ATP and temperature dependent, this suggested that the response was physiological.

To investigate a role for transglutaminase as a Ca^{2+} target, the effect of a range of competitive amine substrates of transglutaminase on Ca^{2+}-stimulated insulin release was examined using electropermeabilsed islets. These compounds were found to inhibit Ca^{2+}-stimulated insulin in a dose dependent manner whilst the non-inhibitory analogues of these compounds had no affect. Since these compounds were unable to inhibit basal insulin release at 10nM Ca^{2+} these results appeared to confirm a role for the enzyme during Ca^{2+}-stimulated insulin release.

When the action of these compounds was tested on PMA-, cAMP/IBMX and arginine stimulated insulin release, it was found that they had no inhibitory effect. Taken together with the result from Ca^{2+}-stimulated insulin release this suggests that either different mechanisms exist for the stimulation of insulin release by the various secondary messengers or alternatively that transglutaminase acts at a step during the secretory mechanism, prior to these other activators.

In order to define a role for transglutaminase during Ca^{2+}-stimulated insulin release attempts were made to identify transglutaminase substrates in both homogenised and electropermeabilised islets, using [^{14}C]-methylamine as a probe. Previous work has identified the major substrate of transglutaminase as being a high molecular weight polymer that was unable to traverse a 3%(w/v) polyacrylamide stacking gel following SDS-polyacrylamide gel electrophoresis (Bungay et al, 1986). Using homogenised islets the existence of this high molecular weight polymer was confirmed. Subcellular fractionation studies showed the polymer to be predominantly associated with membrane containing fractions in particular, the plasma membrane.

Since it is thought that the polymer is formed by the covalent crosslinking of lower molecular weight substrates by transglutaminase, attempts were made to identify these by fluorography. This showed that a whole range of proteins became radiolabelled, with the majority being found in the cytosol fraction following subcellular fractionation of labelled homogenates. Since these experiments were performed in homogenised islets and at supra-physiological concentration of Ca^{2+}, it was not possible to correlate the activation of transglutaminase and the subsequent labelling of its substrates with insulin release. These studies were therefore repeated in electropermeabilised islets. This work showed that although transglutaminase activity could be measured, it was not significantly increased during Ca^{2+}-stimulated insulin release (10nM-10μM) and only when the concentration of Ca^{2+} was raised to 1mM did a significant increase in the enzyme activity occur which correlated in the loss of insulin release. These results could be explained by the saturation, at 10nM Ca^{2+} and 10μM Ca^{2+}, of available γ-glutamyl residues in which radiolabelled methylamine could be incorporated by transglutaminase, as has been reported by Gomis et al (1985). However, as the concentration of Ca^{2+} is raised to 1mM, this may release more substrates to the enzyme as a result of the activation of proteases or phosphatases. Alternative, at 10nM Ca^{2+} and 10μM Ca^{2+}, localized concentrations of Ca^{2+} may be available to the enzyme when the applied Ca^{2+} concentration is low or, conversely, the concentration applied to the permeabilised islet does not become available to the enzyme within the cell because of Ca^{2+} clamping in the micro-environment surrounding the enzyme.

Finally, the endogenous substrates that become labelled with radiolabelled methylamine through the action of transglutaminase were examined at different Ca^{2+} concentrations in electropermeabilised

islets. During Ca^{2+}-stimulated insulin release labelled methylamine was found in three proteins of approximate molecular weights 78kDa, 34kDa and 32kDa. At supra-physiological concentrations (100μM and 1mM) the [^{14}C]-methylamine was also incorporated into the high molecular weight polymer that had previously been identified in islet homogenates.

Overall this work suggests that transglutaminase may play two functions in the B-cells of the rat islets of Langerhans. At physiological Ca^{2+} concentrations, the enzyme appears to be important to the process of Ca^{2+}-stimulated insulin release, whilst at higher Ca^{2+} concentrations the enzyme maybe important to the process of programmed cell death (apoptosis). A role for the enzyme in mediating the effects of intracellular messenger signals is supported by investigations of transglutaminase in other tissues. Loewy et al, (1981a; 1981b) has reported an increase in the formation of intracellular ε-(γ-glutamyl)lysine crosslinks in tissue-cultured embryonic chick heart myofibrils and from cultures of the slime mold, Physarium polycephalum, following the addition of ATP. Recently, Achyuthan & Greenberg (1987) have reported that guinea pig liver transglutaminase is able to bind GTP at physiological concentrations, and that this results in loss of enzyme activity. Lee et al (1989) later demonstrated that the enzyme possesses GTPase activity. These observations have been confirmed by Bergamini (1988) using erythrocyte transglutaminase who suggests that the inhibitory effect of GTP binding is a result of this nucleotide reducing the enzymes affinity for Ca^{2+}. A Ca^{2+}- and GTP-dependent tissue transglutaminase could therefore be an ideal mediator of the intracellular secondary messenger systems of the islet B-cell.

Although at present, there is still little information as to

the function of transglutaminase during Ca^{2+}-stimulated insulin release, a recent report by Ando et al (1989) has demonstrated that transglutaminase is able to crosslink and activate lipocortin I (calpactin II) in human epidermoid carcinoma A431 cells. The crosslinking of lipocortin I, which has a molecular weight of approximately 35kDa, results in the formation of higher molecular weight lipocortins of molecular weights of approximately 75kDa and 160kDa. This crosslinking enhances the Ca^{2+} sensitivity of lipocortin for phospholipid binding by reducing the free Ca^{2+} required for 50% binding to phosphatidylserine vesicles from 20μM to 3μM. It is therefore possible that the 32kDa and 34kDa substrates of transglutaminase may be lipocortins (calpactins) which are crosslinked and activated by transglutaminase to produce the 78kDa protein. Lipocortins are members of the Ca^{2+} and phospholipid binding proteins, collectively known as annexins. At least 5 distinct but closely related Mammalian annexins have been identified in a wide range of tissues and cells, and it is expected that they will be found to be ubiquitous in all cell types (Burgoyne & Geisow, 1989). It is therefore likely that all or at least a subset of the proteins will also be present in the B-cells of islets. The exocrine adrenal chromaffin cells have been demonstrated to contain the annexins p70, calpactin I, calpactin II, endotoxin I and two forms of endotoxin II (Geisow & Burgoyne, 1982; Creutz et al, 1983; 1987; Geisow et al, 1984).

