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# ADENYLATE CYCLASE IN THE HUMAN DUODENUM

by

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## ABSTRACT

### ADENYLATE CYCLASE IN THE HUMAN DUODENUM

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The studies undertaken in this work take advantage of a source of human duodenal tissue, in the form of biopsies, from patients attending the endoscopy clinic at the City Hospital, Nottingham. The work utilises two different biopsy preparations, a particulate membrane preparation and isolated mucosal epithelial cells. The regulation of adenylate cyclase was examined with respect to the effects of calcium, calmodulin and EGTA in membrane preparations and the regulation of VIP stimulated cAMP synthesis was studied in cells.

The concentration dependent effects of $Ca^{2+}$ on adenylate cyclase activity in particulate membranes were measured. Adenylate cyclase activity was inhibited by $Ca^{2+}$ concentrations greater than 90nM. There was no stimulatory phase indicative of calmodulin stimulated adenylate cyclase activity. Addition of calmodulin to EGTA washed membranes did not stimulate enzyme activity. Inhibition of adenylate cyclase activity by the calmodulin antagonist TFP and IODO 8 did not inhibit enzyme activity at concentrations specific for calmodulin antagonism. The $Ca^{2+}$ chelator EGTA increased the enzyme's sensitivity to $Ca^{2+}$. In the presence of 1μM free $Ca^{2+}$, EGTA inhibited adenylate cyclase activity irrespective of stimulus. In the absence of added $Ca^{2+}$, EGTA inhibited NaF stimulated enzyme activity but stimulated basal, forskolin and GMP-PNP stimulated enzyme activity. These data suggested EGTA removes membrane bound $Ca^{2+}$ associated with adenylate cyclase activity which was not associated with calmodulin.

Greater than 80% of isolated epithelial cells were viable based on trypan blue exclusion. VIP stimulated cAMP production in isolated epithelial cells ($EC_{50}$=1.78pmol). Maximum stimulation was elicited by 100nM VIP. VIP stimulated cAMP production was not inhibited by TFP or IODO 8 at concentrations as high as 100μM.

The results from both approaches suggest that human duodenal adenylate cyclase is not calmodulin dependent and the physiological significance of $Ca^{2+}$ inhibition of adenylate cyclase is unclear in this tissue.

*TO MY FATHER*

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**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 presented here was carried out by the candidate. Due acknowledgement has been made of all assistance received.

Signed J. A. Smith

(Candidate)

Signed R G Long

(Director of Studies)

## PUBLICATIONS

Smith JA, Griffin M, Long RG (1989) Calmodulin dependence of human duodenal adenylate cyclase. Eur J Clin Invest *19:* 221

Smith JA, Griffin M, Long RG (1989) Isolation of human duodenal enterocytes; Effect of vasoactive intestinal peptide. Eur J Clin Invest *19:* 221

Smith JA, Griffin M, Mireylees SE, Long RG (1990) Preparation of isolated enterocytes from human duodenal biopsies and their response to vasoactive intestinal peptide (VIP). Gut *31:* A610

Smith JA, Griffin M, Mireylees SE, Long RG (1990) Effects of vasoactive intestinal peptide on cyclic adenosine monophosphate production in enterocytes isolated from human duodenal biopsy specimens. Gut *31:* 1350-1354

Smith JA, Griffin M, Mireylees SE, Long RG (1991) Vasoactive intestinal peptide (VIP) stimulated cyclic AMP production in epithelial cells isolated from human duodenal biopsy specimens is not calmodulin dependent. Clinical Science *81: (supplement 25)* 34P

Smith JA, Griffin M, Mireylees SE, Long RG (1991) Calmodulin independence of human duodenal adenylate cyclase. Gut (in press)

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## ABBREVIATIONS

Adenylate cyclase subunits

| | |
|---|---|
| $R_s$ | Receptors that stimulate adenylate cyclase |
| $R_i$ | Receptors that inhibit adenylate cyclase |
| G protein | Heterotrimeric guanine nucleotide binding protein - $\alpha\beta\gamma$ |
| $\alpha_s$ | Stimulatory $\alpha$ subunit of G proteins |
| $\alpha_i$ | Inhibitory $\alpha$ subunit of G proteins |
| $\beta\gamma$ | G protein subunit |
| C | Catalytic Unit of adenylate cyclase |
| ATP | Adenosine triphosphate |
| [$\alpha$-$^{32}$P]ATP | adenosine 5'-[$\alpha$-$^{32}$P] triphosphate |
| ADP | Adenosine diphosphate |
| BSA | Bovine serum albumin |
| CaM | Calmodulin |
| cAMP | Adenosine 3', 5'- cyclic monophosphate |
| [$^3$H] cAMP | [8-$^3$H]adenosine 3', 5'- cyclic monophosphate |
| cpm | Counts per minute |
| DNA | Deoxyribose nucleic acid |
| DMSO | Dimethylsulphoxide |
| DABA | 3' 5' diaminobenzoic acid |
| EDTA | Ethylenediaminetetra-acetic acid |
| EGTA | Ethyleneglycol bis(b-aminethylether) N,N'-tetra-acetic acid |
| Fura-2 | 1 - (2(5 - carboxyoxozol - 2 - yl) - 6 - ammobenzo - Furan - 5 - oxy) - 2 - (2'- amino - 5'- methylphenoxyl) - ethane - N,N,N',N'- tetra - acetic acid |
| GDP | Guanosine diphosphate |

| | |
|---|---|
| GIP | Gastric inhibitory peptide |
| GMP-PNP | Guanylyl-imidodiphosphate |
| Gpp(NH)p | Guanylyl-imidodiphosphate |
| GTPγS | Guanosine-5'-O-(3'thiophosphate) |
| GRF | Growth hormone-releasing factor |
| GTP | Guanosine triphosphate |
| HBH | HEPES-bicarbonate buffered Hanks balanced salt solution |
| HEPES | N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid |
| $IC_{50}$ | The concentration at which an agent induces 50% inhibition of enzyme activity |
| IODO 8 | N - (8-aminohexyl) - 5 - IODO - 1 - naphthalene - sulphonamide |
| $IP_3$ | Inositol 1, 4, 5, trisphosphate |
| $I_{sc}$ | Short circuit current |
| MEH | $Mg^{2+}$/EGTA/HEPES buffer |
| MH | $Mg^{2+}$/HEPES buffer |
| NAD | Nicotinamide adenine dinucleotide |
| NSB | Non-specific binding |
| PBB | Phosphodiesterase boiled blanks |
| PDE | phosphodiesterase |
| PHI | Peptide histidine isoleucine amide |
| PHM | Peptide histidine methionine amide |
| SDS | Na-lauryl(dodecyl)sulphate |
| TCA | Trichloroacetic acid |
| TFP | Trifluoprazine |
| Tris | 2 - amino - 2 - (hydroxymethyl)propane - 1, 3 diol (tris) |
| VIP | Vasoactive intestinal peptide |

# CHAPTER 1

# INTRODUCTION

## 1.1 GENERAL INTRODUCTION

Cyclic adenosine 3' 5' monophosphate (cAMP) is formed from ATP by adenylate cyclase. Adenylate cyclase is a complex membrane bound multicomponent enzyme. Its constituent units include receptors, guanine nucleotide regulatory proteins and a catalytic unit; each part represents many potential sites of regulation.

In the intestine, adenylate cyclase is highly responsive to vasoactive intestinal peptide (VIP). Stimulation of adenylate cyclase by VIP, and the subsequent increase in intracellular cAMP in the intestinal epithelial cell, results in an increase in fluid and electrolyte secretion into the gut lumen. The clinical consequence is secretory diarrhoea.

VIP regulates adenylate cyclase activity via specific receptors on intestinal epithelial cells. Modulation of adenylate cyclase activity by other agents can occur by direct activation (or inhibition) of the enzyme. $Ca^{2+}$ and calmodulin are two such agents shown to regulate the enzyme in other tissues. Calmodulin may play a pivotal role in the actions of a number of $Ca^{2+}$ and cyclic nucleotide dependent intestinal secretagogues, including VIP.

It is the involvement of $Ca^{2+}$ and calmodulin in the regulation of adenylate cyclase in the human duodenal mucosa that is the main topic of this thesis. This work takes advantage of duodenal biopsy as a source of human intestinal material. Two different preparations have been developed, a particulate membrane preparation of whole biopsies and an isolated epithelial cell preparation. In the membrane preparation the direct effects of $Ca^{2+}$ and calmodulin were measured on adenylate cyclase activity. In the isolated epithelial cell preparation VIP stimulated cAMP accumulation was studied. Evidence is

presented, from both preparations, which support the hypothesis that human duodenal mucosal adenylate cyclase activity is calmodulin-insensitive. The involvement of $Ca^{2+}$ in the direct regulation of adenylate cyclase activity has also been explored together with the influences of the divalent cation chelator, EGTA.

By way of introduction the characteristics of the tissue under study and the different mechanism of adenylate cyclase regulation will be reviewed.

## 1.2 THE STRUCTURAL ORGANIZATION OF THE INTESTINAL MUCOSA

The epithelium of the small intestine is a highly differentiated structure. The constituent cells of the epithelium possess complex morphological specializations that facilitate diverse functions (Nelson 1989). For example, not only do the products of digestion gain access to the mucosal blood and lymph vessels by traversing the intestinal epithelium but also many of the secretions delivered to the intestinal lumen are synthesized and assembled within intestinal epithelial cells.

The luminal surface is organized to increase the available surface area. At the macrostructural level there are 1cm high and 5cm long folds. These folds are most numerous in the distal duodenum and proximal jejunum. The increase in surface area is also reflected in the microstructural organization of the intestinal mucosa. Finger like processes called villi increase the surface area of the intestine 7 to 14 fold. In the distal duodenum and proximal jejunum the villi may be leaf or finger shaped and range in height from 0.5 to 0.8mm. In the ileum the villi are finger shaped and rarely exceed 0.5mm in height. The shape and height of villi is influenced by diet, lactation and several disease states (Madara and Trier 1986), (**Figure 1**).

The epithelial cells line the villi and crypts of the luminal surface and sit on a thin basement membrane which separates them from the underlying lamina propria. Epithelial cells in the crypts at the base of the villi are proliferative and undifferentiated. They migrate the length of the villi differentiating into the various constituent epithelial cell types. Once at the tip of the villi they are sloughed into the lumen by

a process called desquamation. It takes approximately 4 to 5 days for a cell to complete it's migration from crypt to villus tip (Castro 1981; Haffen et al. 1986).

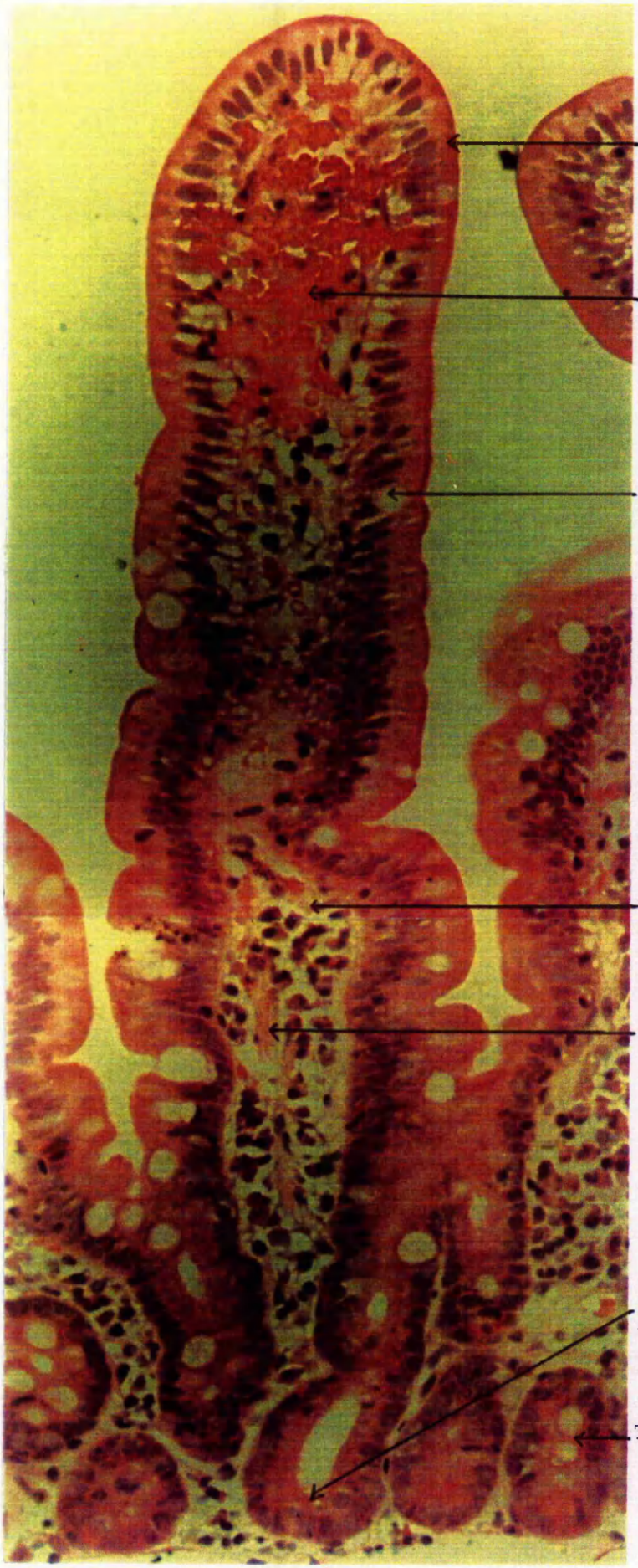
The main cell types of the intestinal epithelium include, in the crypt:- undifferentiated cells, mucus secreting goblet cells, endocrine cells, tuft and Paneth cells. The villus cells include:- the predominant mature absorptive cell type termed enterocytes, mucus secreting goblet cells, a few endocrine cells and tuft and cup cells (Madara and Trier 1986; Neutra and Louvard 1989; Dobbins 1990).

Both endocrine and exocrine functions have been recognized in the cells of the intestinal epithelium. There is exocrine secretion of peptides, mucus, water and electrolytes into the gut lumen and endocrine secretion of regulatory peptides, prostaglandins, neurotransmitters, water and electrolytes into the lamina propria (Castro 1981; Sernka and Jacobson 1983; Noren et al. 1986; Madara and Trier 1986).

One of the main characteristics of transporting epithelia is the high degree of cellular structural and functional polarity (Caplan and Matlin 1989). The plasma membrane of the apical pole (luminal side) of all epithelial cells is organized into microvilli (brush border) which in the intestine has been estimated to further increase the surface area 25 fold. The lateral surface has several points of cell to cell contact, called tight junctions (zonula occludens), that maintain and regulate paracellular permeability to nutrients, water and electrolytes. Tight junctions serve to separate the brush border membrane from the basolateral membrane (Louvard et al. 1986).

**Figure 1 A Duodenal Villus**

A photomicrograph of a single villus and several crypts from a human duodenal biopsy stained with haematoxylin and eosin. Magnification x400.

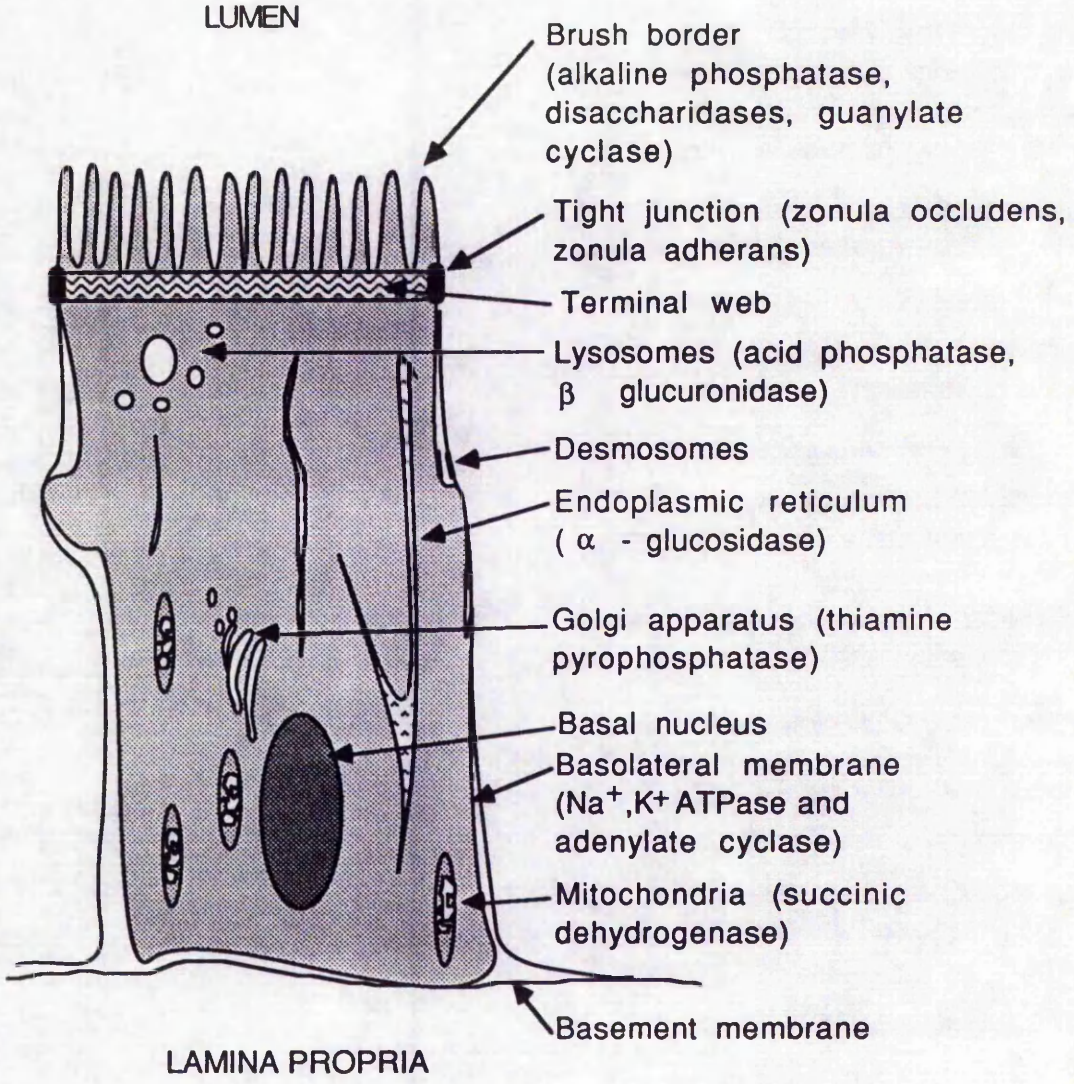

Mature enterocytes with brush border and basally located nuclei
Erythrocytes from biopsy haemorrhage artefact
Mucus containing goblet cell
Lamina propria containing plasma cells and occasional lymphocytes
Lacteal
Base of crypt with proliferative cells
Transverse crypt

The epithelial cell plasma membrane not only has distinct areas of structural organization but also areas of very different biochemical composition that reflect specialized functions. For example, $Na^+,K^+$-ATPase and adenylate cyclase are localized to the basolateral membrane, emphasizing the importance of this membrane in intestinal water and electrolyte transport. Similarly enzymes and proteins important to nutrient degradation and absorption are localized to the brush border membrane (peptidases, disaccharidases, alkaline phosphatase and guanylate cyclase (Noren et al. 1986), (**Figure 2**).

The subcellular distribution of adenylate cyclase, suggested by immunohistochemical localization of cAMP, was predominantly to the basolateral membrane (Ong 1975). More direct evidence came from measurement of adenylate cyclase activity in preparations where enterocyte brush border and basolateral membranes had been separated and the enzyme activity found to be confined to the basolateral membrane fraction (Murer et al. 1976, 1977; Walling et al. 1978). Recently, however, Rambotti et al (1987) concluded that adenylate cyclase and guanylate cyclase were not confined to the different membrane domains. This was based on the distribution of precipitated imidodiphophate enzymic cleavage products of adenylyl and guanylyl imidophosphate.

**Figure 2 Enterocyte polarity**

A diagram of a single enterocyte demonstrating structural and biochemical polarity. Junctional complexes (i.e. zonula occludens, zonula adherens and desmosomes) form and maintain attachment points between adjacent cells. Cells are also attached to the basal lamina through specific receptors and junctions. Transporting epithelia develop and maintain concentration gradients of ions and solutes between the fluid compartments they separate. This requires the polarized distribution of membrane proteins which are synthesized on the rough endoplasmic reticulum and transported through the Golgi apparatus and then targeted to the apical or basolateral domains of the plasma membrane. Proteins are constrained from between the domains by the tight junction (Nelson 1989).


LUMEN
Brush border (alkaline phosphatase, disaccharidases, guanylate cyclase)
Tight junction (zonula occludens, zonula adherans)
Terminal web
Lysosomes (acid phosphatase, β glucuronidase)
Desmosomes
Endoplasmic reticulum ( α - glucosidase)
Golgi apparatus (thiamine pyrophosphatase)
Basal nucleus
Basolateral membrane ($Na^+$,$K^+$ ATPase and adenylate cyclase)
Mitochondria (succinic dehydrogenase)
Basement membrane
LAMINA PROPRIA

## 1.3 SECOND MESSENGERS IN THE INTESTINAL MUCOSA

The regulation of fluid and electrolyte flux by cAMP is the paradigm of second messenger function in the small intestine. The involvement of second messengers in the regulation of nutrient absorption has rarely been documented. One possible reason for this is that the clinical outcome of abnormal fluid and electrolyte secretion (diarrhoea) results in the death of 4 million children under the age of 5 each year (WHO 1989; O'Loughlin and Gall 1989a). It was not until the early 1970's that a connection was made between elevated cAMP levels and secretory diarrhoea in patients with Asian cholera (Field 1974); Kimberg 1974). The activation of adenylate cyclase by cholera toxin leads to an increase in the cAMP concentration of the intestinal mucosa (Chen et al. 1971; Field 1974).

Another classical pathological example of stimulated adenylate cyclase and elevated cAMP induced diarrhoea is pancreatic cholera. So called because the changes in fluid and electrolyte secretion are indistinguishable from those produced by the cholera enterotoxin and they are usually associated with an islet adenoma of the pancreas (Rambaud et al. 1975; Gaginella et al. 1979; Bloom et al. 1973). That ganglioneuroblastomas could also produce similar symptoms was subsequently established (Long et al. 1981; Bloom et al. 1988). Hormones and neurotransmitters are released from these tumours into the circulation in large amounts (Bloom et al. 1973 and 1988; Long et al. 1981 and 1982; Fahrenkrug et al. 1986). In contrast to cholera enterotoxin, which is thought to increase adenylate cyclase through direct G protein activation, these peptides influence intestinal electrolyte transport processes through receptor mediated

mechanisms, (see section 1.4.2) (Christophe et al. 1986). Vasoactive intestinal peptide (VIP) and peptide histidine methionine (PHM) are neurotransmitters, that have been identified as secretogues released from such tumours (Kane et al. 1983; Fahrenkrug et al. 1986; Magistretti 1990). Whether these peptides exert their effects directly through receptors on the intestinal mucosal cells or via a intramural secretory nerve reflex has yet to be established (Laburthe et al. 1979; Field et al. 1983; Robichon et al. 1987; Eklund et al. 1986; Sjöqvist 1988; Smith et al. 1990).

Cooke (1989) has suggested that intestinal secretion induced by bacterial toxins or other substances may play an important role in flushing the lumen of the invading organisms or noxious substances. This has intriguing implications, rather than intestinal secretion being an unfortunate pathological consequence, it may be a defence strategy where the intestinal mucosa has evolved mechanisms which utilise the by-products of invading organisms. This is an idea supported by evidence suggesting tissue specificity for cholera toxin. In contrast to the findings with the jejunum, cholera toxin has no significant effect on fluid volume or adenylate cyclase activity in colonic segments (Simon et al. 1981).

In the intestinal epithelial cell, intracellular increase in cAMP is considered a key occurrence in a series of poorly understood events which culminate in elevated secretion of chloride, bicarbonate and fluid and in the inhibition of sodium absorption, (Wolosin et al. 1989; Farack et al. 1988). The pathological consequence of which is that the gut (or at least some portion of it) is changed from an organ of net absorption to one of net secretion. That this secretion results from an increase in the $Cl^-$ permeability of the mucosal brush border has

been demonstrated (Field 1974; Kimberg 1974; Ilundain and Naftalin 1979; Binder 1984; Steward and Case 1989; Thillainayagam and Farthing 1990).

In the duodenal villus cells there is a bicarbonate transport mechanism independent of luminal $Cl^-$ that is under the control of cAMP. Norepinephrine, VIP and cholinergic agonists alter bicarbonate secretion but it is unknown whether these effects are mediated by direct action on the epithelial cells or on their innervation (Cooke 1989).

In the normal gut both secretion and absorption occur simultaneously with absorption predominating. These two processes are thought to be confined to two different cell populations along the villus, i.e. absorption predominates in the mature villus tip cells and secretion in the crypt cells (Donowitz 1984a; Krejs 1987; Hubel 1989). Concurrent with this is the crypt-villus distribution of adenylate cyclase. The highest adenylate cyclase activity has been reported in the crypt cells (Long et al. 1986). Paradoxically the development of VIP receptors, that stimulate adenylate cyclase activity, has been found to increase as the cells develop and migrate to the villus tip (Weiser 1973; Laburthe et al. 1987). There is further evidence to support the idea of VIP receptors on mature villus tip cells. Only mature cells and not crypt cells, where secretion is believed to occur, respond to VIP stimulation with increasing cAMP production (Krejs 1987). It is possible that cells, which under normal circumstance are absorptive, become secretory given the appropriate stimulatory physiological conditions or disease states, (Turnberg 1981, 1991).

The model of fluid and electrolyte regulation, under non-stimulated conditions, in the enterocyte is depicted in **Figure 3**. How this system is modified by diseases which activate adenylate cyclase is compared in **Figure 4**.

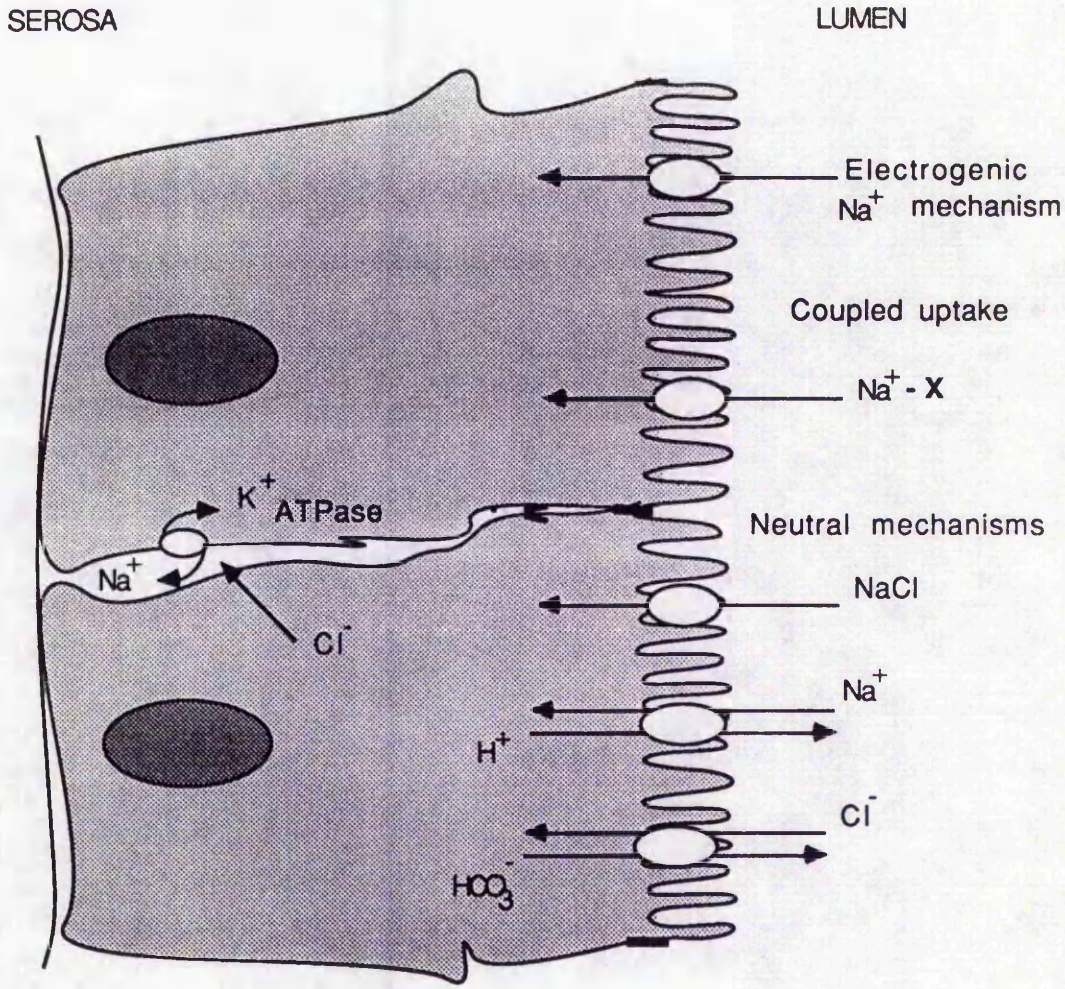
**Figure 3 Fluid and electrolyte regulation**

Electrogenic, neutral and coupled mechanisms are involved in the active absorption of $Na^+$ together with the passive uptake because of solvent drag (solute movement secondary to water flow).

$Na^+$,$K^+$-ATPase on the basolateral membrane of enterocytes generates and maintains an electrochemical gradient. The intracellular environment is negative relative to the lumen and $Na^+$ moves passively down the electrochemical gradient.

With the neutral mechanism there is no net movement of charge, $Na^+$ is transported with a cation or in exchange for another anion. (e.g. $Na^+$ $Cl^-$ linked transport or one for one $Na^+/H^+$ exchange or $Cl^-/HCO_3^-$ exchange). It is the neutral sodium chloride linked absorption that is inhibited by increases in the intracellular concentration of cAMP and free calcium.

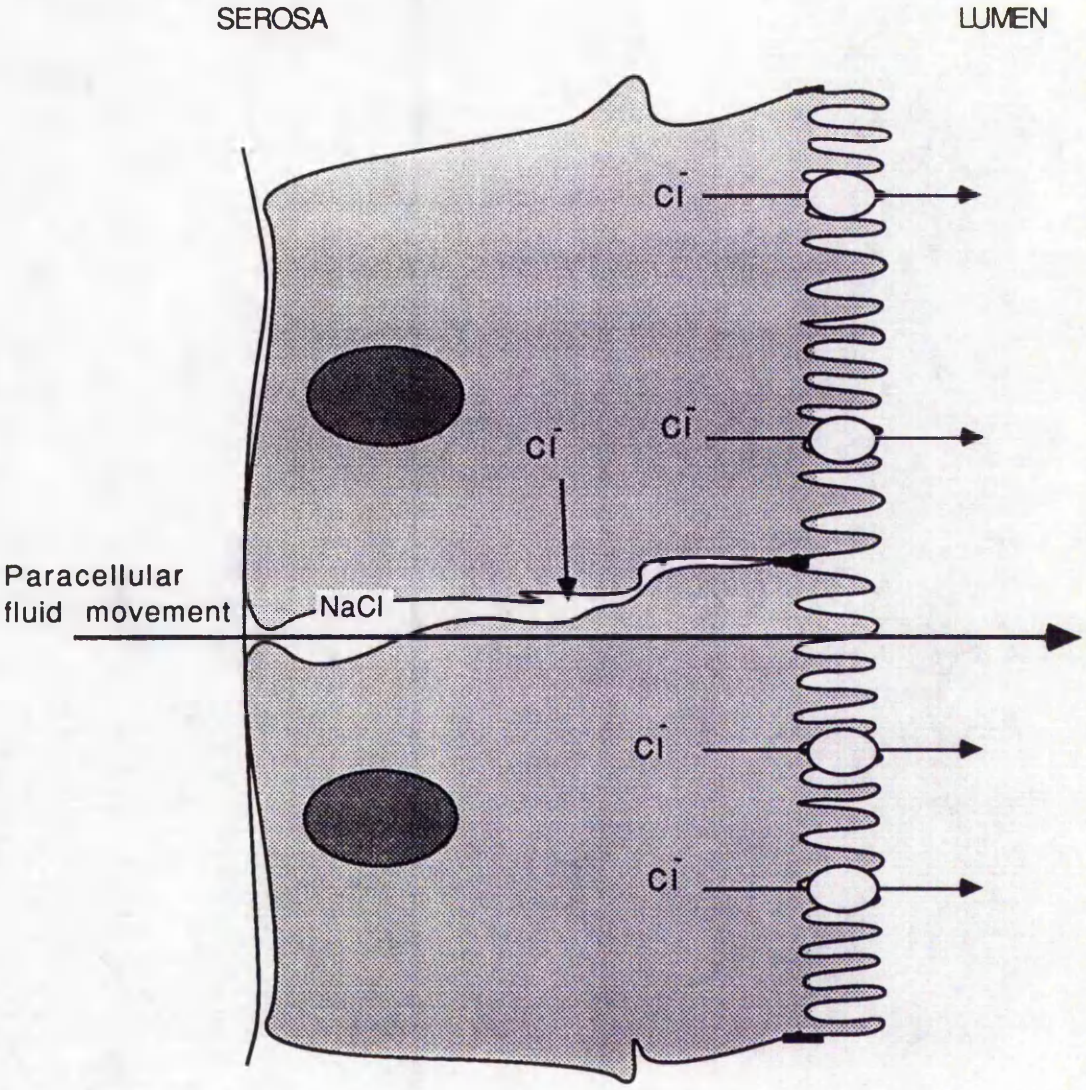
With the coupled mechanism $Na^+$ is absorbed via carrier proteins in the luminal brush border in combination with the end products of digestion (e.g D - glucose, D - galactose, L amino acids, dipeptides, tripeptides and nucleosides) (Binder 1984; Thillainayagam and Farthing 1990; Alpers et al. 1990).




- X = D-glucose, D-galactose, L-amino acids

**Figure 4 Mechanism of intestinal secretion**

Under normal conditions $Cl^-$ secretion is slow because the luminal brush border is relatively impermeable to $Cl^-$. This ensures that $Cl^-$ taken up by the cell at the luminal brush border, by the secondary active processes described in **Figure 3**, does not leak passively back into the lumen. Brush border membrane permeability changes induced by elevated cAMP or free calcium, allowing $Cl^-$ to move out of the cell down its electrochemical gradient into the lumen. NaCl is maintained at hypertonic concentrations in the lateral intercellular spaces of the intestinal mucosa by the activity of the $Na^+,K^+$ ATPase. $Cl^-$ then leaves the cell down the electrochemical gradient. Passive leakage of NaCl from the lateral intercellular spaces into the mucosal bathing solution follows $Cl^-$ secretion. The resultant osmotic pressure exerted across the tight junction results in paracellular fluid movement in the intestine (Sernka et al. 1983; Bakker et al. 1984; O'Loughlin and Gall 1989a,b). The permeability of tight junctions has also has been shown to be influenced by cAMP and free calcium (Madara et al. 1984).


SEROSA
LUMEN
Cl⁻
Cl⁻
Cl⁻
Paracellular
fluid movement
NaCl
Cl⁻
Cl⁻

Mechanisms, distinct from adenylate cyclase and cAMP production, which operate via G proteins have recently been shown to influence intestinal transport, e.g cGMP, the inositol phospholipid metabolites diacylglycerol and $IP_3$ and the involvement of $Ca^{2+}$ has long been recognised.

Stimulation of brush border guanylate cyclase by heat stable bacterial enterotoxin increases cGMP concentrations which in turn increases fluid and electrolyte secretion via mechanisms similar to those of cAMP (de Jonge 1984; O'Loughlin and Gall 1989b).

Elevation of intracellular $Ca^{2+}$ has two recognised modes of action in intestinal epithelial cells. Firstly activation of protein kinase C by $Ca^{2+}$ released from endoplasmic reticulum leads to protein kinase C becoming incorporated into the brush border membrane and a reduction in neutral NaCl absorption (Fondacardo et al. 1985; Yeo et al. 1989; Rood and Donowitz 1990). Secondly $Ca^{2+}$ has long been implicated in regulation of intestinal secretion via the activation of calmodulin. $Ca^{2+}$-calmodulin is thought to have a direct effect upon the intestinal cell brush border membrane $Cl^-$ conductance and to inhibit neutral NaCl absorption possibly by direct inhibition of the brush border $Na^+/H^+$ exchanger (Ilundain and Naftalin 1979; Powell 1984; Donowitz 1984; Gilman 1989; Rood and Donowitz 1990). Further discussion on $Ca^{2+}$ and calmodulin is given in section 1.5.

The brush border therefore contains several different types of protein kinase (i.e. cAMP, cGMP and $Ca^{2+}$ Calmodulin dependent protein kinases and protein kinase C) whose functions seem to converge onto a common regulatory pathway.

There is therefore considerable redundancy in this system because increase in either cAMP, cGMP or free $Ca^{2+}$ result in the same physiological response (Rasmussen 1982) i.e. an increase in brush border membrane permeability to $Cl^-$ and a decrease in the neutrogenic absorption of $Na^+$ and $Cl^-$. The apparent redundant control of second messenger interactions makes it very difficult to organize and decipher experiments involving intestinal tissue (Hundain and Naftalin 1979; McCabe et al. 1985) because there seems to be a common final pathway leading to activation of specific protein kinases and the phosphorylation of specific brush border, basolateral membrane proteins and cytosolic proteins (Shlatz et al. 1978, 1979; Sharp et al. 1984). This in turn induces conformational changes in ion channels or carriers or specific regulatory proteins that lead to a modification of ion transport (de Jonge 1984; O'Loughlin and Gall 1989a, b; Petersen and Gallacher 1989; Thillainayagam and Farthing 1990).

Simultaneous stimulation of both the cAMP and $Ca^{2+}$ systems however, may result in a potentiation of the cellular response. When $T_{84}$ cells (a cell line derived from a human colonic carcinoma) were stimulated with a $Ca^{2+}$-acting effector (eg ionomycin) together with VIP or $PGE_1$ the observed rise in short circuit current ($I_{sc}$) was greater than the predicted sum of the two effectors acting individually (McCabe et al. 1989; McRoberts and Barrett 1989). Another example of cAMP and $Ca^{2+}$ augmenting an intestinal cellular response was demonstrated in chief cell pepsinogen production when the two systems were simultaneously stimulated with VIP and cholecystokinin-8 (Ballantyne et al. 1986).

The actions of adenosine indicate that there may be a fourth

mechanism controlling $Cl^-$ permeability in enterocytes. Adenosine can stimulate $Cl^-$ secretion at concentrations that do not cause detectable increases in cAMP, cGMP or intracellular free $Ca^+$ (McRoberts and Barrett 1989).

In other cell types the different second messenger systems interact to produce co-ordinated, hierarchical, antagonistic and sequential control of cellular events (Rasmussen 1982 and 1986). These, together with the redundant control thought to exist in intestinal cells, have been summarised and compared in **Figure 5.**

Other gastrointestinal functions that have been reported to be influenced by elevated cAMP are listed in **Table 1**

**Figure 5 Synarchic control of second messengers**

A summary of the synarchic control between the $Ca^{2+}$ and cAMP signal transduction mechanisms proposed by (Rasmussen 1981).

