INHIBITION OF SECRETORY ACTIVITY IN CELLS ISOLATED FROM THE RAT STOMACH.

by

Mark M Atwell

A thesis submitted for the
degree of
Doctor of Philosophy

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The overall aim of this study was to further understanding of the mechanisms by which inhibitors of secretory activity mediate their action in isolated stomach cells. One objective was to determine whether a G-protein sensitive to inactivation by pertussis toxin was involved in the action of the following inhibitors of histamine-stimulated acid secretion: prostaglandin E_2 (PGE_2), somatostatin, epidermal growth factor (EGF) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA), an activator of protein kinase C. The site and mechanism by which EGF inhibited acid secretion and its effects on pepsinogen secretion were also of interest. Further objectives were to determine whether TPA could induce down-regulation of protein kinase C in parietal cells and to examine the inhibitory action of cyclic GMP on acid secretion. Acid secretion was estimated by the accumulation of the weak base aminopyrine in parietal cells.

Experiments in which cells were preincubated with pertussis toxin indicated that PGE_2, somatostatin and EGF mediated their inhibitory action against histamine-stimulation via an inhibitory G-protein of the "G_i-like" family. Stimulation of PGE_2 production by EGF also involved a pertussis toxin-sensitive G-protein. EGF inhibited acid secretion stimulated by forskolin, but only in the absence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). This action of EGF was sensitive to inactivation by pertussis toxin. It is suggested that the effect of EGF was due to an increase in low Km cyclic AMP phosphodiesterase activity, rather than an effect on the histamine (H_2) receptor. EGF did not inhibit pepsinogen secretion.

TPA exerted only a small part of its inhibitory action by a mechanism sensitive to pertussis toxin. TPA was unable to induce detectable down-regulation of protein kinase C. Acid secretion stimulated by near-maximally effective concentrations of histamine plus IBMX, dibutyryl cyclic AMP (dbcAMP) and K^+ was inhibited by dibutyryl cyclic GMP (dbcGMP).

Key Words: Gastric acid secretion. Prostaglandin E_2 (PGE_2). Somatostatin. Epidermal growth factor (EGF). Protein kinase C.

This work is dedicated to

Mrs E. G. Atwell

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Firstly I would like to thank Dr P. J. Hanson for the truly excellent quality of his supervision.

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ABBREVIATIONS.

The following, non-standard abbreviations were used throughout this work.

ANOVAR	Analysis of varience
ANP	atrial natriuretic peptide
APR	aminopyrine accumulation ratio
BSA	bovine serum albumin
CCK	cholecystokinin
CCK-8	cholecystokinin octapeptide
dbcAMP	dibutyryl cyclic AMP
2',5'DDA	2',5'-dideoxyadenosine.
df	degrees of freedom
DMEM	Dulbecco's minimal essential medium
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EGF	epidermal growth factor
Fig.	figure
IBMX	3-isobutyl-1-methylxanthine
kDa	kilodalton (dalton=$^{1}/_{12}^{th}$ of the mass of 1 atom of nuclide ^{12}C)
Mr	relative molecular mass
M. Wt	molecular weight
PGE_2	prostaglandin E_2
PIA	N^6-phenylisopropyladenosine
protein kinase A	cyclic AMP-dependent protein kinase
protein kinase C	calcium-sensitive, phospholipid-dependent protein kinase
SDS	sodium dodecyl sulphate
S.E.M.	standard error of the mean
somatostatin antagonist	cyclo[7-aminoheptanoyl-Phe-D-Tryp-Lys-Thr (Bzl)]
TBS-T	Tris buffered saline with Tween-20
TBS	Tris buffered saline
TGFα	transforming growth factor α.
TPA	12-*O*-tetradecanoylphorbol 13-acetate

Chapter 1

INTRODUCTION

1.1 CELL TYPES IN THE RAT GASTRIC MUCOSA.

The rat stomach is a single-chambered sac which serves as a reservoir and a digestive organ. The rat stomach can be broadly divided into several regions. The non-glandular or cardiac region, which is distal to the entrance to the oesophagus, takes no part in the secretion of acid. Extending down to the pyloric region and duodenum is the glandular region which includes the fundic and antral glandular regions. Gastric glands which contain acid-secreting parietal cells are present in the fundus. Pyloric glands are located in the antrum, which is closer to the pylorus, and contain gastrin-secreting G-cells.

The surface of the gastric mucosa consists of mucus- and bicarbonate-secreting columnar epithelial cells which also extend down into the gastric pits. The surface of the stomach is punctuated with about 100 gastric pits per mm^2 and at the bottom of these pits are gastric glands. Gastric pits increase the surface area by approximately 20-fold. Gastric glands contain a number of different cell-types (Fig.1.1). At the neck of the gland another type of mucous cell is found, the mucous neck cell. Parietal cells secreting HCl are scattered throughout the gland but predominate in the isthmus. Chief cells, which are primarily located at the base of fundic glands, have been shown to contain pepsinogen by immunofluorescent techniques (Samloff, 1971). As many as nine different types of endocrine cell have been identified (Grube and Forssmann, 1979). These endocrine cells are scattered throughout the the gland. Endocrine-like cells which secrete histamine have been located in the rat fundic mucosa.

1.1.1 Morphological changes associated with stimulation of the parietal cell.

Parietal cell morphology has been reviewed by Ito (1987). The parietal cell is a large cell containing a large centrally located nucleus and many mitochondria, which may account for up to 34% of the cell volume. Secreting parietal cells contain a network of narrow canals termed canaliculi which run from the apical (luminal-facing) membrane through much of the cell. Numerous finger-like projections or microvilli protrude into these canals. Secretory canaliculi are poorly developed in the resting cell. An elaborate system of tubular and vesicular membranes called tubulovesicles is prominent in resting cells.

Ultrastructural changes within the parietal cell that take place upon stimulation have been reviewed recently by Forte and Wolosin (1987). Profound morphological changes become apparent as soon three minutes after secretagogues are added to the resting cell (Forte *et al.*, 1981) (Fig 1.2). The secretory canaliculi become more prominent and the microvilli elongate. Stimulated parietal cells have a 6 to 10 times larger apical membrane surface area than resting cells. The increase in the surface area

Figure 1.1

A simplified diagram illustrating some of the cell types present in a gastric gland (Ito, 1987).

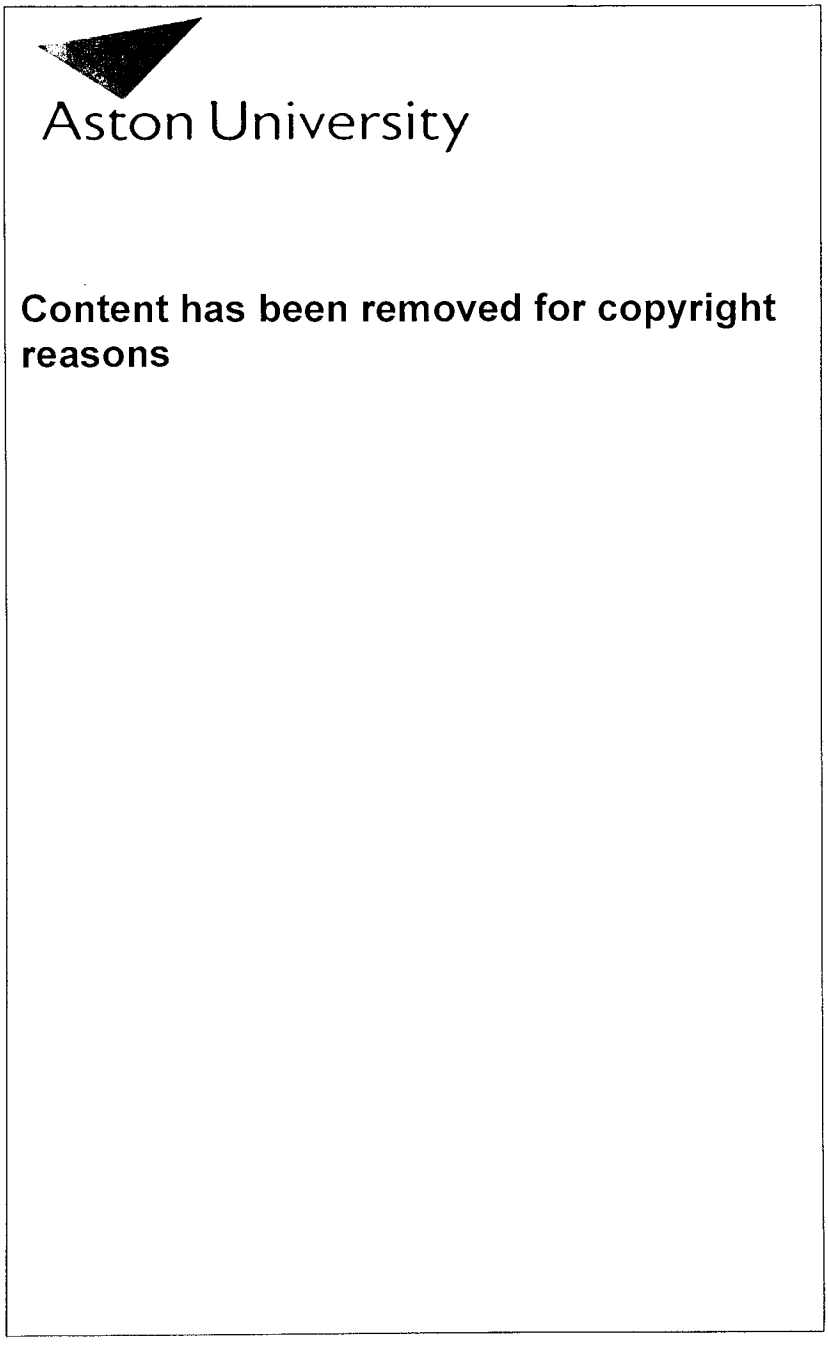
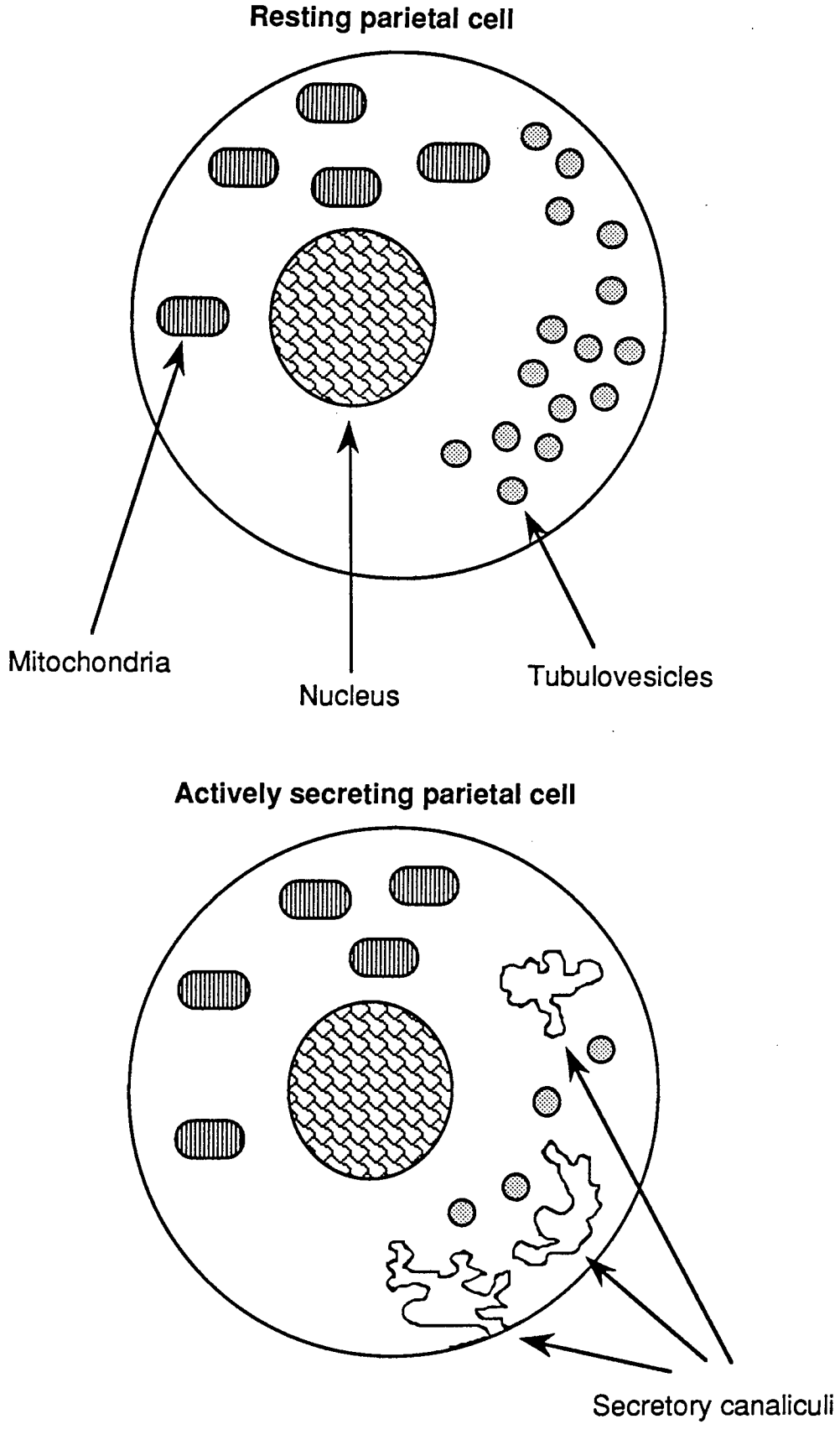


Figure 1.2

Diagrammatic representation of the morphological changes that take place in the parietal cell upon stimulation.



of the apical membrane is almost quantitatively matched with a decrease in surface area of the tubulovesicles (Helander and Hirschowitz, 1972). Two alternative explanations have been proposed to explain this transformation.

The first is the osmotic expansion theory of Berglindh *et al.* (1980) which proposes that the tubulovesicles are collapsed canaliculi. With stimulation HCl accumulates within the lumen of the acid-secreting spaces. This produces a hypertonic environment which causes water to move out into the canaliculi and leads to their expansion. However, Gibert and Hersey (1982b) induced salt and water accumulation within tubulovesicles and found vacuolar swellings but no apical surface expansion. Thus it appears that osmotic expansion alone cannot explain the observed changes.

Secondly the membrane-recycling hypothesis of Forte *et al.* (1977) suggests that the cytoplasmic tubulovesicles migrate to the apical membrane upon stimulation and fuse with it. Evidence in favour of this hypothesis has come from immunocytochemical studies which have shown that the H^+/K^+-ATPase (or proton pump) is predominantly located in the tubulovesicles in the resting cell, but upon stimulation this enzyme becomes detectable on the microvilli of the secretory canaliculi (Smolka *et al.*, 1984). Further evidence for this hypothesis comes from studies with macromolecular tracers and freeze-fracture electron microscopy techniques (Forte and Wolosin, 1987). It is likely that a combination of both these hypothesized mechanisms occur under physiological conditions.

1.1.2 Chief cell morphology.

Chief cell morphology has been reviewed by Ito (1987). Studies on the chief cell have been rather limited in comparison to the parietal cell. The luminal surface of the chief cell has a thin fuzzy coating of glycoprotein called a glycocalyx and contains stubby microfilaments. The lateral membrane is quite smooth and a thin basement lamina underlies the cell, as with all epithelial cells. Zymogen granules containing pepsinogen are usually present in the apical cytoplasm. The granules often appear to be enclosed within a trilaminar membrane. Pepsinogen is released by exocytosis.

1.2 MECHANISM OF ACID AND PEPSINOGEN SECRETION.

1.2.1 Mechanism of acid secretion.

The mechanism by which the parietal cell secretes acid has been reviewed by Forte and Wolosin (1987). In a low-density microsomal fraction isolated from parietal cell homogenates it appears that H^+: K^+ exchange occurred and was accompanied by ATP hydrolysis (Lee *et al.*, 1974). It was later discovered that ATPase-dependent efflux of the K^+ substitute Rb^+ paralled H^+ uptake and this suggests that the process

was electroneutral (Sachs *et al.*, 1976). The enzyme responsible for the movement of H^+ and K^+ became known as the gastric H^+/K^+-ATPase. Thus it was suggested that the H^+/K^+-ATPase had a central role in gastric acid secretion.

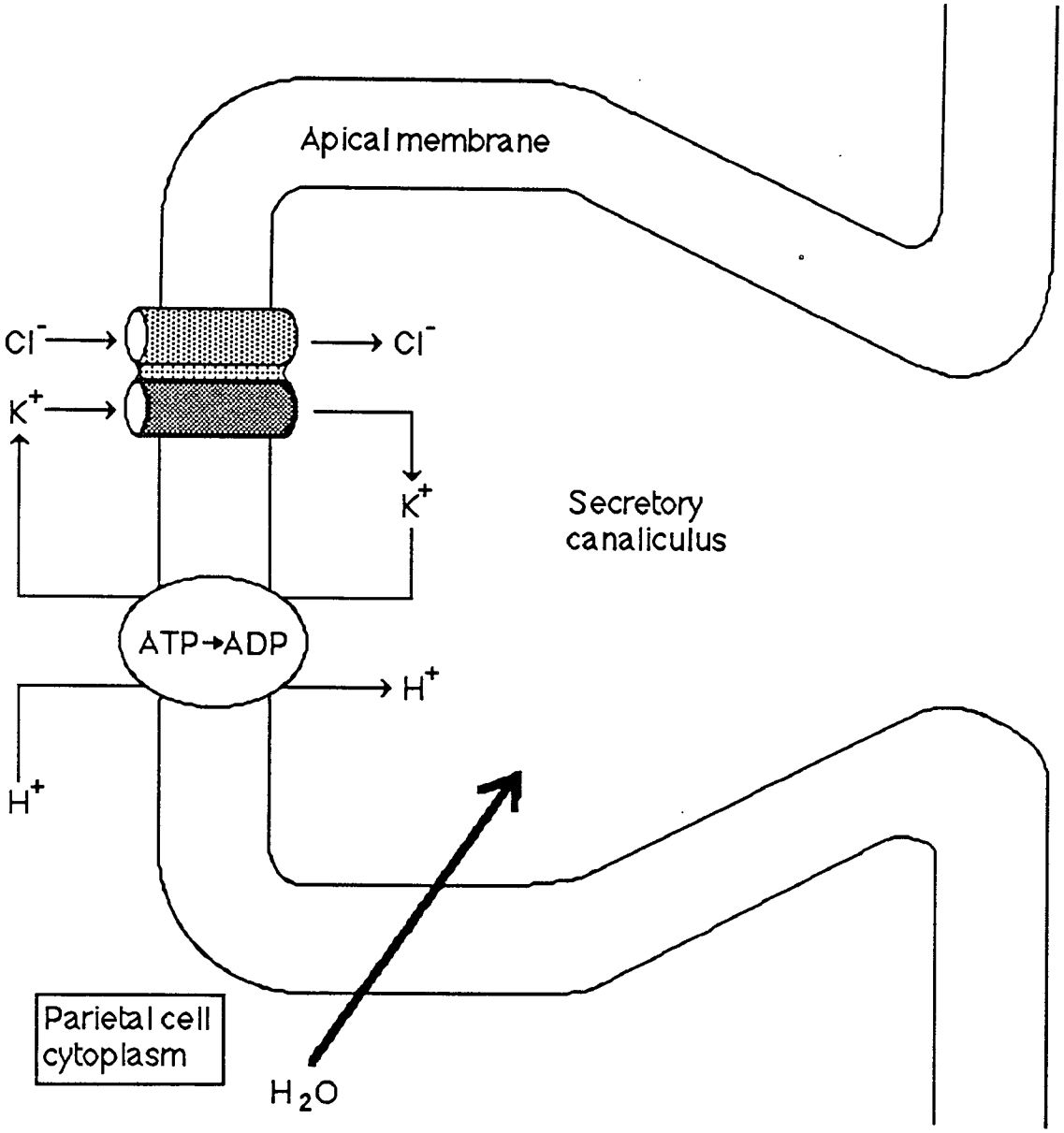
In the non-secreting stomach the H^+/K^+-ATPase was found predominantly in the tubulovesicles, isolated as the light microsomal membrane fraction. In the stimulated stomach most of the H^+/K^+-ATPase activity was localized to a much heavier and larger membrane fraction which is believed to be derived from the apical membrane of the stimulated parietal cell (Wolosin and Forte, 1981a; Hirst and Forte, 1985). The vesicles derived from these membranes were termed stimulation-associated vesicles. Vesicles from the light microsomal membrane fraction have displayed a low permeability to K^+. In contrast to light microsomal vesicles, stimulation-associated vesicles were readily permeable to K^+(Wolosin and Forte, 1981b; Hirst and Forte, 1985).