Subcellular fractionation studies and immunocytochemical studies have suggested that the p70 and calpactin are localized below the plasma membrane and associated with the detergent insoluble sub-plasmalemmal matrix and that they bind to fodrin and actin in a Ca^{2+}-dependent manner, at millimolar concentrations of Ca^{2+} (Greenberg &

Edelman, 1983; Lehto et al, 1983; Gerke & Weber, 1984; Raeymaekers et al, 1985). This has led to the suggestion that the one of the functions of annexins may be the linking of cytoskeletal elements to the plasma membrane. The annexins have also been implicated in the process of exocytosis since all of the proteins have been reported to be able to aggregate secretory vesicles and liposomes in the presence of Ca^{2+} (Geisow & Burgoyne, 1982; Creutz et al, 1883; Creutz et al, 1987). Recently, Nakata et al (1990) has reported that following the stimulation of adrenal chromaffin cells, calpactin I complexes were visible between the plasma membrane and secretory vesicle membrane and appeared to form thin crosslinks between the two. They therefore suggest that the calpactin may be involved in the mechanism of exocytotic fusion.

It is therefore possible that the action of transglutaminase during Ca^{2+}-stimulated insulin release results from the crosslinking and activation of calpactin. This reduces the Km for Ca^{2+} of binding of the calpactin into the micromolar range, permitting the calpactin complex to bind to the inner side of the plasma membrane and induce exocytotic fusion. Alternatively, the redistribution of calpactin to the plasma membrane and its subsequent binding to actin or fodrin could result in the disruption of the actin network beneath the plasma membrane thereby permitting fusion of the secretory vesicles, as has been suggested by Burgoyne & Cheek (1987). At millimolar concentrations of Ca^{2+}, as occurs during the terminal stages of programmed cell death or following necrosis, transglutaminase could be involved in the extensive covalent crosslinking of the annexins and other membrane proteins to the cytoskeleton beneath the plasmalemma, to form the high molecular weight polymer or apoptotic body as has been reported by Fesus et al (1989). This proposal is supported by many

reports of cytoskeletal proteins as being substrates for transglutaminase (Introduction 1.2.4.5). This would also explain why such a large number of proteins were labelled by transglutaminase at high concentrations of Ca^{2+} in homogenised islets and in permeabilised islets at 1mM Ca^{2+}.

Whether these two functions are performed by the same transglutaminase or whether two types of the enzyme exist, with different Km for Ca^{2+} and different substrate specificities, is unknown. Recent work has suggested that tissue transglutaminase may exist in both a cytosolic form and particulate form (Chang & Chung, 1986; Knight et al, 1990b). Whether the particulate enzyme is an inactive form of the cytosolic enzyme that is activated following proteolytic cleavage or whether the particulate enzyme is active and has a different function, is uncertain. In epidermal cells, membrane transglutaminase has been reported to be involved in envelope formation during terminal differentiation (Thacher & Rice, 1985; Schmidt et al, 1985) whilst the cytosolic enzyme has been implicated in the programmed cell death (Fesus & Thomazy, 1988). However, several reports have suggested that the particulate enzyme found in keratinocytes may be proteolytically cleaved from the membrane (Schmidt et al, 1988; Rice et al, 1988; 1990). To further complicate the situation, Ando et al (1988) has demonstrated that proteolytic cleavage of two cytosolic transglutaminases in porcine skin results in a 20-fold increase in their activity, suggesting that the activity of cytosolic transglutaminases may also be regulated by proteolysis.

At the present time, although this work has been able to implicate transglutaminase in insulin release from the B-cells of the rat pancreatic islets, we can only speculate as to the function the enzyme during this process. These conclusions, especially the

identification of the enzyme substrates are further confused by the fact that islets of Langerhans are not an homogeneous clump of cells. Future work needs to confirm these results using homogenous suspensions of B-cells. However, it is my belief that this ubitiqious enzyme, which is the only enzyme presently known to cause the post-translational modification of proteins through covalent crosslinking, will be found to be important to many intracellular processes.

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Transglutaminase Involvement in the Secretion of Insulin from Electropermeabilised Rat Islets of Langerhans

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Ca^{2+}-Induced insulin release from electropermeabilised islets is inhibited by the transglutaminase inhibitors monodansylcadaverine, glycine methylester, methylamine and cystamine but not by the control compounds dimethyl monodansylcadaverine and sarcosine methylester which lack the primary amine group. Neither monodansylcadaverine nor glycine methylester inhibited insulin secretion induced by either cAMP or the phorbol ester PMA at basal levels (10 nM) of Ca^{2+}. These data provide further evidence for the involvement of transglutaminase in Ca^{2+} induced insulin secretion, they also suggest that insulin secretion induced by either cAMP or PMA may act in part by a mechanism independent of that induced by Ca^{2+}.

KEY WORDS: transglutaminase; calcium; insulin release.

INTRODUCTION

There is now increasing evidence to indicate that a rise in the concentration of cytosolic Ca^{2+} is required for the stimulation of insulin release from the pancreatic β-cell [1, 2]. However increases in other intracellular mediators such as cAMP and activators of protein kinase C may also stimulate insulin release through a mechanism still not fully understood but which may act in part by a Ca^{2+} dependent effect, through changing the sensitivity of the secretory apparatus to Ca^{2+}, and also by Ca^{2+}-independent effects [3, 4, 5, 6]. Although the exact pathway for these different mechanisms is unknown it is thought that their effects are exerted by altering the phosphorylation state of β-cell proteins by activation of protein kinases [7, 8]. The rise in the concentration of intracellular Ca^{2+} may also lead to a change in the activity of other specific Ca^{2+}-binding proteins which

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are important in the stimulation of insulin release. One such possible Ca^{2+} target is the enzyme transglutaminase [EC2.3.2.13.], whose presence in the β-cell has been demonstrated and whose possible role in glucose stimulated insulin release has been suggested [9, 10, 11].

In the following study we have used isolated rat islets permeabilised with high voltage discharge, thus enabling the intracellular concentration of the β-cell to be controlled by Ca^{2+}/EGTA buffers. Using this model we have investigated the link between Ca^{2+} induced insulin secretion and transglutaminase activation.

METHODS

Islets of Langerhans were isolated from the pancreas of 250–400 g Sprague-Dawley rats of both sexes by collagenase digestion as previously described [10].

Islets were permeabilised using the previously published method of Jones *et al.* [3] except a BIORAD Gene pulser was used.

For measurement of the effect of transglutaminase inhibitors on insulin release, groups of 3–5 permeabilised islets were pre-incubated at 4°C for 15 min in 0.9 ml of permeation buffer [3] at 10 nM Ca^{2+}. Transglutaminase inhibitor was included where necessary. Islets were then incubated at 37°C for 30 min after addition of 0.1 ml of permeation buffer which included where necessary the correct amount of $CaCl_2$ needed to give a stimulatory Ca^{2+} concentration of 10 μM. The Ca^{2+} concentration of the buffer was calculated using the dissociation constants previously described by Yaseen *et al.* [12].