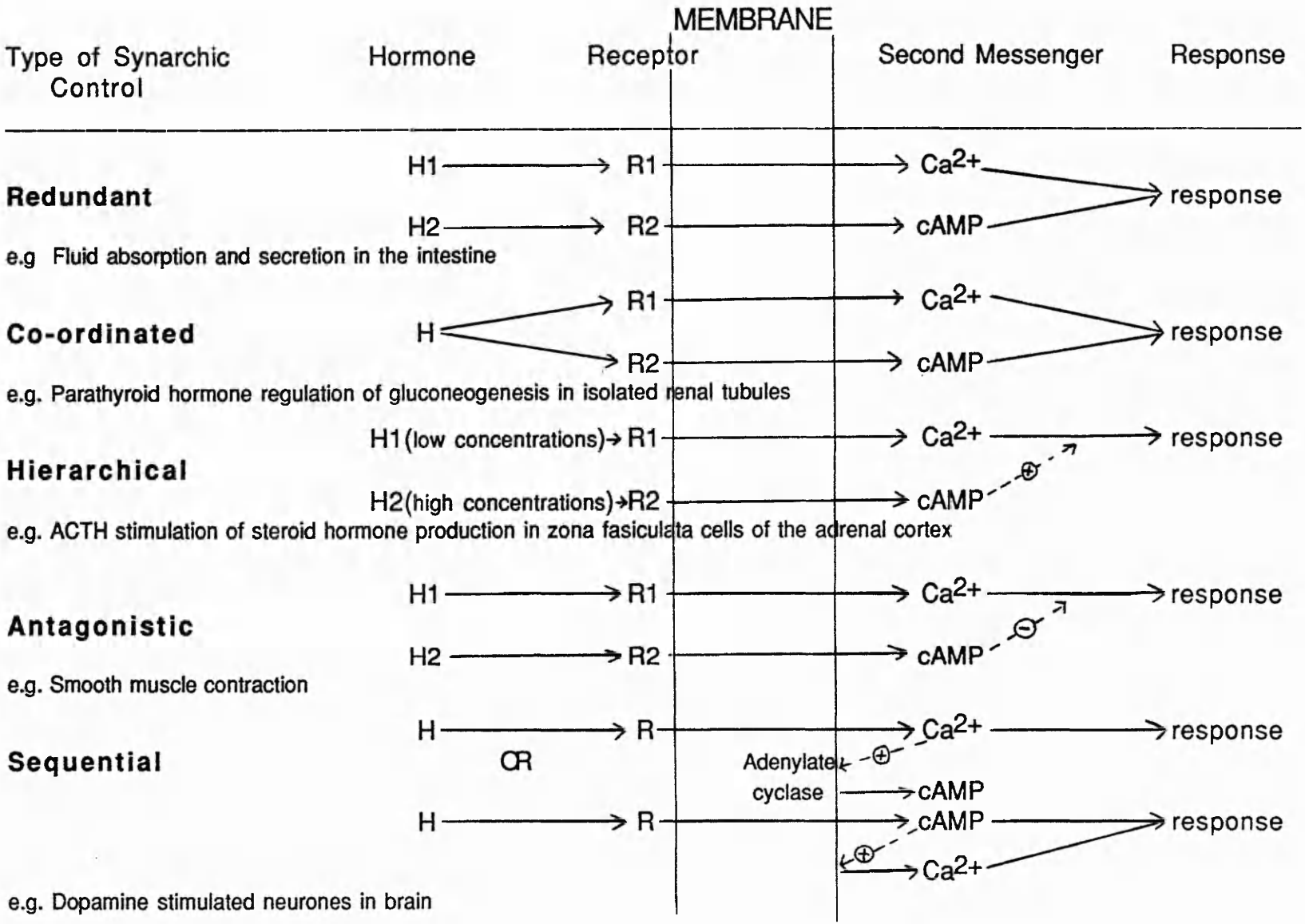

MEMBRANE
Type of Synarchic Control
Hormone
Receptor
Second Messenger
Response
Redundant
H1
R1
Ca2+
H2
R2
cAMP
response
e.g Fluid absorption and secretion in the intestine
Co-ordinated
H
R1
R2
Ca2+
cAMP
response
e.g. Parathyroid hormone regulation of gluconeogenesis in isolated renal tubules
Hierarchical
H1 (low concentrations)
H2 (high concentrations)
R1
R2
Ca2+
cAMP
response
e.g. ACTH stimulation of steroid hormone production in zona fasiculata cells of the adrenal cortex
Antagonistic
H1
H2
R1
R2
Ca2+
cAMP
response
e.g. Smooth muscle contraction
Sequential
H
R
OR
Adenylate cyclase
Ca2+
cAMP
response
e.g. Dopamine stimulated neurones in brain

**Table 1 Cyclic AMP functions in the gastrointestinal tract**

| Physiological Response | Species and Tissue Preparation | Reference |
|---|---|---|
| Mucosal blood flow | Anaesthetized rat | Whittle (1972) |
| Gastric acid secretion | Parietal cells | Thompson (1977) |
| Pepsinogen secretion | Chief cells | Ballantyne (1986) |
| Inhibition of glucose oxidation | Isolated rat enterocytes | Vidal (1988) |
| Mucus secretion | Human cell line CL.16E | Laburthe (1989) |

## 1.4 ADENYLATE CYCLASE

### 1.4.1 Historical Perspective

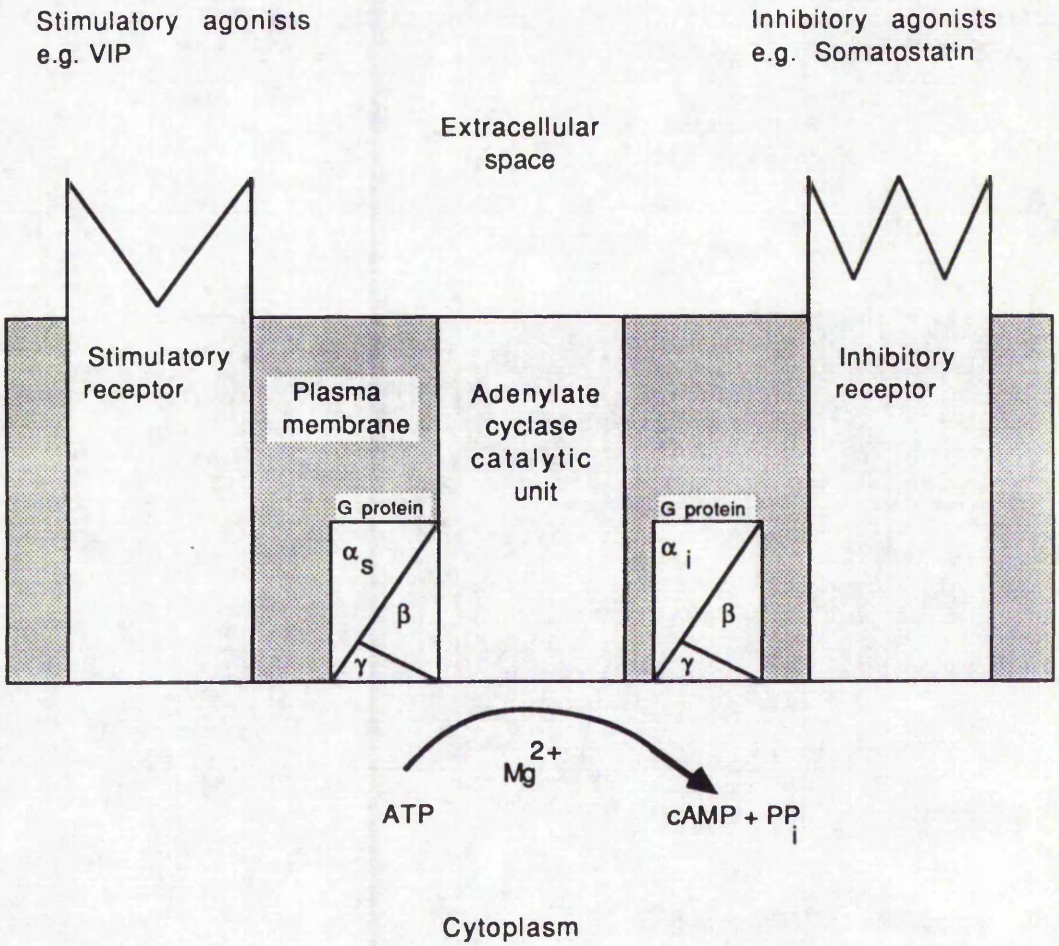
Thirty years ago speculation about the structure of the adenylate cyclase system consisted of two hypotheses (Perkins 1973). A two component model was suggested by (Robison, Butcher and Sutherland 1967). The model assumed the existence of only two plasma membrane subunits. A receptor on the cell surface was proposed to interact directly with a catalytic unit on the inner cytoplasmic cell surface when bound by hormone. The second model was suggested by Hechter and Halkerston 1964; Hechter 1965. It differed in that they proposed a three component system consisting of intermediate moieties between the hormone receptor and the catalytic unit within the plasma membrane. It is this model that forms the basis of current dogma. The intermediate moieties have subsequently been identified as the guanine nucleotide binding proteins (G proteins) considered to be responsible for signal transduction across the plasma membrane of a cell (Northup 1980; Sternweis 1981). G proteins have been the focus of intense research for the past ten years and the adenylate cyclase system has been found to be far more complicated than first imagined. This work revealed hormone sensitive adenylate cyclase to consist of five components; stimulatory and inhibitory hormone receptors designated $R_s$ and $R_i$, stimulatory and inhibitory G proteins designated $G_s$ and $G_i$ and the catalytic component, C (Levitzki 1990).

The understanding of the proposed mechanism by which occupied hormone receptors stimulate cAMP formation requires an

explanation of both the structural and functional properties of the adenylate cyclase system. In the following section each of the adenylate cyclase components and how they interact, will be discussed. Where possible examples will be drawn from what is known about the system in the gastrointestinal tract.

**Figure 6** summarsies the components of the adenylate cyclase system and their proposed interactions.

**Figure 6 Schematic diagram of the adenylate cyclase system**



### 1.4.2 Receptors

By necessity this discussion will be confined to receptors on intestinal epithelial cells for which there is evidence and with particular attention to VIP receptors.

Direct evidence of specific intestinal epithelial cell receptors based on binding studies exists for very few receptor types (VIP, PHI or PHM and $PGE_2$, somatostatin, acetylcholine and noradrenaline) and details for human intestinal epithelial cell receptors are even more scarce (Cooke 1986; Laburthe 1986 1989; Smith 1987). However, based on the influence of neurotransmitters on intestinal secretion and/or the secretion of substances from intestinal endocrine cells, receptors have been predicted for a variety of peptides, prostanoids and steroids (Sjolund 1979; Bryant 1979; Limbird 1988).

The direct and indirect evidence for the different intestinal epithelial cell receptors is summarized in **Table 2**

## Table 2 Intestinal epithelial cell receptors

| Hormone | Species/Tissue | Observed Response | Receptor | Reference |
|---|---|---|---|---|
| Bradykinin | Guinea pig ileum<br>Rabbit colon<br>Human HCA-7 cells | Increased $Cl^-$ secretion | $\beta_2$ | Gaginella (1989) |
| Substance P | Guinea pig isolated enterocytes | Decrease $Na^+$-$H^+$ exchange | ? | Cook (1989) |
| *Bombesin | Rat oxyntic cells | Increased growth and gastrin release | ? | Dembinski(1990) |
| VIP | Rat enterocytes | VIP binding | VIP | Prieto (1979) |
| | Human colonic mucosa | Increased AC activity | " | Simon (1980a) |
| | Human CaCo-2 cell line | Increased AC activity and receptor numbers | " | Laburthe (1987) |
| | Human CL.16E cell line | Increased AC, cAMP and mucin production | " | Laburthe (1989) |
| | Rat submandibular gland | Receptor binding | " | Turner (1987) |
| VIP and Secretin | Guinea pig epithelial cells | VIP binding and increased cAMP | " | Binder (1980) |
| PHI | Rat enterocytes | increased cAMP | VIP | Bloom (1983) |

* The mammalian counterpart of bombesin is gastrin-releasing peptide which has a similar structure and biological activity

| Hormone | Species/Tissue | Observed Response | Receptor | Reference |
|---|---|---|---|---|
| Somatostatin | Cat parietal and peptic cells | Inhibition of acid and pepsin secretion | ? | Newman (1987) |
| Prostaglandin $E_1$ | Rat epithelial cells | Increased cAMP | PGE? | Smith (1987) |
| Prostaglandin $E_2$ | " | " | " | |
| Prostaglandin $I_2$ | Human colonic mucosa | increased AC activity | PGE? | Simon (1980a) |
| Acetylcholine | Rat colonic cells<br>Turtle villus cells | increased $[Ca^{2+}]_i$<br>decreased salt absorption | M | Cook (1990) |
| Adrenergic | Rat jejunum | inhibition of VIP and $PGE_1$ stimulated secretion | $\alpha_2$ | Nakaki (1982) |
| Seratonin | Rabbit crypt cell membranes | increased permeability to $Ca^{2+}$ | 5HT | Furman (1989) |
| Histamine | Dog parietal cells | increased acid production | $H_2$ | Thompson (1977) |
| Aldosterone | Rat perfused segments | increased $Na^+$ transport | ? | Franco (1988) |

1.4.3 VIP and VIP receptors

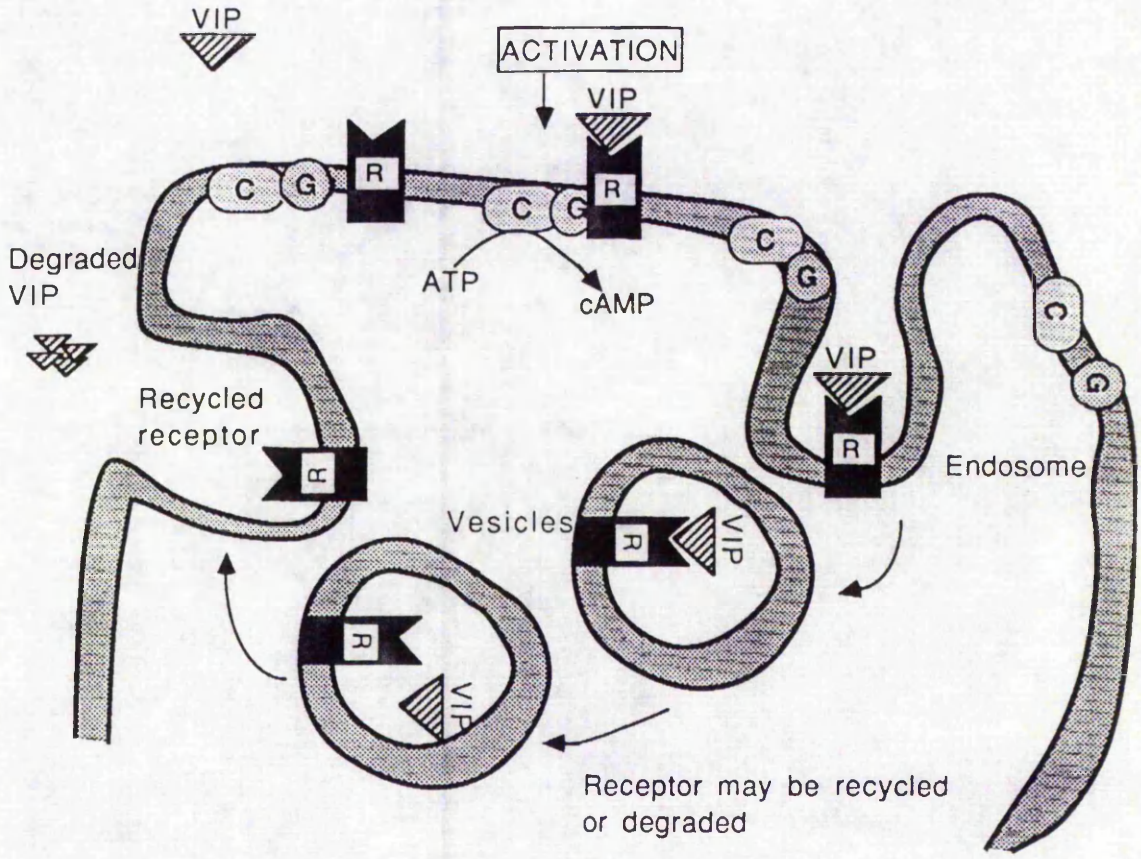
VIP is a 28 amino acid basic peptide originally isolated from porcine small intestine (Said et al.1972). It is a member of a family of peptides that include secretin, glucagon, GIP, PHI (and PHM its human equivalent) (Said et al. 1972; Larsson 1982). VIP has been found, so far, to be the most potent stimulator of adenylate cyclase in the intestinal mucosa and the sequential activation of adenylate cyclase, protein kinase A and cAMP specific phosphodiesterase has been demonstrated (Amiranoff et al. 1978; Marchis-Mouren et al. 1988).

VIP is released from nerve terminals in close proximity to the enterocyte basolateral membrane. VIP stimulates adenylate cyclase through binding to specific cell surface receptors. These receptors have been extensively studied in rat and human intestinal epithelium using natural and synthetic VIP analogues. Secretin, PHI/PHM and GRF but not glucagon nor GIP were able to interact with VIP receptors, although with lower affinity than VIP. Significant species differences have been observed between rat and human VIP receptors to recognize natural VIP related peptides and partial VIP sequences (Laburthe et al. 1982, 1986; Robichon et al. 1987). These differences have been taken as an indication of the higher degree of specificity of the recognition sites to have evolved in man (Laburthe et al. 1986). The complete sequence of VIP (1-28) is necessary for full potency in both receptor binding and adenylate cyclase activity (Robichon et al. 1987).

Kinetic experiments have indicated that [$^{125}$I]-labelled VIP, bound to cell surface receptors, is internalized and degraded within lysosomes. The receptor may then be recycled back to the cell surface or

degraded (Nau et al 1987; Rosselin et al. 1988; Marchis-Mouren et al. 1988). Furthermore, the internalized VIP-bound receptor is no longer bound to or capable of stimulating adenylate cyclase (Rosselin et al. 1988) (**Figure 7**). The formation of an inactive metabolite of VIP, [des-$His^1$]-VIP, by an aminopeptidase on the intestinal epithelial cell outer surface membrane is another documented mechanism of VIP inactivation (Nau at al. 1987).

**Figure 7 VIP receptor internalization**




A schematic diagram of VIP receptor membrane binding, internalization, recycling and degradation. Redrawn from Rosselin et al. (1988). Although not shown most endocytotic vesicles fuse with primary lysosomes (Albert et al. 1983a). There is evidence to suggest that VIP may not be internalized as the intact peptide but as the [des-His$^{1}$] metabolite with greatly reduced biological activity (Nau et al. (1987)

1.4.4 Guanine nucleotide binding proteins (G proteins)

G proteins are a group of ubiquitous regulatory proteins found within the plasma membrane of all eukayotes so far studied. The earliest and most studied G proteins are those involved in the modulation of adenylate cyclase. Rodbell et al. (1971a,b) were the first to observe a requirement for GTP in the glucagon activation of adenylate cyclase in membranes from rat liver cells. They also demonstrated that GTP enhanced the rate of dissociation of radiolabelled glucagon from its receptor binding sites. That GTP was involved in the regulation of adipocyte adenylate cyclase was reported by Harwood (1973a,b) and again later by Yamamura (1977). These basic observations have been found for all cells that possess hormonal regulation of adenylate cyclase (Northup 1985).

In the intestine, Calvo et al. (1989) has successfully isolated VIP/VIP-receptor/$G_s$ as a 152Kd complex and demonstrated GTP regulation of this complex in isolated plasma membranes from rat enterocytes. G proteins, therefore, not only bind GTP and regulate the catalytic activity of adenylate cyclase but also hydrolyse GTP and regulate hormone affinity for receptors.

Some of the G proteins and their proposed functions are summarized in **Table 3**

**Table 3 Summary of G proteins and their interactions**

The family of G proteins is expanding very rapidly. This table, therefore, is not intended to be an exhaustive list but is a summary of the best known G proteins. (Gilman 1987; Birnbaumer et al. 1990)

CHARACTERISTICS

| | $G_s$ | $G_i$ | $G_t$ | $G_{olf}$ | $G_o$ | $G_{plA}$ | $G_{plC}$ | $G_{plD}$ | $G_z$ |
|---|---|---|---|---|---|---|---|---|---|
| Heterotrimeric G proteins | α(52) (44) | α(42) | α(39) | α(45) | α(39) | α | α | α | α |
| Subunits (MW x$10^{-3}$) | β(36) (35) | β(36) (35) | β(36) (35) | β | β(36) | β | β | β | β |
| | γ(8-10) | γ(8-10) | γ(8-10) | ? | γ(8-10) | ? | ? | ? | ? |
| Effector | AC/$Ca^{2+}$ /channel | AC/$K^+$ /channel | cGMP-PDE | AC | Ion channels e.g $Ca^{2+}$ | Phospho-lipase $A_2$ | Phospho-lipase C | Phospho-lipase D | ? |
| Toxin ADP-Ribosylation | C | P | C&P | ? | P | ? | C&P tissue differences | ? | None |
| Effect | Stim | Inhib | Stim | Stim | Inhib | Stim | Stim | Stim | ? |
| Example Receptor/ Agonist | β-adrenergic Glucagon ACTH VIP Serotonin | Somato -statin muscarinic | Light sensitive Rhodopsin | Olfactory signals | Neuro-peptide Y | Gonado-trophin Releasing Hormone | Histamine Gastrin | $\alpha_1$-adren -ergic | ? |
| Example Target cells/ Organ | Heart, Fat Skeletal Muscle Intestinal Mucosa | Heart Liver Renal Cortex Intestinal Mucosa | Rods Cones | Olfactory Cilia | Sensory Ganglia Leydig | Gonadotrophs Granulosa CNS | Chromaffin Cells Parietal Cells | Liver | ? |

C = cholera toxin P= pertussis toxin

G proteins are thought to be heterotrimeric molecules consisting of an $\alpha$ subunit and two smaller subunits ß and $\gamma$ that are tightly coupled together (ß$\gamma$). The $\alpha$ subunits have a single, high affinity binding site for guanine nucleotides, possess GTPase activity and contain NAD-dependent ADP-ribosylation sites. There are both stimulatory ($\alpha_s$) and inhibitory ($\alpha_i$) forms of $\alpha$ which mediate the action of stimulatory and inhibitory receptors (Gilman 1987, 1989; Levitzki 1990).

The functional characteristics of ß$\gamma$ subunits are less well understood. Unlike the separation of $\alpha$ from ß$\gamma$ separation of ß from $\gamma$ is only possible under denaturing conditions with the result that information about the functional role of each oligomer is scant. The presence of ß$\gamma$ is essential for the interactions of G proteins with receptors and they are thought to anchor the $\alpha$ subunits to the membrane (Mattera et al. 1987; Gilman 1987, 1989). ß$\gamma$ may also regulate effector enzyme activity, e.g. phospholipase $A_2$ (Bourne 1989; Kaziro 1990) (see below).

Both hormone binding to receptors and GTP binding to G proteins are required for the activation (or inactivation) of the catalytic unit that produces cAMP (Cerione et al. 1984a, b). Hormone-receptor complexes activate G proteins by catalysing replacement of bound GDP by GTP. Binding of GTP to the $\alpha$ subunit causes the dissociation of ß$\gamma$, thereby generating two potentially active subunit, $\alpha$-(GTP) and ß$\gamma$ (Bourne 1989). The active state is reversed as the intrinsic GTPase activity of $\alpha$ hydrolyses GTP to GDP and $P_i$. $\alpha$-(GDP) then reunites with ß$\gamma$. The non hydrolysable analogues of GTP (Gpp(NH)p and GTP$\gamma$S) thus persistently activate the enzyme.

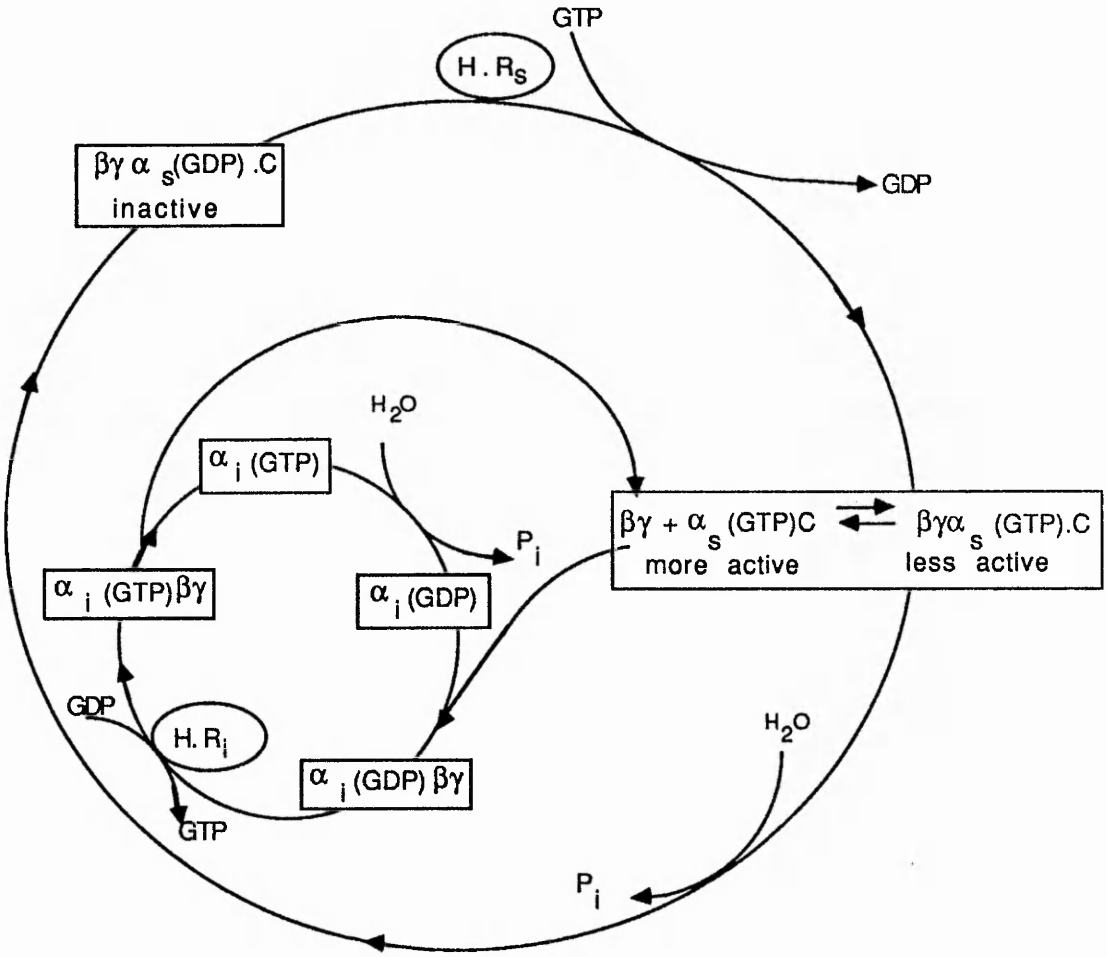
Current debate centres on whether the components of the system dissociate when activated and reassociate when deactivated within the membrane. The "collision coupling" model proposes that $\alpha_s$ in the presence of bound hormone receptor, GTP and $Mg^{2+}$ dissociates from the $\beta\gamma$ subunit and associates with the catalytic unit thereby activating it to generate cAMP. Evidence for this model is derived from experiments in which the system was reconstituted from highly purified detergent disrupted membranes. However, detergent isolated $\alpha_s$ has little GTPase activity and where GTP analogues lead to subunit dissociation and $\alpha_s$ activation, GTP does neither. Restoration of GTPase activity and GTP activation was established when the system was reconstituted with hormone-receptors (Brandt et al.1983; Cerione et al.1984a, b). There is no evidence that subunit dissociation occurs in native membranes (Mattera 1987). Levitzki (1988) favours the idea that $\alpha_s$ is permanently coupled to the catalytic unit *in vivo* because it co-purifies with $\alpha_s$, and kinetic analysis indicates the interaction between $\alpha_s$ and C is not rate limiting as might be expected if activation of C depended on collision with $\alpha_s$ (Arad et al. 1984; Levitzki 1990). The "modified dissociation" model of G protein activation of adenylate cyclase is summarized in **Figure 8.**

The $\alpha$ subunits have been divided into four groups based on their response to the bacterial enzyme toxins of *Vibrio cholera* and *Bordetella pertussis* (Neer et al. 1988). The toxins from these bacteria alter the G proteins by covalently adding an ADP-ribose group to a specific site on the $\alpha$ subunit from NAD. As a consequence the GTPase activity of the G protein is inhibited causing its persistent activation. The G proteins thus act as substrates for these toxins and it

is their characteristics as substrates which have been instrumental in identifying the different $\alpha$ subunits, (Spiegel 1990) (**Table 3** ).

Firstly there are the cholera stimulated $\alpha$ subunits, which irreversibly stimulate adenylate cyclase in the presence of toxin; these are the $\alpha_s$ subunits discussed above. The $\alpha_s$ of intestinal epithelial cells is the natural target for this toxin and it is this interaction that results in the pathogenesis of the disease. However the activation mechanism of the toxin was worked out in cell systems more amenable to experimental manipulation and analysis than is the intestinal epithelium (Field 1980; Binder 1984).

**Figure 8 G protein activation cycle**




The G protein modified dissociation model as proposed by Alexander Levitzki (1990)

$G_s$ was established as the stimulating regulatory component of adenylate cyclase by the use of a mutant cell line, derived from S49 murine lymphoma cells called *cyc*$^-$, deficient in $G_s$. These cells had no adenylate cyclase activity even though the catalytic unit of the enzyme was present. Restoration of adenylate cyclase activity was achieved by the addition of wild type membranes in which adenylate cyclase had been inactivated but $\alpha_s$ was intact (Ross 1977, 1978, 1980; Houslay 1983). The intestinal epithelium poses some intriguing problems for the proposed mechanisms of adenylate cyclase activation by cholera toxin stimulated $G_{s\alpha}$. *In situ* cholera toxin binds to the brush border of the mucosal cells but adenylate cyclase is localized to the basolateral membrane. There is, therefore, a logistic problem of how the cholera toxin accesses the adenylate cyclase in these cells. Dominguez et al. (1987) has demonstrated the presence of $\alpha_s$ subunits in isolated brush border membranes. He postulates that:-

"It is conceivable that after interaction of cholera toxin with brush border membranes the A1 promoter (the active subunit of cholera toxin) dissociates from the holotoxin within the bilayer, therein catalyzing the ADP-ribosylation of $G_s$ components. Then the dissociated $\alpha$ subunit of the modified $G_s$ component would reach in a yet-undefined way the catalytic subunit in the basal lateral membrane."

In the mouse intestinal mucosa immunofluorescence microscopy showed the binding of cholera toxin predominantly at the apical membrane. Some labelling also appeared in the cytoplasm and in the basolateral membrane suggesting internalization and translocation of the cholera toxin (Lonnroth 1984). Whether this internalized toxin is associated with a G protein is as yet unknown. Movement of G proteins, in the plane of the membrane, from the brush border to the basolateral membrane is a less likely mechanism

than transcytosis (a process of membrane invagination, cytoplasmic transport and membrane fusion) because a prerequisite for the maintenance of polarized transporting epithelia is that protein traffic between the two domains of the plasma membrane is restricted (Louvard et al. 1986; Caplan and Matlin 1989).

However, this proposal conflicts with Levitzki's (1990) suggestion based on kinetic evidence, from other cell types, that $\alpha_s$ is permanently associated with the catalytic unit (Arad 1984). The mechanism of cholera toxin activation of adenylate cyclase in intestinal epithelial cells is therefore unclear. It is possible that a mechanism worked out for one cell type may not be applicable to all cell types (Cantiello 1989). In light of Cooke's (1989) suggestion, that intestinal secretion may be a defence mechanism to rid the intestine of invading organisms, it may be that intestinal cells have evolved a particular mechanism to ensure cholera toxin makes contact with their adenylate cyclase.

Secondly there are the pertussis stimulated $\alpha$ subunits, $\alpha_i$. When $\alpha_i$ was treated with pertussis toxin (islet activating factor) a rise in adenylate cyclase activity was observed or response of adenylate cyclase to inhibitory agonists was prevented (Bokoch et al.1983; Codina et al.1983; Gill 1984; Mattera et al. 1987). The inhibitory regulation of adenylate cyclase was discovered through the effects of GTP on the enzyme (Birnbaumer 1973; Harwood et al 1973a,b; Yamamura et al.1977). The same *cyc*$^-$ variant used to identify $\alpha_s$ was used to identify $\alpha_i$. To detect inhibition of adenylate cyclase in *cyc*$^-$ membranes the residual adenylate cyclase activity was amplified with forskolin, a diterpine which is thought to stimulate the C unit directly

(Seamon and Daly 1981, 1982). Inhibition of forskolin activated adenylate cyclase was demonstrated at low concentrations of GTPγS (Hildebrandt 1982, 1983b) and Gpp(NH)p (Seamon et al. 1983; Katada et al. 1984c).

Inhibition of adenylate cyclase activity in the presence of GTP by somatostatin, a peptide which binds $R_i$, also indicated the presence of an inhibitory G protein in *cyc*$^-$ (Jakobs 1983; Roof et al.1985). Treatment of membranes derived from human platelets with pertussis toxin resulted in the abolition of guanine nucleotide and receptor mediated inhibition of adenylate cyclase activity (Katada et al. 1984a, b; Jakobs et al. 1984). Adrenaline has a dual effect, stimulating adenylate cyclase through activation of $\alpha_s$ by binding to $R_s$ and inhibiting adenylate cyclase activity through activation of $\alpha_i$ by also binding $R_i$ type receptors. Because the two G proteins have different affinities for GTP the net effect of adrenaline on adenylate cyclase activity depends on the GTP concentration (Gill 1984; Levitzki 1990).

An intestinal example of dual control of adenylate cyclase may be found in histamine stimulated gastric acid production in parietal cells. Individually $PGE_2$, somatostatin, secretin and histamine all stimulate gastric mucosal adenylate cyclase activity. However, $PGE_2$, somatostatin and secretin inhibited histamine stimulated enzyme activity (Becker et al 1982). The influence of GTP or its analogues have not been studied in this system.

How inhibition of C is mediated by α is unknown. It has been proposed that inhibition results from $\alpha_i$ reversing the stimulatory effects of $\alpha_s$ on C by increasing the local concentration of βγ when, upon

activation, $\alpha_i$ dissociates from βγ. When GTPγS was used to demonstrate $\alpha_i$ activity, the presence of $\alpha_s$ was found to be essential and most of the inhibition was related to βγ and not $\alpha_i$ (Gilman 1987, 1989). βγ derived from either $\alpha_s$, $\alpha_i$ or $\alpha_o$ is capable of inhibiting $\alpha_s$-C indicating a common pool of βγ (Birnbaumer et al. 1985; Gilman 1987). However, in the *cyc*$^-$ system, where there is no $\alpha_s$, addition of βγ does not inhibit C activity; but inhibition was detected with low concentrations of GTPγS implying direct involvement of $\alpha_i$ in the inhibition of C (Katada et al. 1984a,b,c, 1986). These are not mutually exclusive mechanism and both $\alpha_i$ and βγ mediated inhibition of adenylate cyclase may operate under physiological conditions (Neer et al. 1988; Gilman 1989)

Thirdly, the tranducins, a category of G proteins that couple light activated rhodopsin to cGMP phosphodiesterase in rods and cones, are stimulated by both toxins (Kaziro 1990b) and the fourth group of G proteins are those not influenced by either toxin (Neer et al. 1988).

The α, β and γ subunits are highly polymorphic. So far nine different genes that encode for G proteins have been found but there are 12 polypeptide products of these genes and specific functions for all of them have not yet been determined (Gilman 1989). Gilman (1989) has speculated that at the end of the day there could be several hundred different G protein oligomers.

The membrane arrangement of G proteins is thought to be within the inner face of the plasma membrane because no transmembrane spanning protein sequences have been identified. Although the $\alpha_i$ has a myristic tail which is thought to anchor the subunit to the

membrane, $\alpha_s$ and βγ have not (Neer et al. 1988). There is evidence to suggest that the βγ may be important for the attachment of α. For example, detergent solubilized α behave as hydrophilic molecules whereas βγ molecules aggregate (Huff et al. 1985; Sternweis 1986; Gilman 1987). The significance of these findings is that the site of action of α may not be confined to the plasma membrane (Rodbell 1985). Interestingly, Nagata et al. (1989) has demonstrated the presence of G proteins in the cytosol of human platelets and Nakano et al. (1989) has discovered a novel G protein in the endoplasmic reticulum and Golgi apparatus of *Saccharomyces cerevisiae* thought to be involved in transcellular protein transport. Both of these findings may have particular relevance to the problems discussed above with regard to signal transduction in intestinal epithelial cells. Indeed, the protein cofactor, arf, required for efficient *in vitro* ADP-ribosylation of $\alpha_s$ by cholera toxin has turned out to be a small monomeric GTP-binding protein important in the regulation of Golgi structure and dynamics (Balch 1990).

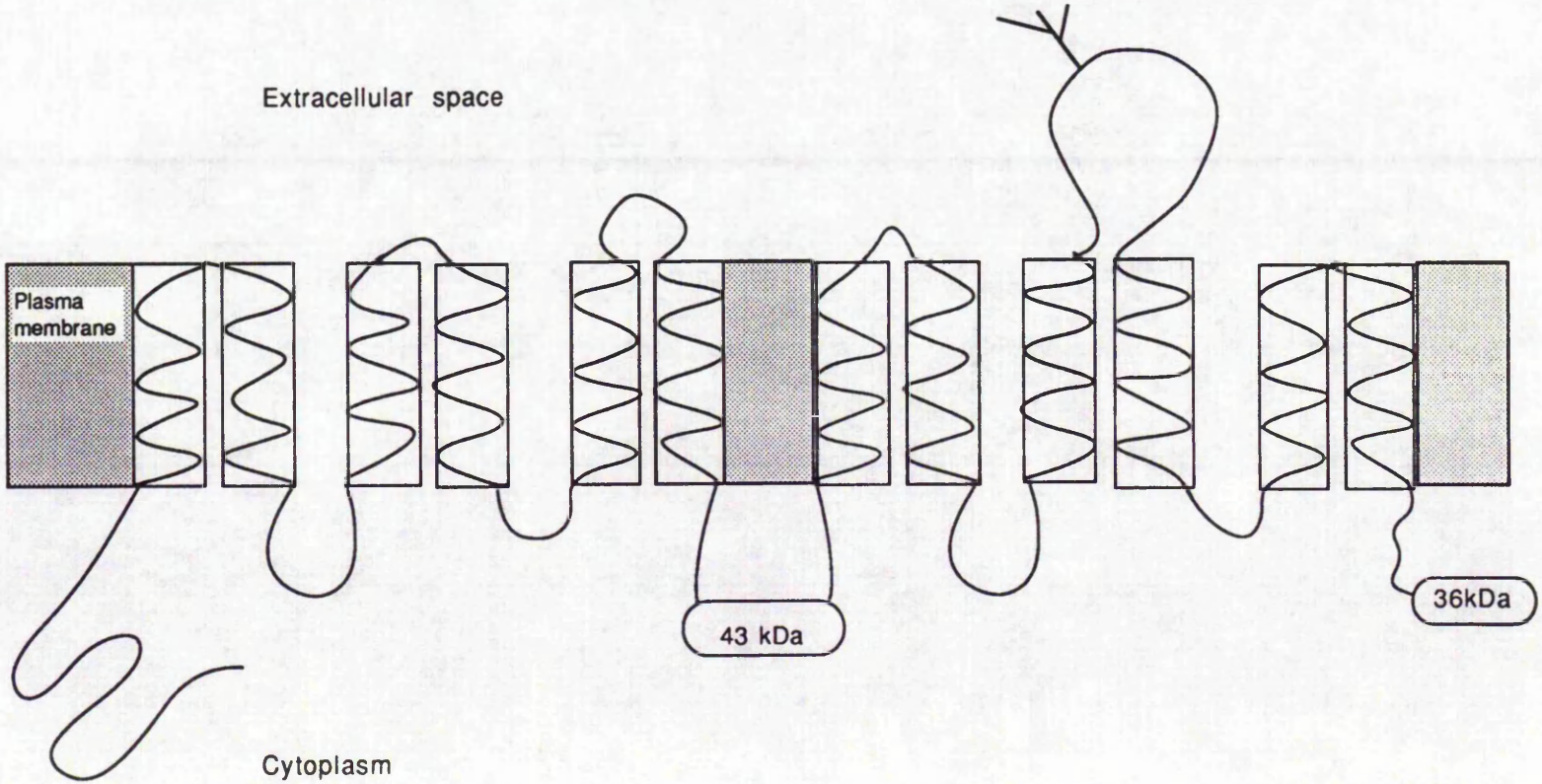
1.4.5 Adenylate cyclase catalytic unit

Much less is known about the catalytic unit of the adenylate cyclase system than about the G proteins although the enzyme has recently been sequenced (Krupinski et al.1989; Mirzoeva 1989; Lipkin et al. 1989). One of the main difficulties is that the purified enzyme is unstable. The unstimulated form is more unstable than the stimulated form bound by $\alpha_s$. Stability also seems to depend on the tissue source of the enzyme, brain material being the most stable so far tested (Pfeuffer 1985; Smigel 1986). There are multiple forms of adenylate cyclase based on genetic and biochemical evidence (Livingstone et al 1984; Pfeuffer et al 1985; Smigel 1986; Mollner et al 1988; Minocherhomjee 1987; Mirzoeva 1989). Two forms have been identified which differ in their requirements for calmodulin. This is an important characteristic of the enzyme exploited for its isolation by utilising forskolin-Sepharose and calmodulin-Sepharose affinity columns. The calmodulin-sensitive form from bovine brain, sequenced by Krupinski et al. (1989), is thought to be a single glycoprotein with a molecular weight of about 150,000 and 1134 amino acids long (Gilman 1987). There are two alternating hydrophobic regions and two hydrophilic regions. Each of the two hydrophobic domains contains six transmembrane spans. The two hydrophilic domains are thought to be orientated into the cytoplasm of the cell and represent nucleotide binding sites. The topographical arrangement of the enzyme resembles that of various channel and transporting membrane proteins indicating a so far undetermined function for adenylate cyclase, e.g. transport of cAMP out of cells (Krupinski et al.1989; Schofield and Abbott 1989; Gilman 1989) (**Figure 9**). The calmodulin insensitive form from bovine brain has only partially been sequenced, and appears to be more unstable in the

purified form than the calmodulin sensitive enzyme (Mirzoeva 1989; Lipkin et al. 1989). Both forms of the enzyme appear to be gycoproteins, regulated by G proteins and hormone bound receptors. The significance of the calmodulin dependent form in the regulation of cAMP production is unclear. Nor is it clear whether the two forms represent two different post-translational modifications of the same polypeptide chain or two different, but closely related polypeptide chains (Mollner and Pfeuffer 1988).

**Figure 9 Molecular arrangement of adenylate cyclase**

A schematic two dimentional model of adenylate cyclase in the plasma membrane. Adapted from Krupinski et al. (1989)



## 1.5 THE RELATIONSHIP BETWEEN CALCIUM, CALMODULIN AND ADENYLATE CYCLASE

### 1.5.1 Introduction

That calcium plays a critical role in the regulation of normal cellular functions and that calmodulin is one of the main mediators of this regulation is well established (Walker et al. 1984; Tomlinson et al. 1984; Dedman 1984; Carafoli 1987).

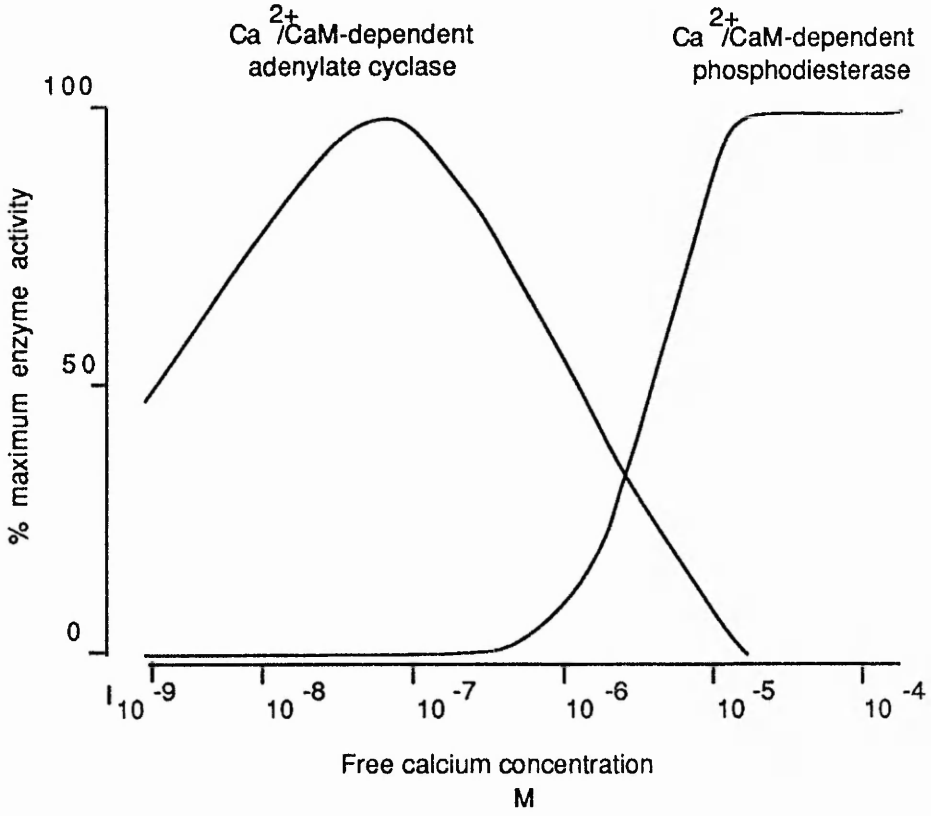
Calmodulin is a highly conserved acidic calcium binding protein that is present in considerable amounts in all eukaryotic cells. When bound by $Ca^{2+}$ calmodulin undergoes a conformational change and interacts with many cellular enzymes. Calmodulin itself has no known enzymatic activity (Cheung and Storm 1982; Dedman 1984; Van Eldik et al. 1985; Carafoli 1987). At rest intracellular $Ca^{2+}$ concentrations have been estimated to be $<10^{-7}M$ (Petersen and Gallacher 1989). At this concentration of $Ca^{2+}$ calmodulin is in the inactive state. An increase in intracellular $Ca^{2+}$ above resting levels (approximately $10^{-6}M$) may be derived from intracellular stores such as endoplasmic reticulum or influx from outside the cell via $Ca^{2+}$ channels e.g. voltage or receptor operated channels. Once bound to $Ca^{2+}$, calmodulin forms a more helical structure, becomes active and binds reversibly to its target apoenzyme, eg. phosphodiesterase or adenylate cyclase, resulting in the formation of an active holoenzyme (Brostrum and Cheung 1980; Jarrett and Medhavan 1991). Calmodulin has 4 $Ca^{2+}$ binding sites. In some calmodulin-sensitive enzyme systems occupation of all 4 sites may not be necessary for the appropriate conformational changes to induce enzyme activation (Manalan and Klee 1984). The sequence of

events depicted in **Figure 10** show that it is $Ca^{2+}$ and not calmodulin which is the rate-limiting factor.