The currently accepted model by which acid is secreted across the apical membrane is shown in Fig 1.3 (Wolosin, 1985). K^+ and Cl^- enter the lumen of the canaliculus via conductance pathways in the apical membrane. Studies with ^{36}Cl and ^{86}Rb suggest the pathways for K^+ and Cl^- entry appear to be functionally independent (Wolosin and Forte, 1985). However the divalent cations Zn^{2+} and Mn^{2+} inhibited both Rb^+ and Cl^- movement to the same extent and with the same inhibition constants, suggesting that the two conductances may reside within the same physical unit (Wolosin and Forte, 1985). HCl forms when K^+ is recycled back into the cell by the H^+/K^+-ATPase in exchange for H^+ with the metabolism of ATP. The presence of HCl in the canaliculus then causes water movement from the cell by osmosis (Forte and Wolosin, 1987). Some of the secreted HCl remains in the secretory canaliculi in isolated parietal cells and glands. This allows the weak base aminopyrine to accumulate within the acidic spaces and to act as an index of parietal cell secretory activity (section 2.3). Berglindh et *al.* (1980) proposed that the basement membrane, connective tissue and muscularis mucosa restrict cellular swelling and thus the secreted HCl is forced from the cell into the lumen of the gastric gland in intact tissue.

The initial location and the means of activation of the K^+ conductances are not known. It has been suggested that Cl^- conductances are present with the H^+/K^+-ATPase and that upon stimulation the Cl^- conductance is activated and the tubulovesicles fuse with the apical membrane simultaneously (Takeguchi and Yamazaki, 1986). Alternatively the conductances may be permanently located in the apical membrane (Forte and Wolosin, 1987). It has also been proposed that they are located in a totally separate population of cytoplasmic membranes in the resting cell which, with the tubulovesicles, fuse with the apical membrane when stimulated (Im *et al.*, 1985). Ion conductances may be regulated by protein phosphorylation (Im *et al.*,

Figure 1.3

Diagrammatic illustration of the transport mechanisms and HCl generation across the apical membrane of the secretory canaliculus.



A single physical unit is believed to contain independent pathways for K^+ and Cl^-. It is via this pathway that K^+ and Cl^- enter the lumen of the secretory canaliculus. The H^+/K^+-ATPase extrudes H^+ into the secretory canaliculus in exchange for the inward movement of K^+ with the consumption of ATP. The pump activity leads to an increased ion concentration in the secretory canaliculus which is the driving force for H_2O transfer and volume flow.

1987). Work using Cl^- conductance inhibitors indicated that acid secretion may be modulated by the ion-conductances (Malinowska and Cuppoletti, 1988). Clearly appearance of K^+ at the luminal face of the proton pump precedes activation of the H^+/K^+-ATPase. It has been suggested that the Cl^- conductance is modulated by cyclic AMP-dependent protein kinase (Soumarmon *et al.*, 1980).

The parietal cell must closely regulate its intracellular pH to maintain homeostasis. Thus when the parietal cell is secreting H^+ ions an equivalent amount of base must be extruded from the cell. This requirement is met by the operation of a Cl^-/HCO_3^- exchanger (Fig. 1.4) (Forte and Wolosin, 1987). When the parietal cell is not secreting acid, H^+ is produced intracellularly as a product of metabolism and may be extruded by the Na^+/H^+ exchanger. So when resting the Na^+/H^+ exchanger may be important in the regulation of intracellular pH (Madshus, 1988). Recent studies by Muallem *et al.* (1988) have suggested that the Na^+/H^+ exchanger was also activated by secretagogues. This in conjunction with the activated H^+/K^+-ATPase will decrease intracellular pH. This change in pH may then activate the Cl^-/HCO_3^- exchanger. Work by Wenzl and Machen (1989) showed that the activity of the anionic exchanger was indeed sensitive to intracellular pH but that this alone did not appear to account for the total activity of the Cl^-/HCO_3^- exchanger. Thus both the Cl^-/HCO_3^- and Na^+/H^+ exchangers may be activated upon stimulation by secretagogues.

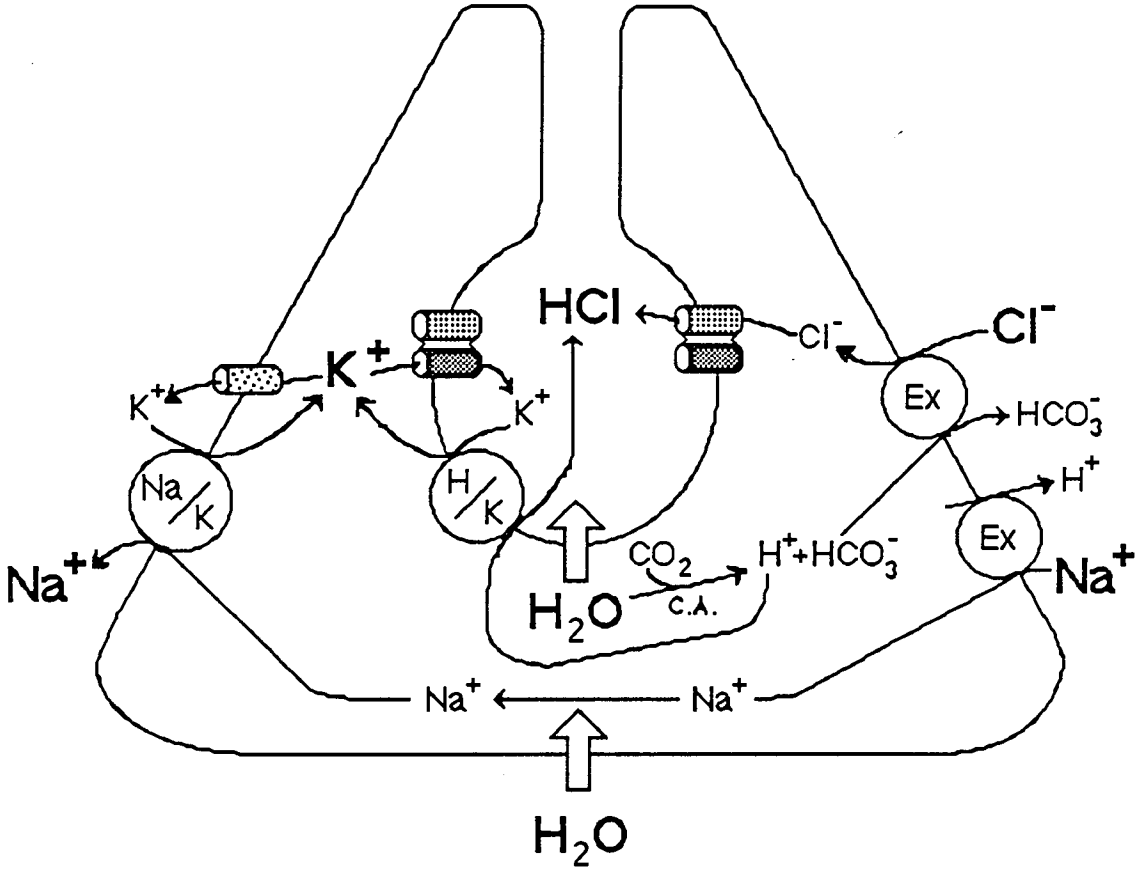
During secretion the parietal cell releases Cl^- ions. Gastric juice also has a K^+ concentration 2 times that of plasma K^+. Therefore there is also a homeostatic requirement for a mechanism for K^+ as well as Cl^- ion uptake. During acid secretion a high intracellular K^+ and low Na^+ concentration is maintained by the Na^+/K^+-ATPase. This electrogenic pump is believed to be mainly responsible for the negative intracellular membrane potential. The low intracellular Na^+ concentration may drive the Na^+/H^+ exchanger, which together with H^+/K^+-ATPase activity may raise intracellular base levels. High base levels may provide the driving force for Cl^- uptake via the Cl^-/HCO_3^- exchanger (Forte and Wolosin, 1987).

1.2.2 Mechanism of pepsinogen secretion.

The mechanism by which pepsinogen is secreted from the chief cell has been reviewed by (Hersey, 1987). To establish that pepsinogen is secreted by exocytosis the loss of secretory granules accompanying secretion and the observation of exocytotic or fusion figures must be demonstrated. The loss of peptic granules has been demonstrated both *in vivo* and *in vitro*. Using electron microscopy exocytotic figures have been demonstrated in chief cells of the rat (Helander, 1965) and in gastric glands of the rabbit (Gibert and Hersey , 1982a). Granule to granule fusion or compound exocytosis has been observed, as well as the fusion of granules with the

Figure 1.4

Diagram illustrating ion transport and homeostasis in the secreting parietal cell (after Forte and Wolosin, 1987).



Key: H/K = H^+/K^+-ATPase; Na/K = Na^+/K^+-ATPase; Ex = exchanger; ⬭ = conductance; C.A., carbonic anhydrase.

The left side of the diagram describes K^+ homeostasis while the right side that of Cl^-. K^+ is accumulated due to the action of the Na^+/K^+-ATPase. Accumulated K^+ leaks through conductance pathways at both the apical and basolateral membranes. K^+ in the luminal space is recycled back into the cell by the activity of the H^+/K^+-ATPase. Cl^- enters the parietal cell electroneutrally in exchange for base. The driving force of this being the increase in base generated by H^+ extrusion at the apical surface, or excess base when cellular H^+ is extruded in exchange for Na^+ moving into the cell down its electrochemical gradient. Cl^- is accumulated within the cell above its electrochemical potential gradient and this provides the driving force for Cl^- movement from cell to lumen via conductive channels.

cell membrane, in rabbit glands (Gibert and Hersey , 1982a). Thus it appears that pepsinogen is secreted from chief cells by exocytosis. At present the mechanisms underlying this process are poorly understood and largely unknown.

Secretion of pepsinogen appears to occur in two phases. Initially there is a rapid output of pepsinogen which was associated with exocytotic figures in rabbit glands, and this was followed by a slower rate of continuous secretion (Gibert and Hersey , 1982a). It is not clear if the slow phase involves exocytosis or some other mechanism.

It has been suggested that osmotic swelling is required for the initiation of exocytosis. This was postulated following the observed loss of electron density of peptic granules during exocytosis in chief cells (Gibert and Hersey , 1982a). Further evidence was provided by the finding that exposure of chief cells to hyperosmotic medium stimulates secretion (Gibert and Hersey , 1982a). Membrane ion transport processes or changes in cell composition also appear to be important in the secretory process (Gibert and Hersey, 1984). Thus pepsinogen secretion seems to be sensitive to pH (Norris and Hersey, 1983), membrane transport inhibitors (Gibert and Hersey, 1984), certain ionophores and the ionic composition of the incubation medium (Norris and Hersey, 1985; Gibert and Hersey, 1984; Norris and Hersey, 1983). It appears that the granules can maintain a pH gradient and may possess a proton transport mechanism (Peerce *et al.*, 1984). The importance of a pH gradient is not known but it may maintain pepsinogen in a condensed form prior to release (Fimmel *et al.*, 1984).

1.3 STIMULATION OF ACID AND PEPSINOGEN SECRETION BY DIRECT ACTION ON SECRETORY CELLS.

This thesis concerns work performed with isolated gastric cells. In this system indirect interactions between cells are minimised by the dilution of paracrine agents in the incubation medium, and there is no scope for indirect neural or endocrine control pathways. Consequently the brief review below only considers in detail those agents which stimulate secretory activity by acting directly on the cell concerned.

1.3.1 Stimulation of acid secretion.

Parietal cell secretory activity may be stimulated by a number of agents. The modes of delivery of these agents which stimulate secretion varies. Gastrin displays endocrine delivery as it is released from G-cells in the antral mucosa and in the first part of the duodenum, into the bloodstream from where it appears to activate parietal cells directly in the dog. Acetylcholine exhibits neurocrine delivery as it is released by post-ganglionic nerve endings on or close to the parietal cell. Histamine on the other

hand shows paracrine delivery as it is released from endocrine-like cells in the rat or mast cells in dog and man (Debas, 1987).

Initially the events leading to induction of acid secretion by eating will be briefly discussed and then the intracellular events which transpire as a consequence of secretagogue action on the parietal cell will be considered.

1.3.1.1 The phases of acid secretion.

The phases of acid secretion have been reviewed by Debas (1987). The cephalic phase is mediated by the vagus and may be initiated by the sight, smell or taste of food. Vagal stimulation effects the release of acetylcholine which has a direct stimulatory effect and it also appears to stimulate gastrin release from antral G-cells in the dog. The gastric phase is initiated by gastric distension and the chemical constituents of the food. Distension activates long (vagovagal) and short (intramural) reflexes which then stimulate acid secretion. The digestion products of proteins may also stimulate acid secretion via gastrin release. The intestinal phase of acid secretion is induced by the presence of digestion products of proteins and distension (Debas, 1987).

1.3.1.2 The action of histamine on parietal cell secretory activity.

The effects of histamine on parietal cells have been reviewed by Soll and Berglindh (1987) and Hanson and Hatt (1989). Histamine stimulates aminopyrine accumulation, oxygen consumption and glucose oxidation in parietal cells and gastric glands (Soll and Berglindh, 1987). Receptor binding studies have been complicated by the occurrence of histamine uptake by parietal cells in for example guinea-pig (Albinus and Sewing, 1981). Cimetidine, an H_2-receptor antagonist, can competitively and completely inhibit acid secretion stimulated by histamine (Soll, 1980a). Thus, histamine appears to stimulate parietal cell acid secretion by interacting with an H_2-receptor on the plasma membrane. The magnitude of the response to histamine varies considerably between species, for example histamine elevates aminopyrine accumulation much more in rabbit than dog parietal cells (Soll and Berglindh, 1987).

The evidence available strongly implicates cyclic AMP as the second messenger for histamine. Thus histamine elevated the cyclic AMP content of parietal cells in many species including for example the rat (Schepp *et al.*, 1983a). Furthermore the cyclic AMP analogue dbcAMP (Soll, 1980a) and forskolin (Chew, 1983a), a direct activator of adenylate cyclase, both elicited a similar response to that exhibited by histamine. Histamine has been found to stimulate adenylate cyclase in

sonicates from rat parietal cells (Thompson *et al.*, 1981). Adenylate cyclase is directly activated by the $G_{s\alpha}$ subunit of G-proteins (Gilman, 1987). It seems likely that histamine activates adenylate cyclase via a $G_{s\alpha}$ protein.

Histamine elevated intracellular Ca^{2+}, as determined by using the fluorescent indicators Quin-2 and Fura-2 in rabbit glands (Chew, 1986) and in parietal cells (Chew and Brown, 1986) respectively. Confirmation of this finding came from Negulescu and Machen (1988a) using microspectrofluorimetric measurements on individual parietal cells in rabbit glands. The pattern of the Ca^{2+} response to histamine was qualitatively the same as that with carbachol. Thus an initial spike of intracellular Ca^{2+} was observed, which was independent of extracellular Ca^{2+}, and then the intracellular Ca^{2+} returned to control levels in the absence of extracellular Ca^{2+} or to a level significantly above that of the control in the presence of extracellular Ca^{2+} (Negulescu and Machen, 1988a). The Ca^{2+} spike in response to histamine was quantitatively less than that found with carbachol. DbcAMP plus the phosphodiesterase inhibitor IBMX can stimulate acid secretion without elevating intracellular Ca^{2+} (Negulescu and Machen, 1988a). This suggests that the cyclic AMP pathway can stimulate acid secretion independently of Ca^{2+}.

The links between the elevation of intracellular cyclic AMP and the increased activity of the H^+/K^+-ATPase have been poorly identified. It is probable that this involves protein phosphorylation as histamine has been found to activate type 1 cyclic AMP-dependent protein kinase (Chew, 1985). There has generally been a lack of agreement concerning the molecular weight of the phosphorylated proteins and/or their location within the cell. For example in the 100,000g supernatant from enriched parietal cells 148, 130, 47 and 43-kDa proteins were found to be phosphorylated upon stimulation by histamine. However, the 130 and 47-kDa proteins were also found in the 100,000g pellet (Malinowska *et al.*, 1988). In the 100,000g supernatant Oddsdottir *et al.* (1988) found that stimulation by histamine resulted in the phosphorylation of a 30-kDa protein which was not detected by Malinowska *et al.* (1988). Using antibodies raised to an 80-kDa phosphoprotein, which was found to be phosphorylated upon stimulation by histamine, Hanzel *et al.* (1989) indicated that this phosphoprotein seems to be located in the parietal but not surface, mucous neck or chief cells. Immunohistological localisation of this phosphoprotein suggest that it is located in the apical membrane. One source of variation may be caused by proteolysis during homogenization before subcellular fractionation. Using a highly enriched parietal cell preparation from the rabbit (98% parietal cells), and a full range of protease inhibitors, histamine was demonstrated to increase the phosphorylation of 27 and 40-kDa proteins (Chew and Brown, 1987).

1.3.1.3 The action of carbachol, an acetylcholine analogue, on parietal cell secretory activity.

The action of carbachol as a muscarinic cholinergic receptor agonist has been reviewed by Hanson and Hatt (1989). Carbachol appears to interact with muscarinic M3-receptors on isolated parietal cells (Pfeiffer *et al.*, 1990). Activation of muscarinic receptors on the parietal cell has been associated with the activation of phospholipase C and the metabolism of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol polyphosphates. As such, carbachol increased the inositol trisphosphate (Puurunen and Schwabe, 1987) and diacylglycerol content (Pfeiffer *et al.*, 1987) of rat parietal cells. Diacylglycerol activates protein kinase C which then mediates its intracellular action via protein phosphorylation. The action of protein kinase C is reviewed in Chapter 5.

Experiments using Fura-2 loaded rabbit glands and cells have shown that carbachol effected an initial rapid increase in intracellular Ca^{2+} which was derived from intracellular stores (Negulescu and Machen, 1988a and b; Chew and Brown, 1986). This effect was not dependent on extracellular Ca^{2+}. Following this initial Ca^{2+} spike the intracellular Ca^{2+} fell but was maintained above the control level in the presence of extracellular Ca^{2+}. Thus it appears that the initial Ca^{2+} spike was followed by a stimulation of Ca^{2+} influx across the plasma membrane. Inositol 1,4,5-trisphosphate, which is released by phosphatidylinositol 4,5-bisphosphate metabolism, has been found to cause mobilisation of Ca^{2+} from intracellular stores in parietal cells (Tsunoda *et al.*, 1988). It is not certain if inositol polyphosphates are responsible for inducing the subsequent influx of Ca^{2+} across the cell membrane. Carbachol depleted the intracellular stores of Ca^{2+} in rabbit gastric glands after treatment for over 3 minutes (Negulescu and Machen, 1988b). Thus it is possible that intracellular Ca^{2+} was extruded from the cell by a calmodulin-dependent Ca^{2+}-ATPase (Muallem and Sachs, 1985).