In those experiments investigating the effect of transglutaminase inhibitors on insulin release stimulated by the protein kinase C activator phorbol 12-myristate 13-acetate (PMA, Sigma 500 nM) dissolved in dimethylsulfoxide (DMSO) to give final concentration of 0.1% (v/v) DMSO and 500 μM cAMP plus the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 100 μM, Sigma), groups of 10 permeabilised islets were used. Islets were pre-incubated in 1 ml of permeation buffer containing 10 nM Ca^{2+}, the test compounds and either cAMP or PMA for 15 min at 4°C. Islets were then incubated for 30 min at 37°C. DMSO was found to have no significant effect on insulin release at the concentration used. Matched controls without test compound were undertaken in all experiments.

Transglutaminase activity was measured by [^{14}C]-putrescine (Amersham) incorporation in N,N′-dimethylcasein as previously described [10].

For statistical analysis significance tests were undertaken using the student *t*-test.

RESULTS

Comparison of transglutaminase activity in islet homogenates obtained from electropermeabilised islets (3.47 ± 0.47 mU/islet) to homogenates of control islets (3.26 ± 0.47 mU/islet), not permeabilised, indicated the enzyme was not lost from the islet cells during the permeabilisation procedure.

Previous experiments have shown that glucose stimulated insulin release from intact islets may be inhibited by inhibitors of transglutaminase activity [10, 11, 13]. The same compounds were therefore tested for their ability to inhibit Ca^{2+}-induced insulin secretion from permeabilised islets. Raising the intracellular concentration of Ca^{2+} in permeabilised islets from 10 nM to 10 μM led to a 2–3 fold increase in insulin release, a result comparable to that of other workers [3, 12]. Incubation of monodansylcadaverine, methylamine, glycine methylester and cystamine with permeabilised islets led to inhibition of Ca^{2+}-induced insulin release (Table 1). The specificity of this inhibition for effects on transglutaminase was tested by incubating permeabilised islets with the control compounds dimethyl monodansylcadaverine (50 μM) and sarcosine methyl ester (10 mM) which do not possess the primary amine group essential for transglutaminase inhibition. Neither of these compounds were found to inhibit Ca^{2+}-induced insulin release. Test compounds were also investigated for their ability to inhibit basal insulin release at 10 nM Ca^{2+} (Table 1). None of the compounds showed any inhibition of this process, indicating their specific action against the Ca^{2+}-induced event. Incubation of monodansylcadaverine (50 μM) with permeabilised islets under basal conditions did however lead to massive release of insulin of a magnitude approaching 300–400% greater than that found in Ca^{2+} stimulated islets. It would therefore appear that under basal conditions monodansylcadaverine may be toxic to the permeabilised β-cell.

Incubation of permeabilised islets with either 500 μM cAMP in the presence of 100 μM IBMX or 500 nM PMA at 10 nM Ca^{2+} led to rates of insulin secretion (pg/islet/30 min) comparable to those induced by 10 μM Ca^{2+}. However unlike

Table 1. Effects of test compounds on Ca^{2+}-induced insulin release from permeabilized islets

Compound	Concentration (nM)	% of control, 10 nM Ca^{2+}	mean ± SEM (n) 10 μM Ca^{2+}
Methylamine	2.0	—	88 ± 17 (36)
	5.0	74 ± 10 (22)	58 ± 13* (34)
	10.0	—	37 ± 14* (24)
Cystamine	1.0	—	72 ± 12 (30)
	2.5	—	62 ± 11* (31)
	5.0	136 ± 15* (26)	38 ± 10** (34)
Glycine Methyl Ester	1.0	—	72 ± 10 (35)
	2.0	—	39 ± 8** (34)
	5.0	109 ± 12 (25)	30 ± 8** (27)
Monodansylcadaverine	0.050	—	44 ± 13* (12)
	0.100	—	40 ± 15* (12)
Dimethylmono-dansylcadaverine	0.050	—	82 ± 18 (40)
Sarcosine Methyl Ester	5.0	—	82 ± 8 (39)

Data represents mean values ± SEM for the number of observations (n) shown. Mean rate of insulin release from controls (pg/islet/30 min) were 552 ± 23 at 10 nM Ca^{2+} (n = 178) and 1292 ± 50 at 10 μM Ca^{2+} ($n = 169$). *$P < 0.05$; **$P < 0.01$; other differences $P > 0.05$.

Table 2. Effects of test compounds on cAMP and PMA stimulated insulin release from permeabilized islets

Compound	% of control, mean ± SEM (n) 0.5 mM – cAMP	0.5 μM – PMA
5 mM Glycine ethyl ester	94 ± 20 (9)	76 ± 11 (15)
5 mM Sarcosine methyl ester	59 ± 7 (9)*	51 ± 14 (16)*
50 μM Monodansylcadaverine	106 ± 19 (9)	116 ± 46 (9)

Data represents mean values ± SEM for the number of observations (n) shown. Mean rates for insulin release (pg/islet/30 min) were 580 ± 27 at 10 nM Ca^{2+} (n = 32); 1082 ± 95 at 10 μM Ca^{2+} (n = 36); 1278 ± 91 at 10 nM Ca^{2+}/500 μM cAMP (n = 9); 1178 ± 92 at 10 nM Ca^{2+}/500 nM PMA (n = 15). *$P < 0.05$; other differences $P > 0.05$.

that found with Ca^{2+}-induced insulin release the transglutaminase inhibitors monodansylcadaverine (50 μM) and glycine methyl ester (5 mM) were found to have no significant inhibitory effect on either cAMP or PMA induced secretion (Table 2). The control compound sarcosine methyl ester which has no inhibitory effect on transglutaminase activity was found to cause greater inhibition which proved to be significant in both cases.

DISCUSSION

Investigations carried out to determine whether the predominantly cytosolic islet transglutaminase [10] was released from islets during and after permeabilization, indicated that no significant loss of activity occurred from permeabilized cells. With this in mind, we proceeded to investigate the effects of transglutaminase on Ca^{2+}-induced secretion.

Exposure of permeabilised islets to a number of transglutaminase inhibitors previously shown to be capable of inhibiting glucose-stimulated insulin release from intact islets [9, 10], led to inhibition of Ca^{2+}-induced insulin release in a dose-dependent manner. The relative potencies of these compounds resembled their relative potencies as inhibitors of transglutaminase activity [9, 11, 14] and basal insulin release at 10 nM Ca^{2+} was not inhibited by cystamine, methylamine or glycine methylester. This suggests that these compounds inhibit insulin secretion through inhibition of Ca^{2+}-activated transglutaminase activity. Other support for this specific action is that glucose stimulated glucose oxidation, which is known to be inhibited by methylamine and cystamine [10], is not involved as a signal for Ca^{2+}-induced insulin secretion. Furthermore, cAMP- and PMA-induced insulin secretion was unaffected by monodansylcadaverine or glycine methylester, suggesting a specific action on the Ca^{2+}-induced events rather than a non-specific action on the insulin secretory mechanism. The latter observations are also interesting because they suggest that transglutaminase, although required for Ca^{2+}-induced secretion, is not involved in the insulin secretory mechanism evoked by either cAMP or PMA. Furthermore they support the idea that cAMP and PMA may act by a mechanism that does not involve sensitization of the secretory apparatus to Ca^{2+} and this is consistent with the recent finding that in

electropermeabilized islets, both cAMP and PMA may act in part by a mechanism independent of that evoked by Ca^{2+} [6].