Therefore intracellular $Ca^{2+}$ fluctuations play a vital role in regulating calmodulin sensitive enzyme activities (Cheung and Storm 1982). That calmodulin activates both adenylate cyclase, the enzyme that generates cAMP and phosphodiesterase, the enzyme that degrades cAMP seems rather paradoxical. The explanation may be sequential activation of adenylate cyclase in the plasma membrane followed by phosphodiesterase activation in the cytosol (Walker et al. 1984). Furthermore, the $Ca^{2+}$ concentration required to activate calmodulin-sensitive cAMP phosphodiesterase has been found to be in the micromolar range. This concentration is inhibitory to adenylate cyclase activity which requires submicromolar concentrations for activation by calmodulin (MacNeil et al. 1985) (**Figure 11**)

**Figure 10 Schematic representation of a calmodulin (CaM) activated adenylate cyclase (AC) system.**


**Figure 11 $Ca^{2+}$/Calmodulin-dependent enzymes**




The different *in vitro* $Ca^{2+}$ requirements of calmodulin-dependent adenylate cyclase activity compared to the $Ca^{2+}$ requirements of calmodulin-dependent phosphodiesterase activity. Redrawn from MacNeil et al. (1985)

In the intestinal epithelium high concentrations of calmodulin (in the mM range) have been reported, particularly in the brush border membrane and the cytosol. In the brush border, calmodulin is tightly bound within the microvillus core where it is thought to have an important role in buffering luminal $Ca^{2+}$ and regulating NaCl transport via a specific protein kinase II (Thomasset et al. 1981; Mooseker et al. 1984; Powell et al. 1984; Glenney and Glenney 1985; Rood and Donowitz 1990). In the intestine, calmodulin can also activate myosin light chain kinase, $Ca^{2+}$-ATPase as well as activating various enzymatic activities found in other cells, e.g. cAMP phosphodiesterase activity (Glenney and Glenney 1985).

The presence of a calmodulin-sensitive form of adenylate cyclase has been reported in rat (Amiranoff et al. 1983), guinea-pig (Pinkus et al. 1983) and rabbit (Lazo et al. 1984) preparations of intestinal mucosa.
The calmodulin-sensitive enzymes are summarized in **Table 4.**

**Table 4 Calmodulin-Sensitive Enzymes**

| Calmodulin-Sensitive Enzymes | Distribution |
|---|---|
| Adenylate cyclase | Ubiquitous |
| Guanylate cyclase | Protozoa |
| Cyclic nucleotide-phosphodiesterase | Ubiquitous |
| $Ca^{2+}$-$Mg^{2+}$ ATPase | Mammals and plants |
| Multifunctional protein kinases:- | |
| myosin light-chain kinase | Mammals |
| phosphorylase b kinase | e.g muscle, intestinal mucosa |
| glycogen synthase kinase | liver |
| synapsin I kinase | mammalian brain |
| kinase II | mammalian brain<br>intestinal mucosa |
| Ornithine decarboxylase | Ubiquitous |
| Phospholipase $A_2$ | mammals |

Adapted from Walker et al. (1984) and Manalan and Klee (1984)

Regulation of adenylate cyclase by $Ca^{2+}$ has been demonstrated for several tissues and it has been suggested that fluctuations in intracellular free $Ca^{2+}$ may be a possible mechanism for controlling cAMP levels (Bradham and Cheung 1980; MacNeil 1985; Shattuck et al. 1987; Minocherhomjee et al. 1988). For example, the $Ca^{2+}$ ionophore A23187 caused an increase in cAMP in rat colon (Donowitz and Walsh 1987). However, in the upper intestine there are data to suggest the converse relationship i.e. a mechanism for cAMP control of intracellular $Ca^{2+}$ concentrations. In isolated chicken enterocytes Semrad et al. (1987) showed that cAMP stimulated a persistent increase in intracellular $Ca^{2+}$ that was independent of the presence of extracellular $Ca^{2+}$.

The calmodulin-sensitive adenylate cyclase system shows a biphasic response to $Ca^{2+}$. Low concentrations (<1μM) stimulate the enzyme's activity whereas high concentrations (>1μM) inhibit it (MacNeil et al. 1985; Resink 1986) (**Figure 11**). The stimulatory phase of adenylate cyclase activation has been shown to be dependent on calmodulin but the inhibitory phase has been thought to be a direct effect of $Ca^{2+}$ on the enzyme (Hanski et al. 1979; Lasker et al. 1982; Dorflinger et al. 1986; Oldham et al. 1984, 1986; Resink et al. 1986). Some of the data for tissues in which the effects of $Ca^{2+}$ and calmodulin on adenylate cyclase activity have been reported are summarized in **Table 5**.

**Table 5 Tissue variations for calmodulin stimulated/ $Ca^{2+}$ inhibited adenylate cyclase**

| Tissue Source/ Preparation | $IC_{50}$ for $Ca^{2+}$ μM | | Reference |
|---|---|---|---|
| Guinea-pig enterocyte membranes | 500(A) | * | Pinkus(1983) |
| Rat enterocyte membranes | 10(F) | *† | Amiranoff(1983) |
| Rabbit enterocyte membranes | 1(F) | * | Lazo(1984) |
| Rat cerebral cortex | 300 (A) | * | Brostrom (1977) |
| Guinea-pig brain | 0.4(F) | * | Potter(1980) |
| Moth brain | 3.5(F) | * | Bodnaryk(1983) |
| Rat cerebellar | 90(F) | * † | Ahlijanian(1987) |
| Rat pancreatic islets | 600(A) | | Valverde(1979) |
| Guinea-pig sarcolemma | 10-20(F) | † | Tada(1974) |
| Guinea-pig ventricles | 0.4(F) | | Potter(1980) |
| Rabbit heart plasma membranes | >1(F) | * | Panchenko(1984) |
| Dog heart sarcolemma | 0.01(F) | | Cros(1984) |
| Rat kidney | 200-300(A) | *† | Sulimovici(1984) |
| Dog parathyroid | 1.5-8 (F) | | Dufresne(1972) |
| Hog parathyroid | 1.52 and 313 (F) | | Oldham(1984) |
| Crayfish abdominal muscle | 1000(A) | * | Sedlmeier(1983) |
| Rat smooth muscle | 2.5(F) | * | Piascik(1983) |
| Turkey erythrocytes partially purified AC | 80(F) | † | Hanski(1977) |
| Turkey erythrocyte membranes | 250(F) | | Lasker(1982) |
| Human platelets | 0.6-0.75(F) | * | Resink(1986) |
| S49 CYC- cell membranes | 360 (F) | | Lasker(1982) |

The table shows the reported concentrations of free (F) or added (A) $Ca^{2+}$ necessary to inhibit adenylate cyclase activity by 50%
*, indicates experiments in which $Ca^{2+}$ stimulation preceded inhibition
†, indicates $IC_{50}$ values interpreted from author's $Ca^{2+}$ concentration response curves

1.5.2 Calmodulin regulation of adenylate cyclase activity

The mechanism by which calmodulin activates adenylate cyclase is still not worked out in detail but in the presence of optimal $Ca^{2+}$, calmodulin increases the turnover number of the enzyme without affecting the $K_m$ for ATP (Amiranoff et al. 1983).

There is evidence to suggest direct calmodulin interaction with the catalytic component of the enzyme (MacNeil et al. 1985). Calmodulin-sensitive adenylate cyclase purified from brain tissue may be stimulated by calmodulin in the absence of GTP or $\alpha_s$. Both basal and hormone stimulated adenylate cyclase may be stimulated by calmodulin but this stimulation is enhanced by the addition of $\alpha_s$ (Minocherhomjee et al. 1987, 1988; Harrison et al. 1989). The animal intestinal studies have also shown that calmodulin-sensitive adenylate cyclase activity was additive to hormonal stimulation (VIP and $PGE_2$) and GTP activation (and Gpp[NH]p) indicating a regulatory site for calmodulin different from a receptor or G protein mechanism (Amiranoff et al.1983; Pinkus et al. 1983; Lazo et al. 1984).

These studies involved the direct *in vitro* measurement of enzyme activity in response to added $Ca^{2+}$ and calmodulin in membrane preparations from intestinal epithelial cells. The use of calmodulin antagonist such as the neuroleptic compounds, chlorpromazine and trifluoperazine (TFP) as anti-diarrhoeal agents, provides indirect evidence for the presence of calmodulin-sensitive adenylate cyclase in animal and human studies (Ilundain and Naftalin 1979; Simon et al.1981; Zavecz et al.1982; Pinkus et al. 1983; Donowitz et al. 1984; Fedorak et al. 1989).

In the human intestine the work of Simon's group using homogenates

of human colonic biopsies showed that stimulation of enzyme activity induced by cholera toxin, VIP or $PGE_2$ could be completely abolished by TFP (Simon et al. 1981). Another group of compounds used to assess the calmodulin-sensitivity of enzymes is the naphthalenesulphonamide derivatives e.g W7 and W13. The *in vitro* effects of these compounds, on adenylate cyclase activity in the basolateral membranes from chicken mucosa, were compared to the effects of TFP and to the less potent dechlorinated analogs, W5 and W12 (Long et al. 1986). The results of this work indicated that W5 and W12 did not significantly inhibit adenylate cyclase activity but that W7 and W13 (and TFP) did.

The recent work of Fedorak et al. (1989) using a new calmodulin antagonist, CGS 9343B, in isolated rat intestinal loops *in vivo* also supports the presence of calmodulin-sensitive adenylate cyclase in the intestine. CGS 9343B, introduced into the lumen completely inhibited cholera toxin induced secretion and increases in mucosal cAMP. They did not however investigate whether there was an interaction between the adenylate cyclase system and calmodulin.

There is convincing evidence for calmodulin regulation of brush border $Na^+$ and $Cl^-$ permeability to be a direct mechanism on specific calmodulin activated protein kinase II (Thillainayagam and Farthing 1990). The more indirect involvement of calmodulin in the regulation of brush border ion conductance by activation of basolateral membrane adenylate cyclase activity is often suggested (Long et al. 1986; Fedorak et al. 1989; Gaginella and Kachur 1989; Thillainayagam and Farthing 1990). There have been no previous reports of direct measurement of calmodulin-sensitive adenylate cyclase in the human intestine.

1.5.3 Inhibition of adenylate cyclase activity by calcium

Inhibition of adenylate cyclase activity by $Ca^{2+}$ has been reported for a variety of tissues (Bradham and Cheung 1980; Cheung and Storm 1982; MacNeil et al. 1985; Shattuck et al. 1987) (**Table 5**). Evidence from turkey erythrocytes suggests $Ca^{2+}$ inhibits adenylate cyclase activity via specific $Ca^{2+}$ binding sites on the catalytic unit. Kinetic studies showed that the adenylate cyclase inhibiting properties of $Ca^{2+}$ remain unchanged in the presence of varying concentrations of free $Mg^{2+}$ (Hanski et al. 1977). Other evidence from S49 *cyc*$^-$ lymphoma cell membranes implied $Ca^{2+}$ and $Mg^{2+}$ compete for the same regulatory binding sites on the catalytic unit (Lasker et al. 1982). The inhibition of forskolin activated adenylate cyclase activity by $Ca^{2+}$, in membranes from human colonic crypts, was found not to be competitive with forskolin (Seamon and Daly 1986).

Previously it was thought that the inhibition of adenylate cyclase by $Ca^{2+}$ was independent of calmodulin (Shattuck et al. 1987). However, more recent data suggest otherwise. The involvement of calmodulin in the regulation of adenylate cyclase inhibition was proposed by Perez-Reyes et al. (1987), and by Ahlijanian et al. (1987). Interaction between the $\alpha_i$ and the C unit in detergent solubilized EGTA washed membranes of rat cerebral cortex was demonstrated. In these experiments a requirement for free $Ca^{2+}$ was observed for Gpp(NH)p mediated inhibition of adenylate cyclase activity and the calmodulin antagonist, calmidazolium, reversed this inhibition (Perez-Reyes et al. 1987). Regulation of calmodulin-sensitive adenylate cyclase by the $\beta\gamma$ subunit of G proteins binding to calmodulin and thereby inhibiting adenylate cyclase activity has also been reported (Katada et al.1987).

## 1.6 AIMS

Taking advantage of a regular and fresh supply of human intestinal biopsy material the aims of this work were to firstly develop suitable tissue preparations from biopsies and secondly, to ascertain if a form of calmodulin-sensitive adenylate cyclase could be demonstrated in these preparations.

From biopsies of the second part of the human duodenum two types of tissue preparation were used, washed membranes and isolated epithelial cells. Both methods represent the novel use of human intestinal biopsy specimens for research purposes (Smith et al. 1990; Smith et al. 1991).

Washed biopsy membranes were used for the direct assay of adenylate cyclase activity. By manipulating the washing media, tightly controlling free $Ca^{2+}$ concentrations with EGTA buffers and stimulating or inhibiting the enzyme's activity with appropriate agonists/antagonists the characteristics of this enzyme preparation were defined.

In contrast, an isolated epithelial cell preparation was employed to stimulate adenylate cyclase via the VIP receptor and measure cAMP production. Again agonist/antagonists were used to indicate the presence of a calmodulin-sensitive form of adenylate cyclase in the human intestine.

This work therefore divides into two main sections each with a brief introduction and a methods, results and discussion section, i.e.

CHAPTER 2. Studies on adenylate cyclase activity in particulate

membranes from human duodenal biopsies: the effects of $Ca^{2+}$, calmodulin and EGTA

CHAPTER 3. Studies on isolated epithelial cells from human duodenal biopsies: stimulation by VIP and the effects of calmodulin antagonists on VIP stimulated cAMP production.

The results from these two different approaches are then discussed and compared in CHAPTER 4.

# CHAPTER 2

## STUDIES ON ADENYLATE CYCLASE ACTIVITY IN PARTICULATE MEMBRANES FROM HUMAN DUODENAL BIOPSIES: THE EFFECTS OF $CA^{2+}$, CALMODULIN AND EGTA

## 2.1 INTRODUCTION

The primary objective of these experiments was to assess the importance of $Ca^{2+}$ and calmodulin in the regulation of adenylate cyclase in the second part of the human duodenum. This was accomplished by assessing a) the concentration dependent effects of free $Ca^{2+}$ on enzyme activity, b) the effects of adding exogenous calmodulin to the adenylate cyclase assay system and c) the effects on enzyme activity of calmodulin antagonists. All these parameters were tested on basal and NaF stimulated adenylate cyclase activity.

During the course of these investigations, observations were made indicating a direct effect of the $Ca^{2+}$ chelating agent, EGTA on adenylate cyclase activity in biopsy particulate membrane preparations. This effect was explored further by studying adenylate cyclase activity response to increasing concentrations of EGTA, under various stimulating conditions, in the presence and absence of a constant concentration of free $Ca^{2+}$.

Presented below are the general methods for experiments using particulate membrane preparations of duodenal biopsies. Variation for specific experiments are detailed in the figure legends in section 2.4

## 2.2 MATERIALS

All chemicals were of the highest available quality from Sigma Chemical Co. with the exception of [8-$^{3}$H]adenosine 3',5'-cyclic phosphate (ammonium salt, specific activity 1.74MBq/mmol) ([$^{3}$H]cAMP) and adenosine 5'-[$\alpha$-$^{32}$P]triphosphate (triethylammmonium salt, specific activity 1.11TBq/mmol) ([$\alpha$- $^{32}$P] ATP) which were from Amersham Int.(UK). The labelled ATP (PB107) was of the kind specially treated by Amersham for the use in the assay of adenylate cyclase activity to give low blank values (typically 0.001%). The N-(8-aminohexyl)-5-IODO-1-naphthalene-sulphonamide (IODO 8) was kindly given by Ian Coutts and Pam O'Donnell of Nottingham Polytechnic, Nottingham. All other exceptions are given in parenthesis in the text.

## 2.3 METHODS

### 2.3.1 Collection of human duodenal biopsies

Patients presenting at clinic with various gastrointestinal symptoms or iron deficiency anaemia were biopsied as part of the diagnostic investigations to exclude malabsorption. Patients were fasted from midnight and the following morning prepared with lignocaine spray to the pharynx and intravenous Diazepam (diazemuls). Biopsies of the second part of the duodenum were collected by a physician using an Olympus GIF IT endoscope (Keymed, Southend - on - Sea, Essex, UK) with 3.7mm biopsy forceps. This size of forceps gave pieces of duodenal mucosa weighing 12.3$\pm$1.4mg (Smith et al. 1989). Two biopsies were placed in formalin for histological examination (by the Histopathology department at the City Hospital) to exclude villous abnormalities. Biopsies for adenylate cyclase studies were immediately placed in prefrozen polypropylene tubes and immersed in liquid

nitrogen. This took approximately 5 seconds. Only biopsies from patients subsequently found to have normal histology, no discernible underlying upper gastrointestinal disease and not on any medication were used for these studies. Ethical permission for the study was obtained from the Nottingham City Hospital Ethical Committee and patients gave informed written consent.

2.3.2 Preparation of particulate membranes from duodenal biopsies

The presence of extracellular and intracellular fluids in tissue homogenates has been shown to interfere with adenylate cyclase activity (Johnson et al. 1979; MacNeil et al. 1980; Crawford et al. 1980). Biopsy homogenates were therefore washed with a series of homogenization and centrifugation steps.

Four or five biopsies from one patient were homogenized in 2ml ice cold 2mM HEPES buffer, pH7.5, containing 5mM $MgCl_2$ and 5mM EGTA (MEH) using 10 strokes of a Potter S homogenizer (B. Braun) at 1200rpm. The homogenate was made up to 12ml with MEH and was centrifuged at 2500g for 20 minutes at 4°C. The pellet was resuspended in 2ml fresh MEH and the homogenization/centrifugation procedure repeated twice more.

Any large tissue fragments remaining after the second homogenization were removed. Microscopic examination showed them to be underlying muscularis mucosa and not epithelium (Tripp et al. 1978). The third pellet was resuspended in 2.4ml MEH and homogenized a fourth time using an Ultra-Turrax (Janke & Kunkel, IKA-WERK) at maximum speed setting for 15 seconds. This final homogenate was used for assay. Calmodulin may be removed from some membrane preparations by washing them in EGTA buffers (MacNeil et

al. 1985). Therefore, in experiments in which no attempt was made to remove endogenous calmodulin, EGTA was omitted from the tissue buffer (MH). All buffers were made up in double distilled deionised water or Hipersolv (HPLC grade water from BDH).

The cerebral cortex from 2 male Wistar rats were combined and treated as for duodenal biopsies. This washed particulate preparation was used as a positive control to ensure that the assay conditions used were capable of detecting calmodulin dependent adenylate cyclase activity.

2.3.3 Assay of adenylate cyclase activity

Adenylate cyclase was assayed by the procedure of Salomon et al. (1974). The assay can be divided into three stages:- 1) incubation of the enzyme with its substrate $Mg^{2+}$-ATP, 2) purification of the labelled product, [$^{32}$P] cAMP from its labelled substrate, [$\alpha$ -$^{32}$P]ATP and 3) measurement of the isolated [$^{32}$P] cAMP by liquid scintillation counting.

1) Incubation of the enzyme with $Mg^{2+}$-ATP

Reactions were conducted in 2ml polypropylene test tubes. In a total volume of 100µl the standard reaction mixture contained:- an ATP regenerating system of 13mM creatine phosphate and 0.1mg/ml creatine phosphokinase, 1mM ATP labelled with [$\alpha$ -$^{32}$P]ATP (1.11TBq/mmol) to give approximately 1x$10^6$ cpm and 40µl adenylate cyclase assay buffer which consisted of 125mM Tris-HEPES, 0.25% BSA, 5mM $MgCl_2$ and 2.5mM of the phosphodiesterase inhibitor 3- isobutyl-1-methylxanthine (IBMX), at pH7.5. For NaF stimulated activity, reactions also contained 10mM NaF (originally dissolved in 1mM acetic acid). When calmodulin was added to the assay it was dissolved in the adenylate cyclase assay buffer. Calmodulin antagonists (TFP and IODO 8) were dissolved in dimethysulphoxide (DMSO) before addition to the reaction system. Control reaction mixtures contained DMSO only and final DMSO assay concentrations did not exceed 1% (v/v).

Reactions were initiated by the timed addition of approximately 50µg of particulate membrane protein and carried out for 30 minutes at 37°C in a shaking water bath. Reactions were terminated at timed intervals by the addition of 100µl of stopping solution containing:- 10mM ATP, 2% SDS (w/v), 50mM Tris-HCl and 2.4nM[$^3$H]-cAMP (1.74MBq/mmol) to estimate [$^{32}$P] cAMP (25000cpm /100µl) recovery

from stage 2 at pH7.5. Tubes were placed in a boiling water bath for 2 minutes, allowed to cool and made up to 1ml with distilled water.

2) [$^{32}$P] cAMP purification

[$^{32}$P]cAMP may be removed from the labelled substrate, [$\alpha$-$^{32}$P]ATP, and any non-specific $^{32}$P labelled reaction products by the highly sensitive double chromatography procedure of Salomon et al. (1974). Reaction mixtures were sequentially passed over columns (BioRad Econo-Columns 0.5cm IDx10cm) containing the cation exchange resin Dowex Ag50 Wx4 followed by passage over neutral alumina. The columns were supported by two 8x8 column perspex stages so that the Dowex columns could be placed to drain directly onto the corresponding alumina columns. Dowex columns were packed to a height of 10cm and any air blockages were removed. The columns were initially activated with an alternating wash (7mls) of distilled water, 2N NaOH, distilled water, 2N HCl and ending with distilled water. This procedure was also used to regenerate the columns after each experiment.

The alumina columns were packed with 0.6g of dry neutral alumina. Before each experiment they were activated with 7ml 0.1M imidazole-HCl buffer, pH7.4. The columns were washed with distilled water after each experiment. Between experiments both the Dowex and alumina columns were stored clean and regenerated in distilled water.

Each 1ml sample was decanted onto a Dowex column and allowed to drain. The largest loss of sample and therefore reduction in recovered product was found to be the residual sample left in the reaction tubes. The next 2ml of distilled water to be applied to the

Dowex columns was first put into the tubes to recover this residual sample. The columns were allowed to drain and the eluate discarded. The Dowex columns were then mounted above the previously activated alumina columns. The samples were eluted from the Dowex columns directly onto the alumina columns with 4ml distilled water. The Dowex columns were then removed and 5ml of 0.1M Imidazole-HCl buffer, pH7.4. was applied to the alumina columns and the eluate collected directly into 20ml polypropylene scintillation vials.

For $^{32}P$ standards, 10µl of a 1 in 100 dilution of the $^{32}P$-ATP substrate was counted and for $^{3}H$-cAMP standards 100µl of the stopping solution was counted. Imidazole-HCl buffer (5ml) was added to each standard to account for quenching in the samples and 5ml of buffer only for background counts.

3) Liquid scintillation counting

The scintillation vials containing samples and standards were filled with 15ml Optiphase X scintillation cocktail (Pharmacia LKB) and vortex mixed well. A minimum of triplicate samples and standards were dual-label counted in a Packard TRI-CARB 4000 beta counter for 3 minutes.

Calculation of results

The following calculation was applied to results to give the specific activity of adenylate cyclase:-

1. The averaged background (BKG) counts (cpm) from each channel ($^{3}H$ and $^{32}P$) were subtracted from the average counts for the corresponding standards.

2. The degree of channel overlap was calculated by:-

$$\frac{^{3}H \text{ standard - BKG}}{^{32}P \text{ standard - BKG.}}$$

This value was approximately 0.007.

3. The efficiency of the columns to recover cAMP was calculated for each column as the percentage of the total [$^{3}H$] cAMP applied by:-

a. ($^{32}P$ sample cpm - $^{32}P$ BKG cpm) x overlap = Crossover cpm (C)

b. ($^{3}H$ sample - C) - $^{3}H$ BKG = corrected $^{3}H$ cpm

c. $\frac{\text{corrected } ^{3}H \text{ cpm}}{\text{Gross } ^{3}H \text{ cAMP added}} \times 100 = \%$ cAMP recovered

The recovery of cAMP was typically 90%

4. This value was then applied to each $^{32}P$ sample count to estimate the amount of $^{32}P$ cAMP in each sample:-

$$\frac{^{32}P \text{ sample - BKG}}{\% \text{ recovery}} \times 100 = \text{corrected } ^{32}P \text{ cpm}$$

5. From this the specific activity was calculated by:-

$$\frac{\text{corrected } ^{32}P \text{ cpm}}{\text{total } ^{32}P \text{ cpm}/10\mu l} \times \frac{10000(\text{pmol})}{30\text{min.mg protein}}$$

= pmol cAMP/min/mg protein

Assay variation

Intra-assay variation was established using a 1% (w/v) homogenate of 6 biopsies from different patients assayed with 8 replicates for basal and NaF stimulated activity. Five separate lots of 6 biopsies were assayed. The coefficient of variation (mean %±SEM) for basal activity was 10.31±2.24, and NaF stimulated activity was 19.00±7.26.

Inter-biopsy variation was measured with 6 separate 1% (w/v) biopsy homogenates from a single patient in duplicate. The inter-biopsy variation from 6 patients were assessed. The coefficient of variation (mean %±SEM) for basal activity was 25.46%±2.29, and NaF stimulated activity was 19.53%±2.90.

2.3.4 Calmodulin assay

The calmodulin content of the particulate preparations was assayed according to the phosphodiesterase activation method of Thompson et al. (1979). This assay was validated by my colleague Janet Amoah. It is principally a two step procedure, i.e

$$[8,^3H]cAMP \xrightarrow{PDE} [^3H]5'AMP \xrightarrow{5'\text{nucleotidase}} [^3H]\text{adenosine} + P_i$$

Samples containing the calmodulin were incubated with calmodulin-sensitive phosphodiesterase and then with 5'nucleotidase. The amount of labelled adenosine produced was separated from any unreacted [$^3$H]cAMP and measured by liquid scintillation counting.

To a previously prepared pellet of biopsy membranes was added 1.2ml of calmodulin homogenization buffer containing 40mM Tris-HCl, 100μM $CaCl_2$, 50mg/l phenylmethylsulfonyl fluoride (in DMSO) and 50μl/l pepstatin A. The pellet was resuspended with 30 strokes of a hand dounce homogenizer on ice. This homogenate (1ml) was heat treated in a 90°C water bath for 6 minutes, cooled in an ice bath and centrifuged for 10 minutes at 2000g at 4°C. The supernatant was passed through a 0.2μm sterile Millipore filter (Whatman UK). The calmodulin content of the filtrate was assayed. The remaining 200μl of resuspended membranes were set aside for protein determination (see section 2.3.6).

In a total reaction volume of 400μl, a 100μl of filtrate or calmodulin standards (1.25ng to 20ng) were incubated with 40mM Tris-HCl (pH7), 4mM 2-mercaptoethanol, 5mM $MgCl_2$, [$^3$H]cAMP (100000cpm), 100μM cAMP, 25μM $CaCl_2$ at 37°C in a shaking water bath for 15 minutes. The assay was initiated by the timed addition of 100μl

(1.5mU) phosphodiesterase. The reaction was terminated by immersing the tubes in a boiling water bath for 45 seconds at timed intervals. Blank tubes contained all the constituents of the reaction tubes but the phosphodiesterase had been previously boiled for 3 minutes and cooled before addition to the reaction mixture. All tubes were kept on ice between incubations.

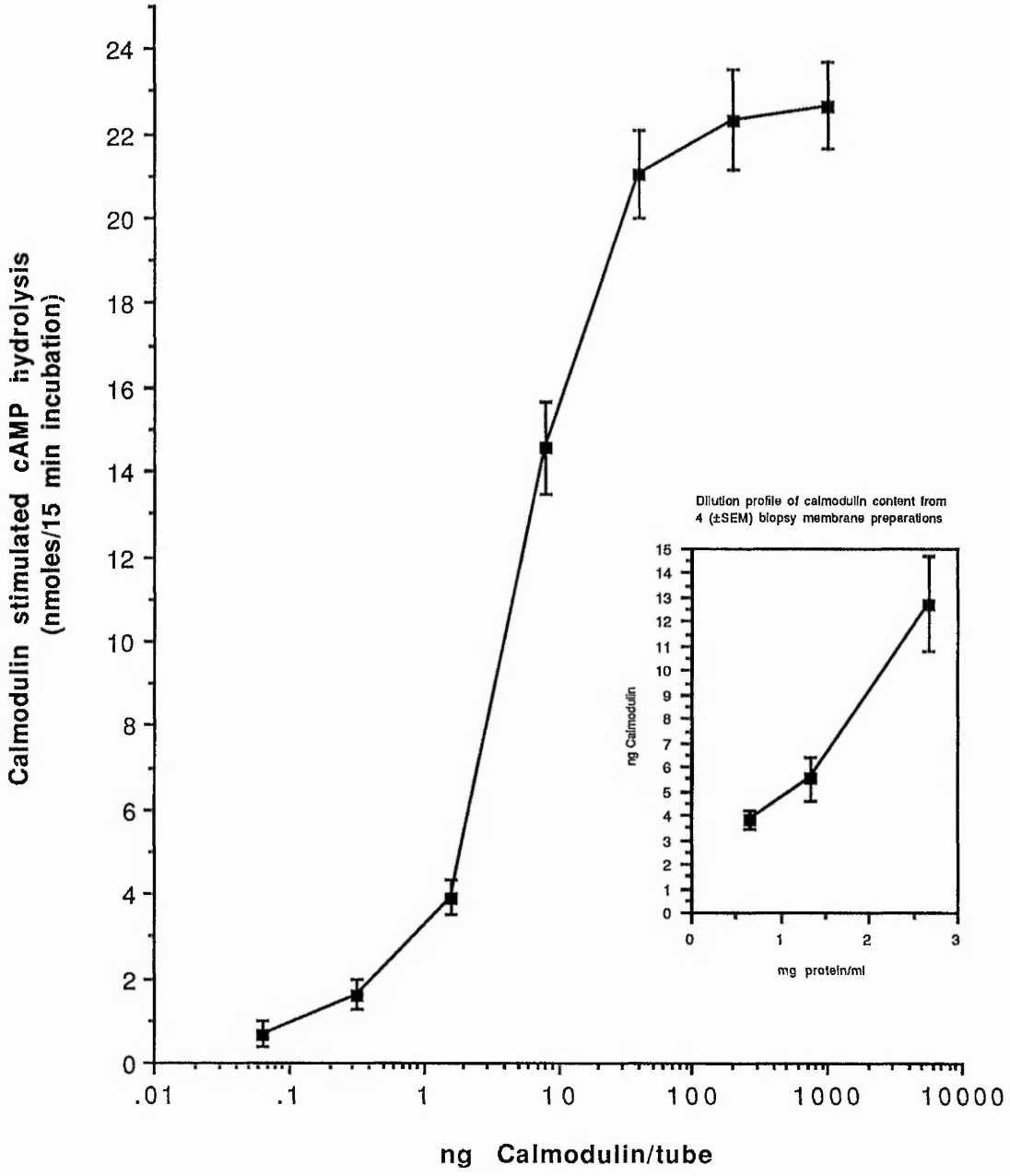
To all tubes 100µl of 1mg/ml 5'nucleotidase (*Crotalus atrox* venom) were added and incubated for a further 10 minutes at 37°C. This reaction was stopped by transferring tubes to an ice bath.

The unreacted [$^3$H]cAMP was removed with the addition of 1ml Dowex anion exchange resin (BioRad 1x8 200-400mesh) in methanol. The resin had previously been washed successively with 0.5M HCl, distilled water, 0.5M NaOH and distilled water until pH5. The resin was finally resuspended and stored in methanol. Tubes were vortexed and centrifuged at 2000g for 15 minutes. The supernatant was decanted directly into scintillation vials containing 10ml Optiphase X scintillation cocktail, vortex mixed and counted in a Packard TRI-CARB 4000 beta counter for 3 minutes. All treatments i.e. samples, calmodulin standards (CaM Std), and phosphodiesterase boiled blanks (PBB) were in triplicate determinations.

Calculation of results

The PBB cpm were deducted from sample cpm and CaM Std cpm and the standard curve plotted (**Figure 12**). The amount of calmodulin per sample tube was read from the standard curve and expressed as µg/mg protein.

**Figure 12 Calmodulin standard curve**




The mean of 4 (±SEM) calmodulin standard curves are shown. PDE activity in the absence of calmodulin was 4.01±1.74SEM nmoles cAMP hydrolysed/15min. Several dilutions of membrane samples were assayed and the dilution which gave cpm which fell on the straight part of the curve were used for calmodulin determinations (inset).

2.3.5 Disaccharidase assay

This assay was originally established as a routine diagnostic test for hypolactasia. The method used was that of Dahlqvist et al. (1970). Disaccharidases (maltase, sucrase and lactase) are brush border enzymes. Consequently their assay can be utilised to assess the distribution of the different membrane domains in biopsy preparation.

The assay is based on the hydrolysis of the substrates maltose, sucrose or lactose, by the corresponding disaccharidase, to glucose. The glucose generated was then assayed based on the reaction:-

$$\text{glucose} + O_2 + H_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-dianisidine} \xrightarrow{\text{peroxidase}} \text{o-dianisidine} + H_2O$$

reduced - colourless  oxidized - brown

Biopsy membranes were prepared as described in section 2.3.2 In addition, a sample of the original homogenate was removed and the supernatants pooled, for disaccharidase assay. Samples, standards and blanks were assayed in duplicate.

In a total reaction volume of 200µl, 100µl of sample (i.e. homogenate, supernatant or membranes) were incubated with 100µl of the substrates (0.056M) maltose, sucrose or lactose in 0.1M sodium maleate buffer, pH6 for 1 hour at 37°C. The reaction was initiated by the timed addition of substrate. The reaction was stopped with 3ml of a solution containing 0.5M Tris-HCl, pH7, 5µg/ml peroxidase, 100µg/ml o- dianisidine, 0.2% triton X-100 (dissolved in 95% ethanol) and 10U/ml

glucose oxidase. This reagent was added to standards containing 5μg to 100μg glucose/200μl tissue buffer and blank tubes of tissue buffer only. It also initiated the colour developing reaction which took a further 1 hour incubation at 37°C. The optical density was read at 530nm with a Gilford spectrophotometer 260.

Calculation of results

The standard curve was plotted (**Figure 13**) and the glucose content of the samples estimated from the curve. Results were calculated as U/mg protein from :-

$$10 \times \frac{\mu g \text{ glucose}}{\text{MW glucose(180)}} \times \frac{1}{60\text{min}} \times \frac{1}{n} \times \text{dilution}$$

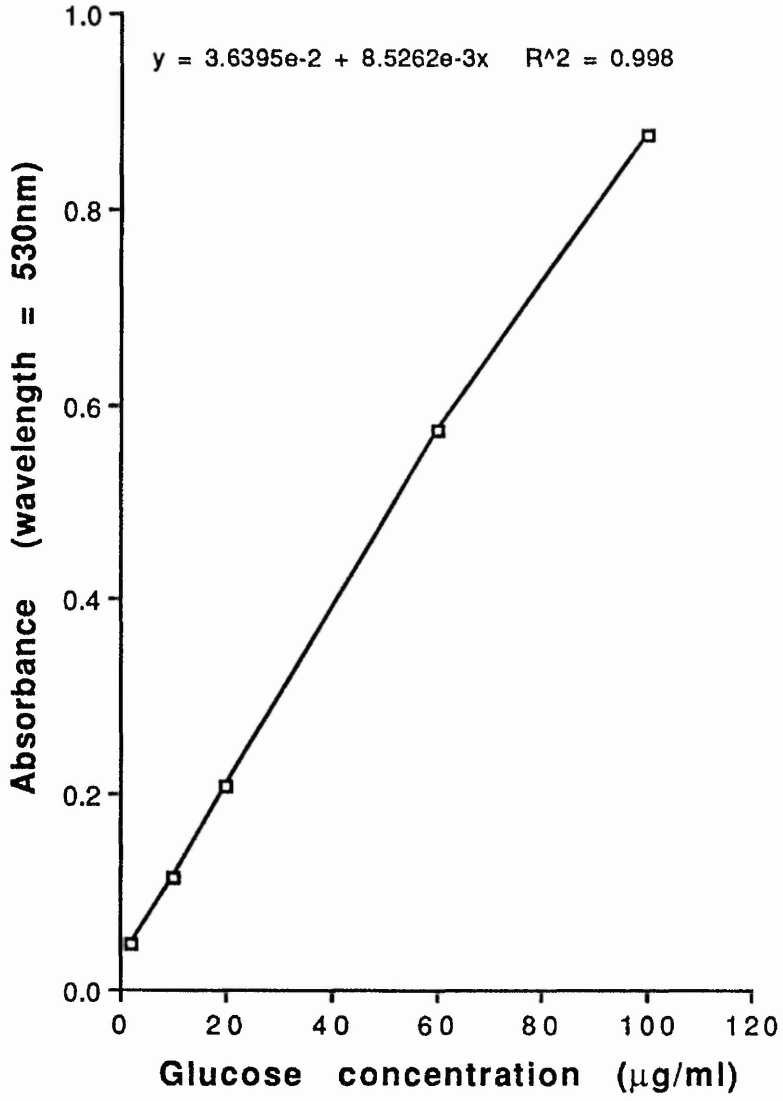
where n = number of glucose molecules liberated i.e. maltose n=2, sucrose and lactose n=1. 1U is equivalent to 1μmol disaccharide hydrolysed per minute

Assay variation

Intra-assay variation was established using a 1% (w/v) homogenate of 6 biopsies from different patients assayed with 12 replicates. Four separate lots of 6 biopsies were assayed. The coefficient of variation (mean %±SEM) for maltase was 5.92±2.66, sucrase 5.23±0.57 and lactase 4.33±1.45.

Inter-biopsy variation was measured with 6 separate 1% (w/v) biopsy homogenates from a single patient in duplicate. The inter-biopsy variation from 5 patients was assessed. The coefficient of variation (mean %±SEM) for maltase was 17.66±4.52, sucrase 32.45±10.12 and lactase 19.45±5.67.

**Figure 13 Glucose standard curve**



2.3.6 Protein determinations

The protein content of the tissue preparations and standards (bovine serum albumin) were determined in duplicate by the method of Lowry et al. (1951). Standards were always suspended in the appropriate tissue buffer and ranged from 25μg/ml to 400μg/ml (**Figure 14**).

Standards and samples (200μl) were incubated at room temperature in 2ml of a solution containing 0.01% (w/v) SDS, 2% (w/v) $Na_2CO_3$, 0.1N NaOH, 0.5% (w/v) $CuSO_4$ and 1.0% (w/v) Na-K tartrate. After 10 minutes 200μl of 1N Folin and Ciocalteu's Phenol reagent was added, vortexed and incubated for a further 30 minutes at room temperature. The optical density was read at 650nm with a Gilford spectrophotometer 260.

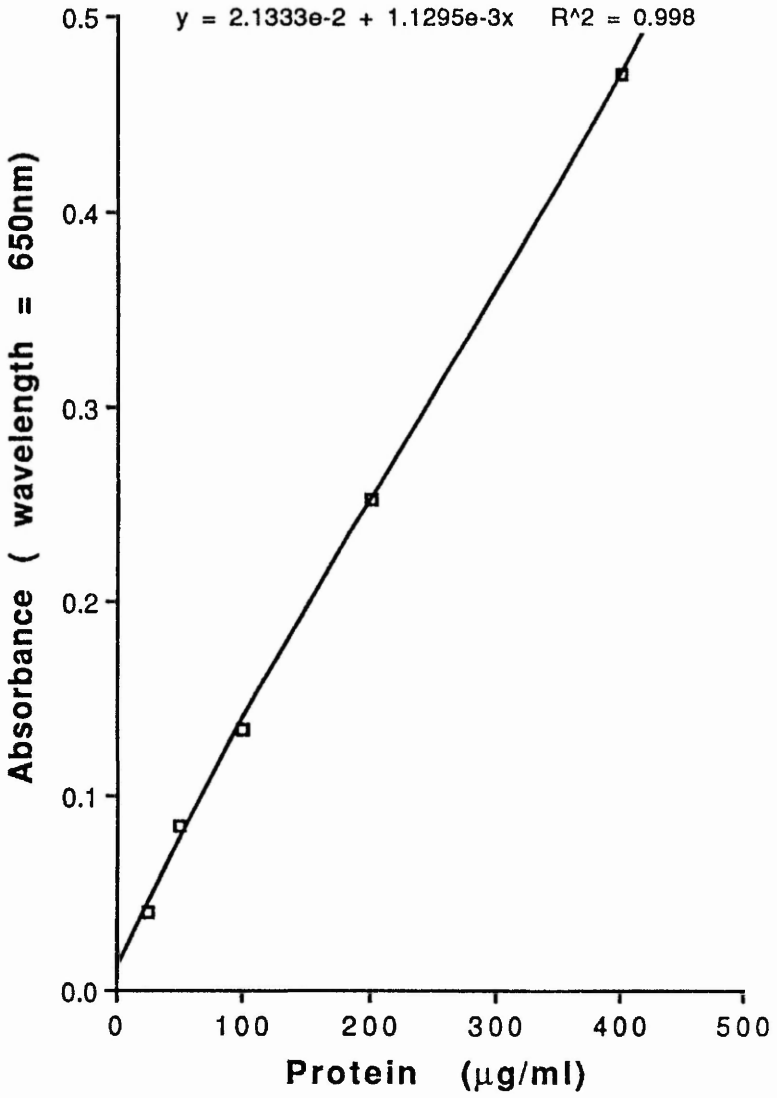
The protein content of the membrane preparations was approximately 1.6mg/ml.