Carbachol may effect changes in protein phosphorylation by at least two mechanisms. The elevated intracellular Ca^{2+} concentration may activate specific Ca^{2+}-sensitive protein kinases (Shaltz *et al.*, 1981). Alternatively carbachol has been found to activate protein kinase C (Park *et al.*, 1987). Protein kinase C phosphorylates cellular proteins and has been shown to phosphorylate an 89-kDa protein in the 100,000g cytosolic fraction from homogenates of enriched rat parietal cells (Hanson and Hatt, 1989). Treatment of enriched parietal cells from the rabbit with an activator of protein kinase C resulted in the phosphorylation of a 36-kDa protein and two other 66-kDa proteins. These protein were located in both cytosolic and microsomal fractions. Carbachol stimulates the phosphorylation of these proteins and a further 28-kDa protein (Brown and Chew, 1989). Thus there appears to be at least 2 protein

kinases stimulated by carbachol. Activation of protein kinase C may also have inhibitory effects on secretory activity (Anderson and Hanson, 1984) and in this thesis some attention is given to the mechanism by which protein kinase C may exert inhibitory effects.

1.3.1.4 The action of gastrin in parietal cell secretion.

The effects of gastrin on parietal cell activity has been reviewed recently by Hanson and Hatt (1989). There appears to be a gastrin receptor present on dog parietal cells and this receptor exhibits equal recognition of gastrin and cholecystokinin octapeptide (Soll *et al.*, 1984). Gastrin stimulated secretory activity in dog (Soll, 1980a), rabbit (Chew and Brown, 1986) and guinea-pig parietal cells (Tsunoda, 1987). However, there are no consistent reports of gastrin-stimulated aminopyrine accumulation in rats. Gastrin raised intracellular inositol trisphosphate (Chiba *et al.*, 1988) and intracellular Ca^{2+} (Chew and Brown, 1986). It appears that gastrin activates phospholipase C and protein kinase C via diacylglycerol in dog parietal cells (Chiba *et al.*, 1988). It appears that a sustained effect of gastrin requires extracellular Ca^{2+} (Tsunoda, 1987). Thus it seems likely that gastrin activates the same intracellular signalling system as that used by carbachol (Hanson and Hatt, 1989).

1.3.1.5 Potentiation between secretagogues.

When two secretagogues act together to produce a response which is greater than the sum of the individual responses then potentiation is said to take place. Potentiation between histamine and carbachol and between histamine and gastrin has found to take place in canine parietal cells (Soll, 1982). Potentiation has also been found to take place between dbcAMP and carbachol or gastrin. This result suggest that potentiating interactions take place at a sites distal to the production of cyclic AMP (Soll, 1982).

1.3.1.6 The action of K^+ on parietal cell secretory activity.

Acid secretion has a requirement for extracellular K^+ (Berglindh, 1978). Luminal K^+ is believed to be required for the functioning of the H^+/K^+-ATPase (section 1.2.1). Thus in stimulated parietal cells K^+ conductances appear, or are already present and are switched on, allowing K^+ to enter the secretory canaliculi (Fig. 1.3). Physiological media usually have a K^+ concentration of between 4.5 to 5.4mM. Substantial elevation of the K^+ concentration (for example to 100mM), and a compensatory reduction in medium Na^+, leads to an increased level of parietal cell secretory activity as shown by aminopyrine accumulation in rat parietal cells (Hatt and Hanson, 1989) and rabbit gastric glands (Berglindh *et al.*, 1980). It seems that incubation of rabbit gastric glands in a medium containing a high K^+ concentration

induces the accumulation of acid within pre-existing secretory canaliculi (Gibert and Hersey, 1982b), possibly due to the provision of K^+ at the extracellular face of the pump.

1.3.1.7 Other stimulators of parietal cell activity.

A β_2-adrenergic receptor appears to be present on rat parietal cells and isoprenaline and other adrenergic agonists have been found to stimulate aminopyrine accumulation. It appears that this effect is mediated by an increase in adenylate cyclase activity (Hanson and Hatt, 1989).

Gastrotropin alternatively known as porcine ileal polypeptide stimulates gastric acid secretion in dog and pig parietal cells. This peptide may stimulate acid secretion under physiological conditions and it has been found to increase cytoplasmic Ca^{2+} in guinea-pig cells (Hanson and Hatt, 1989).

1.3.2 Stimulation of pepsinogen secretion.

Pepsinogen secretion has been reviewed by Hersey (1987). Pepsinogen secretion, as with acid secretion, appears to be controlled by both cyclic AMP-dependent and calcium-dependent cellular mechanisms (Chew, 1983a; Koelz *et al.*, 1982; Raufman *et al.*, 1983). Pepsinogen secretion is stimulated by a variety of secretagogues (Table 1.1).

Cholinergic agonists appear to mediate their action via an increase in intracellular Ca^{2+}. Conflicting results have been obtained with pentagastrin, thus pentagastrin stimulates pepsinogen secretion in the rat (Bersimbaev 1985; see Fig. 1.1) but not chief cells isolated from the dog (Hersey, 1987). Cholecystokinin octapeptide has been found to stimulate pepsinogen secretion *in vitro* in, for example, dog chief cells. Thus in dogs the chief cell, unlike the parietal cell, appears to be able to distinguish between cholecystokinin and gastrin. It appears that cholecystokinin mediates its action via an increase in intracellular Ca^{2+}. Secretin seems to stimulate pepsinogen secretion *in vitro* but to varying degrees, and this may be a result of species differences or the purity of the secretin preparation used. The action of secretin on pepsinogen secretion is believed to be mediated by cyclic AMP. β-adrenergic agonists such as isoprenaline have been found to stimulate pepsinogen secretion in some *in vitro* preparations and this action is believed to be mediated via an increase in intracellular cyclic AMP (Hersey, 1987).

Histamine has been found to stimulate pepsinogen secretion *in vivo* in the dog and other species (Hersey, 1987). However, histamine and other H_2 receptor agonists had no effect on pepsinogen secretion from isolated gastric cells from the rat

Table 1.1

Illustration of the action of various agents on chief cell activity.

Species	Preparation	Agents	Effect on chief cell activity	Reference
Rat	Enriched, isolated chief cells	Histamine plus carbamylcholine	Stimulation	Fatemi *et al.* (1982)
Rat	Enriched, isolated chief cells	DbcAMP, pentagastrin, pentagastrin plus cimetidine	Stimulation	Bersimbaev (1985)
		Histamine	No effect	
Rabbit	Isolated gland	Carbachol, isoprenaline, dbcAMP and crude secretin	Stimulation	Koelz *et al.* (1982)
		Histamine and PGE_2	Inhibition	
Rabbit	Isolated gland	Forskolin	Stimulation	Chew (1983a)

Species	Preparation	Agents	Effect on chief cell activity	Reference
Guinea pig	Isolated chief cells and gastric glands	DbcAMP, forskolin, A23187 Histamine, adrenaline	Stimulation No effect	Vivell *et al.* (1984)
Guinea pig	Isolated gland	TPA, A23187 and dbcAMP	Stimulation	Sakamoto *et al.* (1985)
Guinea pig	Isolated gland	PGA_1, PGA_2, PGE_1, PGE_2 PGD_2, PGI_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$	Stimulation Inhibition	Berger and Raufman (1984)
Guinea pig	Isolated chief cell	NaF, CCK-8 and carbachol	Stimulation	Matozaki *et al.* (1988)

Table 4.1 continued.

(Bersimbaev, 1985). Histamine also had no effect *in vitro* on pepsinogen secretion in the rabbit (Hersey *et al.*, 1983) and guinea-pig (Vivell *et al.*, 1984). It has been suggested that histamine increases pepsinogen output *in vivo* by a "wash out" effect caused by stimulation of electrolyte (HCl) and water secretion. Acidification of the gastric lumen, which will result from histamine-stimulated acid secretion, can also lead to a secondary stimulation of pepsinogen secretion *in vivo* (Hersey, 1987). Thus it appears that histamine does not stimulate pepsinogen secretion directly.

1.4 STRATEGIES FOR THE INHIBITION OF ACID SECETION.

The major intention of this work was to investigate aspects of the signal transduction systems involved in the inhibition of acid secretion. Consequently an overview of strategies for inhibition of acid secretion will be provided here. More detailed consideration of the action of specific direct inhibitors is provided later.

Secretion by parietal cells may be inhibited by agents which act directly on the cell or by indirect mechanisms. Agents which have an indirect inhibitory action can stimulate the release of substances from certain cells which then act directly on the parietal cell to inhibit secretion. Alternatively indirect inhibitors of secretion may act by inhibiting the release of agents which stimulate secretion.

1.4.1 Indirect inhibitors of acid secretion.

Somatostatin, as well as having a direct inhibitory action on parietal cell secretory activity, also has indirect inhibitory effects which bring about the reduction of acid secretion. Thus somatostatin appears to inhibit the gastrin-stimulated release of histamine from cellular stores (Chew, 1983b). This action, if it occurs physiologically, is probably a paracrine one as physiological concentrations of somatostatin inhibit acid secretion whilst having no effect on serum gastrin (Colturi *et al.*, 1984). Somatostatin has also been found to stimulate prostaglandin production (PGE_2 and 6-keto $PGF_{1\alpha}$) in rat gastric epithelial cells in culture (Romano *et al.*, 1988). However, this may not be of physiological significance as high concentrations of somatostatin were required (10^{-5} and 10^{-4}M-somatostatin). Indeed *in vivo* studies in the cat have suggested that prostaglandins do not mediate the action of somatostatin (Albinus *et al.*, 1985).

EGF has a potential indirect inhibitory action as it was found to stimulate PGE_2 secretion from an enriched parietal cell preparation from the rat (section 3.3.8.2). Adenosine in addition to its direct inhibitory action may inhibit acid secretion by blocking histamine and acetylcholine release (Westerberg and Geiger, 1988).

Oxyntomodulin (a cleavage product of the peptide glicentin) is found in the intestine, particularly the jejuno-ileum (Battaille *et al.*, 1982). Oxyntomodulin *in vivo* inhibited acid secretion stimulated by pentagastrin more potently than histamine. In an isolated cell preparation from the rat, oxyntomodulin had no effect on aminopyrine accumulation stimulated by histamine or gastrin. Thus unless the putative gastrin receptor was damaged oxyntomodulin appears to have an indirect action (Hanson and Hatt, 1989). Indeed glucagon (the product after 8 amino acids are cleaved of the C-terminus of oxyntomodulin) was found to increase somatostatin secretion in perfused rat stomach preparations (Chiba *et al.*, 1980). Another peptide derived from preproglucagon, truncated glucagon-like peptide-1, has been found to inhibit acid secretion (Schjouldager *et al.*, 1989). It has also been found to increase somatostatin release, but inhibit gastrin, release in the perfused rat stomach (Eissele *et al.*, 1990). At present it is not known if truncated glucagon-like peptide-1 acts directly on D- and G-cells to affect the release of somatostatin and gastrin.

The peptide secretin is structurally similar to glucagon and vasoactive intestinal polypeptide. Secretin is found in the small intestine, especially in the duodenum and jejunum (Walsh, 1987). Vasoactive intestinal polypeptide has been localised to nerve terminals of the gastric mucosa and is a potential inhibitory neurotransmitter (Walsh, 1987). *In vivo*, secretin inhibited acid secretion stimulated by gastrin, and vasoactive intestinal polypeptide was found to inhibit pentagastrin and histamine-stimulated secretion. In crude parietal cell suspensions from the rat secretin and vasoactive intestinal polypeptide also inhibited secretory activity stimulated by histamine, but these actions occurred at high, probably non-physiological, concentrations (Schepp *et al.*, 1983b). As with glucagon, secretin and vasoactive intestinal polypeptide evoked a dose-dependent increase in somatostatin secretion in the perfused rat stomach (Chiba *et al.*, 1980). Thus it appears that the inhibitory action of secretin and vasoactive intestinal polypeptide are, at least in part, indirect and mediated via somatostatin. Secretin may not be a physiological inhibitor as concentrations of secretin required for inhibition are high (Henriksen *et al.*, 1981).

Cholecystokinin (CCK) is a peptide found in the duodenum and jejunum. It is structurally similar to gastrin, and indeed stimulates acid secretion in the cat. However, in the dog and human CCK-8 is a possible enterogasterone. *In vivo* CCK inhibits acid secretion stimulated by pentagastrin and histamine (Walsh, 1987). In isolated cell preparations from the dog CCK-8 does not appear to act as an inhibitor (Soll *et al.*, 1984). Schepp *et al.* (1983b) found that CCK-8 inhibited a crude suspension of parietal cells from the rat stimulated by histamine. However the CCK-8 preparation was not pure and there is the possibility of an indirect action. CCK-8 and gastrin seem to share the same receptor on dog parietal cells, and the reason for the differences in *in vivo* action may be because CCK-8 is a much more potent stimulator

of somatostatin release than gastrin. Therefore it appears that CCK acts indirectly, at least in part, to inhibit acid secretion by stimulating somatostatin release (Soll *et al.*, 1985).

Calcitonin gene-related peptide has been found in the fundus. Calcitonin gene-related peptide decreased acid secretion stimulated by pentagastrin in the conscious dog and increased somatostatin release (Helton *et al.*, 1989). In isolated rabbit glands calcitonin gene-related peptide appears to mediate at least part of its action by increasing somatostatin release (Zdon *et al.*, 1988). Isolated guinea-pig parietal cells stimulated by histamine, carbachol, pentagastrin and dbcAMP were inhibited by calcitonin gene-related peptide (10^{-12} to 10^{-9}M). It was suggested that calcitonin gene-related peptide may act directly or by increasing somatostatin release (Umeda and Okada, 1987). As with dog parietal cell suspensions calcitonin gene-related peptide had no effect on rat parietal cell suspensions stimulated by histamine (data not shown). In conclusion, in certain species calcitonin gene-related peptide also appears to mediate at least part of its action by acting indirectly to increase somatostatin release. This paracrine action of calcitonin gene-related peptide may be of physiological significance.

1.4.2 Direct inhibitors of acid secretion.

PGE_2 has a direct inhibitory action on dog and rat parietal cell secretory activity stimulated by histamine (section 3.3.3). PGE_2 also appears to directly inhibit secretory activity stimulated by β_2-adrenergic receptors in the rat (Rosenfeld, 1986). Somatostatin appears to have a direct inhibitory effect on parietal cells stimulated by a variety of secretagogues including gastrin, carbachol and histamine (section 3.1.2 and 3.3.4). The physiological significance of the action of PGE_2 and somatostatin on parietal cells will be considered at the end of the thesis.

EGF may have a direct inhibitory action on histamine-stimulated acid secretion (section 3.1.3 and 3.3.6). EGF also inhibited acid secretion stimulated by a muscarinic cholinergic agonist in a rabbit cell suspension enriched in parietal cells (Lewis *et al.*, 1990).

Adenosine may have a physiological role in the direct inhibition of parietal cell secretory activity stimulated by histamine (Gerber and Payne, 1988). This action appears to be mediated by A_1 adenosine receptors (Gerber *et al.*, 1985). However, this effect is only seen in some species and indeed the rat does not appear to have adenosine receptors on parietal cells (section 3.3.1).

Recently a 49 amino acid peptide termed pancreastatin has been detected in the human gastrointestinal tract and in porcine gastric mucosa. This peptide inhibited

secretory activity stimulated by histamine and carbachol in an enriched parietal cell preparation from the rabbit (Lewis *et al.*,1989). Thus it seems likely that pancreastatin acts directly. Although the physiological relevance of pancreastatin remains to be determined it may be a paracrine and/or endocrine inhibitory regulator of parietal cell secretion. In this thesis aspects of the mechanism of action of EGF, prostaglandins and somatostatin will be investigated, and more detailed information is presented in later chapters.

Chapter Two

GENERAL METHODOLOGY.

2.1 PREPARATION OF ISOLATED CELL SUSPENSIONS.

2.1.1 The use of isolated parietal cell preparations.

An isolated cell suspension was used to investigate aspects of the mechanism of regulation of acid secretion. This was used in preference to the stomach *in vivo* or an intact mucosa preparation *in vitro*, as it avoids some fundamental problems that are associated with these systems. Thus it is difficult to determine the exact site of action of the agent using stomach preparations *in vivo* or an intact mucosal preparation *in vitro* as the agent may either be acting directly on the parietal cell, or it may act on another cell type to release an agent which then acts on the parietal cell. In both cases the same effect would be observed, that is a stimulation or inhibition of acid secretion, but the exact site of action of the agent could not be determined. Intact stomach and intact mucosal preparations used in vitro, also have problems associated with oxygenation and the access of certain agents to the parietal cell surface, which is caused by diffusion barriers in intact mucosa. *In vivo* metabolism of the agent in the blood may occur. Furthermore designing complex experiments without using large numbers of animals is difficult *in vivo* but not when using isolated parietal cell suspensions.

Isolated cell preparations have the disadvantage of the loss of intercellular connections and cell polarity which may result in reduced responsiveness. In addition the proteolytic enzymes used in the cell isolation procedure may disrupt receptors on the parietal cell plasma membrane. However there are examples where isolated parietal cells seem to respond to secretagogues as well as gastric glands, for instance Chew (1983b) found similar responses in the rabbit.

2.1.2 Everted stomach sac preparation.

A fed rat (see Appendix A.6) with a body weight of 200-300g was anaethetised by an intra peritoneal injection of sodium pentabarbitone (Sagatal) at 60mg/kg body weight. The stomach was exposed by midline incision and the oesophagus ligated. By cutting above this ligature and across the duodenum, the stomach was removed. The rat was then immediately sacrificed by perforating the diaphragm.

The stomach was then gently rinsed in ice-cold saline (NaCl, 0.9%($^{w}/_{v}$)) while holding the non-glandular region with a pair of forceps. Everted stomach sacs were prepared as in Fig. 2.1 (Dikstein and Sulman, 1965). Pronase was dissolved in medium A (see Table 2.1) at a concentration of 1000PUK/ml. The sac was inflated by injecting this pronase solution using a 26-gauge hypodermic needle (approximately 1.5ml was required).

Figure 2.1

Preparation of an everted stomach sac from the rat stomach.