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Identification of the endogenous substrates of the pancreatic islet transglutaminase

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A potential Ca^{2+}-responsive target in the pancreatic β-cell is the enzyme transglutaminase which catalyses the cross-linking of proteins via $\varepsilon(\gamma$-glutamyl)lysine bridges [1]. Evidence suggests that this enzyme may be involved in the motile events associated with the insulin secretory mechanism [2–4]; however, a full characterization of the endogenous substrates of the enzyme is still to be undertaken. Preliminary studies using the Ca^{2+}-dependent incorporation of [^{14}C]methylamine and [2,5-^{3}H]histamine into islet homogenates indicates the major substrate/product of the enzyme to be a high molecular mass polymer which is unable to traverse a 3% (w/v) acrylamide gel [5, 6]. A further membrane-associated substrate of 84 kDa has also recently been identified [6].

In the following study we have extended this work by: (i) further characterization of the high molecular mass polymer and (ii) investigating other potential transglutaminase substrates in islet homogenates.

Islets were homogenized and incubated with [^{14}C]methylamine for 30 min in the presence of 2.5 mM-$CaCl_2$ or 5 mM-EGTA as previously described [3]. Incubations were fractionated into a 600 $g_{av.}$ pellet, a 71 000 $g_{av.}$ pellet and a particle-free supernatant (PFS), and fractions were characterized by the use of marker enzymes as described previously [7]. SDS/polyacrylamide-gel electrophoresis was undertaken according to the method of Laemmli [8], using a 3% (w/v) acrylamide stacking gel and a 10% (w/v) resolving gel.

Fractionation of islet homogenates previously incubated with [^{14}C]methylamine indicated the majority (61 ± 13%, $n = 3$) of the high molecular mass polymer, as judged by SDS/gel electrophoresis, to be present in the 71 000 $g_{av.}$ fraction with 33 ± 10% ($n = 3$) present in the 600 $g_{av.}$ pellet and 6 ± 8% ($n = 3$) present in the PFS. This pattern was similar to that found for the plasma membrane marker 5′-nucleotidase. A similar pattern for the total radiolabel present in the different fractions was also found. No radiolabelled polymer was found in incubations containing EGTA, similarly the incorporation of radiolabel into islet proteins was inhibited in the presence of 5 mM-cystamine, thus indicating it to be transglutaminase mediated.

Further identification of other radiolabelled islet proteins was undertaken using fluorography of the SDS/polyacrylamide gels obtained from different subcellular fractions. In the 600 $g_{av.}$ pellet only three other proteins were radiolabelled corresponding to >250 kDa (unable to traverse resolving gel), 69 kDa and 14 kDa. The majority of the radiolabelled substrates were found in the PFS and the 71 000 $g_{av.}$ fraction as shown in Fig. 1. The suggestion of these findings, that smaller cytosolic proteins may be translocated to the membrane fraction before polymer formation was confirmed by separate incubation of the PFS and total membrane fraction (71 000 $g_{av.}$ pellet plus 600 $g_{av.}$ pellet) with [^{14}C]methylamine. In the PFS, which contains approximately 90% of the islet transglutaminase activity, no detectable radiolabelled polymer was evident, while in the 71 000 $g_{av.}$ pellet only 15 ± 6% of the radiolabel found in the complete incubated homogenate was found in the high molecular mass polymer.

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Abbreviation used: PFS, particle-free supernatant.

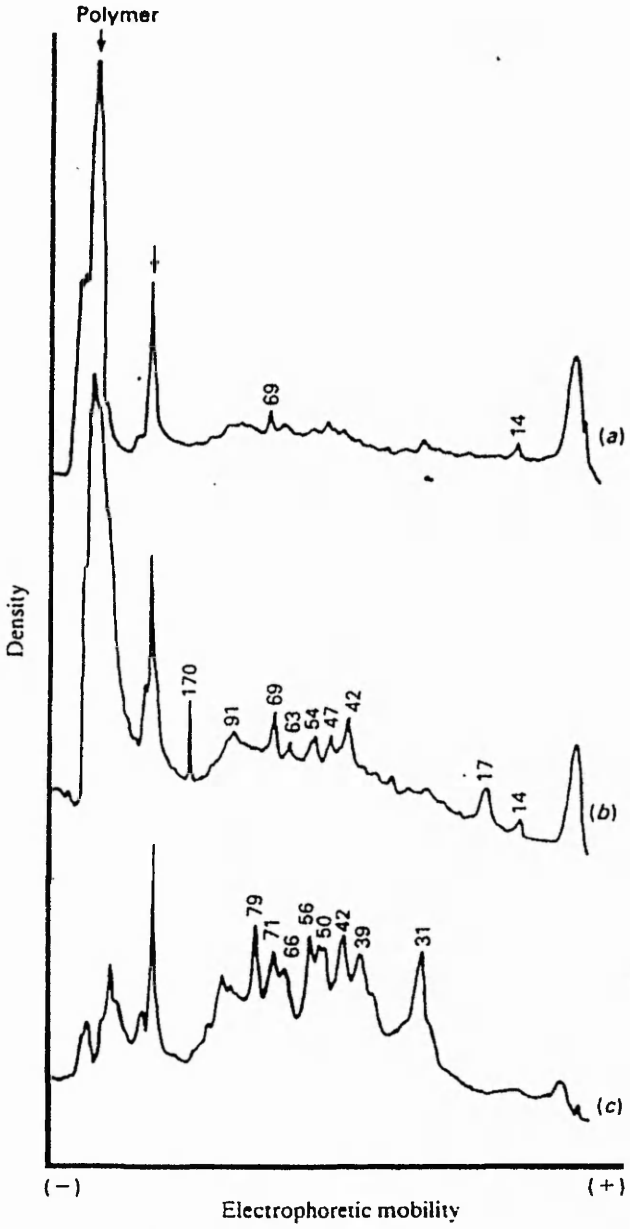

Fig. 1. *Typical densitometric scans of fluorographs of [^{14}C]methylamine radiolabelled proteins present in islet subcellular fractions after separation of proteins by SDS/PAGE*

Islet homogenates were incubated in the presence of [^{14}C]methylamine and 2.5 mM-$CaCl_2$ and then fractionated into (*a*) 600 $g_{av.}$: 10 min, (*b*) 71 000 $g_{av.}$: 45 min and (*c*) PFS. The approximate molecular mass of the radiolabelled proteins was calculated by comparison with the migration of known standards and is indicated on the scans (kDa).