Assay variation

Intra-assay variation was established using a 1% (w/v) homogenate of 6 biopsies from different patients assayed with 12 replicates. Six separate lots of 6 biopsies were assayed. The coefficient of variation (mean %±SEM) was 8.21±2.86.

Inter-biopsy variation was measured with 6 separate 1% biopsy homogenates from a single patient in duplicate. The inter-biopsy variation from 7 patients were assessed. The coefficient of variation (mean %±SEM) was 17.46±3.54.

**Figure 14 Protein standard curve**



2.3.7 Estimation and calculation of free $Ca^{2+}$ concentrations

Contamination of buffered solutions by $Ca^{2+}$ ( in some cases up to 20μM) necessitates the use of the divalent chelator EGTA in controlling and estimating free $Ca^{2+}$ concentration (Dinjus et al 1984; Segal 1986).

For each $CaCl_2$ addition the free $Ca^{2+}$ concentration was computed for ambient pH7 in the presence of $Mg^{2+}$, EGTA and ATP using an updated version of the iterative ligand-metal binding program of Feldman et al. (1972) and Yaseen et al. (1982), kindly provided by Prof. M. Griffin. **Table 6** gives the apparent dissociation constants used by the program given the assay conditions used.

Measuring free $Ca^{2+}$ in the micromolar range and below may be possible using the $Ca^{2+}$ fluorescence indicator Fura-2. Fura-2 exhibits a left shift in peak excitation spectra (nM) to shorter wavelengths in the presence of free $Ca^{2+}$ that is proportional to the $Ca^{2+}$ concentration but independent of Fura-2 concentration (Rao et al. 1985; Grynkiewicz et al. 1985). This characteristic of Fura-2 was utilised in preference to the changes in fluorescence because addition of protein to Fura-2 has a quenching effect on the fluorescent signal.

The contaminating level of free $Ca^{2+}$ was therefore estimated in MH tissue buffer and in a bulk volume of the adenylate cyclase reaction mixture (RM)(see section 2.32). Excitation scans of 2ml of MH or RM containing 5μM Fura-2 were performed in 1cm light path, 4ml fluorimetric cuvettes (Hughes and Hughes) in a Kontron SFM 25 spectrofluorimeter at 37°C. The emission wavelength was set at 505nm. A standard curve of $Ca^{2+}$ concentration against peak excitation wavelength was established using MH tissue buffer containing 0.3mM

EGTA and $CaCl_2$ to give calculated free $Ca^{2+}$ concentrations ranging from 0.00788μM to 104.5μM (**Figure 15**). From the curve the $Ca^{2+}$ contamination in the MH buffer was estimated to be 1.4μM and in the RM was 2.5μM.

**Table 6 Apparent dissociation constants used to calculate free $Ca^{2+}$ concentrations (μM) at ambient pH7**

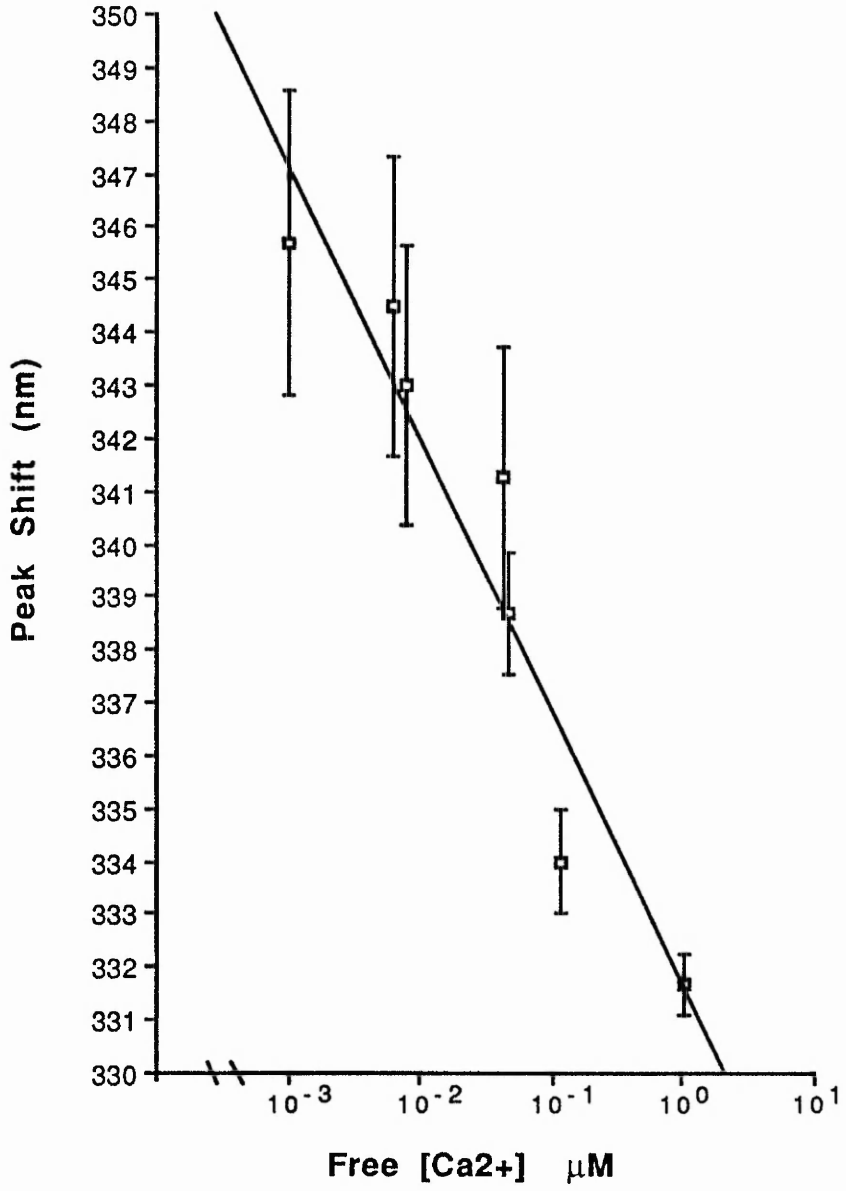
| | $Ca^{2+}$ | $Mg^{2+}$ |
|---|---|---|
| EGTA | 0.207 | 24702 |
| ATP | 173 | 97 |

The $Ca^{2+}$ binding capacity of the biopsy particulate membrane preparations was also estimated using this system and compared to that of EGTA. Six membrane preparations were resuspended in 2ml MH containing 5μM Fura-2 and excitation scanned as described above. For comparison 2ml MH and MEH buffers containing 0.3mM, 1mM and 2.5mM EGTA were also excitation scanned.

2.3.8 Statistics

The one factor ANOVA-repeated measures test was used to test for significance. Probability values $\leq 0.05$ were taken as significant.

**Figure 15 Standard curve of free $Ca^{2+}$**




The mean±SD of 3 separate preparations of $Ca^{2+}$ standards are shown against the shift in peak excitation wavelength (nM). Free $Ca^{2+}$ concentrations were calculated using the ligand - metal binding program of Feldman et al. (1972). The correlation coefficient for shift in peak excitation wavelength and free $Ca^{2+}$ concentrations was 0.955.

## 2.4 RESULTS

### 2.4.1 Characteristics of particulate membrane preparations from human duodenal biopsies

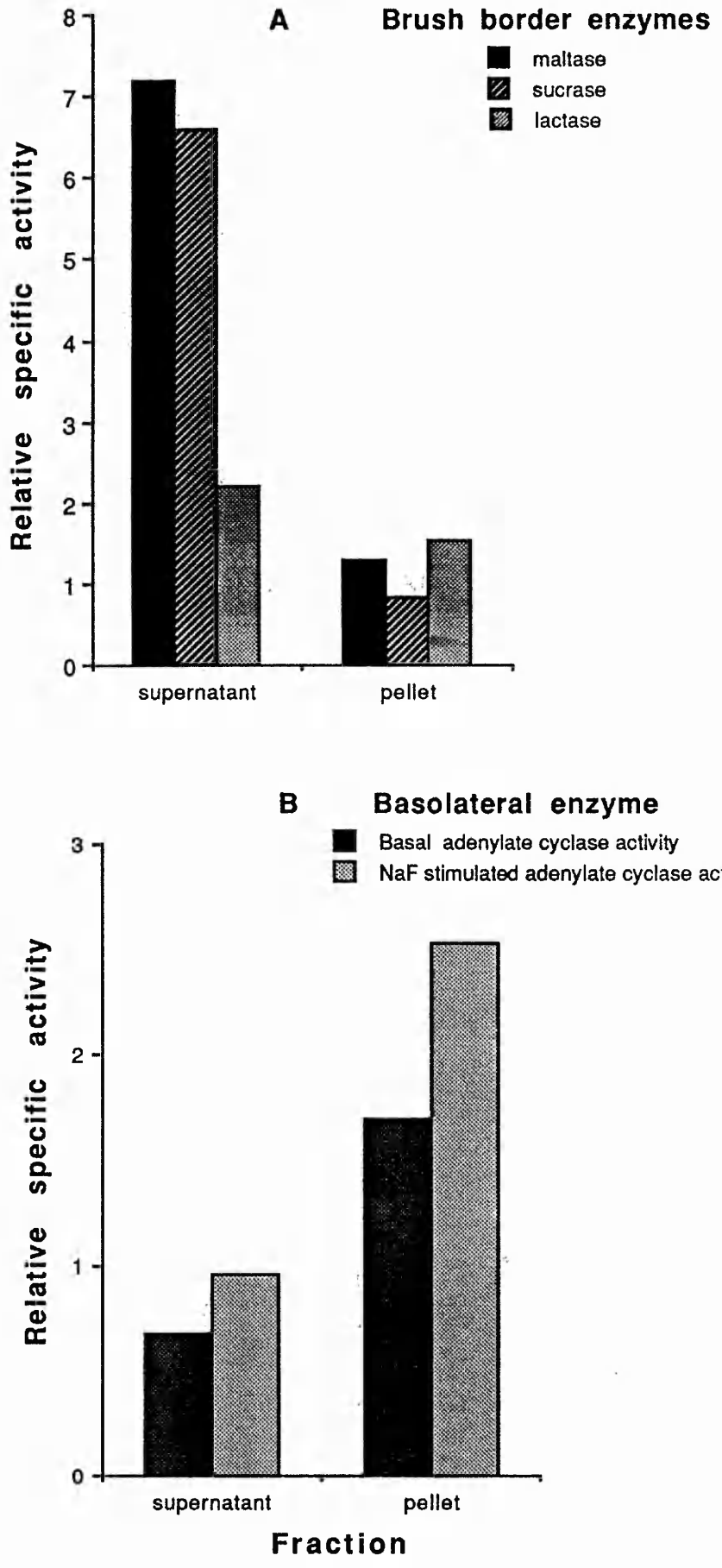
To establish that no adenylate cyclase was being lost by the membrane washing procedure and that there was no undue contamination of the membrane preparation with brush border membranes, the distribution of brush border disaccharidase activity and basolateral membrane adenylate cyclase activity was assessed in the whole homogenate, the supernatant and the membrane pellet.

The relative specific activity of maltase, sucrase and lactase was compared with that of adenylate cyclase in pooled supernatants and particulate preparations from three independent tissue preparations assayed twice. Disaccharidase activity was enriched in the pooled supernatants. Mean (±SEM) enrichment was 7.18±2.31, 6.6±0.87 and 2.21±0.60 times for maltase, sucrase and lactase, respectively (**Figure 16A**). Mean (±SEM) basal and NaF stimulated adenylate cyclase activity was enriched in the particulate fraction by 1.69±0.32 and 2.53±0.09, respectively (**Figure 16B**). There was no enrichment of adenylate cyclase activity in the supernatant. The final protein concentration of the pellet represented 15% of the original homogenate.

**Figure 16 Enzyme distributions in particulate membrane preparations of duodenal biopsies**

The distribution of maltase, sucrase and lactase activity in 3 membrane preparations assayed twice in duplicate is shown, (A). Basal and NaF stimulated adenylate cyclase activity (B) was measured in the same preparations under the standard assay conditions outlined in section 2.3.3.

$$\text{Relative specific activity} = \frac{\%\ \text{enzyme activity relative to homogenate}}{\%\ \text{protein relative to homogenate}}$$

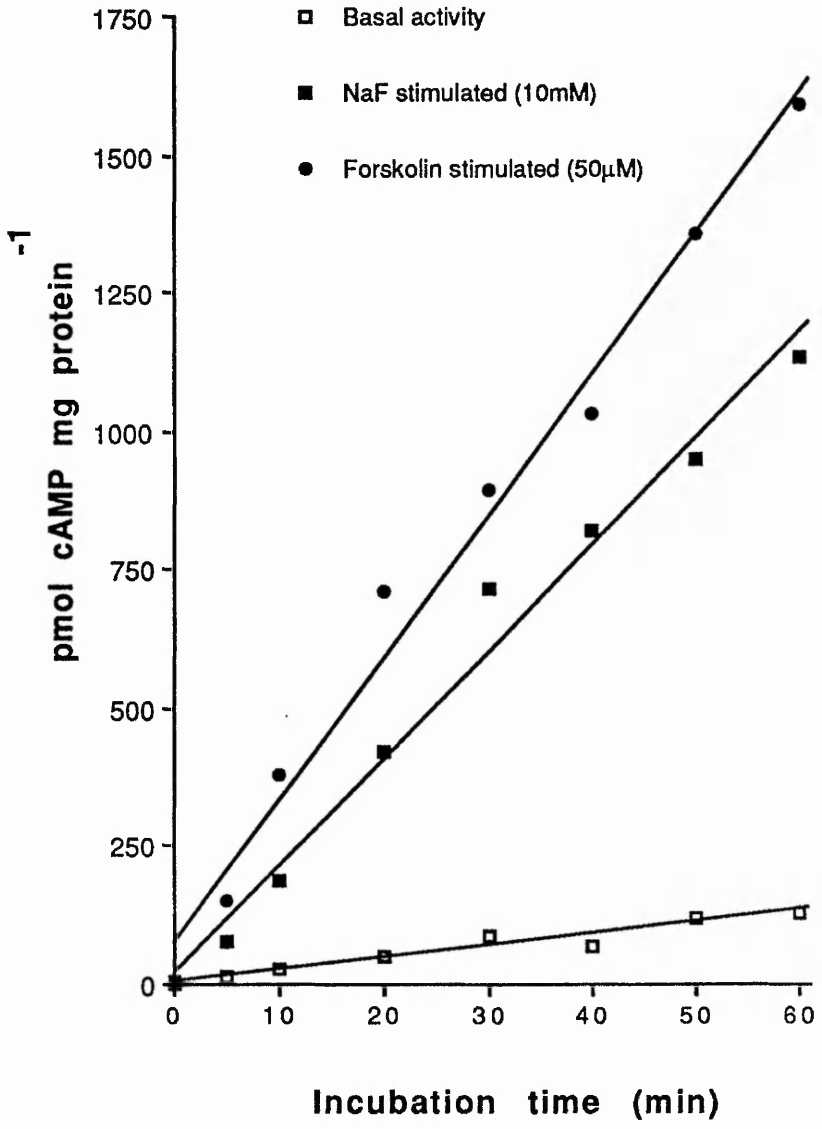

A
Brush border enzymes
maltase
sucrase
lactase
Relative specific activity
0
1
2
3
4
5
6
7
8
supernatant
pellet
B
Basolateral enzyme
Basal adenylate cyclase activity
NaF stimulated adenylate cyclase activity
Relative specific activity
0
1
2
3
supernatant
pellet
Fraction

2.4.2 Adenylate cyclase assay conditions

The adenylate cyclase assay was performed under conditions where the accumulation of cAMP was found to be linear with incubation time (**Figure 17**) and to the amount of enzyme used (**Figure 18**). Other parameters such as assay pH (**Figure 19**), $Mg^{2+}$ concentration (**Figure 20**) and ATP concentration were also measured (**Figure 21**). The assay conditions, detailed in section 2.3.3, were establish for chick duodenal adenylate cyclase activity, and were found to be appropriate for the measurement of adenylate cyclase activity in particulate membranes from human duodenal biopsies, (Dr RG Long personal communication).

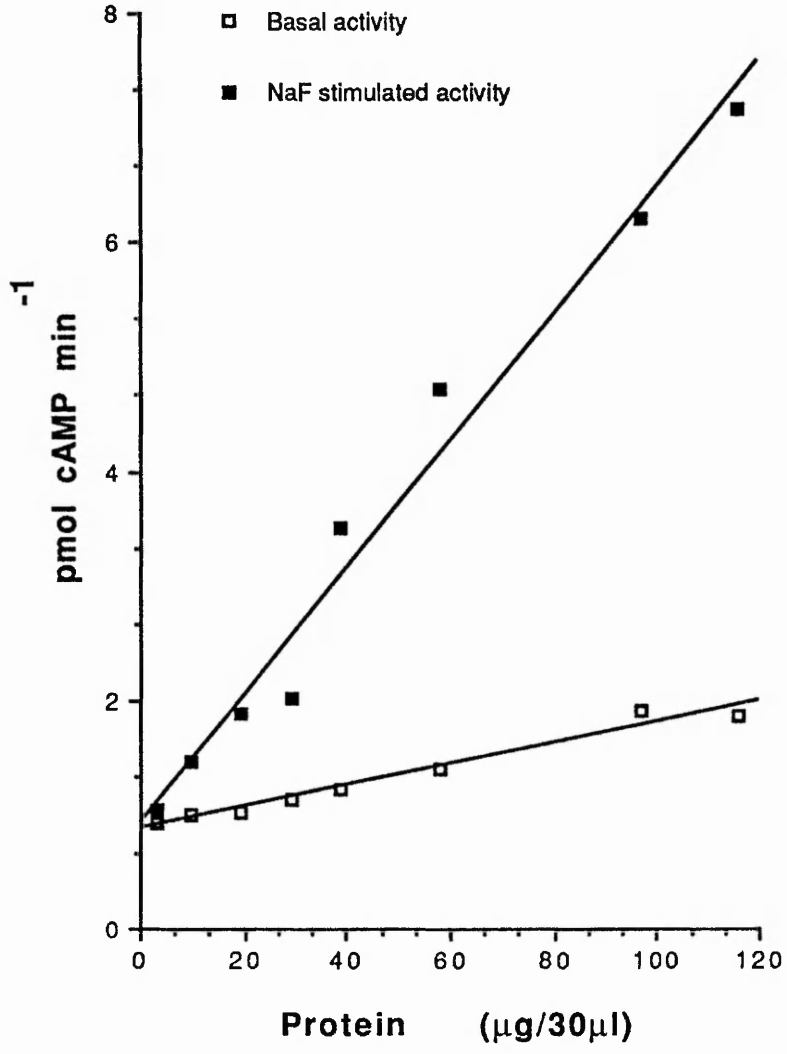
NaF is a non-specific activator of G protein activity and was used throughout the assay of adenylate cyclase activity as a positive control at 10mM. **Figure 22** shows the concentration response of adenylate cyclase activity to NaF. The shape of the curve was like that thought to be indicative of NaF stimulated calmodulin-sensitive adenylate cyclase activity (Brostrom et al. 1977).

**Figure 17 Adenylate cyclase activity with incubation time**



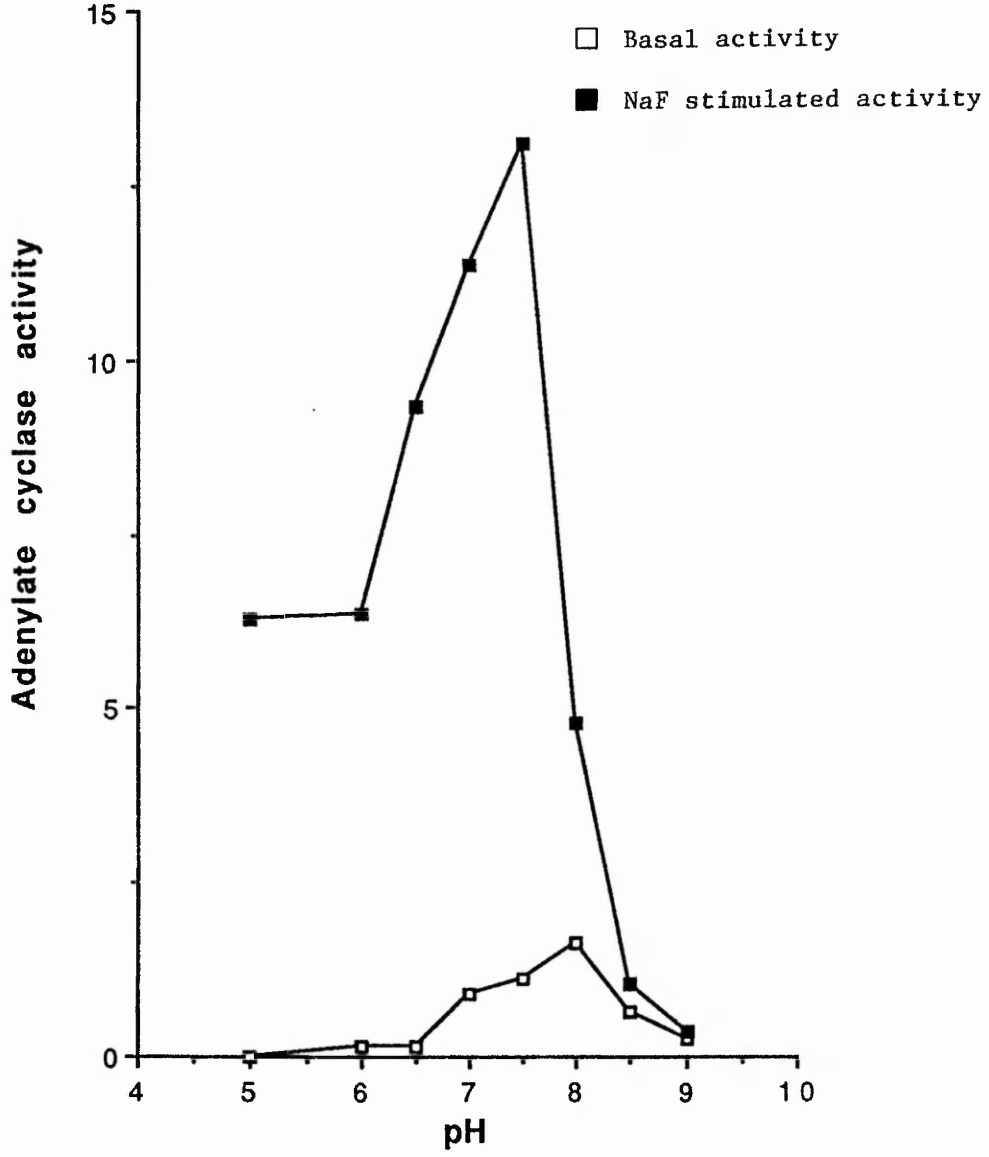

Basal, NaF and forskolin stimulated adenylate cyclase activity was measured under the standard assay conditions given in section 2.3.3. 100μl of bulk reaction mixtures were taken at different incubation time points and transferred directly into 100μl 'stopping solution'. cAMP production was linear up to the maximum time point studied (60 minutes).

**Figure 18 Adenylate cyclase activity with increasing membrane protein**



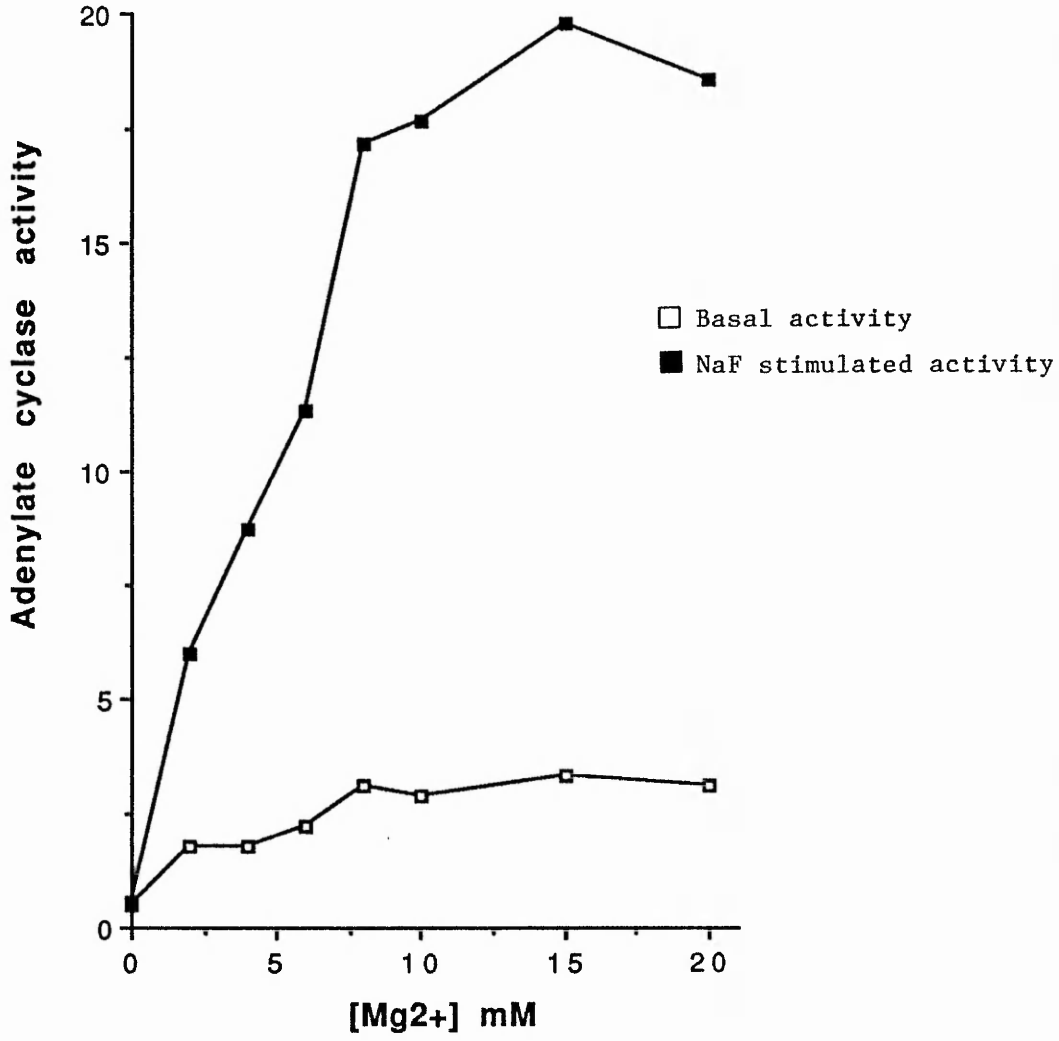

Basal and NaF stimulated adenylate cyclase activity from 2 different membrane preparations were assayed at varying concentrations with the standard assay conditions given in section 2.3.3. Membrane protein was assayed according to the method described in section 2.3.6. Adenylate cyclase activity was proportional to membrane protein. The mean (±SEM) protein content of membrane preparations used in the adenylate cyclase studies described throughout this section was 50.98µg/30µl±4.13 (n=18)

**Figure 19 Adenylate cyclase activity with changing pH**



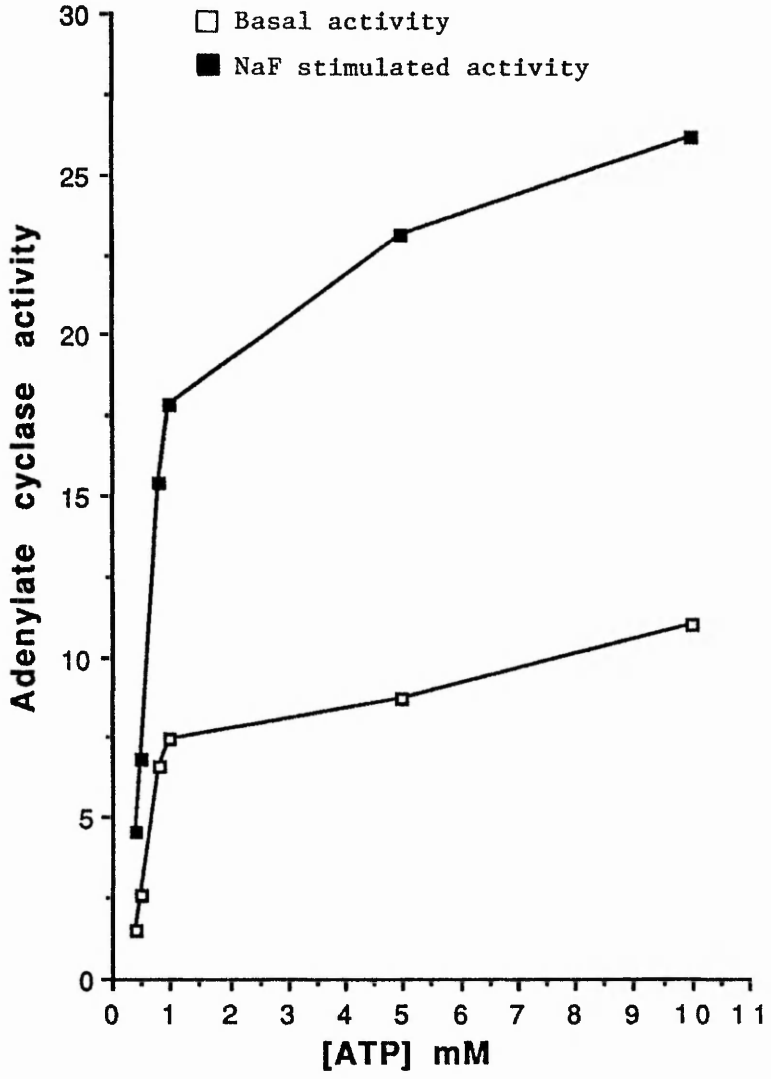

Basal and NaF stimulated adenylate cyclase activity (pmol cAMP $min^{-1}$ mg $protein^{-1}$) in a single membrane preparation (with four replicates) was assayed in the presence of increasing pH. Other assay conditions were those given in section 2.3.3. Maximum enzyme activity was reached between pH7 and pH8.

**Figure 20 Adenylate cyclase activity with increasing $Mg^{2+}$ concentration**



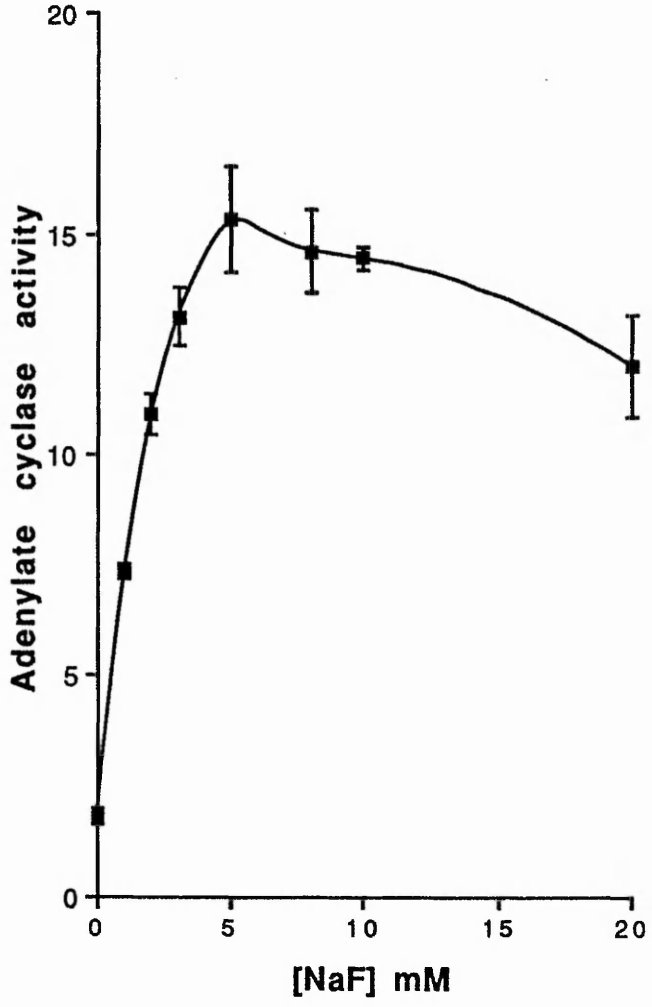

Basal and NaF stimulated adenylate cyclase activity (pmol cAMP $min^{-1}$ mg $protein^{-1}$) in a single membrane preparation was assayed (with 4 replicates) in the presence of the concentrations of $MgCl_2$ indicated on the abscissa. Other assay conditions were those given in section 2.3.3. Maximum enzyme activity was reached by approximately 10mM $Mg^{2+}$.

**Figure 21 Adenylate cyclase activity with increasing ATP concentration**




Basal and NaF stimulated adenylate cyclase activity (pmol cAMP $min^{-1}$ mg $protein^{-1}$) was assayed with increasing concentrations of ATP in the presence of excess $Mg^{2+}$ (15mM) under the otherwise standard assay conditions given in section 2.3.3. A single membrane preparation was assayed with four replicates. A plateau of enzyme activity was reached at approximately 1mM ATP.

**Figure 22 Adenylate cyclase response to changing NaF concentrations**




Adenylate cyclase activity (pmol cAMP $min^{-1}$ mg $protein^{-1}$) was assayed in the presence of increasing concentrations of NaF. The mean±SEM enzyme activity is shown for 2 different membrane preparations. Maximal stimulation of adenylate cyclase activity was given by 10mM NaF under the standard assay conditions given in section 2.3.3.

section 2.3.3.

2.4.3 The effects of $Ca^{2+}$ on adenylate cyclase activity

Stimulation of adenylate cyclase activity at low free $Ca^{2+}$ concentrations has been reported to be an indication of the enzyme's calmodulin dependence, MacNeil et al (1985). Therefore, the concentration dependent effects of $Ca^{2+}$ on adenylate cyclase activity in particulate preparations of human duodenal biopsies was measured. Both basal ($IC_{50}$= 193.75nM ± 57.5nM SEM) and NaF stimulated ($IC_{50}$=188.0nM ± 44.0nM SEM) adenylate cyclase activity was strongly inhibited by free $Ca^{2+}$ concentration greater than 90nM (**Figure 23**). A Free $Ca^{2+}$ concentration less than 90nM neither stimulated nor inhibited adenylate cyclase activity. These data indicate a dramatic reduction in adenylate cyclase activity over a narrow free $Ca^{2+}$ concentration range but do not show any consistent stimulation by $Ca^{2+}$ at submicromolar concentrations characteristic of calmodulin stimulation.

An alternative way of assessing the importance of $Ca^{2+}$ was to measure the concentration dependent effects of the $Ca^{2+}$ chelating agent, EGTA, on adenylate cyclase activity (**Figure 24**). NaF stimulated adenylate cyclase activity was inhibited by EGTA in a concentration dependent manner with 50% inhibition at 2.5mM. Attempts to restore EGTA inhibited adenylate cyclase activity with $Ca^{2+}$ did not result in a recovery of the enzyme's activity indicating direct inhibition of adenylate cyclase activity by EGTA (**Figure 28**). A slight but highly significant increase in basal adenylate cyclase activity was observed at 0.25mM EGTA compared to controls; thereafter a plateau was reached up to the maximum concentration tested (5mM).

**Figure 23 Adenylate cyclase response to changing free $Ca^{2+}$ concentrations**

Basal (A) and NaF stimulated (B) adenylate cyclase activity (pmol cAMP $min^{-1}$ mg $protein^{-1}$) was assayed in particulate preparations of duodenal biopsies from 4 patients according to the method described in section 2.3.3. Enzyme activity was measured in the presence of 2.5mM EGTA and $CaCl_2$ at concentrations which gave calculated free $Ca^{2+}$ concentrations as indicated on the abscissa. Each line represents a single patient and each data point is the mean of 4 replicates. Controls were in the absence of added $CaCl_2$ and in the presence of EGTA.

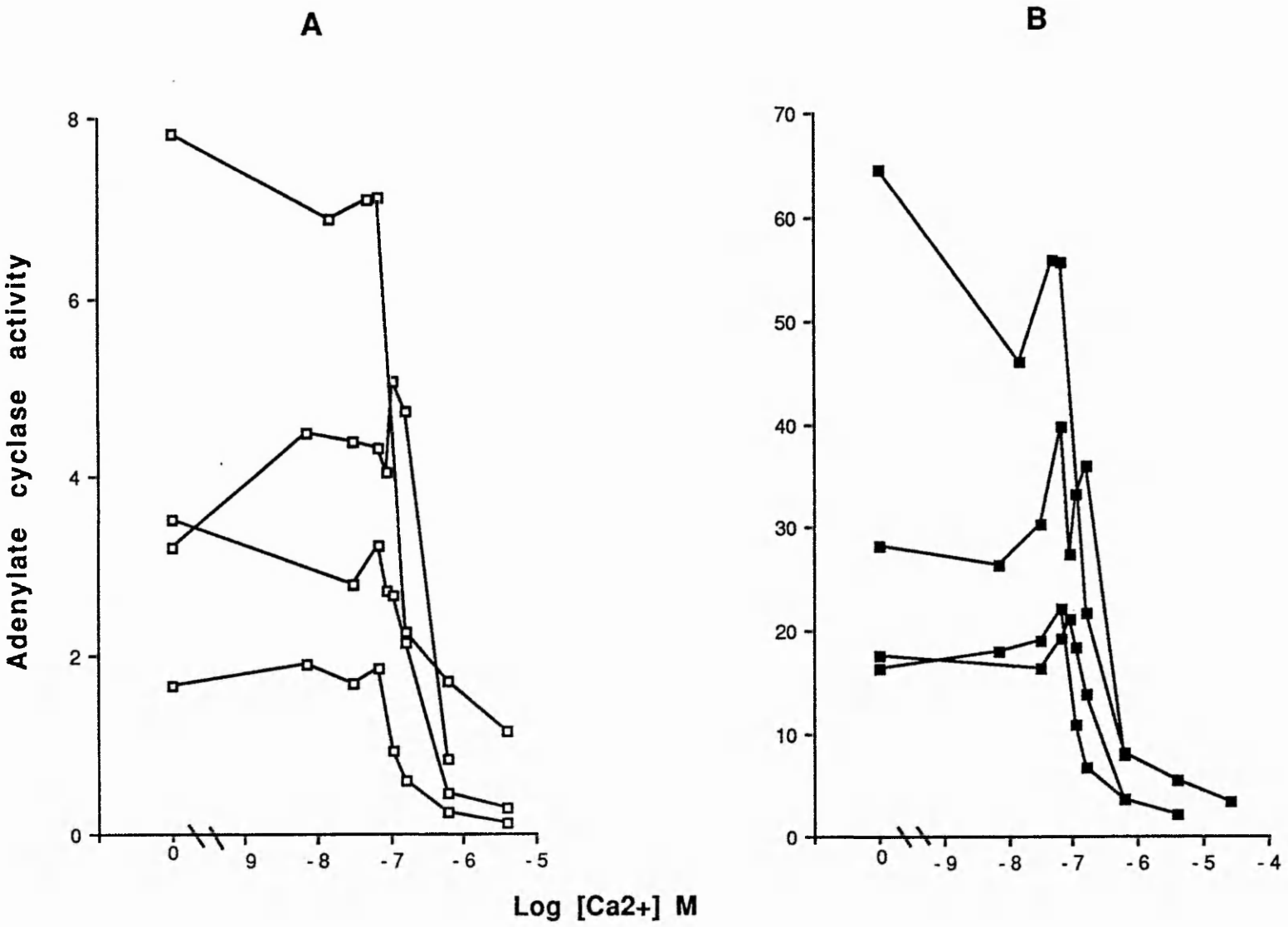
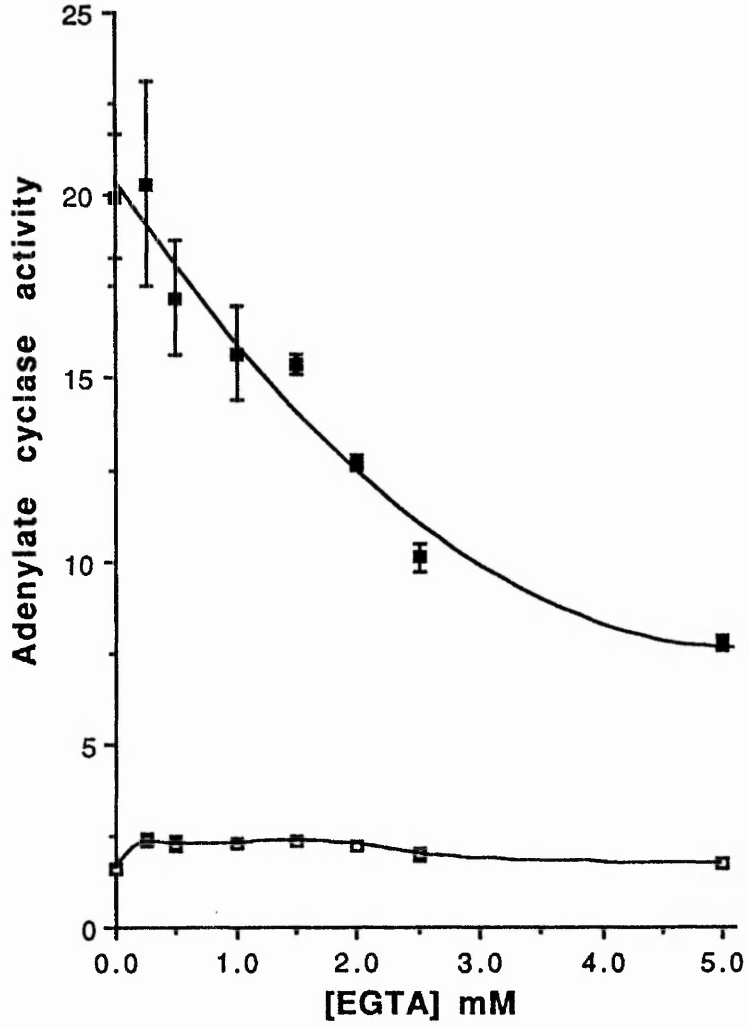

A
B
Adenylate cyclase activity
8
6
4
2
0
70
60
50
40
30
20
10
0
0
9
-8
-7
-6
-5
0
-9
-8
-7
-6
-5
-4
Log [Ca2+] M

**Figure 24 Adenylate cyclase response to changing EGTA concentrations**




Particulate preparations of duodenal biopsies were assayed for NaF stimulated (■) and basal (□) adenylate cyclase activity (pmol cAMP $min^{-1}$ mg $protein^{-1}$) in the absence of added $CaCl_2$ and in the presence of EGTA at the concentrations indicated. Data are the mean±SEM from three experiments (i.e 3 patients) each with 4 replicates.