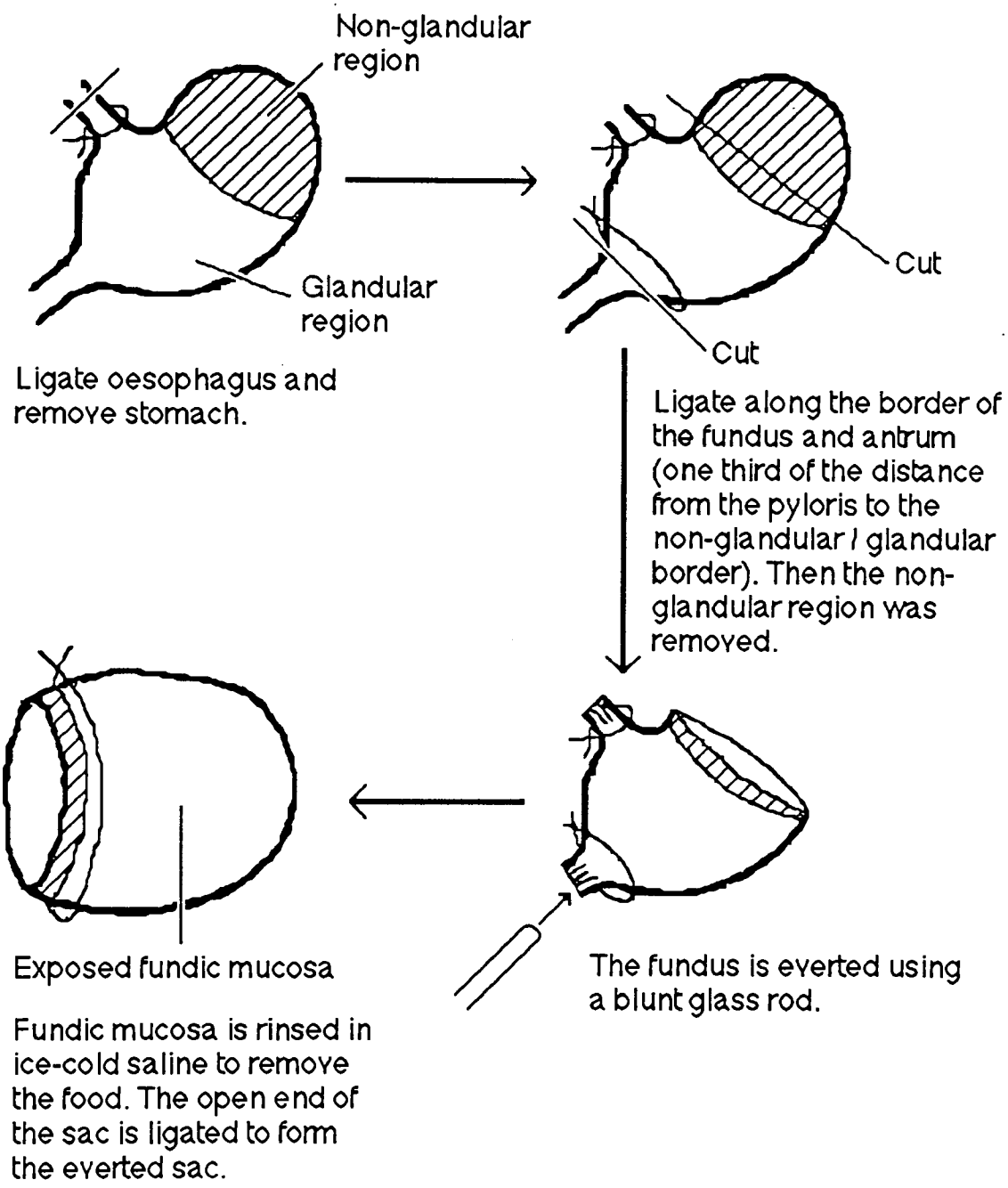


Table 2.1

Media for isolation, incubation and enrichment of rat parietal cells.

The additions specified below were added to Eagle's Minimum Essential Medium containing 25mM-$NaHCO_3$ and 20mM-HEPES (see Appendix A.2.1).

Medium	Additions
A	EDTA (2mM) Soybean trypsin inhibitor (0.1mg/ml) Dextran (30mg/ml)
B	Bovine serum albumin fraction V (30mg/ml)
B'	Bovine serum albumin, fraction V (1mg/ml)
C	EDTA (3.0mM) Dithiothreitol (0.5mM) Bovine serum albumin, fraction V (1mg/ml)

2.1.3 Preparation of crude parietal cell suspensions.

The term "crude" refers to cell suspensions containing approximately 20% parietal cells. Parietal cells were isolated using a method similar to that of Trotman and Greenwell (1979) which is a modification of the methodology by Lewin *et al.* (1974). Two everted stomach sacs were required to provide enough cells for one experiment using a crude parietal cell preparation and 4 sacs were needed for preparations of enriched parietal cells (2.2.2). The everted stomach sacs were incubated in a plastic bottle containing 40ml of medium A (Table 2.1) and incubated for 30 minutes at 37°C with constant shaking (60 cycles/minute), and continuous gassing with 95%O_2/5%CO_2. This mixture was used throughout to gas the airspace above the cell suspension. After digestion, the sacs were removed, blotted free from medium A and added to 20ml of medium B (Table 2.1) in a covered plastic beaker (50ml capacity).

The sacs in medium B were incubated for 30 minutes, with gentle stirring using a magnetic follower, at room temperature with continuous gassing of the airspace above the medium. The cells released into medium B were harvested by filtering the medium through nylon mesh (150μm pore size, Sericol Group Ltd., London) into plastic centrifuge tubes and centrifuged at 15°C for 5 minutes at 100g. The supernatant was discarded and the cell pellet containing parietal cells was carefully resuspended using a plastic transfer pipette (L.I.P. (Equipment and Services) Ltd., Shipley, W. Yorks), in 10ml of fresh medium B before storage at 37°C with gentle shaking (60 cycles/minute) and continuous gassing. The sacs were incubated for a further 2 cycles (2 hours) with incubation in medium A and harvesting in medium B.

The harvested cell fractions were pooled and centrifuged at 15°C for 5 minutes at 100g and the resulting cell pellet resuspended in 10ml of the appropriate incubation medium. The cell suspension was centrifuged again under the same conditions and resuspended in the incubation medium.

This preparation typically produced a cell-fraction containing 20.2 ± 0.5% parietal cells (n = 20 batches of cells) and the average number of cells isolated per stomach was 6.4 ± 1.1 x 10^7 (n = 20 batches of cells).

Schepp *et al.* (1983a and b) isolated a similar number of cells from the rat stomach (8x10^7 cells/stomach) by using an isolation procedure in which there was only one harvesting period and prolonged exposure to EDTA. However, if pieces of rat gastric glandular mucosa are incubated in medium containing 0.75% ($^w/_v$) pronase, a ~~considerably~~ reduced yield is obtained (2.5x10^7 cells/stomach; Dial *et al.*, 1981).

2.1.4 Identification of parietal cells and trypan-blue exclusion test.

Parietal cells were identified, using the light microscope (x400), by their size and morphology. Parietal cells usually have a diameter in excess of 13μm and are therefore the largest cell type present in the preparation. These cells are also distinctive as they possess a large centrally-located nucleus and the cytoplasm contains a high proportion of mitochondria which results in a granulated appearance.

The ability of the cells to exclude the dye trypan blue (M.Wt. 96-Da) was used as an indicator of the structural integrity of the cell membrane. A 20μl aliquot of the cell sample in the appropriate incubation medium was mixed with 20μl of trypan blue (4mg/ml) in saline (NaCl 0.9% ($^{w}/_{v}$)). Cells were counted under a light microscope using a haemocytometer (E.Leitz, Wetzlar). At least 200 cells were counted on each occasion and the percentage of cells which exclude trypan blue was calculated.

Using the isolation procedure described in section 2.1.3, 95.4 ± 0.4% (n = 20 batches of cells) of the cells as a whole and 91.2 ± 0.9% (n = 20 batches of cells) of the parietal cells exclude trypan blue. It should be emphasised that the dye exclusion test only gives an indication of the structural integrity of the cell membrane and it is thus not a direct measurement of viability. Artificially high viabilities may be obtained if there is insufficient cell-dye contact time (Eliot, 1979). High viability readings may also be obtained in the presence of bovine serum albumin (BSA) which can bind trypan blue (Seglen, 1976). However, this test was only performed on cells resuspended in medium B', high K^+ medium and Krebs-Ringer bicarbonate (KRB) medium each of which only contain 0.1% ($^{w}/_{v}$)-BSA.

2.2 ENRICHMENT OF PARIETAL CELLS.

2.2.1 Techniques used for parietal cell enrichment.

A heterogeneous gastric mucosal cell suspension containing approximately 20% parietal cells (as isolated in section 2.1.3) can be used to measure acid secretion by using aminopyrine accumulation as an indirect index of acid secretion (see section 2.3). However, for certain studies, for example cyclic AMP content (2.4.2.2) or prostaglandin production (2.4.3.2) it is necessary to enrich the parietal cell content of the cell suspension. This is achieved by exploiting both the size and density of the parietal cell. Parietal cells are the largest and also one of the least dense of the gastric cell types due to the high proportion of membranes (Soll and Berglindh, 1987). Thus the parietal cell may be separated from the other cell types by velocity and density-gradient separation techniques. Many separation techniques have been used to enrich parietal cells and examples of some of these are shown in Table 2.2.

Velocity sedimentation is based on Stockes' Law (equation 2.1). Cell size is a major influence on sedimentation velocity, as the equation involves the square of the

Table 2.2

Comparison of procedures used to enrich gastric parietal cells.

Procedure	Species	% parietal cells	Reference
Centrifugal elutriation (using elutriator rotor)	Dog	Up to 65%	Soll, 1978
Unit gravity sedimentation	Dog	Up to 95%	Major and Scholes, 1978
Density gradient using Percoll	Rat	84.5 ± 0.7%	This work
	Rat	70-90%	Sonnenberg *et al.*, 1979
	Rat	65-85%	Schepp *et al.*, 1983a, b
Density gradient using Nycodenz	Dog and rabbit	70-90%	Berglindh and Sachs, 1985
Combination of elutriation and Nycodenz density gradient	Rabbit	About 95%	Brown and Chew, 1987

radius, and thus the larger parietal cell sediments more rapidly than other mucosal cells.

$$SV = \left(\frac{2}{9}\right)\frac{r_p^2 (\rho_p - \rho_m)}{\eta} g$$

Equation 2.1

SV = sedimentation velocity.
ρ_p = density of cell
ρ_m = density of medium
r_p = cell radius
η = viscosity of medium
g = gravitational field
$\frac{2}{9}$ = shape factor constant for a sphere

Separation methods based on velocity sedimentation include the use of the elutriator rotor and unit gravity sedimentation.

Density or isopycnic separation depends on variation in cell density. On centrifugation the cells migrate through the medium until their sedimentation velocities reach zero when both the cell and medium densities are equal. Provided the duration of centrifugation and the centrifugation speed are sufficient all cells should reach this equilibrium position. Before reaching this equilibrium cells will separate according to both size and density.

Parietal cell suspensions were prepared by isopycnic centrifugation of a crude cell suspension in Percoll using a method developed by G.P. Shaw and P.J. Hanson which is a development of that used by Sonnenberg *et al.* (1979). Percoll is composed of colloidal silica particles coated with polyvinylprrolidone (PVP) and can form a self-generated density gradient within 10-30 minutes. These particles do not penetrate the cell membrane and are non-toxic to cells (Pertoft *et al.*, 1977). Cell-types of a similar density to the parietal cell, such as the histamine containing cells, may co-purify with the parietal cell using this procedure (Soll and Berglindh, 1987). This contamination is reduced by a subsequent low speed centrifugation step which partially separates the larger parietal cells from other cell types (Ecknauer *et al.*, 1981).

2.2.2 Routine preparation of an enriched parietal cell suspension.

A stock Percoll solution (20ml) was prepared by adding 2ml of 10 x concentrated Eagle's Minimum Essential Medium to 18ml of Percoll. $NaHCO_3$ was added to this medium to provide a final concentration of 25mM. The iso-osmotic

Percoll was gassed at room temperature for 15 minutes with stirring and the pH was adjusted to 7.4 with 3M-HCl. Aliquots of this mixture (6 x 3ml) were placed in separate polycarbonate tubes (10ml capacity) just prior to use. The suspension of crude cells in medium B' (section 2.1.3) was centrifuged at 15°C for 5 minutes at 100g and the pellet resuspended in medium C (Table 2.1) at a final concentration of 4-5x10^6cells/ml (30ml of medium C was usually used). To produce a cell suspension in 40% ($^v/_v$) Percoll, 4.5ml of cell suspension was added to each of the polycarbonate tubes. The tubes were gently inverted to ensure thorough mixing and then centrifuged at 30,000g_{av} for 13 minutes at 4°C using a 20° angle rotor in a MSE Superspeed 50 centrifuge. The top 1.5ml of each tube was carefully removed using a Gilson pipette. This layer corresponds to a density of approximately 1.03g/ml. Each 1.5 ml fraction was placed in a plastic centrifuge tube and the volume made up to 10ml with medium B' (Table 2.1). These tubes were centrifuged at 15°C for 5 minutes at 100g. The cell pellets were resuspended in the appropriate incubation medium (typically supplemented tissue culture medium as in section 2.2.3.2) at a concentration of 1-2x10^6cells/ml. The supernatants containing Percoll and types of cells with a similar density to, but smaller than, parietal cells were discarded.

This procedure for the enrichment of parietal cells routinely produced cell fraction containing 84.5 ± 0.7% parietal cells (n = 10) with a total mean yield of 3.1 ± 0.4 x 10^6cells/stomach.

2.2.3 Short-term culture of preparations enriched in parietal cells.

2.2.3.1 Improved responsiveness of enriched parietal cell suspensions by short-term culture.

Improved responsiveness of enriched canine parietal cells to secretagogues by incubation for 2-18 hours in tissue culture medium with various additions was observed by Soll *et al.* (1986). Results obtained by J.F. Hatt using a similar tissue culture medium to that above with an enriched rat parietal cell suspension supported this observation. Consequently enriched parietal cell preparations were used immediately, or pre-incubated for 2 hours in medium B' (Table 2.1) with 8µg/ml-insulin, 10nM-hydrocortisone, 50µg/ml-gentamicin and 5% ($^v/_v$) foetal calf serum prior to stimulation with 0.5mM-histamine. In contrast to enriched parietal cell preparations cellular responsiveness was not improved in crude parietal cell suspensions which were pre-incubated in supplemented tissue culture medium (as above) (see section 3.3.6). This suggests that the Percoll purification procedure produces a temporary inhibition of cellular responsiveness.

2.2.3.2 Routine short-term culture of enriched parietal cell preparations.

The enriched parietal cell suspension which had been washed free from Percoll (section 2.2.2) was resuspended to a concentration of 1-2x10^6cells/ml in supplemented tissue culture medium (medium B' containing 8μg/ml-insulin, 10nM-hydrocortisone, 50μg/ml-gentamicin and 5% ($^v/_v$) foetal calf serum). The cell suspension (usually 10ml) was then incubated for 2 hours at 37°C with shaking (60 cycles/minute) in a capped polycarbonate conical flask (25ml capacity). The airspace above the cells was also constantly gassed with a slow stream of 95%O_2/5%CO_2. Every 30 minutes the cells were gently resuspended using a plastic transfer pipette. Finally the cells were washed twice by centrifugation at 15°C for 5 minutes at 100g and resuspension in the experimental incubation medium. Assessment of the ability of the cells to exclude trypan blue was performed just prior to incubation with secretagogues and inhibitors.

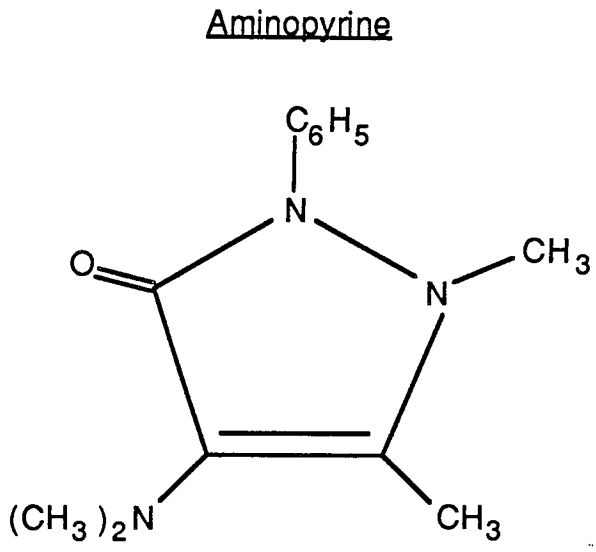
The enrichment procedure followed by the 2 hour pre-incubation seems to have caused some membrane damage as the average ability of cells to exclude trypan blue was significantly lowered (After enrichment 87.7 ± 0.9% (n = 10) of cells were able to exclude trypan blue compared to 95.2 ± 0.6% (n = 10). $P<0.01$ by an unpaired *t*-test).

2.3 THE AMINOPYRINE ACCUMULATION RATIO.

A crude parietal cell preparation containing approximately 20% parietal cells can be used to measure acid secretion by using intracellular accumulation of aminopyrine as an index of acid secretion. This is because aminopyrine becomes trapped only within the acidic spaces of stimulated parietal cells. Aminopyrine exists almost entirely in an unionised form at physiological pH as it is a weak heterocyclic base with a pKa of 5.0. In this form it is able to diffuse across the parietal cell plasma membrane and enter the acidic spaces of the secretory cannaliculi where it becomes trapped upon ionisation (Fig. 2.2). Therefore aminopyrine accumulation is only an indicator of the average pH within the secretory canaliculi which is determined by the sequestration of acid within this region of the parietal cell. Aminopyrine accumulation has been expressed in many ways, though the most popular method of expression is probably the aminopyrine accumulation ratio used by Soll (1980a). The aminopyrine accumulation ratio is determined by dividing the concentration of intracellular aminopyrine by the concentration of aminopyrine in the medium. Results are also sometimes expressed as aminopyrine accumulation per 10^6cells by some workers. However, this method may be regarded as inferior because it depends on the

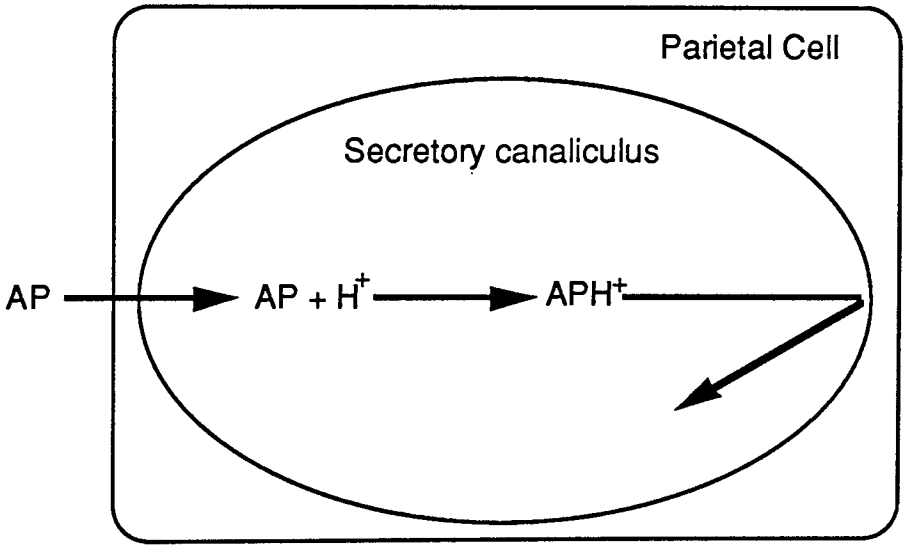
Figure 2.2

The structure of aminopyrine and its sequestration within the parietal cell.



Weak base
Aminopyrine pKa = 5

The protonated form of aminopyrine is trapped inside the cell as below.



concentration of aminopyrine in the medium, which is usually assumed to be the initial concentration.

2.3.1 Determination of the aminopyrine accumulation ratio.

Aliquots of cell suspensions (1.5ml) at a concentration of 2-5x10^6cells/ml were added to either siliconised glass or plastic 20ml incubation vials containing [^{14}C] aminopyrine (0.1μCi/ml: 0.9μM), [^{3}H] polyethylene glycol (M.Wt. 4000-Da) (0.4μCi/ml) and required secretagogues and agents. Before use siliconised glass vials were washed well in double-distilled water and then dried, and plastic vials were soaked in double-distilled water for at least 18 hours and dried. Unless stated otherwise secretagogues and agents were dissolved in saline (NaCl 0.9% ($^w/_v$)) and were present in small volumes (usually 20μl or less) to minimise any dilution of the cell suspension. Equal volumes of vehicle were added to the appropriate vials so that the final volume of all vials was the same. Each vial was gently swirled and gassed for 5 seconds with 95%O_2/5%CO_2 and then incubated for 30 minutes at 37°C in a waterbath with continuous shaking (120 cycles/minute).