Further characterization of the SDS-solubilized [4% (w/v) SDS, 1 mM-dithiothreitol] radiolabelled polymer was undertaken by elution on a Bio-Rad A5M column, previously equilibrated in 1% (w/v) SDS, 50 mM-Tris/HCl, pH 7.4, and 1 mM-dithiothreitol, which indicated the polymer to have a molecular mass equal to or greater than 5 million.

In conclusion, the finding of a large molecular mass polymer which is membrane associated and which may be formed by the cross-linking of smaller cytosolic proteins to those already present in the islet membrane is consistent with

the view that the islet transglutaminase may play an important role in the Ca^{2+}-induced secretory mechanisms of the pancreatic β-cell.

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BENEFIT-RISK RATIO OF THE ALPHA-GLUCOSIDASE INHIBITOR ACARBOSE (BAY G 5421)

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Data was pooled from placebo-controlled studies in NIDDM- and IDDM-patients for judgement of benefit-risk ratio.

Efficacy: In patients (dose: 300 mg/day) mean changes in blood glucose (mmol/l) (fasting, 1 and 2 hours pp) compared with levels before treatment were: Type 1 (n = 276), acarbose: - 0.94, - 1.94, - 2.61, placebo: + 1.33, + 0.44, + 2.05; Type 2 (n = 491), acarbose: - 0.72, - 1.17, - 1.44, placebo: + 0.56, + 0.67, + 0.94. Treatment for a period of over 8 weeks (n = 46 Type 2) resulted in HbA_1 - reduction of 1 % compared with placebo from the initial level of 11.6 %.

Tolerability: Of the 819 cases (Type 1 n = 276, Type 2 n = 491, others n = 52) the number of patients with intestinal side effects (meteorism/flatulence, diarrhoea, constipation, drop outs): acarbose: 44, 8.4, 1.8, 3.8 %; placebo: 17, 3.2, 3.4, 2 %. The number of patients reporting side effects under acarbose decreased constantly with treatment for longer than 3 months although there was no decline in efficacy. Hypoglycemia was reported in 2.7 % of patients (placebo) and 3.1 % (acarbose). There was no difference as regards efficacy and tolerability in patients with liver (n = 457) and renal (n = 232) disease and over the age of 65 (n = 2642) compared with overall findings (n = 5634).

Conclusion: Acarbose is a beneficial drug with minimal risk.

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AMPULLOMETRY AS AN EVALUATING TEST IN DIABETIC NEUROPATHY

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Diabetic autonomic neuropathy (DAN) plays an important role in the pathogenesis of fecal incontinence and diarrhea, determining internal and external anal sphincter dysfunctions (Schiller et Al.,1982; Wald and Tunungguntla,1984; Caviezel et Al.,1986). We studied 10 diabetic patients with symptoms and signs of DAN (8 M; 2 F; 5 IDD; 5 NIDD; age 22-60 yr); 10 diabetics without DAN (7 M; 3 F; 4 IDD; 6 NIDD; age 21-64 yr); 10 controls (8 M; 2 F; age 21-64 yr). Each patient was asked a series of questions about past ano-rectal disease and surgery and underwent ampullometry by means of an urodynamic catheter adapted with a small rectal latex balloon expansible by saline solution, connected with a Wolf cystomanometer in order to evaluate: a)first defecatory stimulus (ampullar sensibility); b)impellent defecatory stimulus; c)ampullar compliance. Diabetics with DAN showed abnormal ampullometric patterns (reduction of sensibility, altered sensations of impellent stimulus; increased compliance). No statistical differences were recorded in the responses of diabetics without DAN vs controls.

In conclusion, ampullometry is a reliable and simple test to detect an impairment of the pelvic responses in diabetic neuropathy.

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TRANSGLUTAMINASE IS IMPORTANT IN THE Ca^{2+}-INDUCED EVENTS ASSOCIATED WITH THE RELEASE OF INSULIN FROM THE PANCREATIC β-CELL.

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Glucose stimulated insulin release from the pancreatic β-cell is thought to be mediated by a rise in the concentration of cytosolic Ca^{2+}. Previous studies have indicated that this rise in the cytosolic Ca^{2+} may lead to the activation of the islet transglutaminase resulting in the post-translational modification of proteins either through their cross-linking or through the incorporation of putrescine. Such reactions may be important in the cytoskeletal/membrane events required for insulin release. Further evidence to suggest that transglutaminase is important in these events has been obtained using isolated rat islets permeabilised by electrical discharge. Incubation of permeabilised islets with the transglutaminase inhibitors/competitive pseudosubstrates monodansylcadaverine, glycine methyl ester, methylamine and cystamine leads to inhibition of Ca^{2+} induced insulin secretion in a dose dependent manner. Using ^{14}C-methylamine as a transglutaminase probe it can be shown that Ca^{2+} induced secretion is accompanied by the incorporation of this radiolabel into specific islet proteins in a Ca^{2+} dependent manner. This incorporation can be inhibited by the active site directed transglutaminase inhibitor cystamine.

These results further confirm that activation of the islet transglutaminase is important in the distal events associated with insulin release from the pancreatic β-cell.

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ACTIVATIONMARKERS OF BLOODCOAGULATION AND FIBRINOLYTIC SYSTEM OF TYPE II DIABETIC PATIENTS:

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In a first phase of our study we found elevated Plasma-Plasmino genactivatorinhibitor(PAI)-capacity in patients with type II Diabetes mellitus (D.m.) and diabetic macroangiopathy. To see whether this finding is specific for these patients we compaired them with other atherosclerotic patients:
I) 141 patients with periperal arterial occlusive disease (POD) including 32 type II) diabetics. This group represents a type of atherosclerosis with widespraed and noumerous leasions. II 118 patients with coronary-heart-disease(CHD)(POD clinically excluded): angiographical signs of stable angina pectoris. This group stands for an atherosclerotic type of disease with a limited expansion . III) 32 Controls without any clinical signs of POD or CHD. We investigated activation markers of coagulation and fibrinolytic system: * Fibrinogen:n.s. *Von Willebrand Factor plasma levels(VWF): Significantly ($p<0.05$) elevated values for POD compaired to CHD and controls. *Thrombin-Antithrombin III-Complexes:Significantly ($p>0.05$) elevated values for POD compaired to CHD and controls. *Tissuetype Plasminogenactivator (tPA) Ag: n.s. *Plasminogenactivatorinhibitor : POD + CHD significantly ($p<0.05$) elevated values compaired to controls. *D-Dimer: POD elevated ($p<0.05$) compaired to CHD and controls.
Conclusions: *Atherosklerotic diseases with limited expansion of vessel leasons CHD do not lead to elevated markers. * Atherotic diseases with widespread vessel leasons as POD show elevated activation markers of the fibrinolytic and coagulation system indepedent of a simultanously existing type II Diabetes mellitus. *Atherosklerotic patients show elevated PAI-capacity and elevated VWF. This could be caused by a situation of endothelial stress. *The actually measured PAI-capacity seems not to mirrorr actual fibrinolytic activity as in our study elevated PAI levels are accompanied by high D-Dimer levels.