2.4.4 The effects of exogenous calmodulin on adenylate cyclase activity

The removal of calmodulin by washing membranes with EGTA buffers and the subsequent stimulation of adenylate cyclase activity by adding calmodulin back to the assay system has been demonstrated for a number of tissues. No stimulation could be demonstrated in the results from similar experiments using human duodenal biopsy particulate preparations. The addition of calmodulin failed to stimulate adenylate cyclase activity in the presence of various combinations of free $Ca^{2+}$, calmodulin and EGTA, **Table 7** . None of the assay conditions shown resulted in a significant stimulation of adenylate cyclase activity over controls in the presence or absence of 10mM NaF. The small increases in activity apparent in some of the $Ca^{2+}$/calmodulin treatments may be attributed to the $Ca^{2+}$ buffering capacity of the calmodulin, removing some of the inhibiting $Ca^{2+}$ from the system. This effect has been interpreted as calmodulin stimulation by other authors eg Pinkus et al. (1983) in guinea-pig enterocytes and Panchenko et al. (1984) in rabbit heart plasma membranes. At no point did the data indicate any significant increase in activity in any of the treatments compared to no added $Ca^{2+}$ or calmodulin.

It was possible that the above assay conditions were missing the critical $Ca^{2+}$ concentration to detect stimulation of enzyme activity. Also the concentration of EGTA (2.5mM) used in the assay may have been interfering with calmodulin- sensitive adenylate cyclase activity (**Figure 23**). Therefore a concentration response to $Ca^{2+}$ in the presence and absence of 8.5μg/ml exogenously added calmodulin was conducted with an EGTA concentration of 0.3mM (**Figure 25**).

Again these data failed to demonstrate any significant stimulation of adenylate cyclase activity by calmodulin.

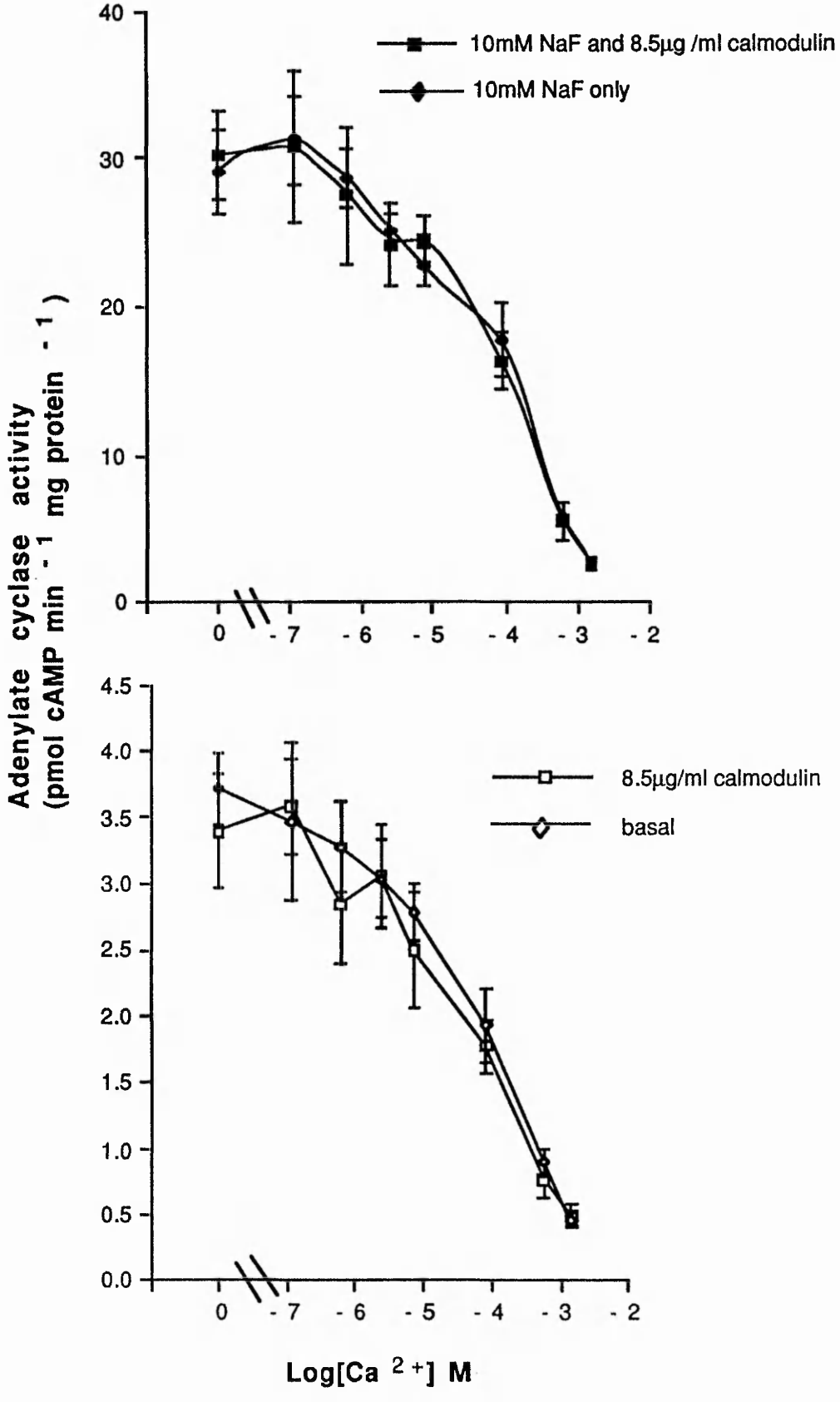
**Table 7 The effect of exogenously added calmodulin on adenylate cyclase activity**

| Added $[Ca^{2+}]$ mM | [calmodulin] µM | [EGTA] mM | calculated free $[Ca^{2+}]$ pCa | Adenylate cyclase activity pmol cAMP $min^{-1}$ mg protein $^{-1}$ basal | NaF stimulated |
|---|---|---|---|---|---|
| 0.16 | 0.15 | 2.5 | 7.76 | 3.20(0.29) | 19.88(1.07) |
| 0.16 | 0 | 2.5 | 7.62 | 2.85(0.24) | 20.77(0.76) |
| 1.16 | 0.15 | 2.5 | 6.52 | 0.54(0.04) | 4.22(0.32) |
| 1.16 | 0 | 2.5 | 6.52 | 0.51(0.05) | 3.50(0.32) |
| 0 | 0 | 2.5 | - | 3.40(0.17) | 20.60(0.49) |
| 0.80 | 3.13 | 2.5 | 6.78 | 3.72(0.03) | 14.98(0.28) |
| 0.80 | 0 | 2.5 | 6.78 | 3.04(0.08) | 13.33(0.45) |
| 0 | 0 | 2.5 | - | 4.25(0.04) | 14.74(0.51) |
| 0.40 | 3.73 | 1.0 | 6.63 | 1.36(0.06) | 11.74(0.12) |
| 0.40 | 0 | 1.0 | 6.63 | 1.88(0.03) | 9.90(0.26) |
| 0 | 0 | 1.0 | - | 2.34(0.05) | 18.73(0.72) |

Basal and NaF stimulated adenylate cyclase activity in particulate membrane preparations of duodenal biopsies was assayed by the method described in section 2.3.3. membranes were washed 3 times in MEH buffer containing 5mM EGTA and finally resuspended in MEH which gave the final assay concentrations of EGTA indicated. Free $Ca^{2+}$ concentrations were calculated using the ligand binding program of Feldman et al. (1972) (section 2.4.1). Data are the results of 3 separate experiments with 4 replicates.

**Figure 25 Adenylate cyclase concentration response to $Ca^{2+}$ in the presence of exogenously added calmodulin**

NaF stimulated and basal adenylate cyclase activity was assayed in particulate preparations of duodenal biopsies. Membranes were washed 3 times in MEH buffer containing 5mM EGTA and finally resuspended in MEH containing 1mM EGTA. Activity was in the presence of 0.3mM EGTA, 8.5µg/ml calmodulin and $CaCl_2$ at concentrations which gave calculated free $Ca^{2+}$ concentration as indicated on the abscissa. Each line represents the mean±SEM of 3 determinations (i.e. 3 patients) in duplicate. Zero $Ca^{2+}$ levels were in the absence of added $CaCl_2$ and in the presence of EGTA.

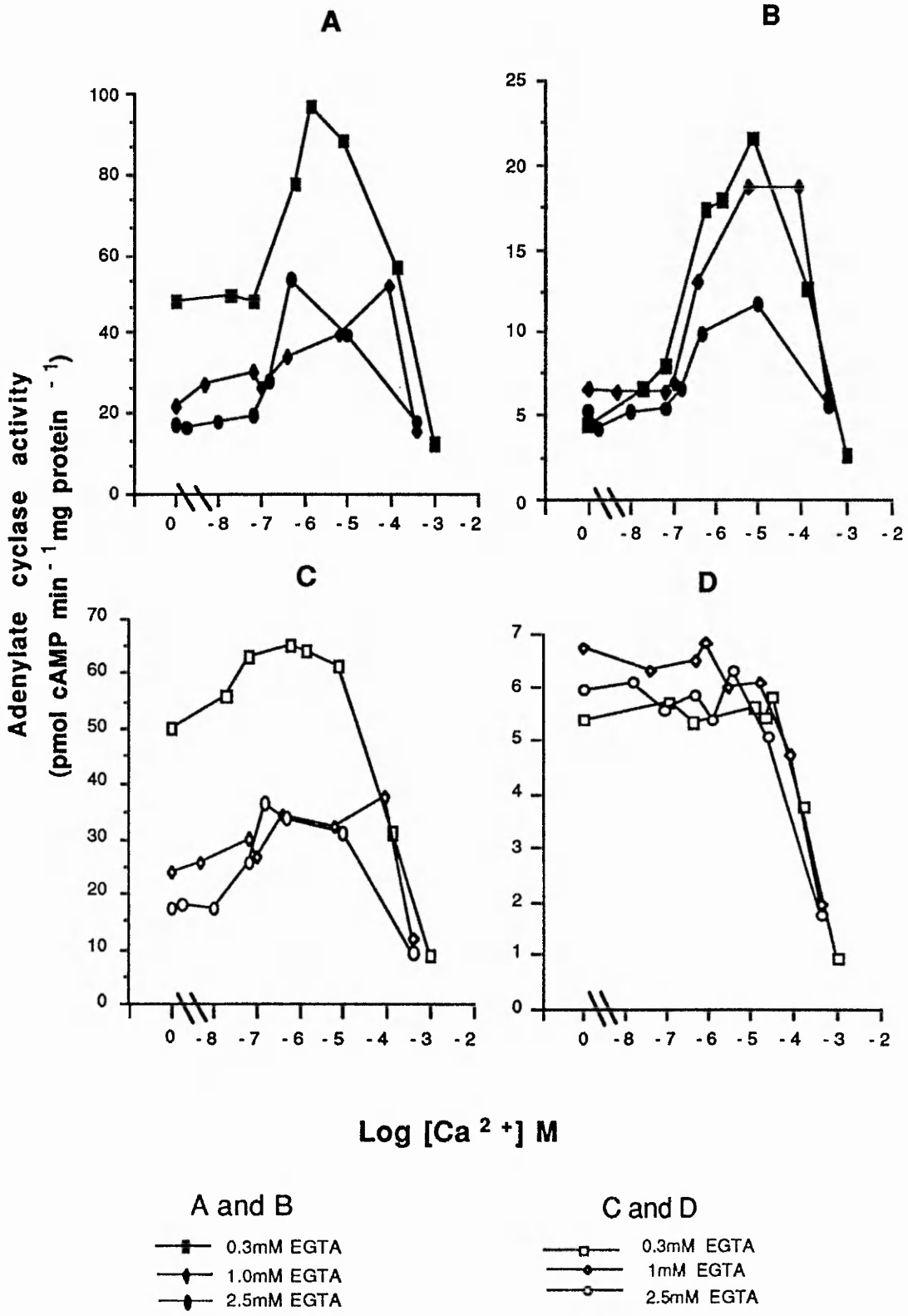

10mM NaF and 8.5µg /ml calmodulin
10mM NaF only
8.5µg/ml calmodulin
basal
Adenylate cyclase activity
(pmol cAMP min - 1 mg protein - 1 )
Log[Ca 2 +] M
40
30
20
10
0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
- 7
- 6
- 5
- 4
- 3
- 2

2.4.5 Verification of assay techniques using particulate membrane preparations of rat cerebral cortex

The possibility existed that the assay methods were in some way incapable of detecting calmodulin dependent adenylate cyclase activity. Adenylate cyclase activity was therefore measured in a tissue well recognized to have a highly active calmodulin-sensitive component i.e. rat cerebral cortex (Shattuck et al. 1987). The preparation and conditions of assay for rat cerebral cortex were conducted in exactly the same way as that for human duodenal biopsies. This tissue clearly demonstrated the following characteristics; a) a biphasic response to $Ca^{2+}$ (activation followed by inhibition) with a maximum requirement for free $Ca^{2+}$ of 1-10μM (**Figure 26A, B and C**); b) the calmodulin content of the preparation was reduced by 25% by the EGTA washing procedure employed and this was sufficient to prevent $Ca^{2+}$ activation of adenylate cyclase (**Figure 26D**); c) the addition of 10μg/ml calmodulin to EGTA washed particulate preparations stimulated adenylate cyclase activity by 30-60% in the presence and absence of 5mM NaF (**Figure 26A and B**). All these characteristics are evident irrespective of the EGTA concentration used but high EGTA concentrations (i.e. 1mM and 2.5mM) inhibited stimulated enzyme activity. These findings are fully compatible with what is known for brain calmodulin-sensitive adenylate cyclase (Shattuck et al. 1987; Boyajian et al. 1991). Verification of the assay methods used was thus established.

**Figure 26 Calmodulin activation of rat cerebral cortex adenylate cyclase activity**

Adenylate cyclase activity was assayed in a particulate preparation of 2 rat cerebral cortices under the following conditions:- A. 10μg/ml calmodulin + 5mM NaF, B. 10μg/ml calmodulin, C. 5mM NaF and D. No addition. $CaCl_2$ was added at concentrations which gave calculated free $Ca^{2+}$ concentrations as indicated on the abscissa. 0.3mM, 1mM and 2.5mM EGTA were used to control free $Ca^{2+}$ concentration. Each point represents the mean of duplicate determinations.


A
B
C
D
Adenylate cyclase activity
(pmol cAMP min -1 mg protein -1)
Log [Ca 2 +] M
A and B
0.3mM EGTA
1.0mM EGTA
2.5mM EGTA
C and D
0.3mM EGTA
1mM EGTA
2.5mM EGTA

2.4.6 The effectiveness of EGTA to remove calmodulin from particulate membrane preparations

The calmodulin content of the EGTA washed particulate preparations were compared with that of preparations washed in the absence of EGTA to assess the effectiveness of the EGTA washing procedure at removing calmodulin. Calmodulin was assayed according to the method described in section 2.3.4.

In the brain tissue there was a 25% (7.49 μg/mg protein to 5.61μg/mg protein) reduction in calmodulin after washing with EGTA containing buffer. The calmodulin content of the biopsy membrane preparations was 3.89±1.50 μg calmodulin/mg protein for EGTA washed membranes compared with 3.59±1.17 μg calmodulin/mg protein for washed membranes in which EGTA was omitted from the buffers. Results are the mean±SEM of 3 different membrane preparations. The calmodulin content of the particulate preparation from human duodenal biopsies was therefore the same irrespective of whether the preparation had been washed in EGTA containing buffer or not. Furthermore, the calmodulin content was approximately 50% lower in the duodenal preparation compared to that of the brain tissue.

2.4.7 The effects of calmodulin antagonists on adenylate cyclase activity

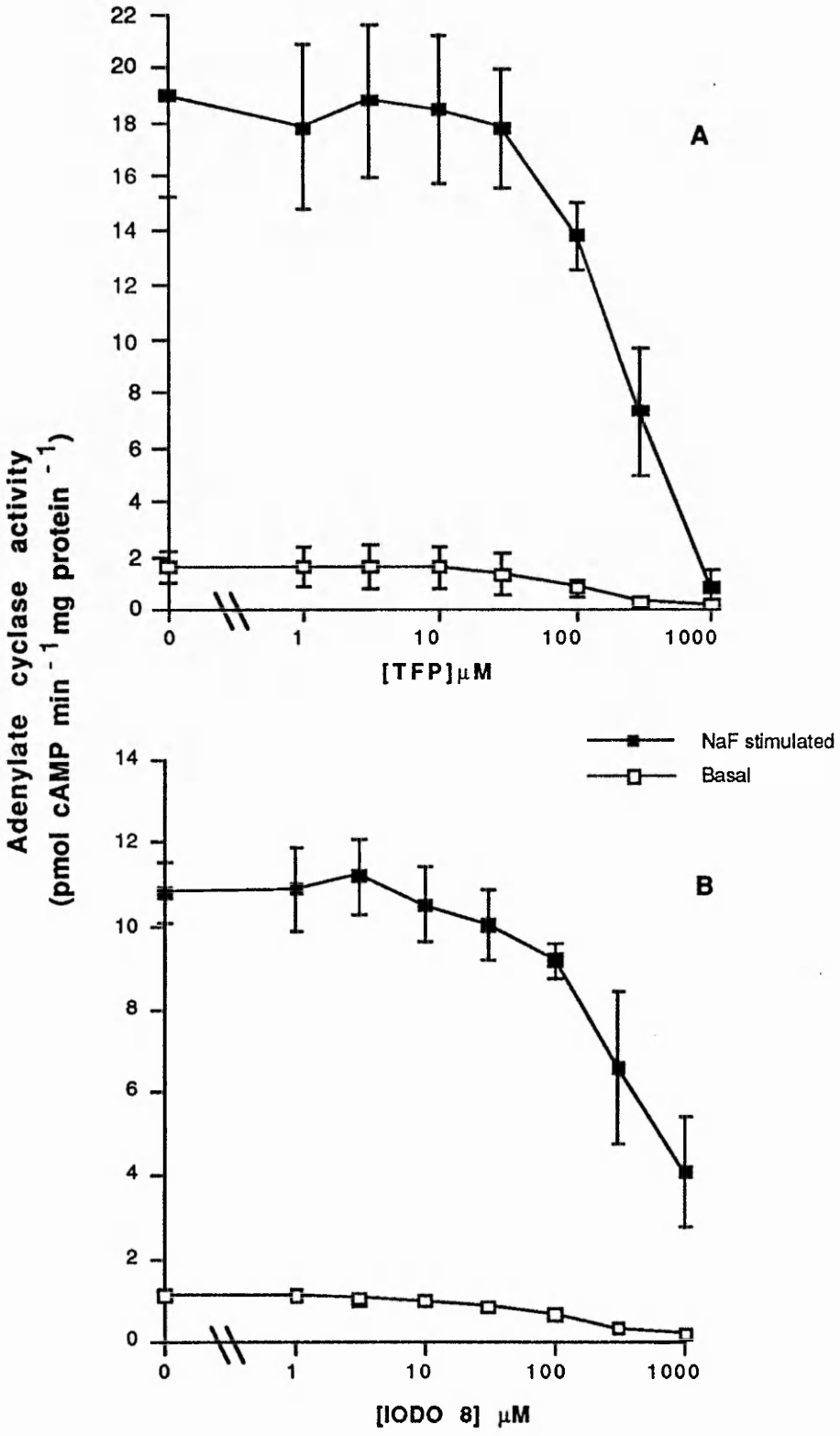
The concentration dependent effects of trifluoperazine (TFP) and IODO 8 were examined to establish at what concentration these calmodulin antagonists exerted an influence on adenylate cyclase activity and further to compare the effects of the relatively nonspecific TFP with that of the more specific IODO 8 (MacNeil et al. 1988). By analysis of variance TFP (**Figure 27A** ) and IODO 8 (**Figure 27B**) did not significantly inhibit basal and NaF stimulated adenylate cyclase activity up to a

concentration of 100μM. The $IC_{50}$ for TFP of NaF stimulated adenylate cyclase activity was 225μM and for basal activity was 125μM. However, corresponding $IC_{50}$ values for IODO 8 were 450μM and 175μM indicating that the NaF stimulated adenylate cyclase activity was inhibited less potently than basal activity. This also shows that IODO 8 is less potent than TFP at directly inhibiting adenylate cyclase activity.

The inhibition of basal activity as well as stimulated adenylate cyclase activity at concentrations of this magnitude represent the nonspecific effects of these calmodulin antagonists (Minocherhomjee et al. 1988).

**Figure 27 Adenylate cyclase response to changing TFP and IODO 8 concentrations**

Biopsy particulate preparations were assayed for NaF stimulated and basal adenylate cyclase activity in the presence of background $Ca^{2+}$ and the TFP (A) and IODO 8 (B) concentrations indicated. No attempt was made to remove endogenous calmodulin from these preparations. TFP and IODO 8 were dissolved in DMSO and diluted to the appropriate concentration with adenylate cyclase assay buffer (see section 2.3.3) Data are the mean±SEM for 3 separate patients with 4 replicates for each calmodulin antagonist concentration.


Adenylate cyclase activity
(pmol cAMP min -1 mg protein -1)
A
[TFP]μM
NaF stimulated
Basal
B
[IODO 8] μM

## 2.5 DISCUSSION

A number of investigators have demonstrated that $Ca^{2+}$ inhibits the activity of adenylate cyclase at concentrations greater than 1μM while at submicromolar $Ca^{2+}$ concentrations activation of the enzyme occurs (Brostrom et al. 1977, 1982; Bradham and Cheung 1980; Potter et al. 1980; Cheung and Storm 1982; Laso et al 1984; MacNeil et al. 1985; Resink et at. 1986; Boyajian et al. 1991). It is thought that this activation of adenylate cyclase is mediated by calmodulin but the inhibition of the enzyme by $Ca^{2+}$ is calmodulin independent and occurs though specific inhibitory site on the catalytic unit (Hanski et al. 1977; Lasker et al. 1982; MacNeil et al. 1985; Oldham et al. 1984, 1986; Boyajian et al. 1991).

The data presented in section 2.4 suggests that human duodenal adenylate cyclase activity is affected by very small changes in free $Ca^{2+}$ concentration but unlike the intestinal adenylate cyclase from rat (Amiranoff et al. 1983) guinea pig (Pinkus et al. 1983) and rabbit (Lazo et al. 1984) it was not calmodulin-sensitive. Evidence to support this statement comes from a number of the findings.

Firstly, it was not possible to detect any consistent $Ca^{2+}$ stimulation of adenylate cyclase activity of a magnitude previously reported in other systems (Bradham and Cheung 1980; Amiranoff et al. 1983; Lazo et al. 1984; MacNeil et al. 1985). The detection of $Ca^{2+}$ stimulation of adenylate cyclase is notoriously difficult because it occurs over a very low and narrow $Ca^{2+}$ concentration range. Therefore, careful manipulation of free ion concentrations by EGTA buffers is necessary (Dinjus et al. 1984; Segal 1986).

The inhibition of adenylate cyclase activity by EGTA implies a requirement of the enzyme for $Ca^{2+}$. This effect was only apparent on the NaF stimulated adenylate cyclase activity which initially suggests that $Ca^{2+}$/calmodulin may be important in the regulation of the stimulated enzyme. Furthermore fluoride ions, which stimulate adenylate cyclase activity by direct activation of G proteins, may also help to distinguish the two forms of adenylate cyclase. *In vitro* the calmodulin-sensitive form of the enzyme is more responsive to fluoride stimulation than is the insensitive form (MacDonald 1975; Brostrom et al. 1977). However, the effects of EGTA could not be reversed by $Ca^{2+}$ (see section 2.6.3). This is an indication that the chelator may have been inhibiting the enzyme directly and not in a way mediated by $Ca^{2+}$ chelation and calmodulin inactivation (Shattuck et al. 1987; Minocherhomjee et al. 1988).

Secondly, it was not possible to stimulate adenylate cyclase activity, in EGTA washed particulate preparations, with exogenously added calmodulin. This may be explained by the inadequate removal of the endogenous membrane bound calmodulin by EGTA containing buffers. Indeed, the calmodulin content of the particulate preparations from duodenal biopsies was not significantly reduced by the EGTA washing procedure. However, the levels of calmodulin in the assay system were comparable to those of other workers using animal intestinal membranes and some have shown calmodulin dependence in the presence of endogenous calmodulin at concentrations similar to those in the present assay system (Amiranoff et al. 1983).

The effective removal of calmodulin from different tissues has been reported to vary considerably (Shattuck et al. 1987; Minocherhomjee et al. 1988; MacNeil et al. 1985). The data suggest there may also be

species differences between the same tissue, an observation that has been noted for other tissues e.g, heart sarcoplasmic reticulum (Cros et al. 1984) kidney (Sulimovici et al. 1984) and pancreatic islets (Thams et al. 1982).

Alternatively the discrepancy in the data between the animal studies and the current results may be because there are differences in the calmodulin regulation of adenylate cyclase activity along the length of the gut. In the animal studies cells were isolated from a length of small intestine whereas in this work tissue was collected from a more specific region of the gut (i.e. the duodenum).

Recent evidence on the distribution of calmodulin in enterocytes indicated that, in the human duodenum, calmodulin was confined to the soluble fractions and to the brush border membrane fractions; no calmodulin was detected in the basolateral membrane fraction (Stoll et al. 1987). This provides indirect support for the current findings in that the distribution of human duodenal adenylate cyclase (a basolateral membrane enzyme) is not coincidental with the distribution of membrane bound human duodenal calmodulin. Interestingly, the distribution of calmodulin in the membranes of rat enterocytes appears to be different from that of human enterocytes. Charpin et al. (1986) reported that in rat duodenal basolateral membrane fractions calmodulin could be detected.

It is conceivable that both brush border membranes and basolateral membranes were present in the assay and that the tightly bound brush border membrane calmodulin became available to influence the basolateral membrane adenylate cyclase. This is a distinct possibility because the microvillus core responds dramatically to $Ca^{2+}$ -

it self destructs - a process called core solation (Powell and Fan 1984). This process happens because of the $Ca^{2+}$ dependent severing action of the core protein villin on actin filaments within the microvilli of the bush border (Mooseker et al. 1984).

However, if this were so, and a fully calmodulin stimulated adenylate cyclase system was being measured, it should have been possible to block the calmodulin effect with a calmodulin antagonist, at concentrations specific for calmodulin, and this was not the case. The $IC_{50}$ for TFP and IODO 8 were well in excess of those reported for other calmodulin dependent enzymes (6-50μM and 3-10μM respectively) (MacNeil et al. 1988; Roufogalis 1985; van Os and Ghijsen 1983).

In combination these data indicate that there is not a calmodulin-sensitive form of adenylate cyclase in the human duodenal mucosa.

Further discussion on the effects of calmodulin antagonist is given in the results of experiments on cells isolated from duodenal biopsies in section 3.5.

## 2.6 RESULTS

### 2.6.1 The effects of EGTA on the inhibition of adenylate cyclase activity by $Ca^{2+}$

The use of the $Ca^{2+}$ chelating agent, EGTA, is an established method for the *in vitro* control of free $Ca^{2+}$ concentrations in enzyme assay systems (Cros et al. 1984; Dinjus et al. 1984; Miller and Smith 1984; Segal 1986; Harrison et al. 1989). It is also used to deplete membrane preparations of $Ca^{2+}$ and/or calmodulin (LeDonne and Coffee 1980; Glenney et al. 1980, 1985; Pinkus et al. 1983; MacNeil et al. 1985). The concentration at which EGTA is used varies considerably from micromolar (Sedlmeier and Dieberg 1983; Amiranoff et al. 1983; Oldham et al. 1984, 1986; Sulakhe and Hoehn 1984; Sulakhe 1985) through to millimolar concentrations (Resink et al. 1986; Segal 1986; Dorflinger et al. 1984; Long et al. 1986; Piascik et al. 1983).

The inhibition of adenylate cyclase activity by EGTA, in brain tissue, is an indication the enzyme has a calmodulin sensitive component and thus a requirement for $Ca^{2+}$. The effective EGTA concentration that inhibits adenylate cyclase activity is subject to variability depending on the source of the tissue under study (MacDonald 1975; Brostrom et al. 1977; Sedlmeier 1983; Sulimovici et al. 1984; Thams et al. 1984; Oldham et al. 1984, 1986; Cros et al. 1984; Segal 1986). The direct effects of EGTA have also been described (Monneron and d'Alayer 1980; Simonin et al. 1980; Sulakhe and Hoehn 1984; Sulakhe 1985).

In the course of the $Ca^{2+}$ studies just described, in section 2.4, it was observed that there appeared to be a shift in the concentration of free $Ca^{2+}$, that inhibited adenylate cyclase activity, depending on the controlling EGTA concentration used. Furthermore, EGTA

inhibited NaF stimulated enzyme activity but stimulated basal activity. In the next series of experiments the effects of EGTA were examined more closely.

The $Ca^{2+}$ concentration response of adenylate cyclase activity in biopsy particulate membranes was repeated with different controlling levels of EGTA. The $Ca^{2+}$ concentration at which adenylate cyclase activity was inhibited by 50% ($IC_{50}$) was deduced from the curves obtained (**Table 8**)

The results show that the free $Ca^{2+}$ concentration, that inhibited adenylate cyclase activity, decreased as the EGTA concentration was increased, i.e. EGTA shifts the adenylate cyclase concentration response to free $Ca^{2+}$ to the left. This effect was observed in both basal and NaF stimulated adenylate cyclase activity. Furthermore this shift was not apparent in membranes prepared from rat cerebral cortex indicating that the lowest EGTA concentration (0.3mM) used was adequate to buffer $Ca^{2+}$. It is also noteworthy that the $Ca^{2+}$ $IC_{50}$ for the brain tissue is at least an order of magnitude greater than the duodenal tissue. In duodenal membranes the same pattern of response was seen irrespective of whether they had been previously washed in EGTA or not.

**Table 8 The effect of EGTA on the inhibition of adenylate cyclase activity by $Ca^{2+}$**

| [EGTA] mM | $IC_{50}$ for basal activity µM | $IC_{50}$ for NaF stimulated activity µM |
|---|---|---|
| 0.0 | 51.32±15.77(n.d) | 62.82±3.76 (n.d) |
| 0.3 | 42.19±10.59(231) | 31.05±0.57(223) |
| 1.0 | 1.50±0.14(237) | 2.99±0.70(275) |
| 2.5 | 0.19±0.057(147) | 0.19±0.04(257) |

n.d = not determined

Basal and NaF stimulated adenylate cyclase activity was assayed in particulate membranes from duodenal biopsies. Three $Ca^{2+}$ concentration response curves were conducted at each of the EGTA concentrations shown. For each curve the $IC_{50}$ was determined and the mean±SEM $IC_{50}$ is shown. For comparison the values in parenthesis are equivalent data from the rat cerebral cortex particulate preparation and were derived from the curves in **Figure 26** .

2.6.2 The $Ca^{2+}$ binding capacity of the particulate membrane preparation

A possible explanation for the left shift effect, just described, is that the membrane preparation has a high intrinsic $Ca^{2+}$ binding capacity; a possibility which might be expected of a tissue that has a significant physiological role in the uptake of $Ca^{2+}$ from the gut lumen. However, experiments using the fluorescent indicator Fura-2 suggest this was not the case. Duodenal biopsy membranes (1.6mg/ml) added to MH tissue buffer containing Fura-2, (in which the contaminating $Ca^{2+}$ concentration was approximately 1μM) caused a shift in the peak excitation spectra to the right (i.e longer wavelength, mean 332.67nm±0.81 SD, n=6) equivalent to a free $Ca^{2+}$ concentration of 0.7μM. EGTA (0.3mM,1.0mM or 2.5mM), on the other hand, caused a shift in the peak excitation spectra equivalent to a reduction in the free $Ca^{2+}$ concentration to 0.003μM (excitation peak = 344nm, n=3). Therefore, the $Ca^{2+}$ binding capacity of the membranes was negligible compared to that of EGTA.

2.6.3 The effect of EGTA on adenylate cyclase activity in the presence and absence of a constant concentration in free $Ca^{2+}$

To investigate further the effect of EGTA, the concentration dependence of adenylate cyclase activity to EGTA in the absence and presence of a constant amount of free $Ca^{2+}$ (1μM) was measured under basal and various stimulating conditions (**Figure 28A-C**). Forskolin (50μM), NaF (10mM) and GMP-PNP (1μM) stimulated enzyme activity approximately 7, 8 and 12 fold above basal respectively without EGTA and /or $Ca^{2+}$. **Figure 28** shows $Ca^{2+}$ did not reverse the effects of EGTA and therefore the inhibitory effects of EGTA were not mediated by $Ca^{2+}$/calmodulin. Furthermore, in the presence of 1μM free $Ca^{2+}$

adenylate cyclase activity was inhibited by EGTA irrespective of which component of the adenylate cyclase system was stimulated i.e. forskolin stimulated catalytic unit or NaF stimulated G proteins. Further the EGTA concentration response data represents a cross section through the $Ca^{2+}$ concentration response at 1μM $Ca^{2+}$ and therefore indicates that the left shift was evident irrespective of stimulus.

In the absence of a constant level of free $Ca^{2+}$, the different components of the adenylate cyclase system no longer exhibit the same pattern of response. Basal adenylate cyclase activity was initially stimulated; thereafter, there was no further effect (**Figure 28A**). The concentration response of forskolin stimulated adenylate cyclase activity showed a similar but enhanced pattern to that of basal adenylate cyclase activity (**Figure 28B**). However, the NaF stimulated response showed the same pattern to that of the EGTA concentration response with 1μM $Ca^{2+}$, only less marked (**Figure 28C**). These data may indicate that the effects of $Ca^{2+}$/EGTA were mediated through the catalytic component but that EGTA exerts an effect directly on the NaF activated G protein that was independent of $Ca^{2+}$.

The experiment was repeated using GMP-PNP, an alternative to NaF for stimulating G protein mediated adenylate cyclase activity. The results showed that the pattern of response was similar to that of basal and forskolin stimulated activity (**Figure 29**).

This result demonstrates that the EGTA stimulation effect observed for basal, forskolin and GMP-PNP stimulated adenylate cyclase activity was reversed by NaF and that the NaF stimulated enzyme's response to EGTA mimics that of the EGTA effect in the presence of $Ca^{2+}$.

**Figure 28 Adenylate cyclase response to changing EGTA concentrations in the presence and absence of 1μM free $Ca^{2+}$**

Adenylate cyclase activity (pmol cAMP $min^{-1}$ mg $protein^{-1}$) concentration response to EGTA in the presence of no added $CaCl_2$ (□) and 1μM free $Ca^{2+}$ (■). Basal (A), 50μM forskolin (B) and 10mM NaF (C) stimulated activities are shown in particulate membranes from human duodenal biopsies. Data are the mean±SEM of at least 3 separate membrane preparations, assayed in triplicate.

* indicates significance at the 95% level compared to no EGTA

** indicates significance at the 99% level compared to no EGTA

+ indicates significance at the 95% level compared to 0.3mM EGTA

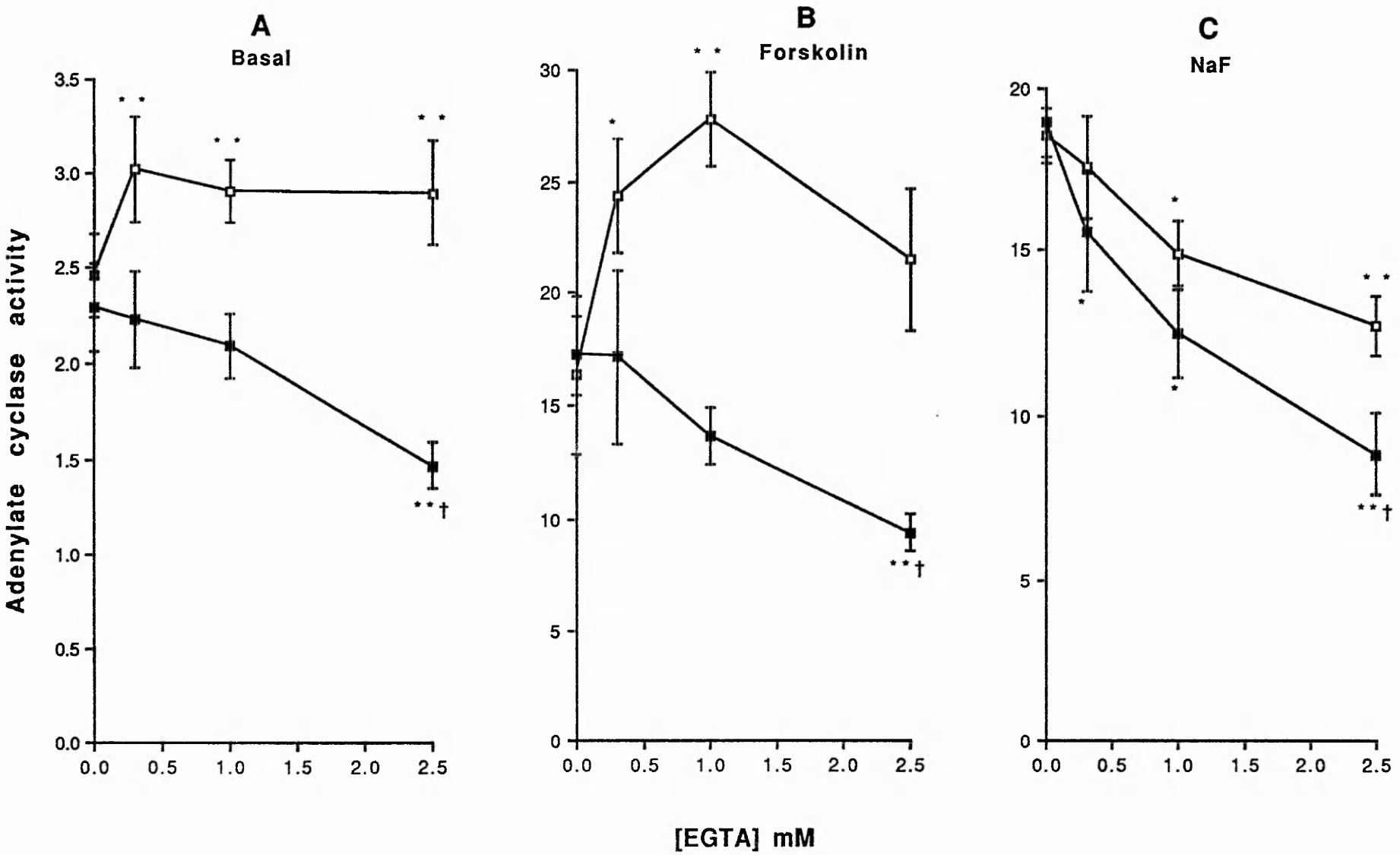
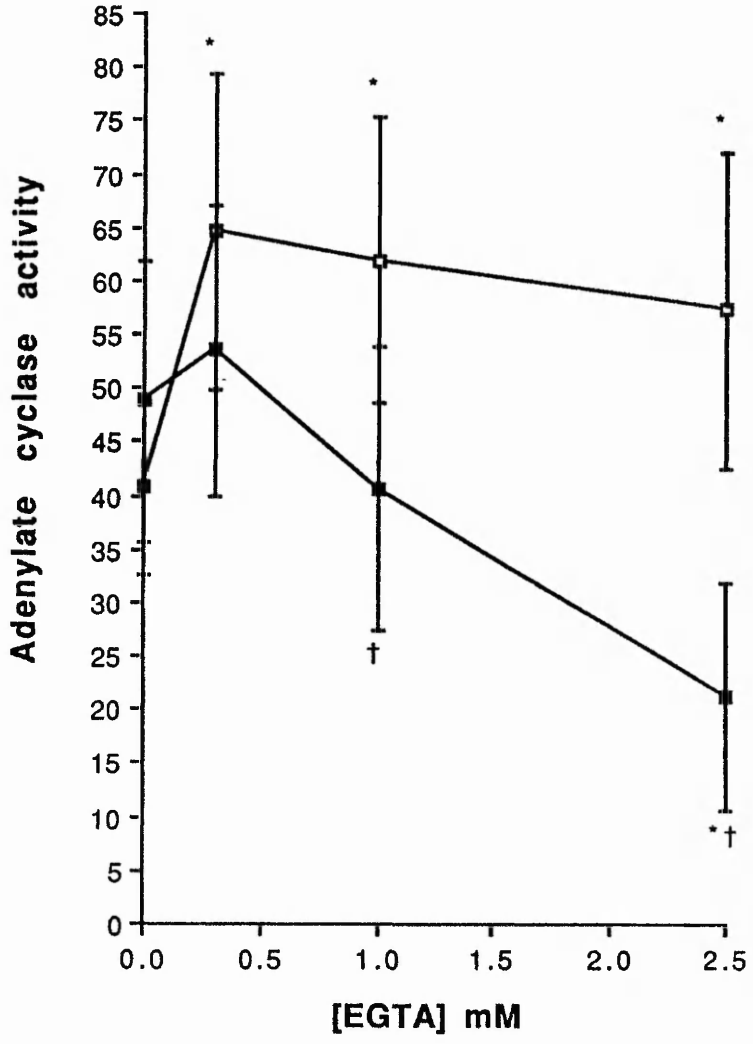

A
Basal
B
Forskolin
C
NaF
Adenylate cyclase activity
[EGTA] mM
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
5
10
15
20
25
30
*
**
†

**Figure 29 GMP-PNP stimulated adenylate cyclase activity concentration response to EGTA**




GMP-PNP (1μM) stimulated adenylate cyclase activity (pmol cAMP $min^{-1}$ mg $protein^{-1}$) concentration response to EGTA in the presence of no added $CaCl_2$ (□) and 1μM free $Ca^{2+}$ (■) in particulate membranes from human duodenal biopsies. Data are the mean±SEM of 3 separate membrane preparations, assayed in triplicate.