After incubation duplicate aliquots of cell suspension were placed into microfuge tubes (L.I.P. Ltd., Shipley, W. Yorks, England) and centrifuged for 45 seconds in a Beckman microfuge at 10,000g. Then a 50μl sample of the supernatant from each of the duplicate microfuge tubes was transferred to a glass scintillation vial. The remainder of the supernatant was removed from each tube by aspiration and discarded. 0.5ml of medium B' was added to each tube which was then centrifuged at 10,000g for 20 seconds in a Beckman microfuge. The supernatants were aspirated and the tip of each tube, containing the pellet, was cut off using a heated scalpel and placed into individual glass scintillation vials. 0.5ml of Protosol was added to each vial (ensuring that Protosol covers each pellet) and the vials were incubated overnight at 37°C. The following morning 10ml of Econoflour was added to each vial and the contents mixed. The radioactivity of all vials was determined by liquid scintillation counting (Appendix A.3).

Calculation of the aminopyrine accumulation ratio (APR) can be carried out using the formula below.

$$APR = \frac{A - \frac{BC}{D}}{CE}$$

Where:
A = pellet [^{14}C] aminopyrine (dpm)

B = pellet [^{3}H] polyethylene glycol (dpm)
C = supernatant [^{14}C] aminopyrine (dpm/μl)
D = supernatant [^{3}H] polyethylene glycol (dpm/μl)
E = volume of intracellular fluid (μl)

The total aminopyrine in each pellet includes that within the cells and that in the extracellular fluid and this must be corrected for. To do this [^{3}H] polyethylene glycol (approx M.Wt. 4,000-Da) was used to estimate the volume of extracellular fluid. The intracellular fluid can be calculated using the relationship that 2μl of intracellular fluid is equivalent to 1mg dry weight of cells (J.F. Hatt personal communication). Dry weights were determined by centrifuging duplicate volumes of cell suspension at 10,000g for 1 minute using a Beckman microfuge followed by aspiration of the supernatant. The tip of the microfuge tube, containing the cell pellet, was removed and placed in an oven for 24 hours at 90°C. The dried cell pellets were weighed on a microbalance (Mettler). The dry weight was corrected for the dilution of the cell suspension upon addition to incubation vials and for the dissolved salts and albumin in the incubation medium associated with the cell pellet (for example medium B' contains 17.54mg/ml). Table 2.3 compares the aminopyrine accumulation ratio obtained in this work with that from other workers.

2.4 MEASUREMENT OF CYCLIC AMP AND PROSTAGLANDIN E_2 CONTENT OF PARIETAL CELL ENRICHED PREPARATIONS BY RADIOIMMUNOASSAY (RIA).

Radioimmunoassay (RIA) combines the specificity of the immune reaction with the sensitivity of radioactive techniques. It is used to quantitatively determine the levels of hormones and other biologically active substances present in body fluids and tissue extracts. The technique is based on the competition between unlabelled antigen and a known amount of isotopically labelled antigen for a limited number of antibody binding sites in a fixed amount of serum. Under standard conditions the amount of labelled antigen bound to antibody will decrease as the amount of unlabelled antigen in the same sample increases, for example

$4Ag^* + 4Ab \rightarrow 4Ag^*Ab$
$4Ag + 4Ag^* + 4Ab \rightarrow 2Ag^*Ab + 2AgAb + 2Ag^* + 2Ag$
$12Ag + 4Ag^* + 4Ab \rightarrow Ag^*Ab + 3AgAb + 3Ag^* + 9Ag$

where

Ab = one equivalent of antibody
Ag = one equivalent of unlabelled antigen
Ag^* = one equivalent of labelled antigen
AgAb = one equivalent of antigen-antibody complex

Table 2.3

Comparison of aminopyrine accumulation ratios from isolated parietal cell preparations stimulated by histamine ± a phosphodiesterase inhibitor.

Species	APR Basal	APR Secretagogues	Percentage parietal cells	Reference
Rat	1.5 ± 0.2(6)	Histamine (0.5mM) +IBMX (0.1mM) 116.8 ± 24.1(6)	18-23	This work
Rabbit	44 ± 7	Histamine (3μM) 271 ± 49	95	Brown and Chew, 1987
Dog	1.8 ± 0.3	Histamine (10μM)+IBMX (10μM) 44.7 ± 6.1	45-60	Soll, 1980a
Guinea pig	0.6	Histamine (10μM)+Ro 20-1724 (100μM) 2.5	70-80	Batzri and Dyer, 1981

Thus the level of radioactivity associated with the antibody-antigen complex is inversely proportional to the concentration of unlabelled antigen in the sample. Using known amounts of unlabelled antigen and a fixed amount of labelled antigen and antibody, a calibration curve for estimating an unknown amount of antigen may be constructed. It is essential that the standards for the standard curve are assayed under identical conditions as the samples because environmental conditions are rarely identical. Antibody-bound and free antigen must be separated so that the radioactivity in one or both fractions may be measured. This enables the proportion of labelled antigen bound to be estimated and thus the amount of unlabelled antigen in the system to be calculated from the calibration curve. Separation of the bound and free antigen can be achieved by a number of methods such as charcoal adsorption, alcohol precipitation or pre-reacted primary antibody / secondary antibody complex.

2.4.2 Radioimmunoassay of cyclic AMP.

2.4.2.1 Introduction.

The cyclic AMP content of enriched isolated parietal cell preparations were determined using the acetylated procedure as set out in the RIANEN^{TM} radioimmunoassay kit (Du Pont). Cyclic AMP was acetylated in the 2' O position to enhance the affinity of the cyclic nucleotide for its antibody (Steiner *et al.* 1972) and this has been found to increases the sensitivity of the radioimmunoassay (Frandsen and Krishna, 1976). Thus it is possible to measure low cyclic AMP levels in samples without the need to purify or concentrate the sample.

The contents of the radioimmunoassay kit were reconstituted as shown in Appendix A4.1.

2.4.2.2 Determination of the cyclic AMP content of preparations enriched in parietal cells.

After incubation samples were extracted using ethanol (final concentration of 50%) and stored at -18°C. Before assay the samples were allowed to thaw at room temperature, mixed thoroughly and centrifuged at 10,000g for 1 minute using a Beckman microfuge to pellet cellular debris. A 0.8ml supernatant sample was placed into a polypropylene test-tube (Luckham Ltd, Burgess Hill, Sussex, U. K.) and evaporated to dryness in a vacuum oven at 55°C. Samples were reconstituted in 100µl of sodium acetate buffer (pH 6.2) and mixed with 5 µl acetylation reagent (2 volumes of triethylamine mixed with 1 volume of acetic anhydride). Tubes were incubated at room temperature for 3 minutes. Then 900µl of sodium acetate buffer was added to each sample which was then mixed well using a vortex mixer.

Modified assay buffer was prepared by adding 50µl of acetylation reagent to 10ml of sodium acetate buffer. The cyclic AMP stock standard reagent (5,000pmole/ml) was diluted in sodium acetate buffer to produce a 40pmole/ml solution. A 20µl sample of this solution was acetylated by adding 10µl of the freshly prepared acetylation reagent and incubating at room temperature for 3 minutes. Then this sample was diluted with 1.8ml of sodium acetate buffer to produce a 4pmole/ml cyclic AMP standard. This standard was used to produce standards with concentrations of 2.0, 1.0, 0.5, 0.25 and 0.10pmole/ml using the modified assay buffer. All samples and standards for radioimmunoassay were prepared on the same day. The cyclic AMP [^{125}I] tracer (succinyl cyclic AMP tyrosine methyl ester) was diluted 1:1 ($^{v}/_{v}$) with the reconstituted cyclic AMP carrier serum immediately before each assay. Additions to polypropylene test-tubes were as shown in Table 2.4 and the contents were mixed well using a vortex mixer. The tubes were then covered and incubated for 16 to 18 hours at 2-8°C. Cyclic AMP precipitator (0.5ml) at 2-8°C was added to all tubes "except" the total counts tubes (tubes 1 and 2) which were placed in the racks ready for insertion into the gamma counter. The contents of each tube were mixed thoroughly using a vortex mixer and then centrifuged at 1250g for 15 minutes at 4°C. The tubes, 12 at a time, were placed in a plastic holder and decanted into the radioactive waste disposal sink. While the tubes were inverted any liquid remaining close to the rim of the tubes were removed by aspiration. The inverted tubes were then placed on absorbent paper and allowed to drain for about 30 seconds. To facilitate removal of the remaining droplets, the rims of the tubes were gently tapped on the paper. After blotting the tubes were placed in racks and the radioactivity in the precipitate of each tube was determined on a Compu-gamma gamma counter (LKB Instruments Ltd, Sweden) with a counting efficiency of 82%. The standard curve with Spline Function (cpm vs log concentration) was plotted and the concentration of cyclic AMP in the samples calculated using the curve fitting package associated with the gamma counter. Table 2.5 shows the performance characteristics of the extraction procedure and the radioimmunoassay as determined by J. F. Hatt. All data were corrected for the recovery of cyclic AMP in the extraction procedure (Table 2.5) and were expressed as pmole cyclic AMP/10^6cells. However medium B' was found to interfere with the assay and gave a cyclic AMP measurement of 0.01 ± 0.00pmole cyclic AMP in the medium associated with 10^6cells (n = 3 separate determinations by J. F. Hatt). This was a small percentage of the total cyclic AMP values (0.25-1.17%) and was a constant for each experiment, thus no correction was made due to this interference. A standard curve is shown in Appendix A 4.1.

2.4.3 Radioimmunoassay of prostaglandin E_2 (PGE_2).

2.4.3.1 Extraction of PGE_2.

Table 2.4

Summary of reagent additions used in the radioimmunoassay of cyclic AMP.

Tube containing	Tube number (µl)	Modified assay buffer (µl)	Standards (µl)	Sample (µl)	Working tracer (µl)	Antiserum complex (µl)
Total counts	1 and 2	---	---	---	100	---
Blank	3 and 4	200	---	---	100	---
0.1pmole/ml standard	5 and 6	---	100	---	100	100
0.25pmole/ml standard	7 and 8	---	100	---	100	100
0.50pmole/ml standard	9 and 10	---	100	---	100	100

Tube containing	Tube number (µl)	Modified assay buffer (µl)	Standards (µl)	Sample (µl)	Working tracer (µl)	Antiserum complex (µl)
1.0pmole/ml standard	11 and 12	---	100	---	100	100
2.0pmole/ml standard	13 and 14	---	100	---	100	100
4.0pmole/ml standard	15 and 16	---	100	---	100	100
Samples	17 and on	---	---	100	100	100

Continuation of Table 2.4.

Table 2.5

Performance characteristics of the cyclic AMP radioimmunoassay and extraction procedure as determined by J. F. Hatt.

Characteristics	Value
Recovery of [^{3}H]	85 ± 1% (n = 6)
Inter-assay co-efficient of variation	8.7% (n = 6)
Intra-assay co-efficient of variation	3.0% (n = 6)
Non-specific binding of cyclic AMP	4.7 ± 0.2% (n = 6)
Cross-reactivity:	
cyclic GMP	0.01%
GMP	0.01%
ATP	0.01%
ADP	0.01%

Prostaglandins are generally produced in nanogram quantities and are present in solution with other biological material in milligram quantities. For this reason and the finding that the presence of certain proteins, especially albumin, can alter the level of detected prostaglandins (Heinson *et al.*, 1987) it is necessary to extract prostaglandin from the incubation medium before radioimmunoassay. Most extraction procedures take advantage of the fact that the carboxylic group on the prostaglandin is ionised at high pH but becomes unionised at low pH. Thus extraction into the organic solvent is most efficient when the aqueous phase is at an acid pH. After extration the required prostaglandin may be purified before radioimmunoassay, but this is not necessary for the measurement of PGE_2. Indeed purification was not performed by many workers using a similar incubation medium, which was derived from isolated cell suspensions, to that used in this study (for example Schepp *et al.*, 1986). The procedure used in this work for extracting PGE_2 was as described by Hatt and Hanson (1988) which was similar to that used by Zenar and Davis (1978).

Aliquots of the cell suspension (0.5ml) were removed from the incubation vials and centrifuged at 10,000g for 1 minute in a Beckman microfuge and immediately placed on ice. Then a 0.4ml sample of the supernatant was rapidly removed from each tube and added to 14µl of 1M-HCL in a polypropylene test-tube. This reduced the pH to 3.0 - 3.3. Each tube was capped after adding 1.2ml of ethylacetate and then vortex mixed for 15 seconds prior to centrifugation at 100g for 1 minute at 15°C. The non-aqueous layer (containing prostaglandin) was carefully removed and transferred to a clean siliconised glass scintillation vial. A second 1.2ml aliquot of ethylacetate was added to the acidified sample which was then vortexed and centrifuged as described. The non-aqueous layer was pooled with the first fraction and evaporated to dryness under a stream of N_2 gas at room temperature. All samples were stored at -20°C for less than one week before radioimmunoassay.

2.4.3.2 Determination of PGE_2 production by RIA.

To quantify PGE_2 production by an enriched isolated parietal cell preparation from the rat a prostaglandin E_2 [^{125}I] radioimmunoassay kit (New England Nuclear) was used. Separation of the bound and free antigen is achieved by precipitation with polyethylene glycol (PEG 6000). The contents of the radioimmunoassay kit were made up as shown in Appendix A4.2.

The dried extracts (from section 2.4.3.1) were reconstituted in 0.5ml of assay buffer (50mM-phosphate pH 6.8) and vortex mixed. PGE_2 standards ranging from 0.25 to 25pg/0.1ml were prepared by serial dilution of a 100ng/ml PGE_2 standard with assay buffer. The [^{125}I] PGE_2 tracer (2.6µCi/ml) was diluted with assay buffer (1:20 ($^{v}/_{v}$)) immediately before each assay. Additions to polypropylene test-tubes were

made as set out in Table 2.6 and the contents vortex mixed. Then all tubes were covered and incubated for 16-24 hours at 2-8°C. After incubation all tubes, except tubes 1 and 2 (total counts), were placed into an ice bath and 1ml of cold precipitating agent containing 16% polyethylene glycol was mixed with the contents of each tube. The tubes were then incubated for 20-30 minutes and then removed from the ice bath and centrifuged at 1,500g for 30 minutes at 4°C. This precipitated the antibody bound tracer. After centrifugation the supernatants were decanted and the precipitates were counted and PGE_2 estimated as for cyclic AMP (2.4.2.2).

Prostaglandins adhere to certain surfaces and to minimise this effect polypropylene test-tubes (Luckham Ltd, Burgess Hill, Sussex, U. K.) and polypropylene pipette tips (L. I. P. (Equipment and Services) Ltd, Shipley, W. Yorks) were used and all glassware was siliconised with dimethyldichlorosilane. As raised temperatures can result in the degradation of prostaglandins, especially the E, A and C series, solvent extraction was carried out in an ice-bath where possible and centrifugation at 4°C. Also after extraction samples were stored at -20°C to minimise degradation.

Performance characteristics of the extraction procedure and the radioimmunoassay are shown in Table 2.7. The efficiency of the extraction procedure was assessed by determining the recovery of [^{3}H] PGE_2 (0.1mCi/ml). Thus an aliquot of [^{3}H] PGE_2 was added to 0.5ml of medium B' and an aliquot of this (100µl) was added to Econofluor and the radioactivity counted on a Packard Tri carb scintilation counter. Then 0.4ml of the remaining medium B'was extracted as scheduled. The residue was reconstituted in medium B', added to Econofluor and the radioactivity counted. By expressing the counts as a percentage of the control counts, the recovery of [^{3}H] PGE_2 could be determined. The figure of 95 ± 3% was similar to that reported by Zenser and Davis (1978) using a similar extraction procedure. The values obtained from the standard curve (see Appendix A.4.2 for standard curve) were routinely corrected for the recovery of PGE_2 and for the interference in the binding of antibody to tracer caused by the incubation medium (0.2-0.3pg PGE_2/0.1ml determined by J. F. Hatt).

Table 2.6

Summary of reagent additions used in the radioimmunoassay of prostaglandin E_2.

Tube contents	Tube number	Buffer	Standard	Samples	Tracer	Antibody
Total counts	1 and 2	---	---	---	100	---
Blank	3 and 4	200	---	---	100	---
0.25pg/0.1ml standard	5 and 6	---	100	---	100	100
0.5pg/0.1ml standard	7 and8	---	100	---	100	100
1.0pg/0.1ml standard	9 and 10	---	100	---	100	100
2.5pg/0.1ml standard	11 and 12	---	100	---	100	100

Tube contents	Tube number	Buffer	Standard	Samples	Tracer	Antibody
5.0pg/0.1ml standard	13 and 14	---	100	---	100	100
10.0pg/0.1ml standard	15 and 16	---	100	---	100	100
25.0pg/0.1ml standard	17 and 18	---	100	---	100	100
Samples	19 and on	---	---	100	100	100

Continuation of Table 2.6.

Table 2.7

Performance of PGE_2 radioimmunoassay and characteristics of the extraction procedure.

Characteristic	Value
Recovery of [^{3}H] PGE_2	95 ± 3% (n = 3)
Inter-assay co-efficient of variation	9.3% (n = 8)*
Intra-assay co-efficient of variation	3.9% (n = 8)*
Non-specific binding of PGE_2	5.4 ± 0.3% (n = 5)*
Cross-reactivity:	
PGE_1	3.7%
6- oxo $PGF_{1\alpha}$	0.03%
PGB_2	0.01%
$PGF_{2\alpha}$	0.01%
Arachidonic acid	0.01%

* Data from J. F. Hatt.

Chapter 3

EFFECT OF PERTUSSIS TOXIN ON THE ACTIONS OF PROSTAGLANDIN E_2, SOMATOSTATIN AND EPIDERMAL GROWTH FACTOR ON PARIETAL CELLS FROM THE RAT STOMACH.

3.1 INTRODUCTION.

Prostaglandin E_2 (PGE_2), somatostatin and EGF inhibit histamine-stimulated secretory activity in rat parietal cells and are possibly of physiological importance. The intention of this work was to establish whether G-proteins were involved in the inhibitory actions of PGE_2, somatostatin and EGF on histamine-stimulated secretory activity in rat parietal cells. It was also of interest to ascertain whether a G-protein was involved in the EGF-stimulated production of PGE_2. In order to achieve these ends *Bordetella pertussis toxin* was used to prove the involvement of G-proteins. A brief review of PGE_2, somatostatin, EGF and G-proteins is set out below to put this work into context.

3.1.1 Prostaglandins.

3.1.1.1 Synthesis of Prostaglandins.

Prostaglandin biosynthesis can be detected in the stomach and every segment of the gastrointestinal tract. There are differences in the ratios of the various prostaglandins detected *in vitro* and this may be caused by species differences and by different pH of the incubation media. Prostaglandins have been found to be released upon nerve stimulation in the rat stomach (Whittle and Vane, 1987). Arachidonic acid is the precursor of all prostaglandins and in cells it is found in esterified to the 2-position of, for example, phosphoinositides. Arachidonic acid can be liberated from phospholipids in two ways. Phospholipase A_2 yields lysophospholipid and free arachidonic acid, and in some cell types activity is regulated by pertussis toxin-sensitive G-proteins (Burch *et al.*, 1986; Wang *et al.*, 1988). Phospholipase C hydrolyses phospholipids to yield diacylglycerol and, in the case of phosphatidylinositol 4, 5-bisphosphate, inositol 1, 4, 5-trisphosphate (Prescott and Majerus, 1983). Then diacylglycerol lipase and monoacylglycerol lipase may catalyse the conversion of diacylglycerol to free arachidonic acid. Phospholipase C can be modulated by membrane bound receptors via G-proteins (Burch *et al.*, 1986; Majerus *et al.*, 1986).