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INAPPROPRIATE RESPONSIVENESS TO MILD SODIUM LOADING IN NOR GLYCEMIC NON-NEPHROPATHIC PATIENTS WITH TYPE 1 DIABETES

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Diabetic sodium retention is a prevalent finding, already fore overt diabetic nephropathy is present, possibly contr buting to its pathogenesis. We examined, therefore, in 10 normoglycemic, non-nephropathic patients with type 1 diabe mellitus (age ($\bar{x} \pm$ SEM), 33 ± 2 years, diabetes duration, ± 3 years) and in 10 healthy controls (age, 28 ± 1 years) effect of physiological sodium loading (750 ml physiologic saline iv) on renal, hormonal and hemodynamic indexes. The were no differences between both groups in stimulation of plasma levels of human atrial natriuretic peptide (hANP), suppression of plasma renin activity and aldosterone level ($p<0.05$), and diuretic and natriuretic responsiveness. Cr tinine clearance remained unchanged in both groups. Howeve whereas heart rate and blood pressure were decreased in co trols ($p<0.05$), they were raised in patients ($p<0.05$). Thus, there is no impairment of renal responsiveness to mi sodium loading in uncomplicated type 1 diabetes as long as normoglycemia is sustained. The increases in heart rate an blood pressure are best explained by impaired function of vagus nerve. In patients with chronic volume expansion due worse metabolic control or with hypertension, a persistent hANP stimulation might ensue resulting, as we could demons te at least in some patients, in the development of hypore nemic hypoaldosteronism.

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INSULIN THERAPY IN PATIENTS WITH TYPE II DIABETES MELLITUS FAILING ON SULFONYLUREA THERAPY.

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In 26 type II diabetics with failure to diet and sulfonylureas (fasting blood glucose levels, FBG, > 7 mmol/l) the effects of 6 months (mo.) insulin therapy on glycaemic control and plasma-lipids (ultracentrifugation) were studied. Mean age ± SD was 58 ± 11 years, with a median known duration of diabetes of 6.5 (range 1-24) years, and a median body mass index of 24.5 (range 18.9-36.3) kg/m^2. Therapy comprised two daily injections of intermediate-acting insulin. After 6 mo., insulin dose was 40 ± 13 U/day. Body weight increased by 3.7 kg ($p<0.01$). FBG fell from 13.4 ± 2.9 to 8.6 ± 2.5 mmol/l after 3 mo., and to 8.0 ± 2.0 mmol/l after 6 mo. ($p<0.01$). Two-hour postprandial BG dropped from 17.2 ± 4.3 to 11.1 ± 2.8 mmol/l after 3 mo., and to 11.2 ± 2.9 mmol/l after 6 mo. ($p<0.01$). Hba_1 decreased from 12.5 ± 2.1 % to 9.5 ± 1.1 % after 6 mo. ($p<0.01$). The increase in body weight showed a good correlation with the decrease in FBG ($r=0.75$, $p<0.01$) and Hba1 ($r=0.73$, $p<0.01$). Fasting C-peptide levels decreased, and free insulin levels increased ($p<0.05$). C-peptide release after iv. glucagon did not change. Insulin therapy did not affect HDL-C or triglyceride (TG) levels. In contrast, median plasma free fatty acids decreased from 0.80 (range 0.42-2.09) to 0.67 (range 0.30-1.19) mmol/l, the LDL/HDL-C ratio from 3.33 to 3.04, and VLDL-TG levels from 0.90 (range 0.06-2.26) to 0.57 (range 0.02-1.35) mmol/l (all $p<0.05$). Almost all patients reported improved well-being. One patient experienced one hypoglycaemia, requiring medical assistance.
We conclude that insulin therapy in type II diabetics failing on sulfonylureas greatly improves metabolic control with a slight improvement of lipid profile, despite a concomitant gain of weight.

195. Characterisation of the endogenous substrates of the pancreatic islet transglutaminase

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Previous studies have indicated that glucose stimulated rat islets contain a high molecular weight protein polymer, the formation of which may be linked to activation of the Ca^{2+}-dependent islet transglutaminase. This protein polymer is both phosphorylated and membrane linked. A similar high molecular weight polymer is formed when islet homogenates are incubated in the presence of Ca^{2+}. Reducing SDS-agarose gel chromatography indicated this polymer to have a molecular size of ≥ 5 million. A similar molecular size was calculated for the phosphopolymer found in glucose stimulated intact islets. Incorporation of [^{14}C]-methylamine into endogenous islet proteins by the islet transglutaminase indicated the majority of radiolabel to be associated with the high molecular weight polymer which was formed concomittantly. Fractionation studies indicated this polymer to be associated with a plasma membrane rich fraction. A small amount of radiolabel was incorporated into islet proteins of molecular size > 250 K, 73 K-75 K and 44 K-46 K found in the plasma membrane rich fraction and into proteins of > 250 K, 73 K-75 K, 57 K-59 K, 44 K-46 K and 31 K-32 K found in the cytosol. Incubation of individual and combined subcellular fractions in the presence of Ca^{2+} further confirmed that the endogenous substrates of the islet transglutaminase are distributed between both the cytosol and membrane fractions.

196. Tissue specific calciproteins associated to cytoskeletal actin are potential substrates for the insulin receptor tyrosine kinase

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Identification of intracellular phosphotyrosine protein substrates for the insulin receptor tyrosine kinase is an important step in the elucidation of the molecular mechanism of insulin action. In order to identify substrates in relevant insulin target tissues we have investigated the ability of the receptor to phosphorylate members of a new family of Ca^{2+}, phospholipid and actin binding proteins (lipocortins or calpactins) in adipose tissue. Lipocortins have been obtained by solubilisation of cells with Triton X-100 and EGTA and further purified by F-actin affinity chromatography. Adipocytes contain a major phosphorylated actin binding protein of 67 kDalton which appears similar to those previously reported calcimodulins in a number of other tissues: hepatocytes, aortic smooth muscle and leucocytes although absent from human erythrocyte. Another subfamily of proteins has been purified from adipocyte and tentatively identified as 32.5 kDalton (endonexin), 34 kDalton and a tetramer complex of 36 + 10 kDalton (a substrate of $pp60^{src}$ oncogene kinase). Among these the insulin receptor phosphorylates preferentially the 36 kDalton protein. These data indicate a possible functional role of actin associated Ca^{2+} binding proteins in the metabolic and/or growth effects of insulin and further supports a potential role of the cytoskeleton in the molecular mechanism of insulin action.