* indicates significance at the 95% level compared to no EGTA

+ indicates significance at the 95% level compared to 0.3mM EGTA

## 2.7 DISCUSSION

In previous studies on intestinal membranes, calmodulin sensitivity of adenylate cyclase has been demonstrated in the presence of micromolar concentrations of EGTA (Amiranoff et al. 1983; Pinkus et al. 1983; Lazo et al. 1984). Brostrom et al. (1977) found that for particulate membrane preparations of rat cerebral cortex the presence of EGTA in the assay was essential for the distinction between calmodulin-sensitive and calmodulin-insensitive forms of adenylate cyclase. However, the direct effects of EGTA have also been described (Monneron and d'Alayer 1980; Simonin et al. 1980; Sulakhe and Hoehn 1984; Sulakhe 1985).

The sensitivity of adenylate cyclase activity to inhibiting free $Ca^{2+}$ concentrations, in particulate membranes of human duodenal biopsies, was increased by the presence of EGTA in the assay medium. Potter et al.(1980) observed a similar effect and recommended the use of high controlling EGTA concentrations (2mM) suggesting that it would eliminate competition between $Ca^{2+}$ and other endogenous divalent cations (e.g. $Mn^{2+}$). The left shift effect has also been reported for brain, liver and heart membranes (Potter et al. 1980; Cros et al. 1984; Sulakhe and Hoehn 1984; Sulakhe 1985). Other investigators propose this reflects the varying ability of different membrane preparations to buffer $Ca^{2+}$. In particular these data support the conclusions of Segal (1986) that in systems with high affinity for $Ca^{2+}$ estimation and preparation of low available free $Ca^{2+}$ concentrations (< 1$\mu$M) should account for both the EGTA and the membrane capacities for $Ca^{2+}$. However, results from the experiments using Fura-2 suggest the biopsy particulate membrane preparation can not compete with EGTA for $Ca^{2+}$.

Other possible explanations include:- a) the $Ca^{2+}$-EGTA complex may be a more active species than $Ca^{2+}$ itself; b) other ions present might antagonise the $Ca^{2+}$ effect but be removed by EGTA and c) contaminating mitochondia in the membrane preparation may sequester $Ca^{2+}$ which may be inhibited by EGTA (Randle et al. 1974).

The left shift effect is conceivably a general characteristic of using chelating agents because there are examples in other enzyme systems and with other chelating agents, e.g. in the absence of EGTA much higher concentrations of $Ca^{2+}$ are necessary for full activation of pig heart pyruvate dehydrogenase phosphate phosphatase (Randle et al. 1974) and the effect may also be observed using the $Al^{3+}$ chelator, nitrilotriacetate, to control $Al^{3+}$ concentrations when studying the effects of $Al^{3+}$ on tranducin (Miller et al. 1989; Chabre 1990).

EGTA, in the absence of a controlled amount of free $Ca^{2+}$, stimulated adenylate cyclase activity. This stimulation was consistent under basal, forskolin and GMP-PNP enzyme activation. The likely mechanism underlying the stimulatory effect of EGTA on enzyme activity may be the chelation of contaminating $Ca^{2+}$ which was estimated to be approximately 1 $\mu$M using Fura-2. If there is only low micromolar contaminating levels of free $Ca^{2+}$ in the assay medium why does it take 0.3 to 1mM EGTA to stimulate the enzyme? Low micromolar levels of EGTA had no detectable effect on adenylate cyclase activity in this system (data not shown). The work of Simonin et al. (1980) using plasmacytoma cells may provide a possible explanation. They demonstrated that increasing concentrations of chelators ( e.g. EDTA or EGTA) successively removed $Ca^{2+}$ and $Mg^{2+}$ linked to the plasma membrane leading to successive activation and inhibition of

adenylate cyclase activity. Therefore, EGTA may have been removing membrane bound $Ca^{2+}$ as well as contaminating free $Ca^{2+}$ from the medium and it was the removal of the membrane bound $Ca^{2+}$ that was stimulatory to adenylate cyclase activity. It is also possible that EGTA may have been chelating another contaminating ion which was inhibitory to enzyme activity (e.g. $Mn^{2+}$) (Sulakhe 1985; Perez-Reyes and Cooper 1987). The removal of membrane bound $Ca^{2+}$ by EGTA has been suggested to induce structural changes in plasma membranes (Wilffert et al 1989).

When a constant level of free $Ca^{2+}$ is maintained in the EGTA concentration response assay, the enzyme was only inhibited by EGTA. This supports the idea that EGTA stimulation is because of chelation of contaminating $Ca^{2+}$. $Ca^{2+}$ does not reverse the inhibitory effects of EGTA; this is an indication that the chelator may have been inhibiting the enzyme directly and not in a way mediated by $Ca^{2+}$ chelation and calmodulin inactivation (Tomlinson et al. 1984; MacNeil et al. 1985).

The inhibition by EGTA is independent of stimulus. Therefore it is conceivable that the mechanism involves interaction between EGTA and the catalytic unit of the enzyme. Interaction of EGTA with a hydrophobic region of particulate adenylate cyclase from rat cerebral cortex has been previously demonstrated. However, it is unknown whether this hydrophobic region represents membrane associated calmodulin or the catalytic unit (Sulakhe and Hoehn 1984; Sulakhe 1985). The data presented here suggests this region is on the catalytic unit of the enzyme.

It is important to appreciate that inhibition of adenylate cyclase

activity by EGTA, thought to be associated with calmodulin inactivation, occurs at low micromolar concentrations of EGTA not millimolar levels as in the present study.

A possible explanation for the lack of stimulation by EGTA, when adenylate cyclase activity was stimulated with NaF, comes from a recent article by Chabre (1990). The mechanism for NaF activation is assumed to be through complexation of $F^-$ with contaminating traces of $Al^{4+}$ ($AlF_4^-$). This complex acts as an analogue of phosphate which binds to tightly bound GDP on the G protein and thus causes activation of adenylate cyclase that by structural and functional criterion is identical to that obtained by GTP or its analogues. However, $F^-$ at millimolar concentrations may mobilize membrane $Ca^{2+}$ pools through the formation of $CaF_x$ complexes analogous to aluminium. It may be that, in the absence of $F^-$, stimulation by EGTA occurs as the membranes are depleted of bound $Ca^{2+}$ and removed from the assay medium. In the presence of $F^-$ membranes may be depleted of $Ca^{2+}$ but the formation of $CaF_x$ complexes reduces $Ca^{2+}$ chelation from the surrounding medium by EGTA. Alternatively NaF may have been stimulating other G proteins linked to $Ca^{2+}$ channels in the membranes so that NaF stimulated-adenylate cyclase response to EGTA mimicked that of $Ca^{2+}$ (Habara et al. 1990; Sjolander et al. 1990).

# CHAPTER 3

## STUDIES ON ISOLATED EPITHELIAL CELLS FROM HUMAN DUODENAL BIOPSIES: STIMULATION BY VASOACTIVE INTESTINAL PEPTIDE (VIP) AND THE EFFECTS OF CALMODULIN ANTAGONISTS ON VIP STIMULATED CYCLIC AMP PRODUCTION

## 3.1 INTRODUCTION

In the previous section membranes from duodenal biopsies were used to study cAMP production. There are several disadvantages to using such membrane preparations.

Firstly the biopsies contain tissues not confined to the mucosa and include some membranes derived from constituent cells of the lamina propria including those of neurological origin (**Figure 1**).

Secondly, the structural disorganization of cells and contamination of the membrane preparation by organelles may have been interfering with the characteristics of adenylate cyclase activity and the possible detection of a calmodulin- sensitive form of the enzyme (Alberts et al. 1983b).

Thirdly, calmodulin is known to bind VIP at nanomolar concentrations (it is unknown if this binding has any physiological significance for cellular processes); therefore receptor mediated stimulation of cAMP production in a membrane preparation known to contain calmodulin is likely to be less sensitive than that possible with intact isolated cells (Simon and Kather 1980d; Anderson and Malencik 1986).

Isolated intestinal epithelial cells have been used for many years in the study of many different aspects of gastrointestinal physiology (Kimmich 1970b; Iemhoff et al. 1970; Simon and Kather 1980d; Hyun and Kimmich 1982; Ilundain et al. 1987; Sepulveda and Smith 1987; Verbost et al.1987; Vidal et al. 1988). Methods of separation of the cells, from the tissues underlying the mucosa, have been developed involving mechanical dispersion, enzymes and chelating agents (Harrer et al. 1964; Stern and Reilly 1965; Huang 1965; Stern 1966; Stern and Jensen 1966; Harrison and Webster 1969; Kimmich 1970a; Weiser 1973; Watford

et al. 1979; Hegazy et al. 1983; Del Castillo 1987; Smith et al. 1990). In animal studies isolated intestinal epithelial cells have been used to study cAMP production with great sensitivity (Laburthe et al. 1979; Lazo et al. 1984; Caemena et al. 1987; Vidal et al. 1988; Smith et al. 1990).

The purpose of this study was to isolate epithelial cells from human duodenal biopsies using the chelating agent, ethylenediaminetetra-acetic acid (EDTA), to assess the effect of VIP in stimulating cAMP production and to determine if calmodulin has an influence on this receptor mediated cAMP formation. Initial studies included the determination of optimal assay concentrations of IBMX, time course studies and VIP concentration response using the methods described below. Details of individual experiments are given in the figure legends in section 3.4.

## 3.2 MATERIALS

All chemicals were of the highest available grade from the Sigma Chemical Company or BDH unless otherwise specified.

## 3.3 METHODS

Biopsies were collected from patients attending endoscopy clinic as described in section 2.3.

### 3.3.1 Preparation of isolated epithelial cells from duodenal biopsies

A modification of a cell isolation technique used in animal studies was developed (Stern 1966; Weiser 1973; Smith et al. 1990). Although enterocytes isolated by the basic method of Weiser (1973) have functional VIP receptors and are capable of generating cAMP, in the absence of an exogenously added ATP regenerating system, only limited success has been achieved in demonstrating their viability in other ways. For example, it was neither possible to block phosphodiesterase activity effectively so that cAMP production approached linearity nor was it possible to show a high percentage of cells capable of excluding trypan blue.

Therefore, several modifications were made in an attempt to improve cell viability based on these criterion. Biopsies were collected into a universal containing 0.9% saline and transported to the laboratory. They were transferred to 10ml citrate buffer (1.5mM KCl, 96mM NaCl, 27mM Na citrate, 8mM $KH_2PO_4$, 5.6mM $Na_2HPO_4$, pH 7.3), prewarmed to 37°C and incubated for 10 minutes in a shaking water bath. The citrate buffer was removed and the biopsies were resuspended in 10ml EDTA buffer (1.5mM EDTA (disodium salt), 0.5mM dithiothreitol, 10mM $NaH_2HPO_4$, 154mM NaCl) at 37°C for 30 minutes. The biopsies were

gently agitated at intervals during this incubation. The cell suspension was separated from the biopsy fragments and centrifuged (5 minutes, 350g, 4°C). The pellet of cells was washed twice in HEPES- bicarbonate buffered Hanks balanced salt solution (Northern Media) (HBH) (1.26mM $CaCl_2$, 5.36mM KCl, 0.44mM $KH_2PO_4$ 0.81mM, $MgSO_4$, 137mM NaCl, 4.17mM $NaHCO_3$, 0.34mM $Na_2HPO_4$, 10mM Hepes, 3mM IBMX pH7.4) at 4°C and gassed with 5% $CO_2$/95% $O_2$ (v/v). Enterocytes in bicarbonate buffer, gassed with 5% $CO_2$/95% $O_2$ (v/v) rather than 100% $O_2$ (v/v), have been shown to have a higher rate of oxidative metabolism compared with enterocytes in other media (Watford et al. 1979; Pinkus et al. 1983). All buffers were supplemented with 5.5mM glucose, 2mM glutamine, 2mg/ml BSA and 1mg/ml soybean trypsin inhibitor (Pinkus et al. 1983). Cells were resuspended in 3ml HBH.

3.3.2 Incubation of isolated epithelial cells with VIP

A 100µl of cells (approximately 1.5 x $10^6$/ml) were preincubated in 3ml polypropylene test tubes for 3 minutes at 37°C. The reaction was initiated with the timed addition of 100µl of synthetic porcine VIP. The VIP was dissolved in HBH including 100µM amastatin, an inhibitor of [des-His] VIP formation (Nau et al. 1987). The cells were incubated with VIP for 5 minutes at 37°C. During the course of the reaction, cells were gassed continually with 5% $CO_2$/95% $O_2$ (v/v). Reactions were terminated with 60% (25µl) ice cold trichloroacetic acid (TCA).

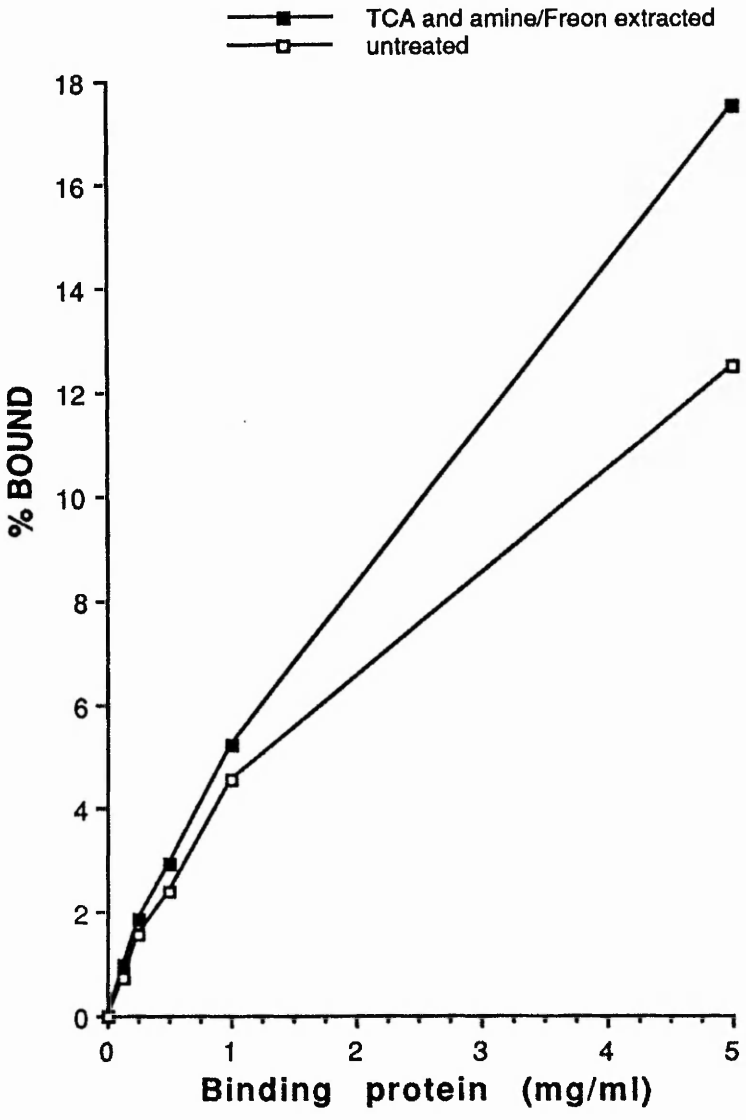
The TCA was removed by amine/Freon extraction (Khym 1975) and cAMP was assayed in the extract by the protein binding assay of Gilman (1970) described below.

3.3.3 Amine/Freon extraction of cAMP

Acid-soluble nucleotides may be extracted from tissues with TCA, but

because TCA may interfere with the subsequent cAMP assay, it was removed from samples before analysis (Khym 1975). The procedure used in these studies involves neutralizing the acidified samples with a water-soluble amine, alamine, contained in a water-insoluble organic solvent, Freon. Amine/Freon (200µl), mixed at a ratio of 1:4 (v/v), was added to the VIP incubated cell suspensions. The tubes were capped and mixed gently for 3 minutes. The phases were separated by centrifugation for 5 minutes at 350g. Three 50µl samples of the surface aqueous phase were transferred to 3ml polypropylene test tubes from each sample for cAMP assay. This procedure did not interfere with the subsequent assay of cAMP (see section 3.3.4) (**Figure 30**).

**Figure 30 Binding protein titration curve**




The % of bound [$H^3$] cAMP in the presence of 10pmol cAMP/50µl was estimated using the cAMP assay system with varying concentrations of binding protein (see section 3.3.4). TCA- amine/Freon extracted cAMP was compared to untreated controls. The apparent slight improvement in protein binding as compared with untreated controls represents the loss of cAMP owing to the extraction procedure. Recovery of cAMP after amine/Freon extraction was approximately 70%

3.3.4 Assay of cAMP

The cAMP assay is based on the competition between unlabelled cAMP and a known, fixed amount of [$^3$H] cAMP for 3',5'-cyclic AMP dependent protein kinase (binding protein). Measurement of the bound isotope enables the amount of unlabelled cAMP in the sample to be calculated (Gilman 1970).

Standards of cAMP (0-16pmol/tube) were suspended in the HBH cell suspension buffer, treated with TCA and amine/Freon extracted in exactly the same way as samples. All assay procedures were conducted on ice at 4°C. Assay buffer (80µl of 50mM Tris-HCl, 4mM EDTA at pH7.5 at 4°C) and 50µl of [8- $^3$H] cAMP (5µl of 0.74-1.1TBq/mmol in 10ml assay buffer gives approximately 25000 cpm) (Amersham Inc.) was added to 50µl of amine/Freon extracted samples and standards. The incubation was initiated by the addition of 20µl of binding protein dissolved in assay buffer and 0.5% (w/v) BSA at a concentration of 0.4-0.5mg/ml. Tubes were mixed well and incubated at 4°C for at least 3 hours but no longer than 18 hours to equilibrate. At the end of the incubation a 100µl of charcoal solution (200mg BSA and 260mg activated charcoal/100ml assay buffer) was added to the tubes. They were then mixed and centrifuged at 1000g for 5 minutes. Care was taken so that the tubes were centrifuged not less than 1 minute or more than 6 minutes after the addition of charcoal to the last tube. The supernatant (200µl) was removed from each sample and standard without disturbing the charcoal sediment and placed into 20ml liquid scintillation vials containing 10ml Optiphase X liquid scintillation cocktail ( Pharmacia LKB).

Non-specific binding (NSB) tubes contained 50µl [$^3$H] cAMP, 150µl assay buffer but no binding protein. Total cpm added to each tube was

measured in a 50µl aliquot of the [$^3$H] cAMP solution in 150µl assay buffer (no charcoal was added to these tubes). Triplicate determinations of all treatments were vortex mixed and then counted for 3 minutes in a Packard TRI-CARB 4000 beta counter.

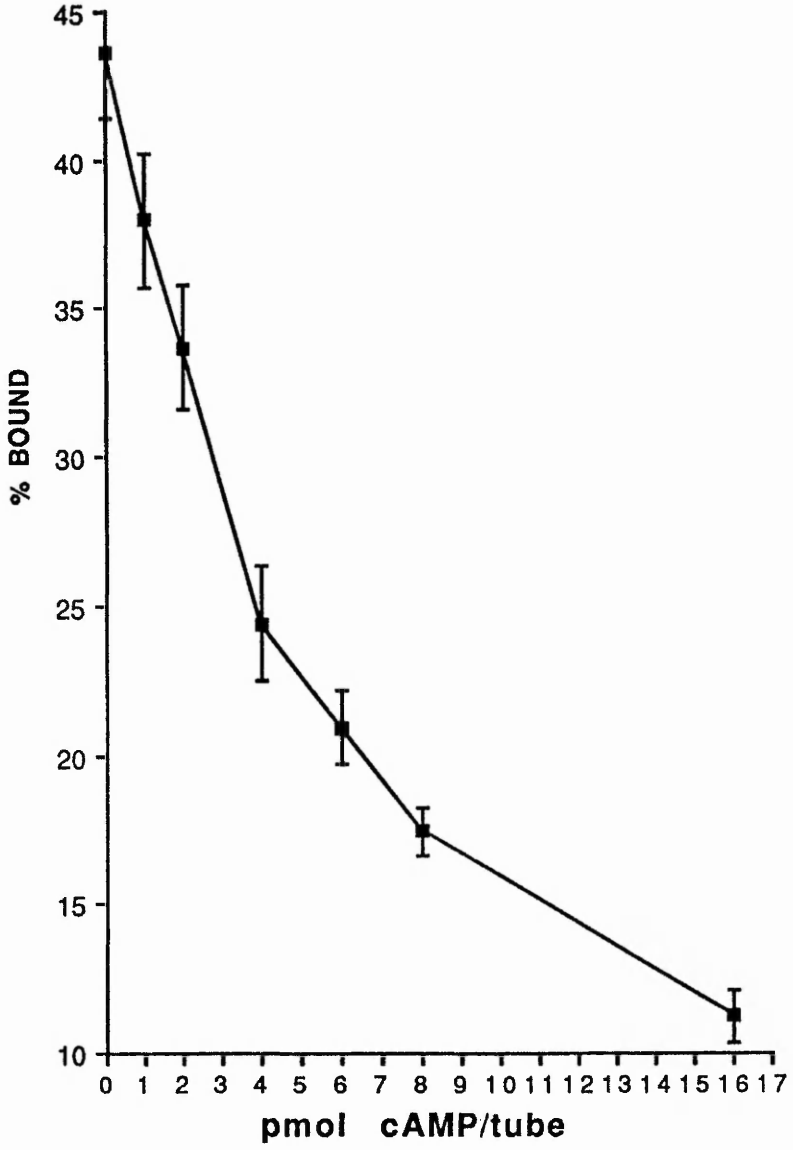
Calculation of results

NSB cpm were subtracted from the cpm of samples and standards and the % bound calculated by:-

$$\frac{\text{Sample (or standard) cpm - NSB cpm}}{\text{total [}^3\text{H]cAMP cpm}} \times 100$$

The % bound was plotted against the concentration of the standards and the unknown samples extrapolated from the curve (**Figure 31**).

**Figure 31 cAMP standard curve**




Each point is the mean± SEM of 5 consecutive TCA-amine/Freon extracted standards. The mean±SEM inter-assay coefficient of variation for % binding was 13.81%±1.1 for any one batch of protein kinase.

### 3.3.5 DNA assay

DNA was assayed in the cell preparations by the method of Kissane and Robins (1958). A sample of cell suspension (50μl) was evaporated to dryness in 2.5ml flat bottomed glass test tubes in a 60°C oven. A DNA standard curve using calf thymus DNA between 6.25 - 100μg/ml was treated in the same way. 3' 5' diaminobenzoic acid solution (DABA) (0.45g/ml distilled water) was decolourised by shaking with activated charcoal (150mg/ml DABA) for 3 hours and then filtered through a 0.2μm Ministart filter (Sartorius). This reagent (0.1ml) was then added to each tube and incubated for a further 45 minutes at 60°C to hydrolyse the DNA. HCl (1.5ml of 1M) was added, mixed well and transferred to 1cm light path 4ml fluorimetric cuvettes. The fluorescence was measured in a Kontron SFM 25 spectrofluorimeter with an excitation wavelength of 401nm and an emission wavelength of 518nm (**Figure 32**).

#### Assay variation

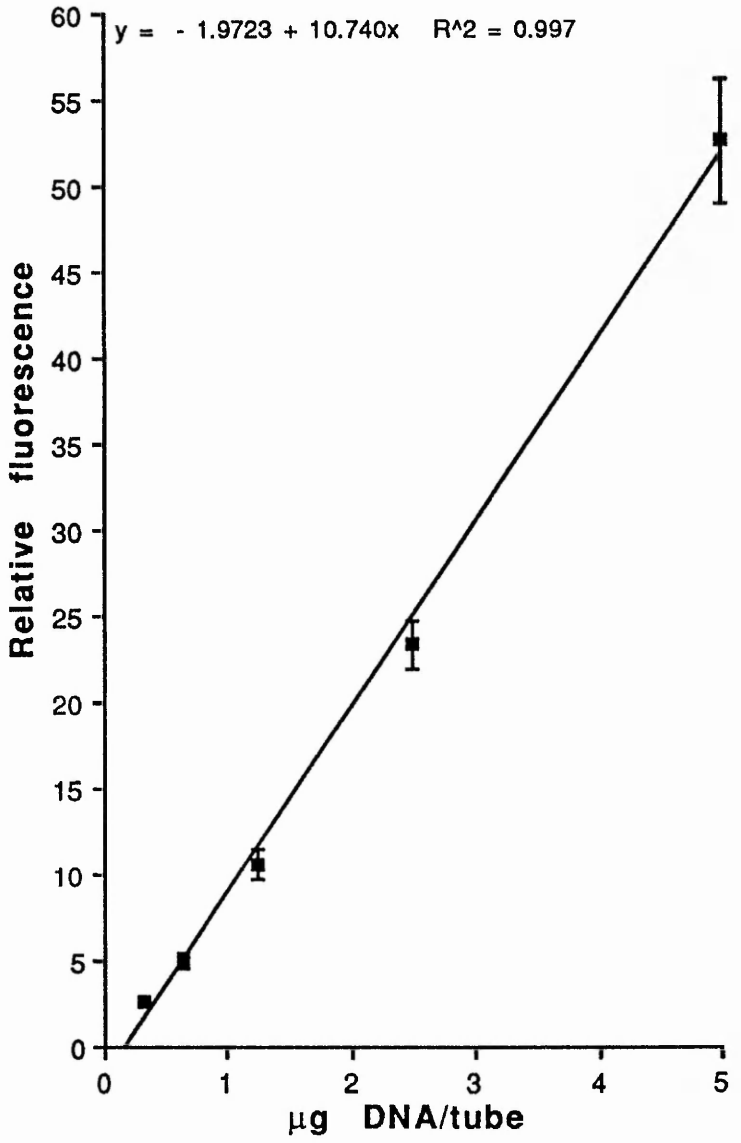
Intra-assay variation was established using a 1% (w/v) homogenate of 6 biopsies from different patients assayed with 6 replicates. Six separate lots of 6 biopsies were assayed.

The mean coefficient of variation was 6.64%±1.55SEM.

### 3.3.6 Statistics

Results are expressed as mean ±SEM. Where appropriate,the one factor ANOVA-repeated measures test was used to test for significance. Probability values $\leq$ 0.05 were taken as significant.

**Figure 32 DNA standard curve**




Each point is the mean of 6 routine consecutive DNA standards ± SEM.
The mean coefficient of variation between assays was 15.97%±1.35.

## 3.4 RESULTS

### 3.4.1 Duodenal epithelial cells from biopsies

Epithelial cells are usually harvested with buffers contained within intestinal sacs, so that the isolating buffer does not come into contact with tissues not of mucosal origin. With the isolation of cells from intestinal biopsies this is not possible and the cut surface of the biopsy is exposed to the isolating medium. Therefore there was the potential for contamination of the cell preparation with cells other than those of the epithelium. Surprisingly , this was not a problem and the majority of the cells were epithelial in origin (Smith et al. 1990). Biopsies have a tendency to curl up with the mucosal surface outermost and the cut surface inward, protected from the buffers. This may explain why cells of the lamina propria were not apparent in the cell preparations. Also the natural propensity of the epithelial cells to be shed from the villous may make these cells more susceptible to isolation by $Ca^{2+}$ chelation compared to the cells of the lamina propria. The largest contaminant was erythrocytes. These cells are devoid of nuclei and have vestigial adenylate cyclase activity, therefore contamination by these cells did not represent a problem for the study of cAMP production. Isolated cells are devoid of neuronal elements and are therefore free from endogenous VIP (Laburthe and Dupont 1982). A mean of $1.95x10^6 \pm 0.93x10^6$(SD) cells/ml was isolated from 6 duodenal biopsies and $1x10^6$cells/ml was equivalent to approximately 30μg DNA (n=14). Greater than 80% of isolated cells excluded trypan blue for at least 2 hours; 86% ± 2.99 SEM immediately after isolation and 84% ± 4.69 SEM 2 hours later (n=15). It did not make any difference to trypan blue exclusion if cells

were kept on ice (91.20%± 4.03SEM) or at 37°C (91.50%± 3.21SEM) (n=11).

3.4.2 Time course studies

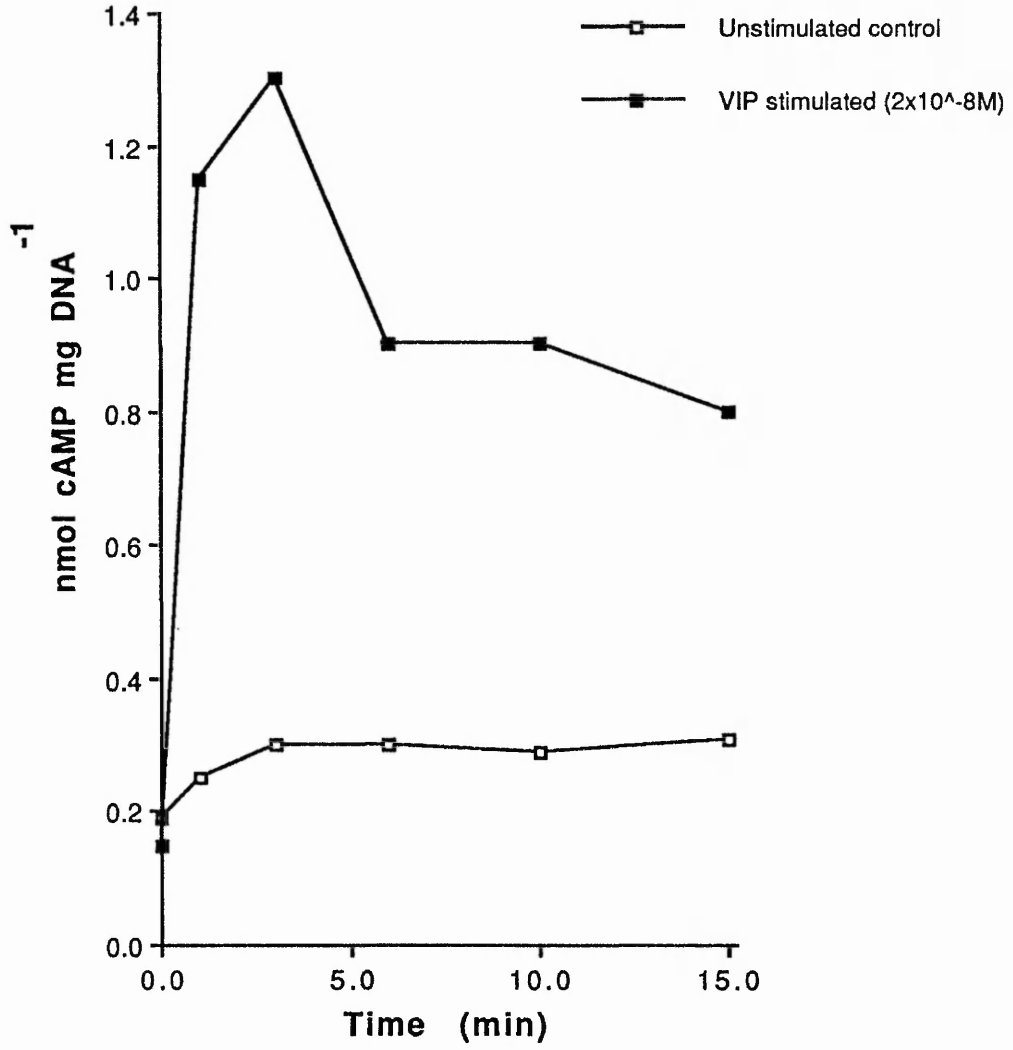
The concentration of cAMP in a cell is dependent on at least four components:- the rate of synthesis and hydrolysis, the rate of cAMP escape from the cell and the proportion of cAMP bound to cellular constituents (Barber and Butcher 1982). In the experiments described below (as with all the experiments in this work) the primary concern is in the regulation of cAMP synthesis. All the cAMP produced by a cell preparation was assayed, whether intra or extracellular, bound or unbound, therefore, these two aspects of cAMP metabolism were not explored. However, cAMP hydrolysis by phosphodiesterase represented a major problem in the measurement of cAMP accumulation.

In preliminary investigations the time course for cAMP production indicated inadequate phosphodiesterase inhibition with 1mM IBMX (**Figure 33**). Therefore, in order to obtain meaningful time course data, from cells incubated with VIP, an IBMX concentration response was conducted to find the maximum IBMX concentration tolerable (**Figure 34**). The results from this series of experiments indicated that an IBMX concentration as high as 3mM may be necessary to block phosphodiesterase activity.

Time course data of cAMP production in the presence of 3mM IBMX, for epithelial cells isolated from duodenal biopsies, is given in **Figure 35**. VIP stimulated cAMP production was proportional to incubation time for the first 5 minutes of incubation and reached a plateau by 10 minutes. The loss of accumulated cAMP associated with

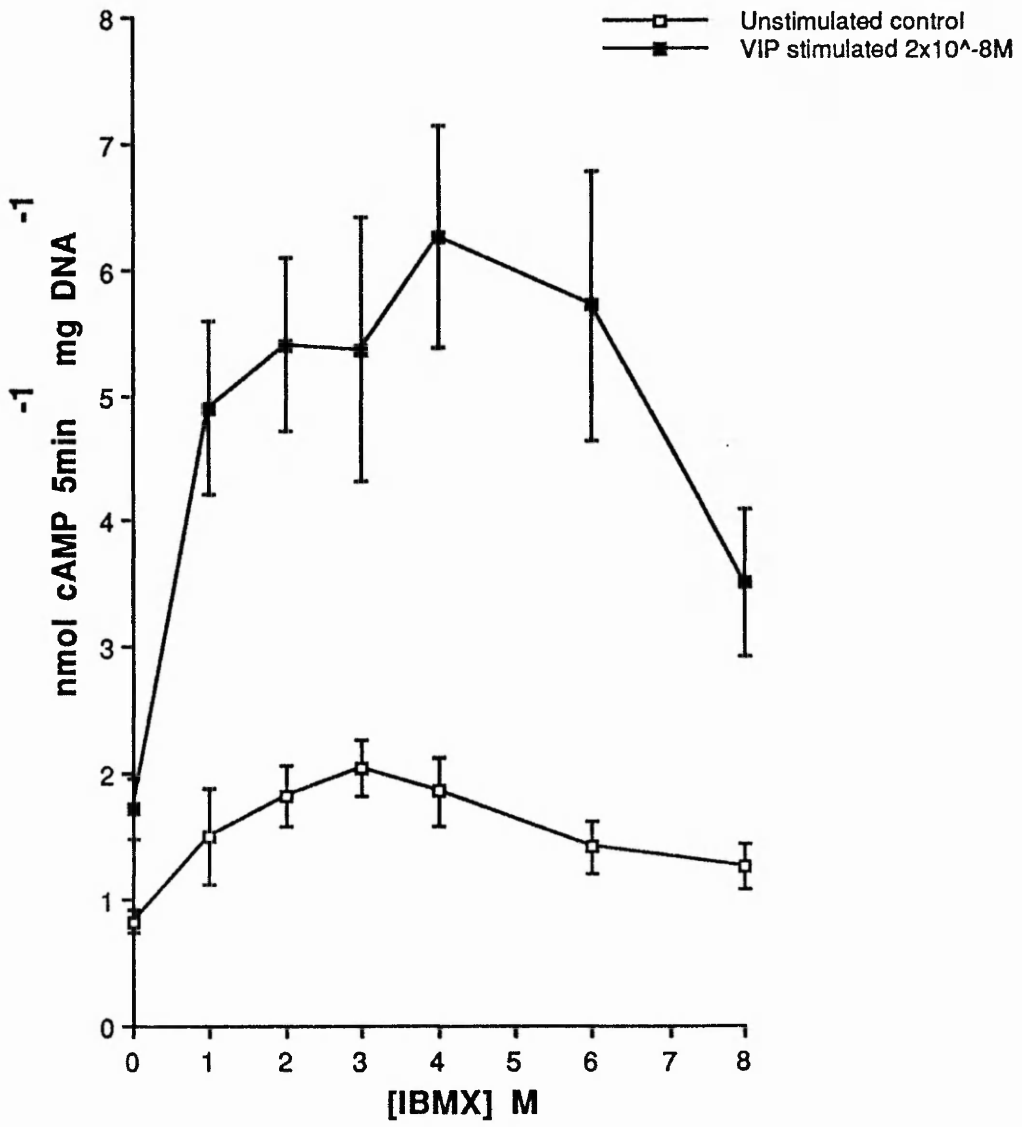
phosphodiesterase activity was no longer evident. A concentration of 3mM IBMX improved the time course so that for the first 15 minutes of incubation it approached a pattern that might be expected with uniform cAMP synthesis and first order elimination (Barber and Butcher 1982). Alternatively the cAMP levels induced by VIP, measured at the plateau (10-15 minutes), indicate that no more cAMP was produced and was that generated during the stimulatory period (1-10 minutes). By 20 minutes incubation a loss of accumulated cAMP became apparent.

**Figure 33 Time course for cAMP accumulation with 1mM IBMX in isolated epithelial cells**



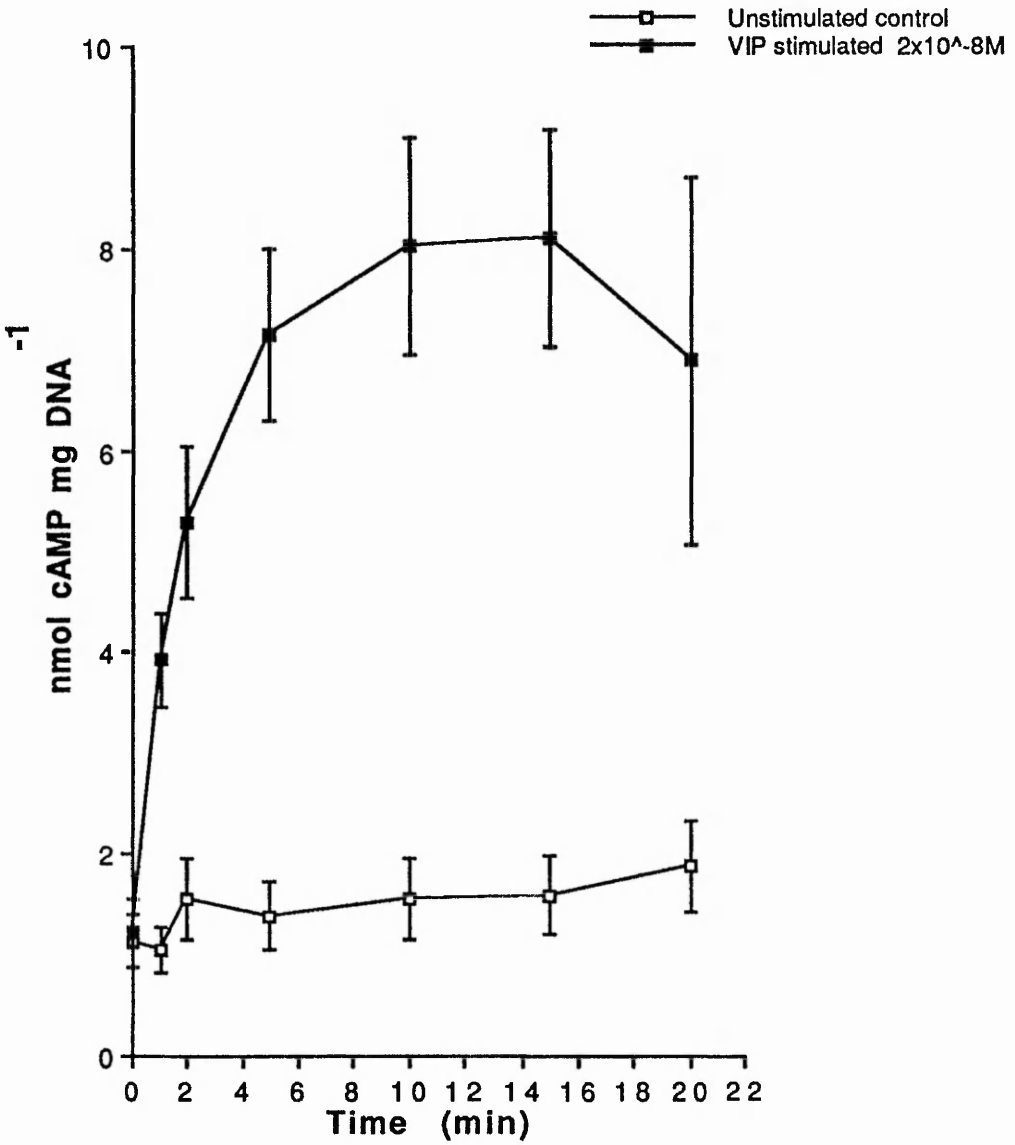

Epithelial cells isolated from duodenal biopsies were incubated for the various time points indicated on the abscissa with $2x10^{-8}$M VIP. VIP was dissolved in HBH and for unstimulated controls, HBH alone was added to incubations. Cells were suspended and washed in HBH supplemented with 1mM IBMX. A time course typical for epithelial cells isolated from duodenal biopsies in which phosphodiesterase activity was insufficiently inhibited is shown. A loss of the cAMP accumulated is evident after 6 minutes for VIP stimulated treatments.

**Figure 34 cAMP accumulation in isolated epithelial cells in response to IBMX**




Epithelial cells isolated from duodenal biopsies were preincubated for 20 minutes with the concentrations of IBMX indicated on the abscissa and then incubated for a further 5 minutes with $2x10^{-8}$M VIP. VIP was dissolved in HBH and for unstimulated controls HBH alone was added to incubations. Data are the mean±SEM from 5 individual patients.

**Figure 35 Time course of cAMP accumulation in isolated epithelial cells with 3mM IBMX**




Epithelial cells isolated from duodenal biopsies were incubated for the various time points indicated on the abscissa with $2x10^{-8}$M VIP. VIP was dissolved in HBH and for unstimulated controls HBH alone was added to incubations. Cells were suspended and washed in HBH supplemented with 3mM IBMX. Data are the mean±SEM from 5 individual patients.