Arachidonic acid is the substrate of the enzyme complex cyclo-oxygenase (Fig. 3.1) which converts it into labile endoperoxides. These can then be readily converted by endoperoxide isomerase to PGE_2 (Hamberg *et al.*, 1975; see Fig. 3.2 for structure).

The precise mechanisms controling prostaglandin production in the stomach are not known. However, it has been shown that muscarinic stimulation by carbachol in canine isolated parietal cells stimulates PGE_2 production by a calcium-dependent mechanism (Payne and Gerber, 1987).

Figure 3.1

Schematic representation of the biosynthesis of prostaglandins from arachidonic acid.

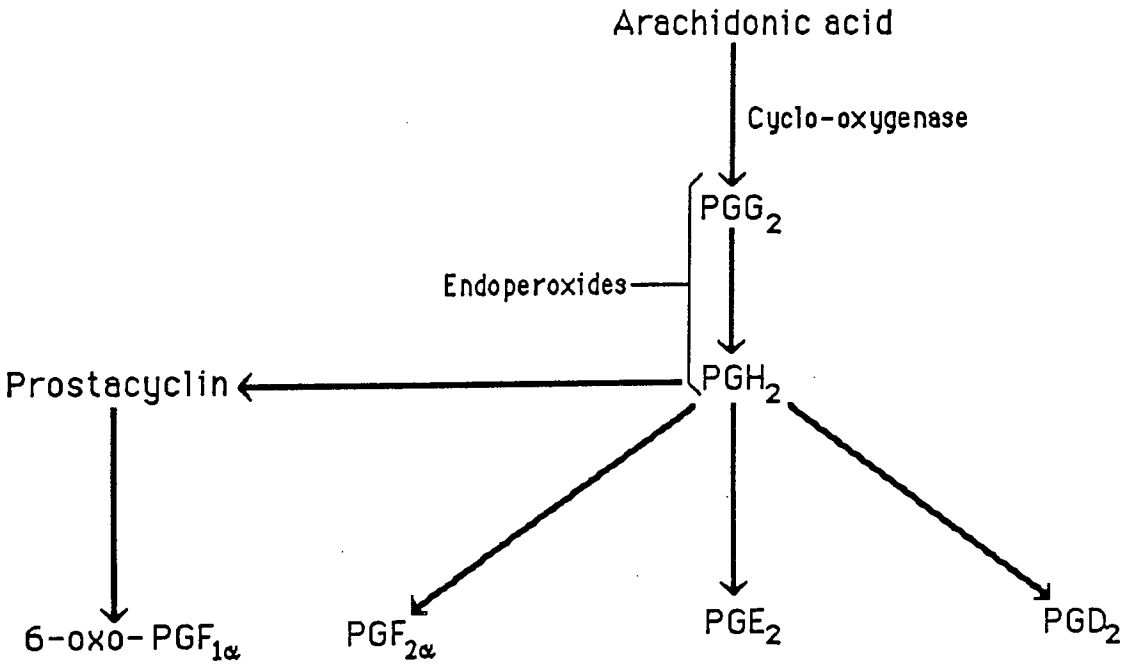
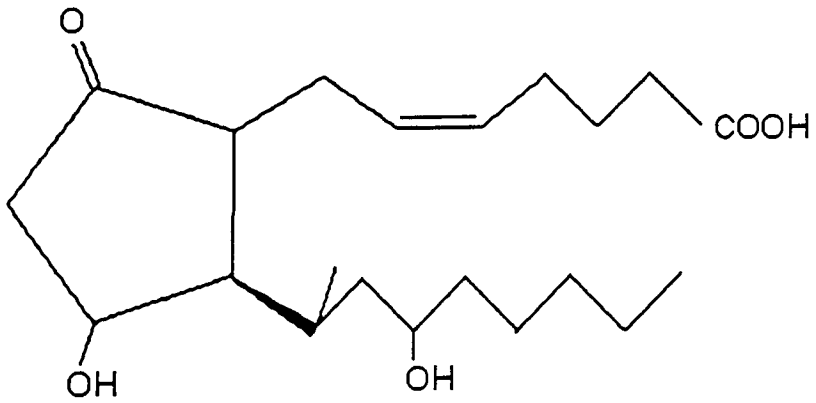


Figure 3.2

The structure of prostaglandin E_2.



3.1.1.2 Prostaglandin receptors.

It has been shown that rabbit isolated parietal cells have true PGE_2 receptors and that PGE_2 binding to the parietal cell was associated with its ability to inhibit histamine-stimulated aminopyrine accumulation in these cells (Barr *et al.*, 1988). More recent studies by Seidler *et al.* (1989) suggest that all prostaglandins mediate their inhibitory effects on rabbit parietal cells by this PGE_2 receptor and the different antisecretory potencies of prostaglandins are determined by their different affinities for this receptor.

3.1.1.3 The action of prostaglandins on gastric acid secretion.

Intravenous infusion of PGE_2 has been shown to inhibit histamine- and pentagastrin-stimulated acid secretion in the rat *in vivo* (Main and Whittle, 1973). PGE_2 inhibits histamine-stimulated aminopyrine accumulation in canine and rat isolated parietal cell preparations (Soll, 1980a; Rosenfeld, 1986). However, PGE_2 has been found to be without effect on isolated canine cells stimulated with carbachol, gastrin or dbcAMP (Soll, 1980b). PGE_2 inhibited forskolin-stimulated aminopyrine accumulation in canine isolated parietal cells (Chen *et al.*, 1988a), but in rat isolated parietal cells forskolin-stimulation was not inhibited by PGE_2 (Rosenfeld, 1986). The fact that the stimulatory effects of dbcAMP are not inhibited by PGE_2 suggest that PGE_2 prevents cyclic AMP generation rather than action, and indeed PGE_2 reduces the cyclic AMP content of parietal cells stimulated by histamine plus IBMX (Major and Scholes, 1978; Soll, 1980b; Schepp *et al.*, 1983a). This effect is probably mediated by an inhibition of adenylate cyclase (Major and Scholes, 1978). Concentrations of PGE_2 above 1 μM increased the cyclic AMP content of isolated gastric cell preparations. However, this has been negatively correlated with the parietal cell content of the preparation and so probably involves activation of adenylate cyclase present in non-parietal cells (Wollin *et al.*, 1979). In isolated canine parietal cells PGE_2 appears to be a more potent inhibitor of histamine-stimulated aminopyrine accumulation than PGI_2, $PGF_{2\alpha}$ or PGD_2 (Skoglund *et al.*, 1982).

Prostacyclin is rather unstable but has been shown to inhibit acid secretion stimulated by histamine in rat gastric mucosa *in vivo* and *in vitro* (Whittle *et al.*, 1978; Soll, 1980b). However, PGD_2 had no antisecretory action on canine isolated parietal cells (Skoglund *et al.*, 1980). In canine isolated parietal cells $PGF_{2\alpha}$ has in some cases an antisecretory effect at high concentrations (Skoglund *et al.*, 1980).

It was not known if inhibitory concentrations of PGE_2 mediated their action against secretory activity via a G-protein, in rat parietal cells stimulated by histamine. Thus the effect that pertussis toxin has on the inhibitory action of PGE_2 on histamine-stimulated parietal cells from the rat was examined.

3.1.2 Somatostatin.

3.1.2.1 Somatostatin structure.

Somatostatin has been reviewed by Walsh (1987). Somatostatin can be isolated in two forms, the tetradecapeptide (SS-14) (Brazeau *et al.*, 1973) and a larger molecular form (SS-28) with 28 amino acid residues (Pradayrol *et al.*, 1980; Fig. 3.3), extended from the amino terminus of SS-14. SS-28 may be a prohormone, having two consecutive basic residues preceding the SS-14 sequence, but it has biological activity and is found in serum. Somatostatin has been detected in nerves and cell bodies in the autonomic nervous system of the gut. The nerve terminals have been identified in the muscle layer of the antrum but not the gastric mucosa in humans. Somatostatin has also been detected in endocrine-like D-cells located in the mucosa and intestine (Walsh, 1987).

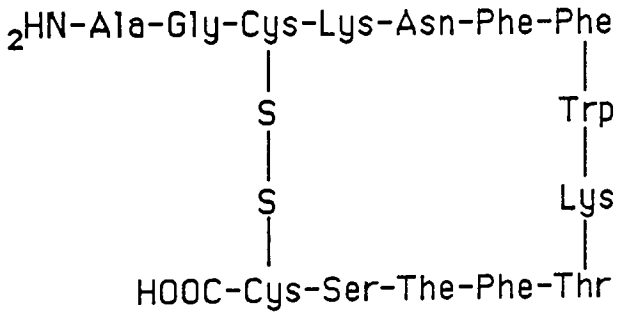
3.1.2.2 Somatostatin receptors

Two classes of somatostatin binding site have been identified on enriched canine parietal cells, one with high affinity and low capacity and the other of low affinity but high capacity (Park *et al.*, 1987). Reyl *et al.* (1977) also found high and low affinity receptors on rat gastric cells but suggested that low affinity receptors were present on parietal cells and that high affinity receptors were present on chief cells. The presence of specific binding sites for somatostatin-14 were demonstrated in the cytosol of rabbit antral and fundic mucosa (Guijarro *et al.*, 1985) and rat parietal cells (Reyle-Desmars *et al.*, 1982). Somatostatin was found to stimulate phosphoprotein phosphatases in rat parietal cells. Consequently it was proposed that somatostatin could mediate its action, at least in part, through stimulation of protein dephosphorylation via intracellular receptors (Reyle-Desmars *et al.*, 1982). However, Park *et al.* (1987) reported that somatostatin binding to canine parietal cells was rapidly reduced by simple dilution (40% reduction within 15 minutes) and suggested that the receptors, in this case, were located on the cell surface only. SS-14 and -28 were equipotent at displacing bound ligand and affecting aminopyrine accumulation. D-cells have been demonstrated to possess long cytoplasmic processes that appear to make contact with gastrin or parietal cells. Therefore it has been suggested that somatostatin may have a paracrine function on gastric acid production (Larsson *et al.*, 1979). However, the number of parietal cells with these connections may be limited as there are far more parietal cells than D-cells (Yamada, 1987). Somatostatin itself appears to autoregulate its own secretion via its own receptors on D-cells (Park *et al.*, 1989).

3.1.2.3 Inhibition of gastric acid secretion by somatostatin.

Figure 3.3

The structure of somatostatin (tetradecapeptide SS-14).



In vivo somatostatin-14 appears to be a more potent inhibitor of acid secretion than somatostatin-28 in, for example, the cat (Hirst *et al.*, 1988). However, *in vitro* somatostatin-14 and -28 inhibited cyclic AMP production stimulated by histamine (Gespach *et al.*, 1983) and aminopyrine accumulation (Park *et al.*, 1987) with similar potencies. This suggests that they both act on a common recognition site on the parietal cell. A direct inhibitory action of somatostatin on histamine-stimulated acid secretion has not been observed in all preparations (Table 3.1). Thus an objective of this chapter was to clarify the effect of somatostatin on rat parietal cells stimulated by histamine. Acid secretion stimulated by gastrin, carbachol and dbcAMP was inhibited by somatostatin in some cases but not others (Table 3.2). In many respects the action of somatostatin resembles that of PGE_2. Like PGE_2, somatostatin inhibited histamine-stimulated adenylate cyclase activity and cellular cyclic AMP levels in rat parietal cells. They also both had inhibitory actions on histamine-stimulated aminopyrine uptake (Schepp *et al.*, 1983b). Despite this, Nylander *et al.* (1985) found that indomethacin did not remove the inhibitory action of somatostatin on histamine-stimulated rabbit glands. Thus it seems unlikely that prostaglandins mediate the action of somatostatin.

In rabbit isolated gastric glands somatostatin inhibited gastrin-stimulated aminopyrine accumulation and oxygen consumption. However, in rabbit isolated parietal cells somatostatin inhibited histamine but not gastrin stimulation. This led to the suggestion that somatostatin was acting indirectly on gastrin stimulation by blocking the gastrin-stimulated release of histamine from other cells (Chew, 1983b).

Contrasting results by Park *et al.* (1987) indicated that somatostatin had a direct inhibitory effect on gastrin- and carbachol-stimulated parietal cell activity in canine isolated parietal cells. 70% of the cells were parietal cells and the remaining 30% were chief or mucous cells, neither of which were likely to influence acid secretion. It was also found that the inhibitory action against gastrin and carbachol took place without altering membrane inositol phospholipid turnover or protein kinase C activity (Park *et al.*, 1987). This suggests that somatostatin exerts its action on gastrin, carbachol and dbcAMP stimulation at a point distal to phosphatidylinositol turnover and cyclic AMP generation. It is possible that somatostatin acts at a site where the various signal transduction systems converge to promote cell activation: such a site might involve protein phophorylation/dephosphorylation (Reyl and Lewin, 1981).

The possibility that a G-protein was involved in the speculated inhibitory action of somatostatin against secretory activity stimulated by histamine, on rat parietal cells, was investigated. To do this the effect of pertussis toxin on the inhibitory action of somatostatin, if present, was explored.

Table 3.1

A comparison of the action of somatostatin on histamine-stimulated aminopyrine accumulation ratio (APR).

Species	Preparation	Secretagogue	Action of somatostatin on APR	Reference
Guinea-pig	Isolated parietal cells (60-70%)	Histamine + RO 20-1724	Inhibitory at 0.2 and 0.4μM-histamine and stimulatory at 1, 10 and 100μM-histamine	Batzri *et al.*, 1981
Dog	Enriched parietal cells	Histamine (10^{-4}M)	No effect	Skoglund *et al.*, 1982
Rabbit	Enriched parietal cells (81 ± 1%)	Histamine (10^{-4}M)	Inhibition	Chew,1983
Rat	Crude (25 ± 1%) and enriched (71 ± 4%) isolated parietal cells	Histamine (10^{-4}M)	Inhibition	Schepp *et al.*, 1983b
Rabbit	Isolated glands	Histamine ($3x10^{-5}$M)	Inhibition	Nylander *et al.*, 1985

Species	Preparation	Secretagogue	Action of somatostatin on APR	Reference
Dog	Isolated parietal cells (76 ± 4%)	Histamine (0.5mM) + IBMX (0.1mM)	No effect	Perez-Reys *et al.*, 1985
Dog	Isolated parietal cells (70%)	Histamine (10^{-4}M)	Inhibition	Park *et al.*,1987

Table 3.1 continued.

Table 3.2

The action of somatostatin on aminopyrine accumulation stimulated by secretagogues other than histamine.

Species	Preparation	Secretagogue	Effect of somatostatin	Reference
Dog	Isolated cell	Pentagastrin Carbachol DbcAMP	Inhibition Inhibition Inhibition	Park *et al.*, 1987
Rabbit	Isolated cell	Gastrin	No effect	Chew, 1983b
	Isolated gland	Carbachol DbcAMP	No effect No effect	
Rabbit	Isolated cell	DbcAMP	No effect	Nylander *et al.*, 1985

3.1.3 Epidermal growth factor (EGF).

3.1.3.1 The EGF molecule.

EGF is a single chain polypeptide consisting of 53 amino acids which was originally isolated from mouse submaxillary glands (Cohen, 1962; see Fig. 3.4). β–urogastrone extracted from human urine was found to be similar to mouse EGF, 37 out of 53 amino acid residues being common to both molecules (Gregory, 1975). Not surprisingly both molecules show similar biological properties (Gregory, 1975) and in cultured fibroblasts β-urogastrone and mouse EGF have been shown to share a common receptor (Hollenberg and Gregory, 1976). After removing eleven amino acid residues from the C-terminal end of human EGF the peptide was still a full agonist although higher concentrations were required (Gregory *et al.*, 1988). EGF has been detected in human saliva, sweat, blood, urine and milk (Carpenter, 1985) and in many other tissues including the stomach and duodenum (Kasselberg *et al.*, 1985). However, whether there are other sites of EGF synthesis apart from the submaxillary gland is not clear.

3.1.3.2 The EGF receptor.

The EGF receptor is a glycoprotein with an approximate molecular weight of 170,000 which is composed of a single polypeptide chain (Carpenter, 1985). Ullrich *et al.* (1984) proposed that the EGF receptor is composed of three functional domains (Fig. 3.5). The EGF receptor appears to exist in high and low affinity states in many cell lines (Livneh *et al.*, 1986). Binding of EGF to its receptor promotes aggregation of the receptor complex in clustered pits which are then internalised. The C-terminal cytoplasmic domain of the EGF receptor seems to play an important role in the aggregation and internalisation of the EGF receptor complex. Most of the bound EGF seems to be internalised after 15 minutes at 37°C (Livneh *et al.*, 1986). Eventually the EGF receptor complex is degraded in lysosomal compartments (Stoscheck and Carpenter, 1984).

Cross linking of EGF receptors by using antibodies has converted low affinity binding sites into higher affinity binding sites (Yarden and Schlessinger, 1985). Therefore, aggregation of EGF receptors may alter their affinity for EGF. In fact clustering of EGF receptors appears to be required for biological activity.

In human A431 epidermoid carcinoma cells, which have unusually high numbers of EGF receptors, EGF stimulates the phosphorylation of the EGF receptor on tyrosine, serine and threonine residues (Iwashita and Fox, 1984). Tyrosine phosphorylation is due to autophosphorylations close to the C-terminal end which is believed to be caused by receptor oligomerization and cross phosphorylation

Figure 3.4

A schematic representation of the secondary structure of human EGF (Cook *et al.*, 1987).