197. Underhydroxylation of kidney cortex collagen in streptozotocin-diabetic rats. Prevention by dietary ascorbic acid

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Diabetic microangiopathy is characterised by accumulation of type IV collagen in the kidney glomerular basement membranes. We have been interested in the post-translational alterations of kidney collagen metabolism, especially proline hydroxylation, since Schneir et al have reported underhydroxylation of skin collagen in diabetes, and its prevention by ascorbate treatment. After 3 months of diabetes induced by streptozotocin (55 mg. kg i.m. at days 0 and 8 in Wistar male rats (150 ± 10 g), we observed a decreased ratio of 4-Hydroxyproline/Collagen and of 3-Hydroxyproline Collagen (collagen being determined by the Sirius Red colorimetric method): total collagen cortex was increased. On the other hand, collagen: UDP-Glc glucosyltransferase specific activity was not significantly elevated ($p < 0.10$) but Glc-Gal-Hyl glucohydrolase specific activity, measured in dialyzed homogenates, was increased. The underhydroxylation of collagen was reversed by dietary ascorbic acid (20 mg/day) given as curative treatment only during the last 5 weeks before killing. Collagen underhydroxylation may be related to intracellular ascorbic acid deficiency in diabetes, possibly due to increased competition between glucose and ascorbic acid for a common transport system, as described in fibroblasts.

198. Influence of fatty acids on insulin secretion in a rat model of insulin-independent diabetes: possible role in B-cell insensitivity to glucose

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We have investigated a possible role of non-esterified fatty acids (NEFA) for abnormal B-cell secretion in insulin-independent diabetes by studying acute and time-dependent effects of NEFA on insulin secretion. Rats were injected with streptozotocin neonatally (STZ) and studied 6-10 weeks later. Pancreatic insulin content in STZ was 33% of non-diabetic rats (C). In perfused pancreas, 10 mmol/l octanoate released 2.9-fold more insulin in STZ than in C when tested with 3.9 mmol/l glucose (16557 ± 3402 vs 5730 ± 1057 μU/20 min, $p < 0.05$). Perfusion with octanoate did not diminish the insulin response to subsequent stimulation with 27 mmol/l glucose. Short-term lowering of NEFA (by 50%) in vivo by nicotinic acid (100 mg/kg i.p. 30 min prior to experiments) failed to affect glucose-induced insulin secretion. To investigate a long-term influence of NEFA 2 mmol/l octanoate or 0.2 mmol/l palmitate was added together with 27 mmol/l glucose during tissue culture of non-diabetic islets for 60 h. Culture with octanoate or palmitate reduced responsiveness to subsequent stimulation with 27 mmol/l glucose by 64 or 51% respectively, $p < 0.05$. Octanoate during culture did not diminish responsiveness to 3-isobutyl-1-methylxanthine (89.8 ± 15 without and 85.0 ± 10.1 μU · islet^{-1} · 60 min^{-1} with previous octanoate). We conclude: 1) insulin responses to NEFA are abnormal in experimental diabetes, 2) long term rather than short term exposure to a combination of high glucose and NEFA can selectively inhibit glucose-induced insulin secretion.

199. Excessive proinsulin vs insulin release from AtT20 (pituitary) cells transfected with a mutant insulin gene (B10-aspartic acid) suggests diversion of the mutant proinsulin from granules to the constitutive release pathway

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Mutation of B10 histidine to aspartic acid results in familial hyperproinsulinaemia. To understand this, we introduced an identical mutation into the rat insulin II gene and stably transfected AtT20 cells with the mutant or native gene (B10Asp-AtT20 and B10His-AtT20 cells respectively). Cell extracts and media were analysed by HPLC/radioimmunoassay. B10His-AtT20 extracts contained 12% proinsulin, 44% conversion intermediate, 44% insulin, compared with 6%, 50%, 44%, respectively, for B10Asp-AtT20. In the medium, B10His-AtT20 showed 7% proinsulin, 13% intermediate 80% insulin, whereas, strikingly, B10Asp-AtT20 showed 50%, 28% and 22% respectively. Release of all three products from B10His-AtT20 was stimulated by cAMP, but predominantly that of insulin and intermediate, with little proinsulin, from B10Asp-AtT20. Following a pulse-label ([^{3}H]leucine; 20 min) only 27% prelabelled material was released from B10His-AtT20 during a 180 min chase (composition: 44% proinsulin, 19% intermediate, 37% insulin) compared with 52% for B10Asp-AtT20 (composition: 87% proinsulin, 2% intermediate, 11% insulin). These data suggest that most native proinsulin in B10His-AtT20 is channeled to granules for conversion and/or release via the regulated pathway. In B10Asp-AtT20 most, but not all, mutant proinsulin appears to be diverted to the constitutive pathway (rapid, non-regulated release of non-converted prohormone) which would explain hyperproinsulinaemia in carriers of the B10Asp mutation.

200. Gallbladder dynamics in Type 1 (insulin-dependent) diabetic patients with autonomic neuropathy

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Gallbladder dynamics was evaluated in Type 1 (insulin-dependent) diabetic patients without autonomic neuropathy (AN) (7F, 9M; age: 28.2 ± 6.0 years), with AN (4F, 12M; age: 33.8 ± 9.3 years) and 10 normal individuals (3F; 7M, age: 28.7 ± 5.2 years) using a scinti-

cAMP produced a variable (10-70%) stimulation. BP-28 levels were nhibited 50% by 300 nmol/l insulin or 3 μmol/l dexamethasone. In acid extracts of cultured tissue BP-28 levels were less than 10% of he amount secreted in 48 h indicating that released BP-28 was not primarily from a storage pool. These studies suggest that intracellular glucose availability is an important regulator of hepatic BP-28 ynthesis.