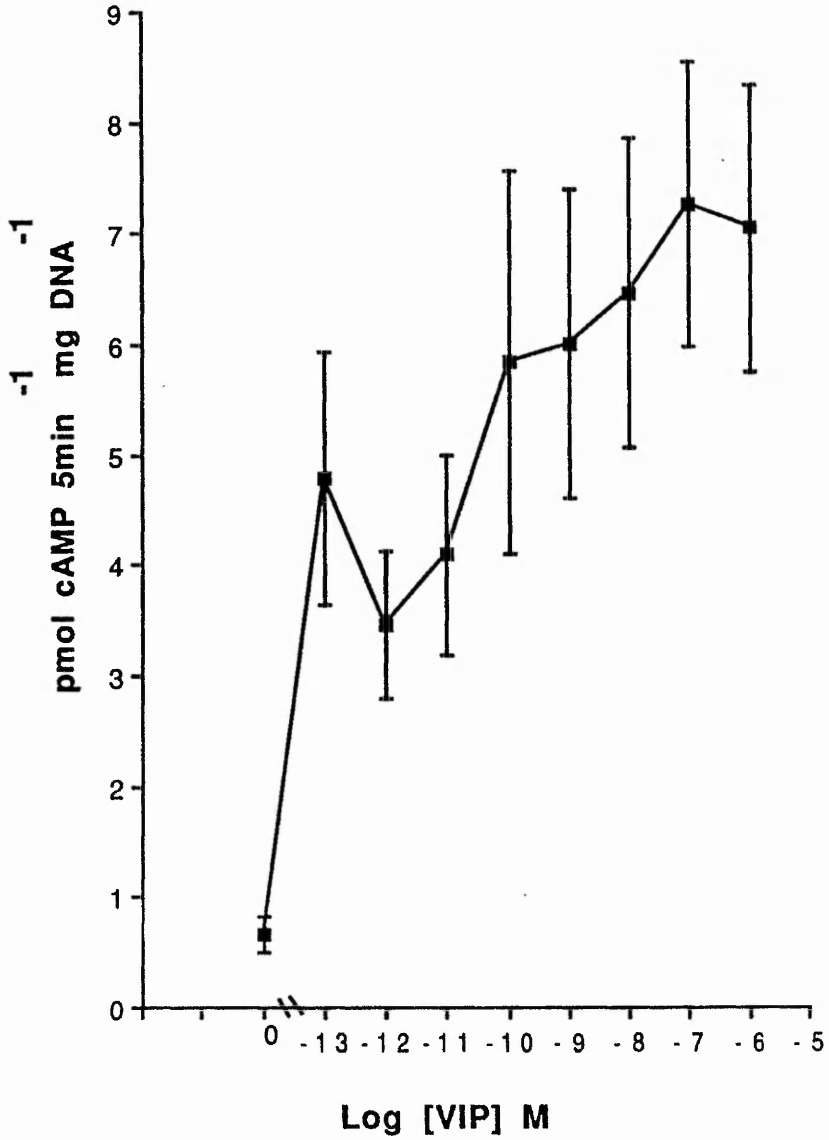
3.4.3 cAMP production in response to increasing concentrations of VIP

The response of duodenal epithelial cells to increasing concentrations of VIP is shown in **Figure 36**. Mean basal cAMP levels were 0.67nmol/5 min/mg DNA (±0.16SEM). The maximum cAMP level was 7.26nmol/5 min/mg DNA (±1.30SEM) which was observed with 100nM VIP. This represented a 10.8 fold increase above the basal level. Half maximal stimulation was elicited by 1.78pmol VIP. An interesting feature of this response was the highly significant 7 fold increase in cAMP synthesis above basal at a VIP concentration as low as 0.1pM.

3.4.4 The effect of calmodulin antagonist on VIP stimulated cAMP production

VIP stimulated cAMP production was not inhibited by TFP or IODO 8 at concentrations as high as 100μM in cells isolated from human duodenal biopsies ( **Table 9**). There was a slight reduction in cAMP formation in the TFP treated cells but this was not statistically significant or related to increasing TFP concentration. Duodenal epithelial cells were preincubated with TFP or IODO 8 for 5 minutes before a 5 minutes incubation with VIP. It was possible that the preincubation time was insufficient to allow the antagonist to be effective. Therefore cells were preincubated for increasing duration and then incubated for 5 minutes with VIP. The results of these experiments are given in **Table 10**. The levels of cAMP produced are consistant with VIP stimulated enzyme activity. Increasing preincubation time had no consistent influence upon the effect of antagonists on cAMP synthesis although there does appear to be inhibition at 20 min induced by the vehicle (DMSO).

**Figure 36 cAMP accumulation in isolated epithelial cells in response to VIP**




Epithelial cells isolated from duodenal biopsies were incubated for 5 minutes with VIP at the concentrations indicated on the abscissa. Cells were washed and resuspended in HBH supplemented with 3mM IBMX. Data are the mean±SEM from 4 individual patients. All VIP concentrations gave cAMP levels above the control (no added VIP) at the 99% significance level by one factor ANOVA-repeated measures analysis.

**Table 9 The effect of TFP and IODO 8 on cAMP accumulation in isolated epithelial cells**

| | | nmol cAMP $5min^{-1}mg$ $DNA^{-1}$±SEM | |
|---|---|---|---|
| Stimulus | [Antagonist]μM | TFP | IODO 8 |
| 0 | 0 | 0.66±0.37 | |
| $2x10^{-8}$M VIP | 0 | 2.85±0.92 | |
| " | 1 | 2.42±1.17 | 2.56±0.92 |
| " | 5 | 2.38±1.05 | 2.91±0.77 |
| " | 10 | 2.71±1.21 | 2.99±1.32 |
| " | 25 | 1.92±0.95 | 2.49±0.76 |
| " | 50 | 2.31±1.04 | 2.72±0.82 |
| " | 100 | 2.25±1.04 | 2.80±0.81 |

Epithelial cells isolated from duodenal biopsies were preincubated with TFP or IODO 8 for 5 minutes and further incubated with VIP for 5 minutes. The TFP and IODO 8 were dissolved in DMSO and the final assay concentration was less than 1%. Data are the mean±SEM of 4 separate cell preparations. There was no significant difference between the control and any of the antagonist concentrations.

**Table 10 The effect of preincubation time with TFP and IODO 8 on cAMP accumulation in isolated epithelial cells**

| Preincubation time (min) | pmol cAMP $5min^{-1}150\mu l^{-1}\pm SD$ Control (DMSO only) | IODO 8 50μM | TFP 50μM |
|---|---|---|---|
| Exp 1 | | | |
| 0 | 7.17±0.63 | 6.37±1.50 | 8.23±0.50 |
| 5 | 7.83±1.04 | 7.50±0.43 | 7.16±1.50 |
| 10 | 5.72±0.10 | *4.07±0.61 | *4.00±0.25 |
| 20 | 4.68±1.03 | 6.80±2.70 | 2.95±0.25 |
| 30 | 8.60±1.50 | 7.7±3.16 | 4.72±0.60 |
| Exp 2 | | | |
| 0 | 11.33±1.66 | 8.75±2.39 | 8.40±1.48 |
| 5 | 8.28±0.20 | 8.38±1.50 | *6.08±0.80 |
| 10 | 8.05±0.13 | #9.93±0.11 | 8.85±1.30 |
| 20 | 5.23±0.32 | 5.76±0.32 | 5.83±0.25 |
| 30 | 10.35±0.54 | 11.57±0.73 | 9.50±1.12 |

The results from two experiments are shown using cells isolated from the biopsies of two patients. After the preincubation times shown, the cells were stimulated with $2x10^{-8}$M VIP and incubated for a further 5 minutes. Data are the mean±SD of triplicate determinations.

* indicates a statistically significant reduction in cAMP accumulation compared to controls at the 95% level by ANOVA- repeated measures analysis.

# indicates a statistically significant **increase** in cAMP accumulation compared to controls at the 95% level.

## 3.5 DISCUSSION

Intestinal epithelial cells may be successfully isolated from human duodenal biopsies by $Ca^{2+}$ chelation and used to study receptor mediated cAMP synthesis. When stimulated with VIP, without adequate IBMX to inhibit phosphodiesterase activity, the cells exhibit a time course for cAMP accumulation typical of that found with other cell types (Barber and Butcher 1982). Problems of inadequate phosphodiesterase inhibition have been previously reported for isolated rat epithelial cells (Laburthe et al. 1979). At 30°C, 0.2mM IBMX was inadequate to fully block phosphodiesterase activity. At 15°C cAMP accumulation reached a plateau by 15 minutes and this was maintained for up to 60 minutes. Therefore, Laburthe et al. (1979), used a lower incubation temperature for VIP incubations. Pinkus et al. 1983) using isolated guinea-pig enterocytes, found that high IBMX concentrations (4mM) were necessary to block phosphodiesterase activity by 89% at 37°C. VIP has also been shown to stimulate phosphodiesterase activity subsequent to adenylate cyclase activity and protein kinase activation (Marchis-Mouren et al. 1988). This provides further explanation for the poor time course of cAMP synthesis without adequate concentrations of IBMX.

Isolated human duodenal epithelial cells are highly sensitive to VIP stimulation. When compared to similar studies, using animal intestinal cells and human cell lines, stimulation of cAMP synthesis at VIP concentrations of 0.1pM, is apparently the lowest reported VIP concentration capable of stimulating cAMP production (Smith et al. 1990). However, the lowest detected increase in cAMP synthesis is usually the lowest concentration tested, (**Table 11**).

The presence of high and low affinity receptors for VIP on epithelial cells has been previously reported using both receptor binding studies and/or stimulating cAMP production (Laburthe 1979 and 1989; Prieto 1979; Binder et al. 1980; Robichon and Marie 1987; Marchis-Mouren et al. 1988). In the human colonic carcinoma cell line, HT29, it has been suggested that VIP receptors exist in two separate states: a small population of high affinity binding sites and a larger population of low affinity binding sites (Marchis-Mouren 1988; Robichon and Marie 1987). The significance of the low affinity sites is unclear. The data presented here indicates that VIP binding to high affinity sites may be more physiologically relevant. Picomolar concentrations of VIP have been shown to produce small but significant changes in $I_{sc}$ indicative of increased $Cl^-$ secretion and reduced $Na^+$ absorption in work using intestinal mucosa in Ussing chambers (personal communication, Dr Alan Young, Research Pharmacologist, Fisons). Therefore, very low VIP concentrations are capable of inducing a physiological response. Receptor binding studies, using this cell preparation, are required to clarify the relationship between VIP stimulation of cAMP synthesis at very low concentrations and the specificity of the receptor that stimulates adenylate cyclase.

**Table 11 Reported effective VIP concentrations for stimulation of cAMP in gastrointestinal tissues**

| Species/tissue | Lowest [VIP] tested pM | Lowest [VIP] to stimulate cAMP formation pM | [VIP] $EC_{50}$ nM | Reference |
|---|---|---|---|---|
| Rat intestinal epithelial cell membranes | 100 | 100* | 4.1 | Amiranoff (1978) |
| Rat intestinal epithelial cells | 100 | 100 | 2.3 | Laburthe et al (1979) Prieto et al (1979) |
| " | 100 | 100 | 3.0 | Carmena et al (1987) |
| Guinea-pig enterocytes | 1000 | 1000 | 45.0 | Pinkus et al (1983) |
| " | 100* | 100* | 1* | Binder et al (1980) |
| Guinea-pig pancreatic acinar cells | 100* | 100* | 40.0 | Robberecht et al (1976) |
| HT29 cell membranes | 1* | 50 | 30.0 | Robichon & Marie (1987) |
| Cl.16E (Human colonic epithelial cells) | 50* | 50* | 0.06 | Laburthe (1989) |
| Human colonic crypt cell membranes | 10* | 10* | 30 | Boige et al (1984) |
| Human duodenal epithelial cells | 0.1 | 0.1 | 0.00178 | present study |

The $EC_{50}$ is the concentration of VIP which resulted in half maximal stimulation of cAMP production
NB The lowest detected increase is usually equivalent to the lowest VIP concentration tested
* Value extrapolated from author's data presented in a figure.

VIP stimulated cAMP production was not inhibited by TFP or IODO 8 at concentrations specific for calmodulin antagonism.

These data are contrary to results from similar experiments using isolated intestinal cells or membranes from other animal species (**Table 12**). In agreement with the results from the membrane work discussed earlier the lack of significant inhibition by either antagonist supports the conclusion that calmodulin does not regulate adenylate cyclase activity in epithelial cells from the human duodenal mucosa. It is possible that $Ca^{2+}$ mobilization, either through receptor operated channels or from intracellular stores, may be necessary to detect any calmodulin dependent adenylate cyclase activity in cells. However intestinal epithelial cells isolated by $Ca^{2+}$ chelation are renowned for being leaky to $Ca^{2+}$ so it is unlikely that calmodulin would be in an inactivated state (Velasco et al. 1986; Sepulveda and Smith 1987).

TFP and IODO 8 have $IC_{50}$ values of about 6-50μM and 3-10μM respectively for the inhibition of cAMP synthesis in other calmodulin-dependent enzyme systems, e.g. phosphodiesterase (Roufogalis 1985; MacNeil et al. 1988; Veilg et al. 1989). Although there may be considerable variability in the inhibitory concentrations between different preparations and putative calmodulin-dependent processes, increases in the $IC_{50}$ values two orders of magnitude above the $K_d$ ( i.e. 1-5μM for TFP) of the antagonist/calmodulin complex should be considered with caution before concluding calmodulin- dependent effects are involved (Roufogalis 1985; Oldham and Lipson 1986; Veigl et al. 1989). Corps et al. (1982) has warned of the limitations of using phenothazines as indicators of calmodulin functions in intact cells because they found TFP had an effect on cellular ATP metabolism. This has significant implications for using TFP to demonstrate calmodulin-

sensitivity of an enzyme system, that uses ATP as a substrate. Differences in $IC_{50}$s may also reflect variations in experimental protocol. For instance, membrane preparations tend to result in lower $IC_{50}$s than whole cell preparations (Ayton et al. 1988; MacNeil et al. 1988).

TFP and IODO 8 binds to calmodulin in a $Ca^{2+}$ dependent manner. At high antagonist concentrations (e.g.100μM and above), binding is independent of $Ca^{2+}$ (Roufogalis 1985). Therefore variations in the $Ca^{2+}$ concentrations of different preparations may also add to the variability of reported $IC_{50}$s. In the present systems (i.e. cells and membranes), the $Ca^{2+}$ concentrations were adequate to allow $Ca^{2+}$ dependent antagonist binding to calmodulin. IODO 8 has a higher specificity and lower $IC_{50}$ than TFP (Roufogalis 1985; MacNeil et al. 1988; Veilg et al. 1989). Therefore one would expect IODO 8 to inhibit calmodulin dependent adenylate cyclase activity at lower concentrations than TFP. The data presented here show no difference between the two antagonists with regard to their effects on adenylate cyclase activity. Phenothiazines and naphthalene sulphonamides are hydrophobic molecules and it is their hydrophobicity which contributes to their antagonist characteristics to calmodulin (Mannhold et al. 1987; Weiss et al. 1982; Tanaka et al. 1982). Therefore, TFP and IODO 8 are poorly soluble in aqueous media and are routinely dissolved in DMSO before addition to assay systems. It is possible that by dissolving these compounds in DMSO ( and thereby making them more soluble in water) their hydrophobic nature and calmodulin antagonistic qualities may be lost or reduced. Unfortunately the problems of solubility are inherent in any experiments in which calmodulin antagonists are used. Other properties such as geometric structure, molecular charge, side-chain length or position and orientation are also important for

antagonist-calmodulin interactions (Weiss et al. 1982; MacNeil et al. 1988). Therefore it seems unlikely that solubilization would have a significant influence on the results from the experiments discussed here. Fortunately calmodulin antagonists with improved properties of specificity and solubility are constantly being sort.

There are many examples of *in vivo* studies using calmodulin antagonists to control induced secretory diarrhoea and it is often postulated one of the mechanisms to be inhibition of calmodulin sensitive adenylate cyclase activity (Ilundain and Naftalin 1979; Zavecz et al.1982; Fedorak et al. 1989; Shook et al. 1989; Thillainayagam and Farthing 1990). These experiments were conducted on laboratory animals and support the *in vitro* findings for the presence of a calmodulin sensitive adenylate cyclase in these species (Amiranoff et al. 1983; Pinkus et al. 1983; Lazo et al. 1984). However, calmodulin antagonist used to treat patients with secretory diarrhoea have been less successful because of the high doses necessary to induce any beneficial effects. The *in vitro* data presented here may provide one explanation why calmodulin antagonists, used as therapeutic agents for secretory diarrhoea in humans, have had little success, Donowitz et al. 1984a, b).

**Table 12 Reported *in vitro* concentrations of calmodulin antagonists for the inhibition of the adenylate cyclase system in intestinal tissues**

| Species/ Preparation | Stimulus | TFP μM (% inhib) | W7 μM (% inhib) | IODO 8 μM (% inhib) | Reference |
|---|---|---|---|---|---|
| Rat/membranes | Calmodulin | 85(50) | - | - | Amiranoff et al (1983) |
| Rat/cells | $PGE_2$ | - | - | 50(51) | Ayton et al(1988) |
| Guinea-pig/cells | $PGE_1$ | 75(50) | - | - | Pinkus et al(1983) |
| Rabbit/membranes | *basal | 200(25) | - | - | Lazo et al(1984) |
| | VIP | 200(29) | - | - | |
| Chick/BLM | *basal | 100(44) | 100(26) | - | Long et al (1986) |
| | NaF | 100(21) | 100(34) | - | |
| Human duodenum | | | | | |
| Membranes | *basal | 125(50) | - | 175(50) | present studies |
| | NaF | 225(50) | - | 450(50) | |
| Cells | VIP | 100(0) | - | 100(0) | |

The data in parenthesis indicates the % inhibition at the given μM antagonist concentration
BLM = purified basolateral membranes
* The inhibition of basal adenylate cyclase activity is an indication that the calmodulin antagonists were acting non-specifically

# CHAPTER 4

# CONCLUSIONS

Methods of studying the regulation of intestinal transport and second messenger function range from *in vivo* perfusion studies to the use of highly purified enterocyte brush border and basolateral membrane vesicles. The use of isolated intestinal segments or isolated enterocytes fall somewhere between these two extremes. Traditionally these methods require large amounts of starting material, consequently there have been relatively few studies with human tissue (Simon et al. 1978, 1980a,b,c; Becker et al. 1983). One of the main problems with obtaining "normal" human intestinal material is that it relies on the opportunistic availability of surgical resectioning procedures or on the less predictable source of organ donation (Shirazi-Beechey 1990). Therefore, the development of gastrointestinal endoscopic biopsy, for clinical diagnosis, represents a valuable and continuous source of human bowel for research purposes (Korn et al. 1974; Perera et al. 1975; Rouff et al. 1981; Aadand et al. 1981; Scott et al. 1981; Becker et al. 1982, 1983; Rachmilewitz et al. 1983; Smith et al. 1990; Smith et al. 1991; Hitchin et al. 1991).

The data presented here support the hypotheses that human duodenal epithelial cell adenylate cyclase is calmodulin independent. This is evident in the results from experiments using both membranes and cells derived from human duodenal endoscopic biopsies.

The regulation of adenylate cyclase activity by $Ca^{2+}$ or calmodulin is often proposed as a potential mechanism for the regulation of intestinal secretion but complex and conflicting evidence means that it is rarely adequately explored in the literature either theoretically or experimentally (Ilundain and Naftalin 1979; Binder 1984; Boige et al. 1984; Semrad and Chang 1987; Fedorak et al. 1989; Thillainayagam and Farthing 1990).

The absence of a calmodulin-dependent form of adenylate cyclase in the intestine is in accord with the redundant synarchic control of the cAMP and the $Ca^{2+}$ second messenger systems proposed by Howard Rasmussen (1981) (**Figure 5**). Increases in intracellular $Ca^{2+}$ do not appear to result in increased intracellular cAMP concentrations as might be expected of a calmodulin regulated system. Membrane bound calmodulin, necessary to stimulate adenylate cyclase activity, does not seem to be present in basolateral membranes from cells of the human duodenal mucosa (Stoll et al. 19). Furthermore, Amiranoff's group who had previously demonstrated a $Ca^{2+}$ stimulatory phase of adenylate cyclase activity in membranes from rat enterocytes could not detect a similar stimulatory phase in membranes from human colonic crypt cells (Boige et al. 1984).

It is very difficult to speculate about specific mechanisms for $Ca^{2+}$ regulation of adenylate cyclase activity in epithelial cells from the results of the present membrane work because of the effects of EGTA. EGTA increased the sensitivity of adenylate cyclase activity to inhibition by $Ca^{2+}$. Therefore, it is impossible to say which $Ca^{2+}$ concentration is applicable in vivo. The $Ca^{2+}$ inhibition of adenylate cyclase activity may be an artefact of using EGTA. Relatively high $Ca^{2+}$ concentrations were necessary (mM) to inhibit adenylate cyclase activity in the absence of the chelator. EGTA may expose high affinity $Ca^{2+}$ binding sites on the catalytic unit of adenylate cyclase. This then begs questions about the significance of these sites in the absence of EGTA in vivo. It may take the movement of $Ca^{2+}$ through the plasma membrane to access these sites rather than intracellular increases in $Ca^{2+}$. In other cell types, marked $Ca^{2+}$ concentration gradients and oscillations are established when increases in

intracellular $Ca^{2+}$ are provoked (Cheek et al. 1991; Turnberg 1991). Therefore the whole range of intracellular $Ca^{2+}$ concentrations may be physiologically relevant to the regulation of adenylate cyclase activity. In membrane preparations, any compartmentalization of processes is disrupted and this may be particularly important in a highly polarized epithelial cell. The complexity of equilibria established in the assay of adenylate cyclase activity between membranes, EGTA and the ionic constituents make explanations for results numerous and obscure.

Epithelial cells isolated from duodenal biopsies, on the other hand, provide a unique opportunity to study many different aspects of human gut physiology. Firstly, the relationship between $Ca^{2+}$ and cAMP synthesis may be further explored with the cellular architecture intact. Secondly, the study of second messenger responses to a variety of different agonists may give further insight to specific human epithelial cell receptors. Thirdly, the highly sensitive nature of the cell preparations to VIP will make them particularly useful for the study of specific human gut responses to the peptide and fourthly, physiological responses such as mucus production, hormone synthesis and ion transport may be examined. The epithelial cells isolated from intestinal tissue represent several different types of cell (e g. enterocytes, goblet cells, endocrine cells) and further work to separate these different cell types may prove fruitful.

Perhaps the last word is best left to the nineteenth century Spanish histologist Santiago Ramón y Cajal (1852-1934) who said

"Discoveries are a function of the methods used"

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# Calmodulin independence of human duodenal adenylate cyclase

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Abstract

The calmodulin and calcium dependence of human adenylate cyclase from the second part of the duodenum was assessed in washed particulate preparations of biopsy specimens by investigating (a) the concentration dependent effects of free [$Ca^{2+}$] on enzyme activity, (b) the effects of exogenous calmodulin on enzyme activity in ethylene glycol bis (b-aminoethyl ether)*N*,*N*′-tetra-acetic acid (EGTA) washed particulate preparations, and (c) the effects of calmodulin antagonists on enzyme activity. Both basal ($IC_{50}$=193·75 (57·5) nmol/l (mean (SEM)) and NaF stimulated ($IC_{50}$=188·0 (44·0) nmol/l) adenylate cyclase activity was strongly inhibited by free [$Ca^{2+}$] greater than 90 nmol/l. Free [$Ca^{2+}$] less than 90 nmol/l had no effect on adenylate cyclase activity. NaF stimulated adenylate cyclase activity was inhibited by 50% at 2·5 mmol/l EGTA. This inhibition could not be reversed by free $Ca^{2+}$. The addition of exogenous calmodulin to EGTA (5 mmol/l) washed particulate preparations failed to stimulate adenylate cyclase activity. Trifluoperazine and *N*-(8-aminohexyl)-5-IODO-1-naphthalene-sulphonamide (IODO 8) did not significantly inhibit basal and NaF stimulated adenylate cyclase activity when measured at concentrations of up to 100 μmol/l. These results suggest that human duodenal adenylate cyclase activity is calmodulin independent but is affected by changes in free [$Ca^{2+}$].

Adenylate cyclase is a basolateral membrane enzyme which catalyses the formation of adenosine 3′–5′ monophosphate (cyclic AMP) from adenosine triphosphate (ATP). In the intestinal epithelium adenylate cyclase and cyclic AMP are involved in the regulation of water and electrolyte transport, particularly $Cl^-$ secretion and $Na^+$ absorption. Changes in intracellular free calcium have also been shown to be important in the regulation of intestinal electrolyte transport.[1] Activation of adenylate cyclase is thought to be important in a number of gut disorders such as the secretory diarrhoea associated with cholera and vasoactive intestinal peptide secreting tumours.[2,3] Calmodulin is an ubiquitous intracellular calcium binding protein which influences many enzymic processes. The possible calmodulin dependence of intestinal adenylate cyclase has potential clinical implications as a large number of drugs which are calmodulin antagonists are now well characterised and available – for example, chlorpromazine, trifluoperazine, haloperidol, α-adrenergic antagonists, antimalarials, anticholinergics, and antihistamines.[4] The use of calmodulin antagonists as antidiarrhoeal agents, however, has had only limited success in humans.[5]

$Ca^{2+}$ and calmodulin in some tissues, such as brain, are known to influence adenylate cyclase activity and therefore cyclic AMP production. Several investigators have shown that $Ca^{2+}$ inhibits the activity of adenylate cyclase at concentrations greater than 1 μmol/l while at submicromolar $Ca^{2+}$ concentrations activation of the enzyme occurs. It is thought that this activation of adenylate cyclase is mediated by calmodulin but the inhibition of the enzyme by $Ca^{2+}$ is calmodulin independent.[6–7] There is now evidence to suggest that some rat,[8] guinea pig,[9] and rabbit[10] intestinal adenylate cyclase activity is $Ca^{2+}$ and calmodulin dependent.

In brain tissue both calmodulin dependent and calmodulin independent forms of adenylate cyclase have been identified. The calmodulin dependent component can be inhibited by micromolar concentrations of the calcium chelating agent ethylene glycol bis (b-aminoethyl ether)*N*,*N*′-tetra-acetic acid (EGTA).[3] Furthermore, fluoride ions, which stimulate adenylate cyclase activity by direct activation of the regulatory guanine nucleotide binding proteins, may also help to distinguish the two forms of adenylate cyclase. In vitro the calmodulin dependent form of the enzyme is more responsive to fluoride stimulation than the independent form.[11]

The objective of this study was to assess the importance of $Ca^{2+}$ and calmodulin in the regulation of adenylate cyclase in the second part of the human duodenum. This was accomplished by assessing (a) the concentration dependent effects of free [$Ca^{2+}$] on enzyme activity, (b) the effects of adding exogenous calmodulin to the adenylate cyclase assay system, and (c) the effects on enzyme activity of the calmodulin antagonists trifluoperazine and *N*-(8-aminohexyl)-5-IODO-1-naphthalene-sulphonamide (IODO 8), a new naphthalene sulphonamide calmodulin antagonist of improved potency and specificity.[12] All these parameters were tested on basal and NaF stimulated adenylate cyclase activity. The results cast doubt on the role of calmodulin in the regulation of human duodenal adenylate cyclase.

## Methods

### COLLECTION OF BIOPSY SPECIMENS

Patients presenting at the clinic with diarrhoea or iron deficiency anaemia were biopsied as part of diagnostic investigations to exclude malabsorption. Biopsy specimens of the second part of the duodenum were collected as previously described.[13] Two specimens were placed in formalin for histological examination to exclude villous

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abnormalities. Those for adenylate cyclase studies were immediately placed in liquid nitrogen. Only specimens from patients subsequently found to have normal histology, who had no underlying upper gastrointestinal disease, and who were not taking any medication were used for these studies. Ethical permission for the study was obtained from the Nottingham City Hospital Ethical Committee and patients gave informed written consent.

### TISSUE PREPARATIONS

The presence of extracellular and intracellular fluids in tissue homogenates has been shown to interfere with adenylate cyclase activity.[14-16] We therefore washed biopsy homogenates with a series of homogenisation and centrifugation steps. Four or five biopsy specimens from one patient were homogenised in 2 ml ice cold 2 mmol/l HEPES buffer, pH 7·5, containing 5 mmol/l $MgCl_2$ and 5 mmol/l EGTA (MEH) using 10 strokes of a Potter S homogeniser (B Braun) at 1200 rpm. The homogenate was made up to 12 ml and was centrifuged at 2500 *g* for 20 minutes at 4°C. The pellet was resuspended in 2 ml fresh MEH and the homogenisation/centrifugation procedure repeated twice more.

Any large tissue fragments remaining after the second homogenisation were removed. Microscopic examination showed them to be underlying muscularis mucosa and not epithelium.[17] The third pellet was resuspended in 2·4 ml MEH and homogenised a fourth time using an Ultra-Turrax (Janke & Kunkel, Ika-Werk) at maximum speed setting for 15 seconds. Calmodulin may be removed from membrane preparations by washing them in EGTA buffers.[5] Therefore, in experiments in which no attempt was made to remove endogenous calmodulin EGTA was omitted from the tissue buffer. All buffers were made up in double distilled deionised water or Hipersolv (high performance liquid chromatography grade water).

Cerebral cortex from 2 male Wistar rats were combined and treated as for duodenal biopsy specimens. This washed particulate preparation was used as a positive control to ensure that the assay conditions used were capable of detecting calmodulin dependent adenylate cyclase activity.

### ASSAY OF ADENYLATE CYCLASE

Adenylate cyclase was assayed by the procedure of Salomon *et al.*[18 19] The assay measures the formation of [$^{32}$P] cyclic AMP from [$\alpha$-$^{32}$P]ATP. In a total volume of 100 µl the standard reaction mixture contained: an ATP regenerating system of 13 mmol/l creatine phosphate and 0·1 mg/ml creatine phosphokinase, 1 mmol/l ATP labelled with [$\alpha$-$^{32}$P]ATP to give approximately $1\times10^6$ cpm, and 40 µl adenylate cyclase assay buffer which consisted of 125 mmol/l tris-HEPES, 0·25% bovine serum albumin, 5 mmol/l $MgCl_2$ and 2·5 mmol/l of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine, at pH 7·5. For NaF stimulated activity, reactions also contained 10 mmol/l NaF (originally dissolved in 1 mmol/l acetic acid). When calmodulin was added to the assay it was dissolved in the adenylate cyclase assay buffer. Calmodulin antagonists (trifluoperazine and IODO 8) were dissolved in dimethyl sulphoxide before addition to the reaction system. Control reaction mixtures contained dimethyl sulphoxide only and final dimethyl sulphoxide assay concentrations were never allowed to exceed 1% (v/v).

Reactions were initiated by the addition of approximately 30 µg of particulate protein and carried out for 30 minutes at 37°C. Adenylate cyclase activity was linear up to the maximum conditions tested for time (60 minutes) and protein concentration (3·9 mg/ml). Reactions were terminated by the addition of 100 µl of stopping solution containing: 10 mmol/l ATP, 1 nmol/l [$^3$H]-cyclic AMP (25 000 cpm/100 µl) to estimate [$^{32}$P] cyclic AMP recovery which was 70–90%), 2% sodium dodecylsulphate (w/v), and 50 mmol/l Tris-HCl at pH 7·5. Tubes were placed in a boiling water bath for 2 minutes, allowed to cool, and made up to 1 ml with distilled water. [$^{32}$P] cyclic AMP was purified, before liquid scintillation counting, by the double column procedure of Salomon *et al.*[18]

### CALMODULIN ASSAY

The calmodulin content of the particulate preparations was assayed according to the phosphodiesterase activation method of Thompson *et al.*[20 21]

### SUCRASE DETERMINATION

The brush border enzyme, sucrase, was assayed by the method of Dahlqvist.[22]

### PROTEIN DETERMINATION

The protein content of the tissue preparation was determined by Lowry's method[23] and was normally found to be approximately 1 mg/ml. Bovine serum albumin was used as standard.

### CALCULATION OF FREE $Ca^{2+}$ CONCENTRATIONS

Contamination of buffered solutions by $Ca^{2+}$ (up to 20 µmol/l) necessitates the use of the divalent chelator EGTA in controlling and estimating the free [$Ca^{2+}$].[24 25] For each calcium chloride addition the free [$Ca^{2+}$] was computed for ambient pH 7 in the presence of $Mg^{2+}$, EGTA, and ATP using an updated version of the ligand-metal binding program of Feldman *et al.*[26 27]

### STATISTICAL ANALYSIS

Where appropriate the Wilcoxon two sample test and the Wilcoxon signed rank tests were used to estimate probability values. Probability values of $<0·05$ were taken as significant.

### MATERIALS

All chemicals were from Sigma Chemical with the exception of [$^3$H]cyclic AMP and [$\alpha$-$^{32}$P] ATP which were from Amersham (UK) and Hipersolv from BDH (Aterstone). The IODO 8 was kindly given by Ian Coutts and Pam

TABLE I *Relative specific activity of sucrase and adenylate cyclase activity in pooled supernatants and particulate preparations of human duodenal biopsy specimens. Results are mean (SEM) from three separate preparations assayed twice*

| | | Adenylate cyclase | |
|---|---|---|---|
| *Fraction* | *Sucrase* | *Basal* | *NaF stimulated* |
| Homogenate | 1 | 1 | 1 |
| Pooled supernatant | 6·60 (0·87) | 0·67 (0·25) | 0·95 (0·20) |
| Pellet | 0·83 (0·02) | 1·69 (0·32) | 2·53 (0·09) |

O'Donnell of Nottingham Polytechnic, Nottingham.

## Results

### CHARACTERISTIC OF PARTICULATE PREPARATIONS FROM HUMAN DUODENAL BIOPSY SPECIMENS

Table I gives the relative specific activity of the brush border enzyme, sucrase, compared with the basolaterally located adenylate cyclase in pooled supernatants and particulate preparations from three independent tissue preparations. Sucrase activity was enriched in the pooled supernatants and adenylate cyclase activity was enriched in the particulate fractions.

The calmodulin content of the EGTA washed particulate preparations was compared with that of preparations washed in the absence of EGTA to assess the effectiveness of the EGTA washing procedure at removing calmodulin. In brain tissue there was a 25% (7·49 μg/mg protein to 5·61 μg/mg protein) reduction in calmodulin after washing with EGTA containing buffer. The calmodulin content of the particulate preparation from human duodenal biopsy specimens, however, was approximately 4–6 μg/mg protein irrespective of whether the preparation had been washed in EGTA containing buffer or not.

### THE EFFECTS OF $Ca^{2+}$, AND EGTA ON ADENYLATE CYCLASE ACTIVITY

Stimulation of adenylate cyclase activity at low free [$Ca^{2+}$] has been reported to be a good indication of the enzyme's calmodulin dependence.[5] Therefore, the concentration dependent effects of $Ca^{2+}$ on adenylate cyclase activity in particulate preparations of human duodenal biopsy specimens was measured. Both basal ($IC_{50}$=193·75 (57·5) nmol/l (mean (SEM)) and NaF stimulated ($IC_{50}$=188·0 (44·0) nmol/l) adenylate cyclase activities were strongly inhibited by free [$Ca^{2+}$] greater than 90 nmol/l (Fig 1). Free [$Ca^{2+}$] less than 90 nmol/l neither stimulated nor inhibited adenylate cyclase activity. These data indicate a dramatic reduction in adenylate cyclase activity over a narrow free [$Ca^{2+}$] range.

An alternative way of assessing the importance of $Ca^{2+}$ in our assay system was to measure the concentration dependent effects of the $Ca^{2+}$ chelating agent EGTA on adenylate cyclase activity (Fig 2). NaF stimulated adenylate cyclase activity was inhibited by EGTA in a concentration dependent manner with 50% inhibition at 2·5 mmol/l. Attempts to restore EGTA inhibited adenylate cyclase activity with $Ca^{2+}$ did not result in a recovery of the enzyme's activity, indicating direct inhibition of adenylate cyclase activity by EGTA. A slight but highly significant increase in basal adenylate cyclase

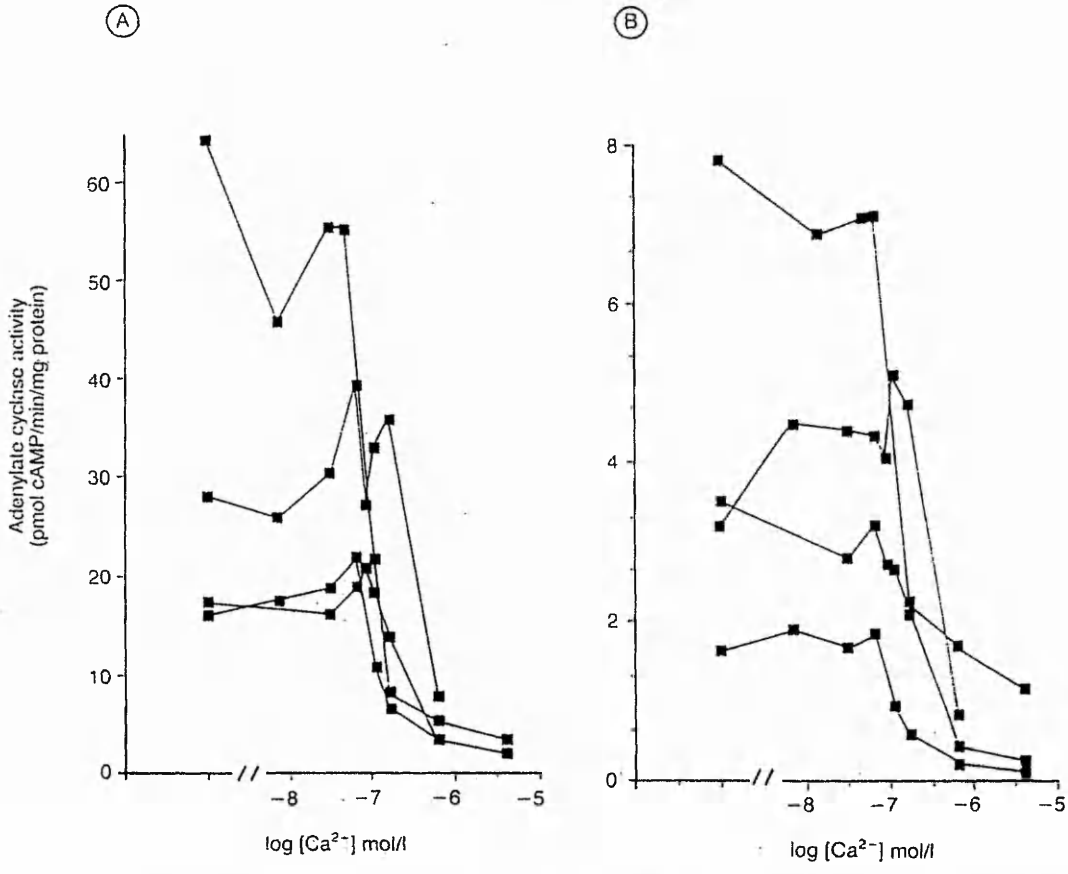


*Figure 1: Adenylate cyclase concentration response to free $Ca^{2+}$. NaF stimulated (A) and basal (B) adenylate cyclase activity was assayed in particulate preparations of duodenal biopsy specimens from four patients. Activity was in the presence of 2·5 mmol/l ethylene glycol bis (b-aminoethyl ether) N,N'-tetra-acetic acid (EGTA) and $CaCl_2$ at concentrations which gave calculated $[Ca^{2+}]$ free as indicated on the abscissa. Each line represents one patient and each data point is the mean of four replicates. Controls were in the absence of added $CaCl_2$ and in the presence of EGTA.*

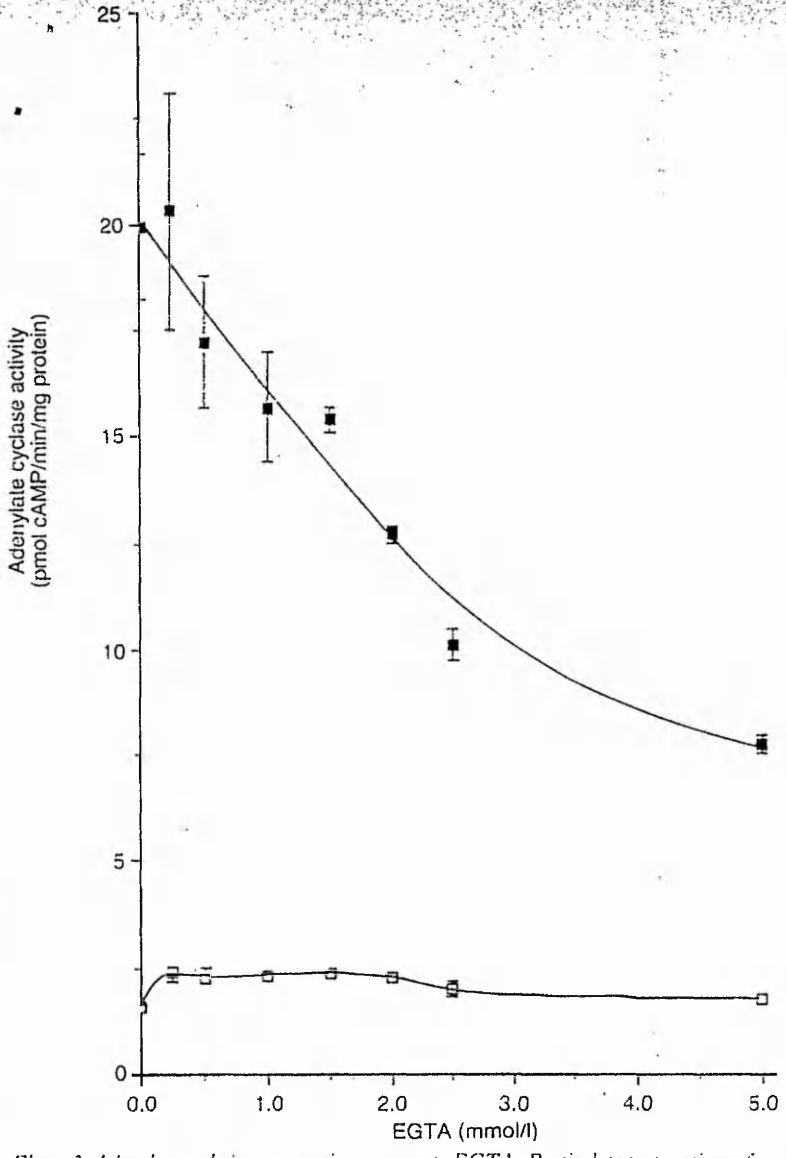


*Figure 2: Adenylate cyclase concentration response to EGTA. Particulate preparations of duodenal biopsy specimens were assayed for NaF stimulated (■) and basal (□) adenylate cyclase activity in the absence of added $CaCl_2$ and in the presence of EGTA at the concentrations indicated. Data are the means (SEM) from three experiments – that is, three patients – each with four replicates.*

TABLE II *Effect of exogenous calmodulin on adenylate cyclase activity in particulate preparation from human duodenal biopsy specimens. Data are the results of three separate experiments each with four replicates*

| Added [$Ca^{2+}$] (mmol/l) | [calmodulin] (μmol/l) | [EGTA] mmol/l | Calculated free [$Ca^{2+}$] (pCa) | pmol cAMP/min/mg protein (SEM) | |
|---|---|---|---|---|---|
| | | | | Basal | NaF stimulated |
| 0·16 | 0·15 | 2·5 | 7·76 | 3·20 (0·29) | 19·88 (1·07) |
| 0·16 | 0 | 2·5 | 7·62 | 2·85 (0·24) | 20·77 (0·76) |
| 1·16 | 0·15 | 2·5 | 6·52 | 0·54 (0·04) | 4·22 (0·32) |
| 1·16 | 0 | 2·5 | 6·52 | 0·51 (0·05) | 3·50 (0·32) |
| 0 | 0 | 2·5 | – | 3·40 (0·17) | 20·60 (0·49) |
| 0·80 | 3·13 | 2·5 | 6·78 | 3·72 (0·03) | 14·98 (0·28) |
| 0·80 | 0 | 2·5 | 6·78 | 3·04 (0·08) | 13·33 (0·45) |
| 0 | 0 | 2·5 | – | 4·25 (0·04) | 14·74 (0·51) |
| 0·40 | 3·73 | 1·0 | 6·63 | 1·36 (0·06) | 11·74 (0·12) |
| 0·40 | 0 | 1·0 | 6·63 | 1·88 (0·03) | 9·90 (0·26) |
| 0 | 0 | 1·0 | – | 2·34 (0·05) | 18·73 (0·72) |

TABLE III *Control experiments in which the effect of exogenous calmodulin on adenylate cyclase activity in particulate preparation of rat cerebral cortex was measured. Data are the results of two separate experiments each with duplicate determinations*

| Added [$Ca^{2+}$] (mmol/l) | [calmodulin] (μmol/l) | [EGTA] mmol/l | Calculated free [$Ca^{2+}$] (pCa) | pmol cAMP/min/mg protein (SEM) | |
|---|---|---|---|---|---|
| | | | | Basal | NaF stimulated |
| 2·30 | 0·61 | 2·5 | 5·40 | 9·93 (4·71) | 53·37 (10·53) |
| 2·30 | 0 | 2·5 | 5·40 | 6·32 (0·25) | 33·48 (3·25) |
| 0 | 0 | 2·5 | – | 5·93 (0·09) | 17·32 (0·26) |
| 0·90 | 0·61 | 1·0 | 5·51 | 18·57 (1·32) | 51·83 (2·61) |
| 0·90 | 0 | 1·0 | 5·51 | 4·73 (1·18) | 37·35 (3·47) |
| 0 | 0 | 1·0 | – | 6·72 (0·26) | 23·94 (0·04) |

activity was observed at 0·25 mmol/l EGTA compared with controls; thereafter the curve plateaued up to the maximum concentration tested (5 mmol/l) (Fig 2).