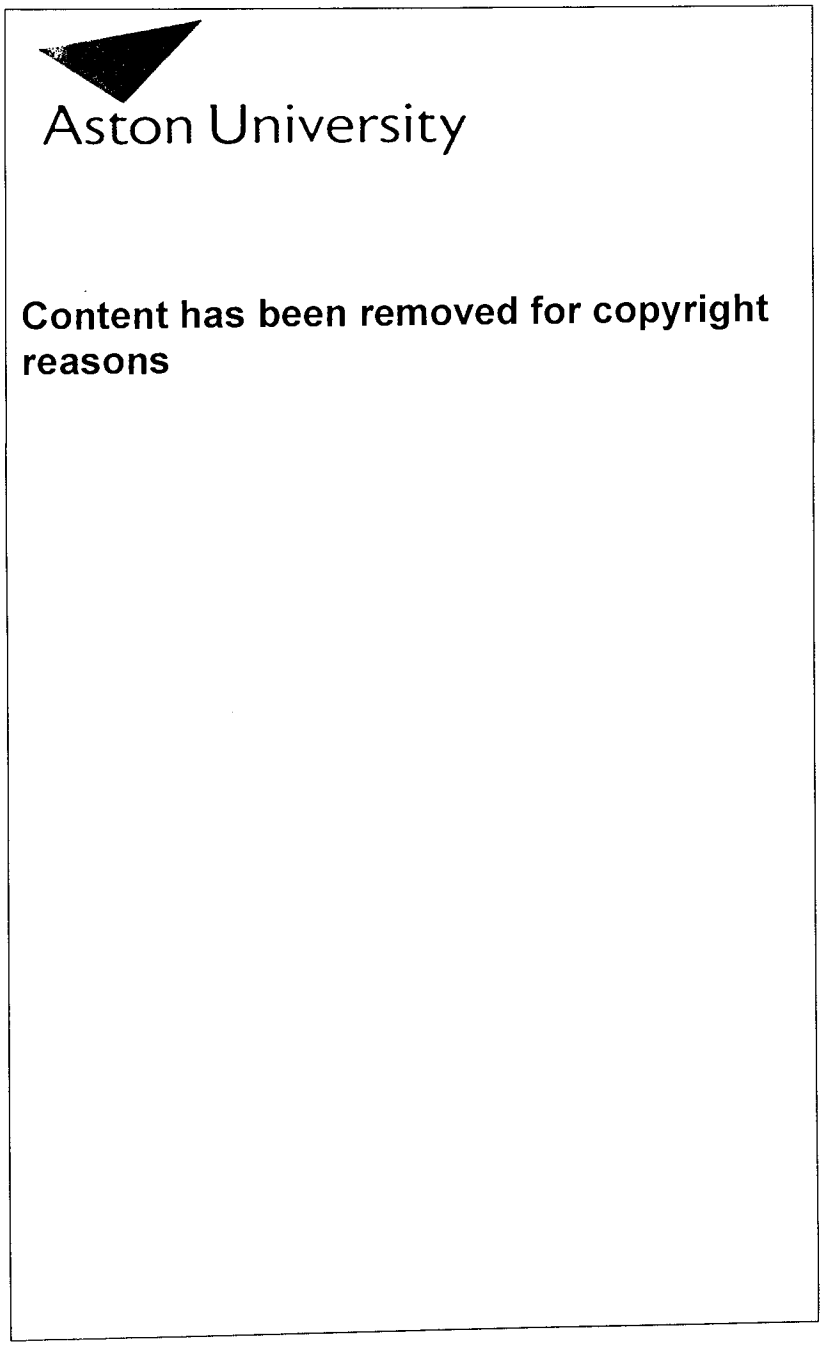
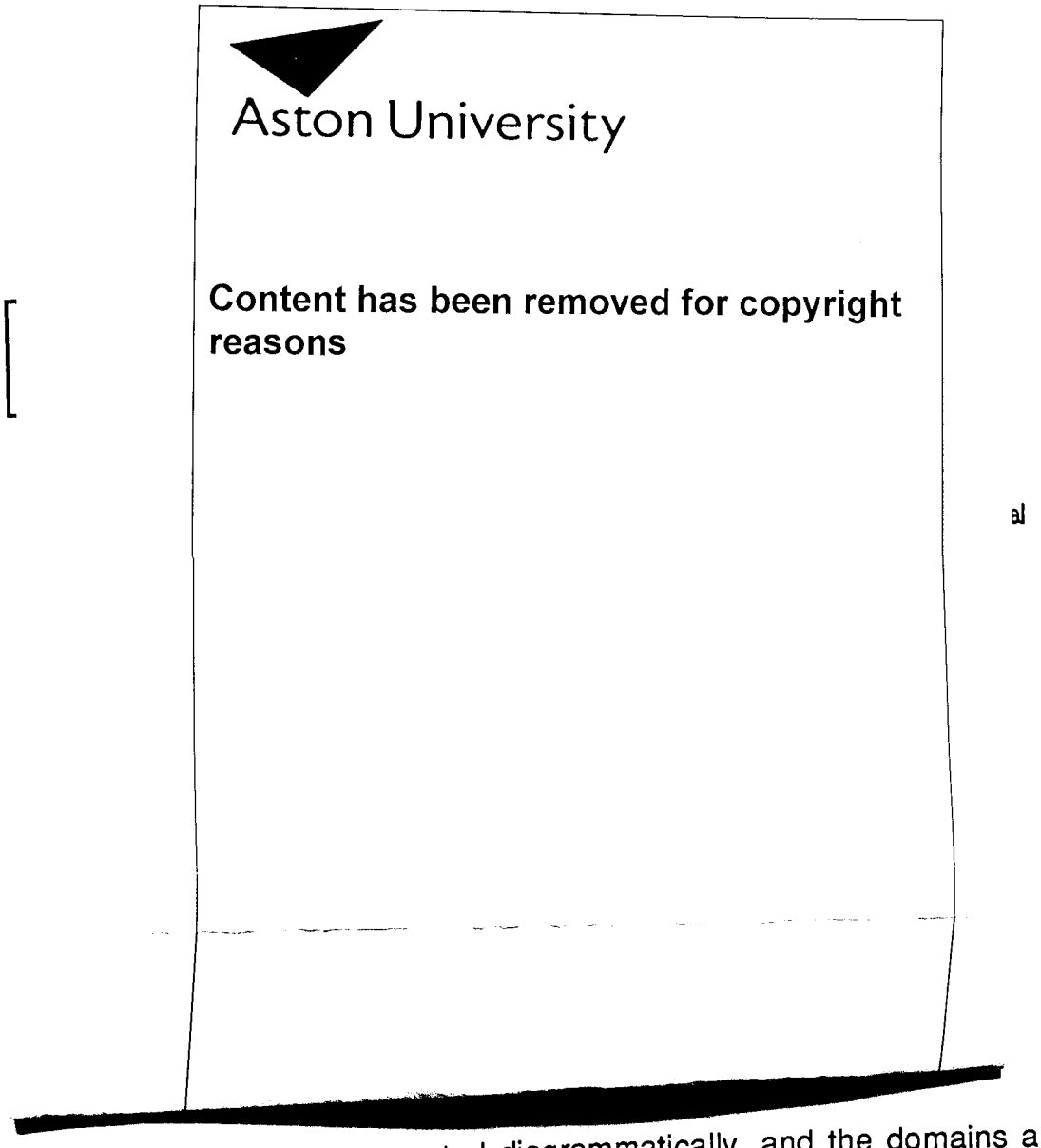


Figure 3.5

Diagrammatic representation of the EGF receptor.



The EGF receptor is represented diagrammatically, and the domains are derived from the amino acid sequences determined by Ullrich *et al.* (1984).

stimulated by EGF binding (Schlessinger, 1989; King and Cooper, 1986). It was suggested that oligomerization results in in receptor activation, which is then followed by receptor autophosphorylation (Schlessinger, 1989). The phosphorylation of both serine and threonine residues was activated by TPA, presumably through the activation of protein kinase C (Cochet *et al.*, 1984). Phosphorylation of the EGF receptor at threonine-654, stimulated by TPA, has been found to inhibit tyrosine autophosphorylation, decrease tyrosine kinase activity (King and Cooper,1986) and cause a reduction in EGF binding affinity (Lin *et al.*, 1986).

High levels of specific ^{125}I-human EGF binding sites have been located over the mucosa and muscularis mucosa of the antrum in adult canine stomachs (Zimmerman *et al.*, 1989). Mori *et al.* (1987) showed that strong EGF receptor-like immunoreactivity was present on canine parietal cells. Strong EGF receptor-immunoreactivity has been demonstrated on the basolateral membrane of the human parietal cell but not on the luminal surface and so it has been suggested that EGF receptors are present on the vascular-facing membrane of these cells (Mori *et al.*, 1987). EGF receptors appear to be present in the rat stomach and seem to be located on parietal cells (Shaw *et al.*, 1987).

3.1.3.3 Effect of EGF on gastric acid secretion.

Parenteral administration of β–urogastrone to dogs with Heidenhain pouches completely inhibited histamine, pentagastrin and cholinergic stimulation of acid secretion (Gregory *et al.*, 1977). This has been confirmed more recently by Konturek *et al.* (1984) who demonstrated that intravenous infusion of EGF inhibited acid secretion in dogs stimulated by sham-feeding, without changing the serum gastrin concentration. This suggests that EGF acts directly on parietal cells. Subcutaneous administration of EGF inhibited acid secretion stimulated by histamine in rats and cats with gastric fistulas (Konturek *et al.*, 1981b). EGF appears to be ineffective when administered intragastrically unless very high concentrations are used (Dembinski *et al.*, 1982; Konturek *et al.*, 1981a). EGF was found to be ineffective on the luminal side, but not serosal side, of guinea-pig mucosal strips stimulated with histamine (Finke *et al.*, 1985). Intravenously administered EGF has been demonstrated to be far more potent than intragastrically administered EGF in dogs (Larson *et al.*, 1988). Although EGF is present in saliva (Starkey and Orth, 1977) it seems from the above not to play a physiological role in regulating acid secretion, under normal circumstances, when the epithelium will prevent its access to the basolateral surface of parietal cells where the EGF receptors are located. Indeed in humans, feeding has been found to increase both plasma and salivary but not gastric EGF. Also although intravenous infusion of pentagastrin raised plasma and salivary EGF, the output of EGF was found to be insufficient to inhibit pentagastrin or feeding-stimulated acid

secretion. This suggested that EGF release is under neuro-hormonal control, but that changes in serum EGF are probably not responsible for the regulation of gastric acid secretion (Konturek *et al.*, 1988).

EGF may be released from platelets to cause an inhibition of acid secretion in conditions of acute damage to the gastric epithelium, and thereby aid the process of repair (Shaw *et al.*,1987). Alternatively luminal EGF may gain access to parietal cells when the epithelium has been damaged.

An antisecretory action of EGF has been observed on gastric glands and isolated cell preparations (Table 3.3). Konturek *et al.*, (1984) and Dembinski *et al.*, (1986) found that EGF had an inhibitory effect on basal secretory activity in gland preparations. In contrast EGF exhibited no effect on basal stimulation in isolated cell preparations (Shaw *et al.*,1987; Hatt and Hanson, 1988).

In isolated cell preparations EGF appears to have a secretagogue-specific action inhibiting histamine-stimulation, in a non-competitive fashion, but having a minimal effect on cholinergic agonists, gastrin and dbcAMP (Shaw *et al.*, 1987; Chen *et al.*, 1984). Stimulation caused by submaximal doses of dbcAMP is inhibited by EGF in gastric glands. As the concentration of dbcAMP increases the inhibitory effect of EGF decreases in a competitive fashion (Dembinski *et al.*, 1986).

The possibility that EGF inhibits acid secretion in rat isolated parietal cells was investigated by Shaw *et al.* (1987). It was demonstrated that the pattern of inhibition of secretion produced by TPA was different from that of EGF, and it was therefore unlikely that protein kinase C was mediating the inhibitory action of EGF.

Hatt and Hanson (1988) found that EGF decreased the histamine-stimulated cyclic AMP content of enriched preparations of rat isolated parietal cells. This correlated with the action of EGF on aminopyrine accumulation ratio in these cells. In other cell types EGF has been shown to inhibit adenylate cyclase activity (Gutierrez *et al.*, 1987).

It was not known if the inhibitory action of EGF on rat parietal cells stimulated by histamine involved a G-protein sensitive to pertussis toxin. Therefore the effect of pertussis toxin on this inhibitory action of EGF was explored.

3.1.3.4 EGF and Prostaglandin Production.

EGF stimulates prostaglandin production in many cell types such as cultured porcine thyroid cells (Kasai *et al.*, 1987) and cultured renal glomerulosa mesangial cells (Margolis *et al.*, 1988). Perfusion of EGF (100nM) through the vasculature of

Table 3.3

A comparison of the action of EGF on secretagogue-stimulated secretory activity in gastric glands and isolated cell preparations.

Species	Preparation	Secretagogue	Action of EGF on secretory activity	Reference
Rabbit	Glands	Histamine Carbachol DbcAMP	Inhibition Inhibition Inhibition	Konturek *et al.*,1984
Rabbit	Glands	Histamine DbcAMP	Inhibition No effect	Reichstein *et al.*,1984
Dog	Isolated cells	Histamine Cholinergic agonists Gastrin Dbc AMP Forskolin IBMX	Inhibition Minimal effect	Chen *et al.*,1984
Rabbit	Glands	Histamine Dbc AMP K+	Inhibition Inhibition No effect	Dembinski *et al.*,1986
Rat	Isolated cells	Histamine Histamine + IBMX Carbachol Dbc AMP	Inhibition No effect No effect No effect	Shaw *et al.*,1987
Rat	Isolated cells	Histamine Histamine + IBMX	Inhibition No effect	Hatt and Hanson,1988

the isolated rat stomach increased the prostaglandin content (PGE_2 and/or PGE_1) of the venous effluent (Chiba *et al.*, 1982).

Using a rat isolated parietal cell preparation, containing more than 80% parietal cells, Hatt and Hanson (1988) showed that EGF stimulated PGE_2 production with a half-maximally effective concentration of 7.5nM. If the cell fraction was depleted of parietal cells (12%) EGF was found to be ineffective in stimulating prostaglandin release, indicating that prostaglandins were released from parietal cells or cells that co-purified with parietal cells on the Percoll gradient. Hatt and Hanson (1988) suggested that the parietal cell was responsible for prostaglandin production because the half-maximally effective concentration of EGF needed to stimulate prostaglandin production was close to that which depressed cyclic AMP. Further evidence was provided by the finding that histamine, which stimulates the cyclic AMP content of parietal cells, inhibited the stimulatory action of EGF on PGE_2 production. The elevation of intracellular cyclic AMP inhibits prostaglandin production in several cell types (Hassid *et al.*, 1982). It seems unlikely that prostaglandins mediate the inhibitory action of EGF on acid secretory activity as cyclo-oxygenase and lipoxygenase inhibitors did not affect the action of EGF (Shaw *et al.*, 1987). Hatt and Hanson (1988) also found that histamine blocked the stimulatory action of EGF on PGE_2 release and so it seems unlikely that EGF inhibits histamine-stimulation via prostaglandins.

The mechanism by which EGF stimulates prostaglandin secretion is not known but may involve the removal of the inhibitory action of lipocortin I on phospholipase A_2 (Pepinski and Sinclair, 1986; Hirata, 1981). This would result in an increased phospholipase A_2 activity and thus prostaglandin synthesis. However, the degree to which lipocortin I inhibits phospholipase A_2 activity under physiological conditions has been questioned (Davidson *et al.*, 1987).

The possibility that EGF mediates its stimulatory effect, on rat gastric cell PGE_2 production, via a G-protein sensitive to pertussis toxin was investigated.

3.1.3.5 Other Biological Effects of EGF.

Incubation of cells for a relatively short period of time (up to 60 minutes) results in a variety of effects such as the phosphorylation of cellular proteins on tyrosine residues (Johnson *et al.*,1986), an increase in intracellular inositol trisphosphates and Ca^{2+} (Sawyer and Cohen, 1981) and activation of metabolic enzymes (for example Bosch *et al.*, 1986). See Table 3.4 for further examples.

Over longer periods of time, at least 5 hours, EGF initiates DNA synthesis in a wide range of cell types such as rat hepatocytes (Richman *et al.*,1976) and human

Table 3.4

Rapid effects of EGF on various types of cell.

Cell-types	Effect of EGF	Reference
Human placenta	Stimulated phosphorylation of G protein β_{35} subunits	Valentine-Braun *et al.*, 1986
Caecum and liver (mouse)	Elevated guanylate cyclase activity	Scheving *et al.*, 1985
A431 cells	Increased tyrosine phosphorylation of lipocortin I	Pepinsky and Sinclair, 1986
A431 cells	Activation of protein kinase C	Sahai *et al.*, 1982

fibroblasts (Hollenberg and Cuatrecasas, 1973). In duodenal gastric mucosa EGF has also been observed to increase the rate of cell division (Dembinski *et al.*, 1982).

Low doses of EGF have a protective effect on gastric and duodenal mucosa (Konterek *et al.*, 1981a and b; Itoh *et al.*,1988). These mitogenic and protective properties of EGF appear to be completely independent from the antisecretory actions, as the doses of EGF required are lower for mitogenic effects and take place from the luminal side of the stomach.

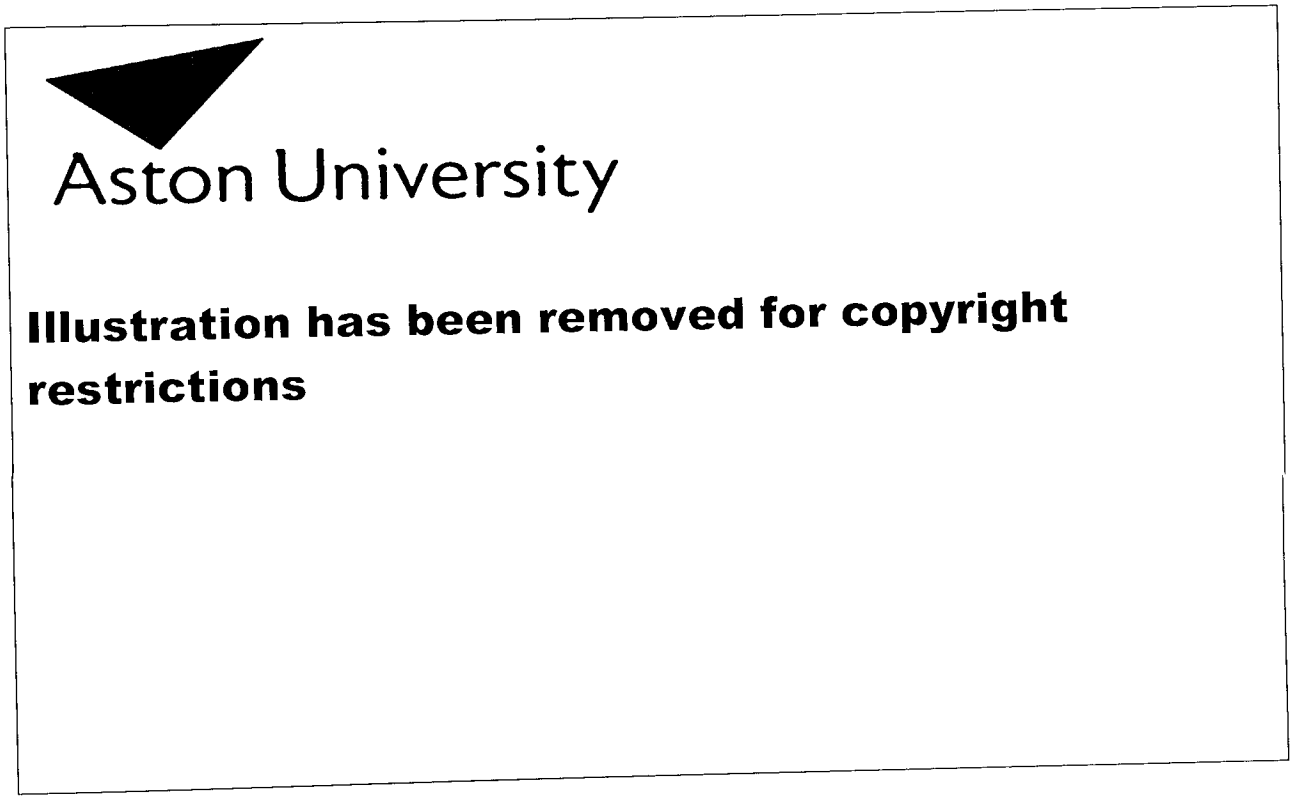
3.1.4 G-proteins.

3.1.4.1 Mode of operation.

A family of guanine nucleotide-binding proteins (G-proteins) intervene between the membrane bound hormone receptors and their effectors. These transducers of receptor-generated signals have been reviewed recently by Graziano and Gilman (1987), Gilman (1987) and Milligan (1988). This family of G-proteins has been sub-divided into four main classes, G_s, G_i, G_o and G_t. This series of G-proteins are composed of three subunits designated α,β and γ (Graziano and Gilman, 1987). The process of coupling agonist-activated receptors to the effector system must be of limited duration, so the G-protein must undergo a cycle of activation followed by subsequent deactivation (Fig 3.6) (Milligan, 1988). The resting state of a G-protein has been suggested to be the holomeric form with GDP bound to the α-subunit. GDP dissociation is slow and appears to be the rate limiting step. Interaction of the G-protein with an appropriate agonist-receptor complex enhances the release of GDP from the α-subunit which is then replaced by the more prevalent GTP. In this state the α-GTP subunit promotes dissociation from the $\beta\gamma$ subunit. In this dissociated state the α-subunit of the G-protein appears to modulate the effector. Slow hydrolysis of the bound GTP to GDP by the intrinsic GTPase activity of the α-subunit is believed to lead to a reassociation of the oligomer and a return to the deactivated state (Gilman, 1987). Pertussis toxin has been found to ADP-ribosylated G_i, G_o and G_t holomeric G-protein on the α-subunit (Milligan, 1987). This takes place close to the C-terminus and occurs on a conserved cysteine residue which appears to be close to the G-protein receptor contact point (Bourne *et al.*, 1987; McKenzie *et al.*, 1988; Hamm *et al.*, 1987). Such ADP ribosylation has been found to inactivate the G-proteins. $G_{s\alpha}$ does not contain this cysteine residue and therefore cannot be ADP-ribosylated (Milligan and McKenzie, 1988). Cholera toxin has been shown to ADP-ribosylate certain G-proteins on an arginine residue close to the GTP binding site on the α-subunit (Van Dop *et al.*,1984; Robishaw *et al.*,1986). This stabilises the stimulatory GTP-bound α-subunit and permanently activates it (Cassel and Selinger, 1977).