290. Potentiation of neurohormone-stimulated insulin secretion in protein kinase C deficient cells

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The precise role of protein kinase C (PKC) in insulin secretion remains unclear. Therefore, we measured insulin secretion, cytosolic Ca^{2+} ($[Ca^{2+}]_i$) and the generation of diacylglycerol, which activates PKC, in RINm5F cells after down-regulation of PKC. After 24-h reatment with 100 nmol/l of phorbolmyristateacetate (PMA) PKC ctivity in membranes and cytosol was no longer detectable. Surprisngly, vasopressin (1 μmol/l) and carbachol (100 μmol/l) elicited nhanced (3- and 2-fold) and more long-lasting insulin secretion rom PMA-treated cells during perifusion relative to control cells. 'asopressin raised $[Ca^{2+}]_i$ (monitored with fura-2) in a biphasic anner. The first phase was prolonged and the $[Ca^{2+}]_i$ oscillations in ie second phase (single-cell recordings) became confluent in PMAeated cells. Diacylglycerol production by vasopressin was more apid and sustained in PKC down-regulated cells. Since Ca^{2+} stimuted insulin secretion from electrically permeabilized cells was not nhanced by PMA treatment, exocytosis does not appear to be sensized to Ca^{2+}. These results suggest that PKC exerts a negative feedack on the generation of the two messengers, Ca^{2+} and diacylglycol. The enhanced insulin secretion in PKC deficient cells points to n inhibitory modulation of the secretory mechanism by PKC. Funcbnal PKC is thus not essential for the initiation of insulin secretion neurohormones.

291. Biosynthesis and metabolism of the two proinsulins, insulins and -peptides in isolated rat and mouse islets

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is not known whether the marked difference in the ratio between sulin I and II in rat islets (2:1) and mouse islets (1:2) is due to difrences in either expression of the insulin genes, proinsulin conver-on rates or intracellular degradation, crinophagy. Therefore, the m of the present study was to evaluate these processes by pulse-ase labelling of isolated rat and mouse islets and quantifying the oinsulins, insulins and C-peptides using RP-HPLC. We found the ne inverse ratio between the newly synthesized proinsulins as for e stored insulin and in both species the conversion rate of proinsu- I was higher than that of proinsulin II. The ratio between the ored C-peptides and insulins was, however, only about 1:3 in rat ets and 1:10 in mouse islets, indicating a pronounced intracellular gradation of the C-peptides. While this process was shown to dend on the culture conditions, the ratio between the two insulins reained unchanged. In conclusion, these results show that the inverse io between the two insulins in rat and mouse islets is neither due species variation in proinsulin conversion nor to differences in inin degradation, but rather to a difference in transcription or transion of the two genes.

292. The importance of transglutaminase in Ca^{2+} induced insulin rese from electropermeabilised islets of Langerhans

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e link between the Ca^{2+} signal, transglutaminase activation and ulin secretion has been investigated in permeabilised islets. The $^{2+}$ induced (10 mmol/l Ca^{2+}) release of insulin from isolated rat ts permeabilised by high voltage electrical discharge may be inited by preincubation of permeabilised islets with the transgluinase inhibitors monodansylcadaverine (25-50 mmol/l), glycine thyl ester (1-5 mmol/l), methylamine (2-10 mmol/l) and cystaie (1-5 mmol/l). The control compound sacrosine methyl ester, ich lacks the primary amino group essential for transglutaminase hibition did not inhibit Ca^{2+} induced insulin release when tested mmol/l. Incubation of permabilised islets with (^{14}C)-methyle led to an increase in the incorporation of this primary amine

substrate into islet proteins in response to an increased tration. This incorporation could be inhibited by cyst mary amine active site directed inhibitor of transglutam fication of the Ca^{2+} induced radiolabelled protein SDS-polyacrylamide gel electrophoresis indicated the molecular weight protein polymers that were formed with methylamine incorporation. These studies using islets further confirm the link between the Ca^{2+}-induc of transglutaminase and the secretion of insulin.

293. Galanin inhibition of insulin secretion: Effects CA^{2+}-efflux from isolated rat islets

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Galanin has been suggested to inhibit insulin secretio linoma cells and B cells from obese mice by a mechan opening of K^+-channels, hyperpolarization and reduc We therefore investigated the effects of galanin on g lated insulin secretion and $^{86}Rb^+$- and $^{45}Ca^{2+}$-efflux fr ase-isolated, overnight cultured, normal rat islets. Whe incubated for 60 min at 8.3 mmol/l glucose, galanin (10 hibited insulin secretion by 98% ($p < 0.001$). From prel galanin at 10^{-7} mol/l did not affect $^{86}Rb^+$-efflux. This at 8.3 mmol/l glucose both in the absence and 1.28 mmol/l Ca^{2+}, and in the presence of tolbutamide (3.3 mmol/l glucose and 1.28 mmol/l Ca^{2+}. Also at 10^{-} anin did not affect $^{86}Rb^+$-efflux. In contrast, diazoxide constantly stimulated $^{86}Rb^+$-efflux markedly under all mental conditions. The effect on $^{45}Ca^{2+}$-efflux of 10^{-7} was then studied by perifusing preloaded islets with glucose. Galanin did not alter the glucose-stimulated neither when added together with glucose, nor at 10 conclusion, galanin inhibits glucose-stimulated insu from cultured rat islets by mechanisms not associated in $^{86}Rb^+$- or $^{45}Ca^{2+}$-efflux from prelabelled islets. This galanin-induced inhibition of insulin secretion does no jor actions on the K^+- or Ca^{2+}-channels in normal isle

294. B cell alkalinisation augments the stimulation of dif secretagogues

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Glucose and pH 8.0 both alkalinize B cells and induce controversy exists as to the role of intracellular pH in in We now show that at 0 mmol/l glucose, mouse islets (released 0.10 ± 0.04 ng insulin/μg dry weight × 60 min 0.51 ± 0.18 ng/μg at pH 8.0. With 20.9 mmol/l K^+, in was 0.24 ± 0.05 (pH 7.4) and 0.76 ± 0.20 ng/μg (p 1 μmol/l glibenclamide, the release was 0.61 ± 0.16 0.24 ng·μg at pH 7.4 and 8.0, respectively. $N = 8$ in all experiments, $p < 0.05$. With 20 mmol/l leucine, secret higher at pH 8.0. In islets perifused with 3 mmol/l glu release increased from 0.004 ± 0.001 to 0.108 ± 0.078 30 min after switching from pH 7.4 to pH 7.9. At 8 mm insulin increased from 0.012 ± 0.002 to 0.121 ± 0.050 Increased pH had no effect at 20 mmol/l glucose. At 1 cose and pH 7.4, K^+ (20.9 mmol/l) induced an initial (fivefold increase in insulin outflow. At pH 7.9, K^+-inc release was not transient but continued to rise for 30 mi All effects were reversible. In conclusion, alkalinisati the effect of different secretogogues but not the maxim nutrient stimuli. Findings with K^+ point to an effect channel activity.

295. Glucose and insulin-like growth factors influence th vival of pancreatic B cells

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It is still unknown whether physiological factors influe time of pancreatic B cells. This question was examined rified rat B cells that were cultured in media of differ tion. More than 80% of the cells survived a 10-da 10 mmol/l glucose. At lower glucose concentrations, a dent decrease in the percentage living cells was obse 8 mmol/l, 54% at 6 mmol/l, 15% at 3 mmol/l). This