### EFFECTS OF EXOGENOUS CALMODULIN ON ADENYLATE CYCLASE ACTIVITY

The removal of calmodulin by washing membranes with EGTA buffers and the subsequent stimulation of adenylate cyclase activity by adding calmodulin back to the assay system has been shown for several tissues.[5] No stimulation could be shown in our results from similar experiments using human duodenal biopsy particulate preparations. The addition of calmodulin failed to stimulate adenylate cyclase activity in the presence of various combinations of free calcium, calmodulin, and EGTA (Table II). None of the assay conditions shown resulted in a stimulation of adenylate cyclase activity over controls in the presence or absence of 10 mmol/l NaF.

The possibility existed that our assay methods were incapable of detecting calmodulin dependent adenylate cyclase activity. We therefore measured adenylate cyclase activity in a tissue well recognised to have a highly active calmodulin dependent component, rat cerebral cortex.[3] The preparation and conditions of assay for rat cerebral cortex were conducted in exactly the same way as for human duodenal biopsy specimens. This tissue clearly showed the following characteristics: (a) the calmodulin content of the preparation was reduced by 25% by the EGTA washing procedure used, (b) a biphasic response to calcium (activation followed by inhibition) with a maximum requirement for free [$Ca^{2+}$] of 1–10 μmol/l, (c) the addition of 10 μg/ml calmodulin to EGTA wash particulate preparations stimulated adenylate cyclase activity by 30–60% in the presence and absence of 5 mmol/l NaF (Table III). These findings are fully compatible with what is known for brain calmodulin dependent adenylate cyclase.[3] Verification of the assay methods used was thus established.

### EFFECTS OF CALMODULIN ANTAGONISTS ON ADENYLATE CYCLASE ACTIVITY

The dose dependent effects of trifluoperazine and IODO 8 were examined to establish at what concentration these calmodulin antagonists exerted an influence on adenylate cyclase activity and further to compare the effects of the relatively non-specific trifluoperazine to that of the more specific IODO 8.[12] By analysis of variance trifluoperazine (Fig 3) and IODO 8 (Fig 4) did not significantly inhibit basal and NaF stimulated adenylate cyclase activity up to a concentration of 100 μmol/l. The $IC_{50}$ for trifluoperazine of NaF stimulated adenylate cyclase activity was 225 μmol/l and for basal activity 125 μmol/l. Corresponding $IC_{50}$ values for IODO 8 were 450 and 175 μmol/l, however, indicating that the NaF stimulated adenylate cyclase activity was inhibited less potently than basal activity. This also shows that IODO 8 is less potent than trifluoperazine at directly inhibiting adenylate cyclase activity. These data

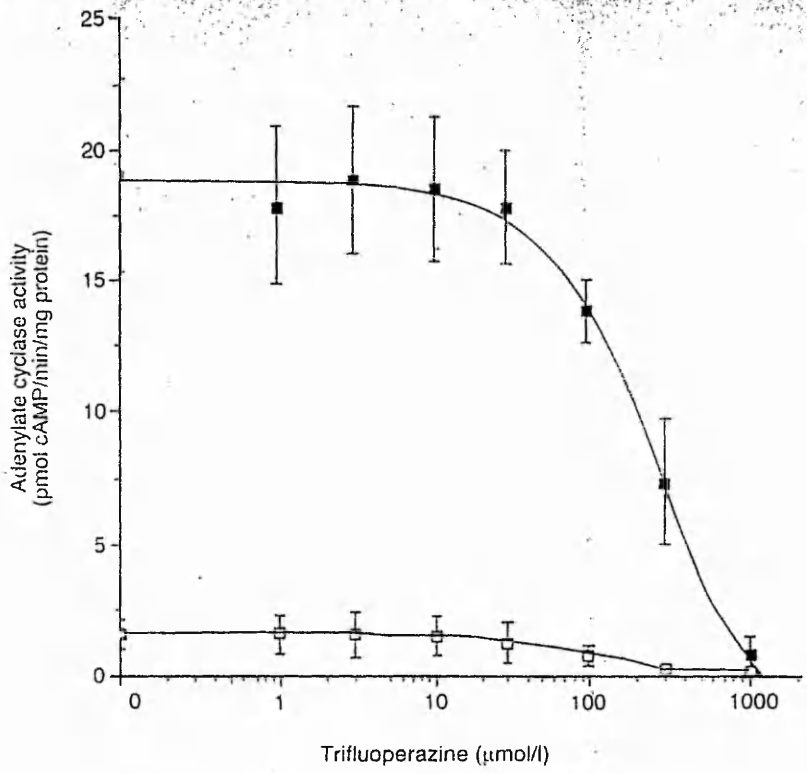


*Figure 3: Adenylate cyclase concentration response to trifluoperazine. Biopsy particulate preparations were assayed for NaF stimulated (■) and basal (□) adenylate cyclase activity in the presence of background $Ca^{2+}$ and the trifluoperazine concentrations indicated. No attempt was made to remove endogenous calmodulin from these preparations. Data are the means (SEM) for three separate patients with four replicates for each trifluoperazine concentration.*

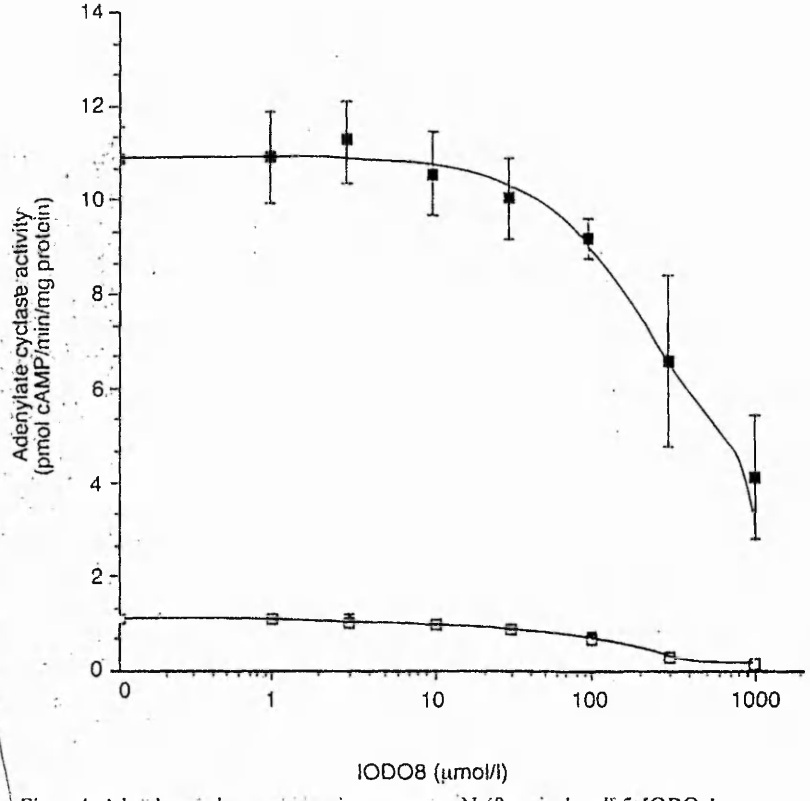


*Figure 4: Adenylate cyclase concentration response to N-(8-aminohexyl)-5-IODO-1-naphthalene-sulphonamide (IODO 8). Biopsy particulate preparations were assayed for NaF stimulated (■) and basal (□) adenylate cyclase activity in the presence of background $Ca^{2+}$ and the IODO 8 concentrations indicated. No attempt was made to remove endogenous calmodulin from these preparations. Data are the means (SEM) for three separate patients with four replicates for each IODO 8 concentration.*

suggest that inhibition of basal as well as stimulated adenylate cyclase activity at concentrations of this magnitude represent the non-specific effects of these calmodulin antagonists.

## Discussion

Data presented in this paper suggest that human duodenal adenylate cyclase activity was affected by small changes in free $[Ca^{2+}]$. Unlike the intestinal adenylate cyclase from rat,[8] guinea pig,[9] and rabbit,[10] it was calmodulin independent. Evidence to support this comes from a number of our findings. Firstly, we were unable to detect any consistent $Ca^{2+}$ stimulation of adenylate cyclase activity of a magnitude previously reported in other systems.[5,8,10] The detection of $Ca^{2+}$ stimulation of adenylate cyclase is notoriously difficult because it occurs at very low $Ca^{2+}$ concentrations. Therefore, careful manipulation of free ion concentrations by EGTA buffers is necessary.[3,4,24,25]

The inhibition of adenylate cyclase activity by EGTA implies a requirement of the enzyme for $Ca^{2+}$. This effect is only apparent on the NaF stimulated adenylate cyclase activity which initially suggests that $Ca^{2+}$/calmodulin may only be important in the regulation of the stimulated enzyme. The effects of EGTA, however, could not be reversed by $Ca^{2+}$. This suggests that the chelator may have been inhibiting the enzyme directly and not in a way mediated by $Ca^{2+}$ chelation and calmodulin inactivation.[3,4] The slight increase in basal adenylate cyclase activity at low EGTA concentrations may be the result of the chelation of an unknown inhibiting ion.[28] The mechanism for $Ca^{2+}$ inhibition of adenylate cyclase has not been elucidated. Some evidence indicates specific $Ca^{2+}$ inhibitory sites,[29] while other evidence implies $Ca^{2+}$ competition for $Mg^{2+}$ binding sites.[30] There is no evidence that the inhibition of adenylate cyclase by $Ca^{2+}$ is mediated through calmodulin.[3,4]

Secondly, we were unable to stimulate adenylate cyclase activity in EGTA washed particulate preparations with exogenous calmodulin. This may be explained by the inadequate removal of the endogenous membrane bound calmodulin by buffers containing EGTA. Indeed, the calmodulin content of our particulate preparations from duodenal biopsy specimens was not significantly reduced by the EGTA washing procedure. The levels of calmodulin in our assay system were comparable to those of other workers using animal intestinal membranes and some have shown calmodulin dependence in the presence of endogenous calmodulin at concentrations similar to those in our assay system.[6,9] The effective removal of calmodulin from different tissues has been reported to vary considerably.[3-5] Our data suggest there may also be species differences between the same tissue. This observation has been noted for other tissues, such as heart sarcoplasmic reticulum,[31] kidney,[32] and pancreatic islets.[33]

Recent evidence on the distribution of calmodulin in enterocytes indicated that in the human duodenum calmodulin was confined to the soluble fractions and to the brush border membrane; none was detected in the basolateral

membrane.[34] This provides indirect support for our findings in that the distribution of human duodenal adenylate cyclase (a basolateral membrane enzyme) is not coincidental with the distribution of membrane bound human duodenal calmodulin. It may be that in our assay system, in which both brush border and basolateral membranes are present, the tightly bound brush border membrane calmodulin becomes available to influence the basolateral membrane adenylate cyclase. But if this were so, and we had been measuring a fully calmodulin stimulated adenylate cyclase system, we would have expected to be able to block the calmodulin effect with calmodulin antagonist (at concentrations specific to calmodulin) and this was not the case. The $IC_{50}$ for trifluoperazine and IODO 8 were well in excess of those reported for other calmodulin dependent enzymes (6–50 and 3–10 μmol/l respectively).[12 35 36] Interestingly, the distribution of calmodulin in the membranes of rat enterocytes seems to be different from that of human enterocytes. Charpin *et al*[37] reported that in rat duodenal basolateral membranes fractions calmodulin could be detected.

Although further investigations are necessary to confirm our findings, these preliminary data indicate that adenylate cyclase in the human duodenal mucosa is calmodulin independent. This implies that increasing the specificity of calmodulin antagonists for use as antidiarrhoeal drugs, which operate through the adenylate cyclase system, would have little pharmacological benefit. It would be interesting to see if human jejunal and ileal tissue adenylate cyclase activity behaves in a similar way.

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*Gut*, 1990, 31, 1350–135

# Effect of vasoactive intestinal peptide on cyclic adenosine monophosphate production in enterocytes isolated from human duodenal biopsy specimens

J A Smith, M Griffin, S E Mireylees, R G Long

Abstract

A modification of a cell isolation technique used in animal studies was developed to remove enterocytes from duodenal biopsy specimens. Citrate-ethylenediaminetetra-acetic acid treatment removed enterocytes from any underlying lamina propria and produced single cells and strips of cells. A mean (SEM) of 4·39 (2·06) $\times 10^6$ cells was obtained from nine duodenal biopsy specimens. Enterocyte recovery was estimated enzymatically using alkaline phosphatase activity and was found to be 61%. Cytological assessment of the cells with CAM 5·2 showed that 98% of the cells isolated were enterocytes with an intact brush border. The cells responded well to vasoactive intestinal peptide stimulation in the absence of an exogenously added adenosine triphosphate regenerating system. The addition of vasoactive intestinal peptide to duodenal enterocytes produced a biphasic dose dependent increase in cyclic adenosine monophosphate production. Stimulation of these cells with $10^{-13}$M vasoactive intestinal peptide resulted in a 50% stimulation over basal value while $10^{-6}$M vasoactive intestinal peptide led to a fivefold increase in cyclic adenosine monophosphate production. We conclude that duodenal biopsy specimens are a good source of human intestinal cells for the study of enterocyte physiology. The cells were viable and highly responsive to vasoactive intestinal peptide.

Vasoactive intestinal peptide (VIP) is a 28 amino acid peptide originally isolated from porcine small intestine by Said and Mutt.[1] It is believed to be a neurotransmitter and is found extensively throughout the central nervous system and gastrointestinal tract.[2 3] It is secreted by non-beta islet cell pancreatic tumours and ganglioneuroblastomas (VIPomas)[2–4] which are clinically characterised by the watery diarrhoea, hypokalaemia, and achlorhydria (WDHA) syndrome.[5] Infusion of VIP into the blood of normal man and animals produces the profuse secretory diarrhoea associated with these tumours.[6] These two pieces of evidence indicate an endocrine role for VIP when high concentrations in plasma are present. Normal plasma values of VIP are thought to be the result of neurone 'dumping' and not to have any physiological function.[7]

The mechanism by which VIP exerts its effects on intestinal secretion is thought to be through the stimulation of adenylate cyclase and therefore cyclic adenosine 3′, 5′—monophosphate production.[4] Raised cyclic adenosine monophosphate values lead to active $Cl^-$ secre tion and decreased $Na^+$ absorption, the overa effect being net intestinal secretion.[8]

Initial in vitro studies with human intestina homogenates failed to show VIP induced adeny late cyclase stimulation[9 10] at concentrations cor sistent with those found in the plasma of patien with VIPomas ($10^{-11}$–$10^{-9}$ mol/l).[11] The po sibility therefore arose that the hypothesis tha VIP from tumours caused direct enterocyt cyclic adenosine monophosphate production intestinal secretion, and diarrhoea was flawed. I animal studies isolated enterocytes have bee used with greater sensitivity.[7 12–14] The purpose t this study was to isolate enterocytes from huma duodenal biopsy specimens using a calciu chelation technique[15] and to assess the effect VIP in stimulating cyclic adenosine mono phosphate production.

## Patients and methods

Patients presenting at clinic with diarrhoea iron deficient anaemia underwent biopsy as pa of diagnostic investigations to exclude malab sorption. Duodenal biopsy specimens from 20 these patients were used to validate the ce isolation technique. All these patients were su sequently found to have normal duodenal hi tology and no underlying upper intestin disease.

The six patients used for the VIP studies we all women with a mean (SEM) age of 40 (7) year After examination, five of the patients we diagnosed as having the irritable bowel sy drome. All had abdominal discomfort or pai three had mild diarrhoea, and one had increase rectus flatus. The final patient had iron de ciency anaemia for which investigations showe no cause. All these patients had histological normal duodenal mucosa and normal lactas sucrase, and maltase values.

The patients were fasted from midnight, ar the following morning they were prepared wi lignocaine spray to the pharynx and intraveno sedation with diazepam (Diazemuls). An Olyn pus G1F IT endoscope (Keymed, Southend-o Sea, Essex) with 3·7 mm biopsy forceps w used. Two biopsy specimens were taken fro the second part of the duodenum for histologic assessment. Subsequent biopsy specimens we put into 0·9% saline at 4°C. Each specim weighed approximately 13 mg.[16] The researc protocol was approved on ethical grounds by t City Hospital Ethical Committee, Nottin ham and the patients gave informed writt consent.

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PREPARATION OF ISOLATED ENTEROCYTES

A modification of a cell isolation technique used in animal studies was developed.[15 17] The biopsy specimens were transferred to 10 ml citrate buffer (1·5 mM KCl, 96 mM NaCl, 27 mM Na citrate, 8 mM $KH_2PO_4$, 5·6 mM $Na_2HPO_4$, pH 7·3), prewarmed to 37°C, and incubated for 10 minutes in a shaking water bath. The citrate buffer was removed and the specimens were resuspended in 10 ml ethylenediaminetetraacetic acid (EDTA) buffer (1·5 mM EDTA, 0·5 mM dithiothreitol, 10 mM $NaH_2HPO_4$, 154 mM NaCl) at 37°C for 30 minutes. The cell suspension was separated from the biopsy fragments and centrifuged for five minutes at 350 *g*. The pellet of cells was washed twice in TRIS buffer (15 mM TRIS, 120 mM NaCl, 5 mM KCl, 1·6 mM $MgSO_4$, 2 mM $NaH_2PO_4$, 1·2 mM $CaCl_2$, 10 mM glucose pH7·4) at 4°C and gassed with 5% $CO_2$/95% $O_2$. Cells for the study of cyclic adenosine monophosphate were resuspended in the above TRIS buffer with the addition of 0·1% bovine serum albumin and 1 mM 3-isobutyl-1-methylxanthine to prevent cyclic adenosine monophosphate degradation. Enterocyte viability was assumed because the adenylate cyclase responded well to VIP stimulation in the absence of an exogenously added adenosine triphosphate regenerating system.

Time course studies showed that once the cells were isolated the rate of both basal and VIP stimulated cyclic adenosine monophosphate production was constant for the first 10 minutes of incubation. The brush border membrane enzyme, alkaline phosphatase, was assayed to assess enterocyte recovery from the biopsy specimens. Alkaline phosphatase activity was assayed in the cell suspensions and homogenates of the biopsy specimens from which the cells had been harvested by the method of Hausamen.[17 18] Cytological assessment of the enterocytes was by staining with CAM 5·2,[19] a monoclonal antibody used as a marker for normal human secretory epithelia. Deoxyribonucleic acid assay[20] showed approximately 50 μg deoxyribonucleic acid was equivalent to $1\times10^6$ cells.

INCUBATION OF ISOLATED ENTEROCYTES WITH VIP

Synthetic porcine VIP was dissolved in the same TRIS buffer used to suspend the cells with the addition of 4 KIU/ml aprotinin. Cells (approximately $1·5\times10^6$/ml) were preincubated for three minutes at 37°C. The incubation was for a further seven minutes at 37°C and the reaction was initiated with the addition of VIP at concentrations ranging from $10^{-13}$–$10^{-6}$ M. The reaction was terminated with sufficient ice cold trichloroacetic acid to produce a final concentration of 6%. The trichloroacetic acid was removed by amine/Freon extraction.[21] Cyclic adenosine monophosphate was assayed in the extract by a protein binding assay.[22]

MATERIALS

All chemicals were of the highest available grade from the Sigma Chemical Company with the exception of ($^3$H) cyclic adenosine monophosphate which was from Amersham Inc and CAM 5·2 from Becton Dickinson.

STATISTICS

Results are expressed as mean (SEM). The Wilcoxon signed rank test was used to test for significance.

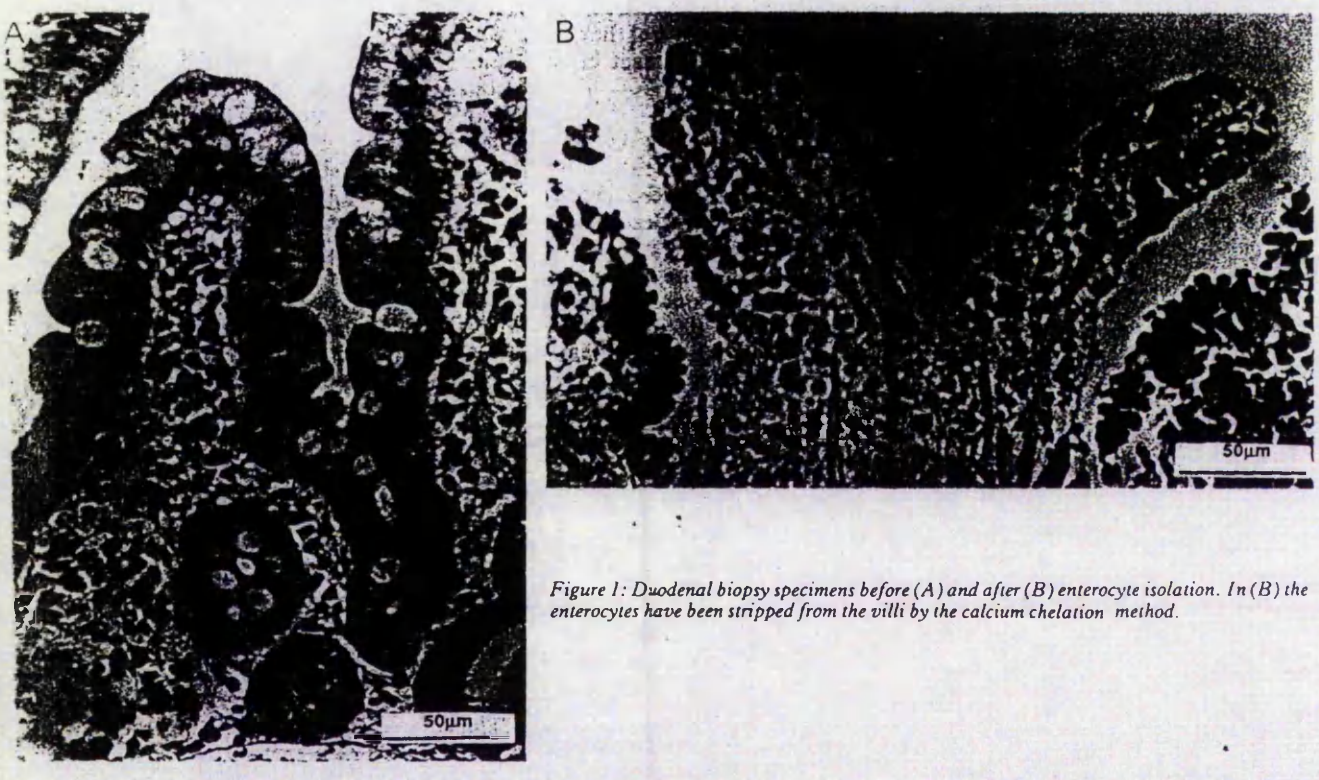


*Figure 1: Duodenal biopsy specimens before (A) and after (B) enterocyte isolation. In (B) the enterocytes have been stripped from the villi by the calcium chelation method.*

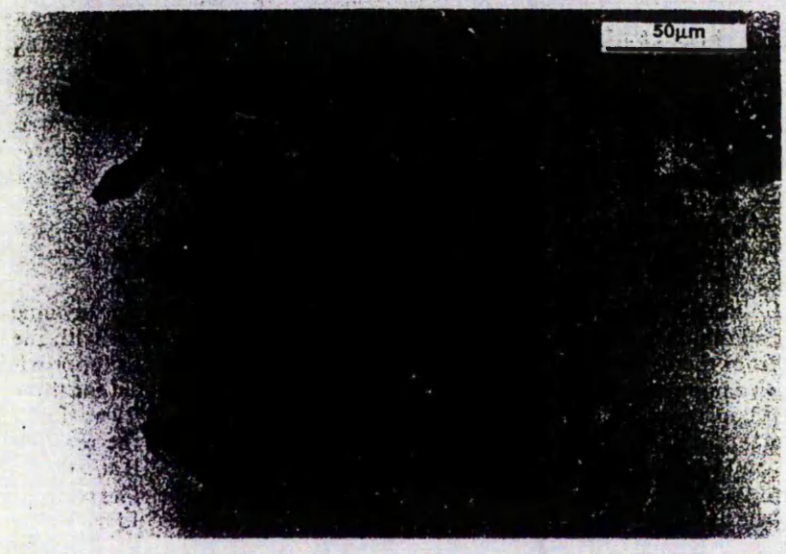


*Figure 2: Enterocytes isolated from duodenal biopsy specimens. The darker staining end of the cells is the intact brush border membrane.*

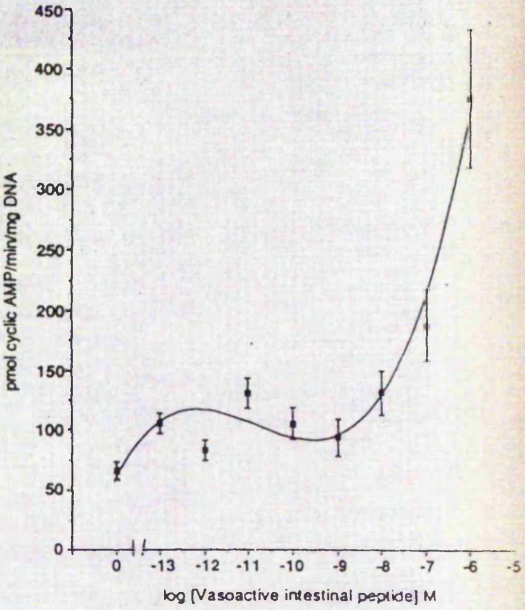


*Figure 3: The dose dependent effect of vasoactive intestinal peptide on human duodenal enterocyte cyclic adenosine monophosphate (AMP) production. Data are mean (SEM) of six experiments.*

## Results

### ISOLATED ENTEROCYTES

Figure 1 shows two duodenal biopsy specimens before and after the cell isolation procedure. The citrate/EDTA treatment removed enterocytes from the underlying lamina propria and produced single cells or strips of small numbers of cells. A mean (SEM) of 4·39 (2·06)$\times 10^6$ cells were removed from nine duodenal biopsy specimens (n=20). Alkaline phosphatase activity is expressed as nmol p-nitrophenol/min/mg protein (n=5). Mean (SEM) alkaline phosphatase activity was 1458 (534) in the cell preparations from the biopsy specimens and 1026 (578) in homogenates of the specimens after the cell isolation procedure. This represented an enterocyte recovery of 61%. The monoclonal antibody, CAM 5·2, showed that 98% of the cells isolated were enterocytes with an intact brush border (Fig 2).

### VIP AND HUMAN DUODENAL ENTEROCYTES

The addition of VIP to isolated duodenal enterocytes seems to produce a biphasic dose dependent increase in cyclic adenosine monophosphate production. Cyclic adenosine monophosphate concentrations in response to $10^{-13}$ M–$10^{-11}$ M VIP were not dose dependent, but they were 50% higher ($p<0·05$) than basal values indicating that even at these very low VIP concentrations there may be an upregulation of adenylate cyclase activity. From $10^{-9}$ M–$10^{-6}$ M, VIP isolated duodenal enterocytes exhibited a linear dose dependent response in cyclic adenosine monophosphate production. At $10^{-6}$ M VIP there was a fivefold increase in cyclic adenosine monophosphate production compared with the basal value (Fig 3).

## Discussion

In this study we have used a calcium chelation technique[15] to remove cells from human duodenal biopsy specimens. This technique allows the isolation of cells that are metabolically active and have intact VIP receptors, and should be applicable to other lines of physiological and pharmacological research. It may also be applicable to other disease states, such as treated coeliac disease, in which enterocytes may be recovered from biopsy specimens and used to ascertain primary lesions. However, unpublished efforts by us using biopsy specimens from patients with villous atrophy have not surprisingly failed to produce cells. Other possible techniques are likely to be less successful. Mucosal scrapes from operative or necropsy specimens taken at the time of organ donation will include additional lamina propria cells. Enzyme techniques – for example trypsin, collagenase, or hyaluronidase – produce intact viable cells but the enzymes seem to damage hormone receptors.[23] Cells in sufficient numbers may be obtained from biopsy material by calcium chelation without undue contamination from underlying non-epithelial tissue. The isolated cells are devoid of neuronal elements and are therefore free from endogenous VIP.[24]

This study provides evidence that VIP stimulates cyclic adenosine monophosphate production in isolated human duodenal enterocytes. The concentration at which VIP stimulation of cyclic adenosine monophosphate could be detected using this cell isolation technique was much lower than that previously reported from human intestinal homogenates[9 10] and more sensitive than other isolated epithelial cell preparations.[25] The shape of the dose response curve is similar to that shown for guinea pig pancreatic acinar cells.[26] The stimulation of cyclic adenosine monophosphate production seemed to occur in two steps with a first plateau of low amplitude for low VIP concentrations and a second one of larger amplitude for higher VIP concentrations. The curve differs from that of pancreatic acinar cells, however, in that the effective concentra-

tions of VIP were 100 to 1000 times lower. This seemingly biphasic effect of VIP on human duodenal epithelial cells was different to the monophasic responses reported with rat isolated jejunoileal epithelial cells[12] and isolated human colonic crypt cells.[27] The shape of the dose response curve suggests the presence of high and low affinity VIP receptors on these cells. Stimulation of low affinity receptors may be more relevant to the neuronal control of gut function, where nerve terminals in close proximity to epithelial cells could attain high VIP concentrations.[28] There is, however, some doubt about the physiological relevance of the low affinity VIP receptors.[29] Stimulation of the high affinity receptors at very low VIP concentrations that give rise to a small but significant cyclic adenosine monophosphate increase fall within both normal blood values and those capable of eliciting intestinal secretion in patients with the VIPoma syndrome.[11] This suggests that either VIP may not have a direct influence upon enterocyte cyclic adenosine monophosphate production in the VIPoma syndrome or that cyclic adenosine monophosphate is not the mediator of intestinal secretion in this disease, and there is some evidence to support both of these ideas.[29 30–32] However, the increase in plasma VIP in VIPoma patients will be in addition to the localised transient neurological VIP already present at the enterocyte basolateral membrane. The increased background values of VIP in combination with the neuronal values may then be sufficient to stimulate the low affinity VIP receptors and generate the large cyclic adenosine monophosphate response and resultant diarrhoea seen in VIPoma patients.

VIP is believed to be cosecreted with at least two other peptides – peptide histidine methionine and peptide histidine valine.[33 34] VIP has been shown to induce small intestinal and colonic secretion of water and electrolytes in human volunteers at plasma values mimicking those seen in patients with the VIPoma syndrome.[35] A similar peptide to peptide histidine methionine and peptide histidine valine – peptide histidine isoleucine – which is found in porcine intestine, has also been infused into normal volunteers and shown to induce intestinal secretion.[36] Recent human infusion experiments of VIP, peptide histidine methionine and peptide histidine valine to similar concentrations to those seen in the VIPoma syndrome have shown much higher ileal secretion in response to VIP than to the latter peptides.[37] This has led to the conclusion that VIP is the major mediator and most appropriate marker of the VIPoma syndrome. There is, however, still some doubt about the mechanism of VIP induced secretory diarrhoea.

We conclude that duodenal biopsy material provides a good, readily available source of human enterocytes for future work on gut physiology. More work is necessary using these cell preparations to discover what other cellular responses are induced by VIP and cyclic adenosine monophosphate.

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J A Smith, M Griffin and R G Long. Calmodulin dependence of human duodenal adenylate cyclase. Eur J Clin Invest 1989;19:221.

# Abstract

CALMODULIN DEPENDENCE OF HUMAN DUODENAL ADENYLATE CYCLASE
J A SMITH, M GRIFFIN*, R G LONG. CITY HOSPITAL AND *TRENT POLYTECHNIC, NOTTINGHAM.

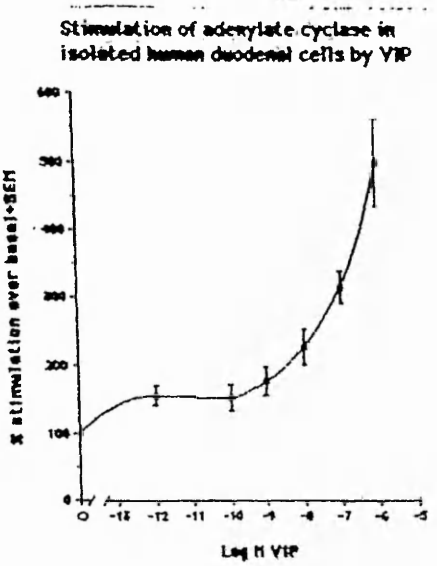
The effects of the calmodulin antagonist N-(4-aminohexyl)-5-chloronaphthalene-1-sulphonamide (W7), ethylene glycol bis ( -aminoethylether) N,N,N',N'-tetraacetic acid (EGTA) and calcium on adenylate cyclase(AC) in washed membranes of human duodenal biopsies were analysed. Dose response of AC to EGTA showed that EGTA concentations greater than 1mM were necessary to inhibit AC activity by 41% and that concentrations less than 1mM had no effect. The addition of 2-4uM calmodulin failed to stimulate both basal and fluoride stimulated AC in the presence of 1mM EGTA. This indicates a system already saturated with calmodulin despite washing the membranes several times with EGTA containing buffer prior to assay. Addition of 80-100uM W7 in the presence of 1mM EGTA failed to inhibit basal (control:2.23+0.58, W7:2.02+0.55) and fluoride stimulated (control:12.85+1.42, W7:12.84+2.26) AC. Results are expressed in pMolcAMP/min/mg protein+SEM. A peak of AC activity was observed at a free calcium concentration of 30nM and concentrations greater than 100nM calcium inhibited the enzyme. This finding is consistent with the effects of calcium on AC reported in other tissue.

We conclude that a large proportion of human duodenal AC is calmodulin independent and that the calmodulin, present in washed membrane preparations that may influence AC, is tightly bound.

J A Smith, J A Smith and R G Long. Isolation of human duodenal enterocytes; Effect of vasoactive intestinal peptide. Eur J Clin Invest 1989;19:221.

# Abstract

ISOLATION OF HUMAN DUODENAL ENTEROCYTES: EFFECT OF VASOACTIVE INTESTINAL PEPTIDE. J A SMITH, J A SMITH, R G LONG, CITY HOSPITAL, NOTTINGHAM. The aim of this study was to assess the possibility of isolating human intestinal cells from duodenal biopsies by calcium chelation. The number and type of cells isolated was determined to estimate contamination by non-epithelial cells. DNA and protein concentrations were correlated with cell number. Alkaline phosphatase, a marker enzyme for the enterocyte brush border, was measured to estimate cell recovery. The effect of vasoactive intestinal peptide (VIP), a potent stimulator of adenylate cyclase in the intestine, on the isolated enterocytes ability to produce cyclic AMP was assessed. On average $4.39 \times 10^6 \pm 2.06$ SEM cells were recovered from nine duodenal biopsies 98% of which were enterocytes. This represented a mean recovery of 61% as assessed by alkaline phosphatase activity. The VIP dose response curve showed that the cells responded in a linear manner between $10^{-9}$ to $10^{-6}$M. However, cyclic AMP levels in response to $10^{-12}$M VIP (68.95 ±16.36) were significantly higher ($p<01$) than basal (49.53 ±15.8). Results are expressed as pMol cyclic AMP/min/mg DNA ±SEM. We conclude that duodenal biopsies are a good source of human intestinal cells for the study of enterocyte physiology.



Smith JA, Griffin M, Mirylees SE, Long RG (1990) Preparation of Isolated enterocytes from human duodenal biopsies and their response to vasoactive intestinal peptide (VIP). Gut 31:A610

# Preparation of isolated enterocytes from human duodenal biopsies and their response to vasoactive intestinal peptide (VIP)

J A SMITH, M GRIFFIN*, S E MIREYLEES*, AND R G LONG (*Medical Research Centre, City Hospital, Nottingham NG5 1PB; Nottingham Polytechnic*, Nottingham NG11 8NS)* A modification of a cell isolation technique used in animal studies was developed to remove enterocytes from human duodenal biopsies. Citrate/EDTA treatment removed enterocytes from any underlying lamina propria and produced single cells and strips of small numbers of cells.

A mean of $4{\cdot}39\times10^{6}\pm(2{\cdot}06)\times10^{6}$ SEM cells were obtained from duodenal biopsies from each patient (n=20). DNA assay showed approximately 50 μg DNA was equivalent to $1\times10^{5}$ cells. Enterocyte recovery was estimated enzymatically using alkaline phosphatase activity and found to be 61%. Cytological assessment of the cells with a monoclonal antibody for normal human secretory epithelia (CAM 5·2 showed that 98% of the cells isolated were enterocytes with an intact brush border.

The cells were viable because adenylate cyclase activity, the enzyme responsible for the production of cyclic AMP from ATP, responded well to VIP stimulation in the absence of an exogenously added ATP regenerating system. The addition of VIP to duodenal enterocytes produces a biphasic dose dependent increase in cyclic AMP production. Cyclic AMP concentrations in response to $10^{-11}$M to $10^{-11}$M VIP were 50% higher ($p<0{\cdot}05$) than basal levels. From $10^{-9}$M to $10^{-6}$M VIP duodenal enterocytes exhibited a linear dose dependent increase in cyclic AMP production.

We conclude that duodenal biopsies are a good source of human intestinal cells for the study of enterocyte physiology. The cells are viable and highly responsive to VIP.

Smith JA, Griffin M, Mireylees SE, Long RG (1991) Vasoactive intestinal prptide (VIP) stimulated cyclic AMP production in epithelial cells isolated from human duodenal biopsy specimens is not calmodulin dependent. Clinical Science 81:(supplement 25) 34P

# VASOACTIVE INTESTINAL PEPTIDE (VIP) STIMULATED CYCLIC AMP PRODUCTION IN EPITHELIAL CELLS ISOLATED FROM HUMAN DUODENAL BIOPSY SPECIMENS IS NOT CALMODULIN DEPENDENT

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VIP is the most potent stimulant of cyclic AMP production in the human intestine so far described. This study examines the dependence of VIP stimulated cyclic AMP production on the calcium binding protein, calmodulin, using the calmodulin antagonists trifuoperazine (TFP) and N-(8-aminohexyl)-5-IODO-1-naphthalene-sulphonamide (IODO 8) in epithelial cells from the human duodenum.

Epithelial cells were isolated from biopsies (Smith et al. GUT (1990) *31*: 1350-1354) with the addition of 1mg/ml soybean trypsin inhibitor, 2mM glutamine, 2mg/ml BSA and 3mM 3-isobutyl-1-methylxanthine in the buffers. 86%±18.8SD (n=15) of isolated cells excluded trypan blue for at least 2hrs after isolation.

Results are the mean±SEM of a minimum of 4 determinations expressed as nM cyclic AMP/5min incubation/mg DNA. $2x10^{-8}$M VIP stimulated cyclic AMP production was linear for the first 5min of incubation; thereafter it plateaued (7.15±0.85). VIP concentrations as low as $10^{-13}$M gave 4.78±1.14 which represents a 7 fold increase above basal (0.67±0.16). $10^{-6}$M VIP gave 7.05±1.30 a 10.5 fold increase above basal. VIP stimulated (2x10-8M) cyclic AMP production was not inhibited by either calmodulin antagonist at concentrations as high as 100μM. Reported concentrations of TFP and IODO 8 necessary to inhibit other calmodulin dependent enzyme systems by 50% are 6μM and 3μM respectively.

The results show that in duodenal epithelial cells cyclic AMP production is highly sensitive to VIP stimulation and this is not regulated by calmodulin.