Figure 3.6

Diagrammatic representation of the activation and deactivation of a G-protein (Milligan *et al.*, 1988).



3.1.4.2 Structure of G-proteins sensitive to pertussis toxin and cholera toxin.

G_α

There are a family of highly homologous but distinct α-subunits (Table 3.5). The cholera toxin sensitive α-subunit of G_s, the G-protein which stimulates adenylate cyclase, exists in two forms which appear to represent two differentially spliced products of a single mRNA precursor (Bray *et al.*, 1986; Robishaw *et al.*, 1986). It has been suggested that both forms of $G_{s\alpha}$ can stimulate adenylate cyclase (Graziano *et al.*, 1987).

There are at least 3 pertussis toxin sensitive $G_{i\alpha}$ subunits termed G_{i1}, G_{i2} and G_{i3} which are products from three separate genes (Codina *et al.*,1988). Some cells express multiple forms of $G_{i\alpha}$ subunits (Jones and Reed, 1987). G_i has been shown to inhibit adenylate cyclase in many tissues (Bokoch *et al.*, 1983; Katada *et al.*, 1984). Opening of a potassium channel has also been stimulated by a G_α protein, possibly a form of $G_{i\alpha}$, in guinea-pig atrial cells (Logothetis *et al.*, 1987). Evidence put forward by McKenzie *et al.* (1988) using anti-peptide antiserum against G_i on a neuroblastoma x glioma cell line indicates that a form of G_i is involved in the modulation of calcium channels. The term G_i-like has been used in the Results and Discussion in this work to include G_{i1}, G_{i2} and G_{i3}.

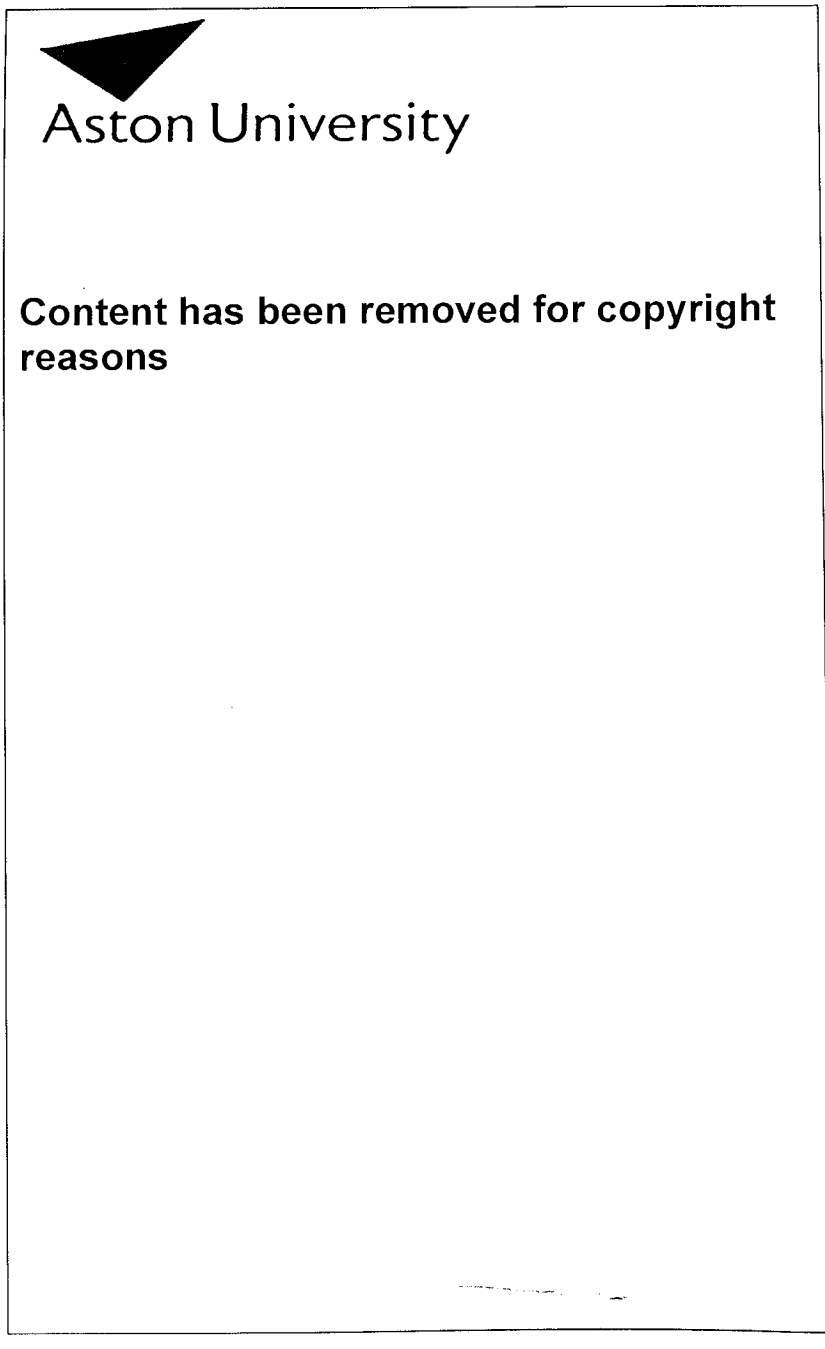
G_o has been found to be the predominant G-protein in bovine brain (Neer *et al.*,1984), but little is known about this pertussis-toxin-sensitive protein. Using anti-G_o antisera Gierschik *et al.* (1986) showed that the α-subunit of G_o is very similar to but distinct from $G_{i\alpha}$ subunits. There may be two separate genes for G_o, or differential splicing of a single gene for G_o, as two separate mRNA strands hybridise with a G_o specific probe (Jones and Reed, 1987). The G_t G-proteins (or transducins) are often pertussis toxin-sensitive and couple rhodopsin receptors to a cyclic GMP-specific phosphodiesterase (Stryer, 1986). Two different forms of $G_{t\alpha}$ have been identified. One is apparently confined to the retinal rods and the other to cones (Lerea *et al.*, 1986).

$G_{\beta\gamma}$

Two forms of the ß subunit, $ß_{35}$ and $ß_{36}$, have been identified from many G-proteins and two different amino acid sequences have been determined from cDNA clones (Fong *et al.*, 1987; Gao *et al.*,1987; Sugimoto *et al.*, 1985). Only $ß_{36}$ has been detected in association with retinal rods.

Table 3.5

The G-protein subunits (Graziano and Gilman, 1987).



Multiple forms of the γ-subunit exist and the G_t γ-subunit is distinguishable from that of G_s and G_i. The G_s and G_i γ-subunits were indistinguishable by peptide mapping (Hildabrant *et al.*, 1985).

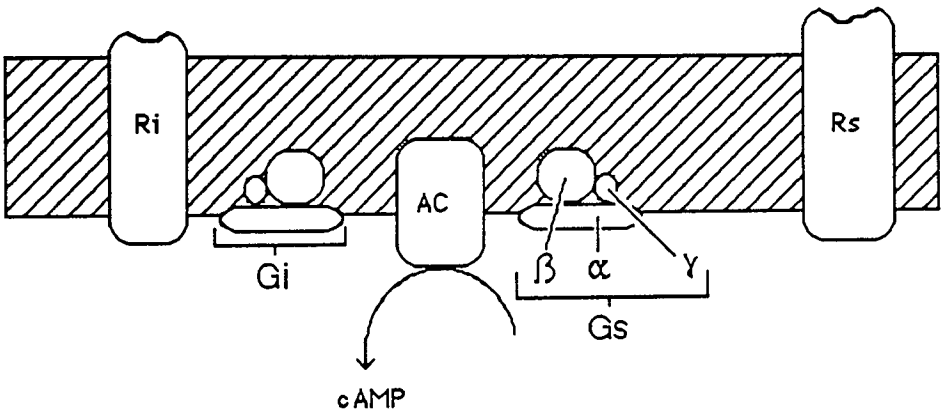
3.1.4.3 Modulation of adenylate cyclase.

G_s has been shown to be the stimulatory regulator of adenylate cyclase and without G_s adenylate cyclase activity cannot be observed (Fig 3.7). Thus reconstitution of G_s into membrane preparations from the G_s deficient S49 cyc^- cell line has restored stimulated adenylate cyclase activity (Ross *et al.*, 1978).

Receptor mediated inhibition of adenylate cyclase takes place through G_i. Reconstitution studies have shown that the ßγ subunits were responsible for most of the inhibition which is probably mediated by the inactivation of G_s (Cerione *et al.*, 1986). However, somatostatin was able to produce a receptor mediated inhibition of adenylate cyclase in the G_s deficient S49 cyc^- cell line suggesting a direct role for G_i (Jacobs *et al.*, 1983).

Figure 3.7

Diagrammatic representation of G-protein regulation of transmembrane signalling involving adenylate cyclase.



AC represents adenylate cyclase.

Gs represents the stimulatory G-protein which is composed of α_s, β and γ subunits.

G_i represents the inhibitory G-protein which is composed of α_i, β and γ subunits.

R_s receptors are a group of molecules which all interact with G_s, but differ in their size and specificity of their hormone binding sites.

R_i receptors are as Rs only they interact with G_i. Single cells may contain various R_i receptors.

3.2 METHODOLOGY.

3.2.1 Preparation of reagents.

Pertussis toxin was dissolved in 50mM-tris-chloride buffer (pH 8.0) containing 1M-NaCl. Histamine, 8-phenyltheophylline, 2',5'-dideoxyadenosine, N^6-phenylisopropyladenosine and the somatostatin antagonist cyclo[7-aminoheptanoyl-Phe-D-Trp-Lys-Thr(Bzl)] were dissolved in saline (NaCl 0.9%($^w/_v$)) and where appropriate a small volume (10-100µl) was added to the incubation vials. PGE_2, IBMX and Ro 20-1724 were dissolved in absolute ethanol and a small volume (2µl) was added to incubation vials. Ethanol was also added to control vials so that the final concentration was 0.125%($^v/_v$) in all vials. EGF was stored at -20°C in aliquots at a concentration of 32µM in saline. A fresh aliquot or an aliquot which had been partly used and refrozen once was used in experiments.

3.2.2 Effect of pertussis toxin on the inhibitory actions of PGE_2, somatostatin and EGF on aminopyrine accumulation in crude preparations of parietal cells.

Parietal cell preparations which consisted of approximately 20% parietal cells were isolated as described in section 2.1.3. After isolation the cell pool in medium B' with gentamycin (50µg/ml) was split in two, one portion containing pertussis toxin (100ng/ml) and the other contained the pertussis toxin carrier solution alone. These cells were preincubated in a shaking waterbath (76 cycles/minute) at 37°C for 2 hours and were constantly gassed with a slow stream of 95%O_2/5%CO_2. Then each cell pool was centrifuged for 5 minutes at 175g at 15°C (MSE Chilspin). The supernatants were discarded and the cell pellets carefully washed twice by resuspending in 10ml of medium B' and recentrifuging. The pellets were finally resuspended in the appropriate volume of B' at a final concentration of 2-5x10^6cells/ml. 1.5ml aliquots of the cell suspension were added to the incubation vials and the airspace above the cells gassed for 5 seconds with 95%O_2/5%CO_2. Treatment vials were then incubated for 30 minutes at 37°C with shaking at 120 cycles /minute. The aminopyrine accumulation was carried out as previously described in section 2.3.

3.2.3 Effect of pertussis toxin on PGE_2 production.

Enriched parietal cell preparations were prepared as described in section 2.2.2. The purified cells were washed free from Percoll by centrifugation and preincubated for 2 hours in medium B' plus gentamycin (50µg/ml) with and without pertussis toxin. The cells were washed twice and resuspended in the appropriate volume of B' at a final concentration of 2-5x10^6cells/ml. Duplicate 0.5ml aliquots were removed and rapidly centrifuged at 10,000g for 30 seconds in a Beckman microfuge and then

stored on ice until PGE_2 extraction. Aliquots of 1.5ml were added to the incubation vials and the airspace above the cells gassed for 5 seconds with 95%O_2/5%CO_2 before capping the vials. Treatment vials were incubated for 30 minutes at 37°C with shaking at 120 cycles/minute and then 0.5ml aliquots were removed from each vial and rapidly centrifuged at 10,000g for 30 seconds in a Beckman microfuge. Samples were placed on ice and then PGE_2 extracted from the medium and measured by radioimmunoassay (section 2.4.3).

Schepp *et al.* (1986) showed that PGE_2 was released into the medium rather than being retained within gastric mucosal cells. As a consequence PGE_2 was measured in the incubation medium only. It was presumed that increased medium prostaglandin content reflected increased biosynthesis of prostaglandins. This procedure has been used by many workers to determine prostaglandin biosynthesis using cell preparations (for example Margolis *et al.*, 1988; Richelsen, 1987).

3.2.4 Analysis and presentation of data.

To overcome the variation in aminopyrine accumulation between cell batches, the data have been normalised and expressed as % inhibition as defined below. Before normalisation the basal (unstimulated) aminopyrine accumulation ratio was subtracted from the stimulated value.

$$\text{\% inhibition by inhibitor} = \left(1 - \frac{\text{value in presence of inhibitor}}{\text{value in absence of inhibitor}}\right) \times 100$$

3.3 RESULTS AND DISCUSSION.

3.3.1 The effect of preincubation with pertussis toxin on the aminopyrine accumulation ratio.

It was initially established that the inhibitory action of PGE_2 on histamine (0.5mM) plus IBMX (0.1mM)-stimulated aminopyrine accumulation was substantially blocked by preincubation with pertussis toxin (100ng/ml) for a period between 90 and 180 minutes (Table 3.6). The period of incubation with pertussis toxin (100ng/ml) was standardised at 2 hours.

The increase in the aminopyrine accumulation ratio observed upon stimulation with histamine (0.5mM) and histamine (0.5mM) plus IBMX (0.1mM) (Table 3.7) was similar to that obtained by Shaw *et al.* (1987) using the same preparation. Pretreatment with pertussis toxin for 2 hours had no effect on basal aminopyrine accumulation, or that produced by incubation with histamine plus IBMX (Table 3.7). In contrast, the aminopyrine accumulation ratio stimulated by histamine alone was significantly increased by preincubation with pertussis toxin (Table 3.7).

It was thought possible that both IBMX and pertussis toxin blocked the action of an endogenous inhibitor of secretory activity. Endogenous adenosine inhibited secretory activity by acting on A_1 receptors on canine isolated parietal cells (Gerber and Payne, 1988). Both pertussis toxin (Berman *et al.*, 1986) and IBMX (Daly, 1985) interfer with the action of A_1 receptors. So preincubation with pertussis toxin may prevent the inhibitory action of endogenous adenosine and thereby cause the elevated response to histamine. If adenosine was the cause of this effect 8-phenyltheophylline an adenosine A receptor antagonist should raise the aminopyrine accumulation ratio for incubations without pertussis toxin pretreatment to that approaching pretreatment with pertussis toxin. However, the expected rise in aminopyrine accumulation ratio with increasing concentrations of 8-phenyltheophylline was not observed (Table 3.8). The possible action of adenosine at an internal inhibitory P-site was eliminated as the adenosine internal P-site agonist 2',5'-dideoxyadenosine (2',5'DDA) (10μM) did not inhibit the histamine (0.5mM) stimulated response [histamine-stimulated aminopyrine accumulation ratios (means ± S.E.M., with the number of determinations in parentheses, using a single batch of cells containing 20% parietal cells): 2',5'DDA absent, 5.6 ± 0.7(4); 2',5'DDA present 6.3 ± 0.2(4)]. Subsequently it was found that N^6-phenylisopropyladenosine (PIA) (10μM), an adenosine receptor agonist, had no effect on the aminopyrine accumulation ratio in cells with histamine (0.5mM) or histamine plus the phosphodiesterase inhibitor Ro 20-1724 [stimulated aminopyrine accumulation ratio (mean ± S.E.M., with the number of determinations in parentheses, using a single batch of cells containing 20% parietal cells): histamine (0.5mM)

Table 3.6

Effect of preincubation with pertussis toxin (100ng/ml) for 90 and 180 minutes on secretory activity by rat isolated parietal cells incubated under various conditions.

Preincubation time with pertussis toxin (min)	APR Histamine (0.5mM) + IBMX (0.1mM)	APR Histamine (0.5mM) + IBMX (0.1mM) + PGE_2 (10^{-7}M)	% inhibition by PGE_2 (10^{-7}M)
0	123.3 ± 7.6(3)	42.3 ± 1.5(4)	65.7
90	120.3 ± 3.2(4)	110.0 ± 4.8(4)	8.6
180	43.6 ± 3.3(4)	44.0 ± 1.2(4)	-0.9

Aminopyrine accumulation ratios (APR) are presented as means ± S.E.M., with the number of cell samples in parentheses. Data are from a single batch of cells containing 19% parietal cells.

Table 3.7

The effects of preincubation with pertussis toxin (100ng/ml) on the aminopyrine accumulation ratio (APR) in parietal cells.

Secretagogues	*n*	APR Control	APR Pertussis toxin
None	13	1.5 ± 0.2	1.4 ± 0.2
Histamine (0.5mM)	6	7.5 ± 1.7	17.9 ± 5.3*
Histamine (0.5mM) + IBMX (0.1mM)	14	122 ± 17	135 ± 21

Cells were preincubated with pertussis toxin (100ng/ml) for 2 hours, washed and then incubated with aminopyrine in the presence and absence of secretagogues for 30 minutes. Aminopyrine accumulation ratios are presented as means ± S.E.M. *n* is the number of cell batches. The effect of pertussis toxin has been analysed by a paired *t*-test. * $P<0.05$.

Table 3.8

Effect of the concentration of 8-phenyltheophylline on the aminopyrine accumulation ratio stimulated by histamine in normal and pertussis toxin preincubated parietal cells isolated from the rat.

8-phenyltheophylline (μM)	APR Control	APR Pertussis toxin
0	3.6 ± 0.4(3)	9.5 ± 0.3(4)*
0.02	2.6 (2.6, 2.6)	9.0 (8.9, 9.0)
0.2	2.8 (3.0, 2.7)	8.8 (9.7, 8.0)
2.0	3.7 (3.7, 3.7)	10.1 (11.0, 9.3)
20.0	2.5 ± 0.3(4)	9.9 ± 0.3(4)*

Control and pertussis toxin (100ng/ml) preincubations were for 2 hours. Control cells were preincubated in medium B'. Cells were stimulated with histamine (0.5mM) and the aminopyrine accumulation ratios are presented as either means ± S.E.M. with the number of cell samples in parentheses, or as means with both samples in parentheses. Results are from a single batch of cells containing 22% parietal cells. * $P<0.001$ for the effect of pertussis toxin (data assessed by unpaired *t*-test where repetition sufficient).

stimulated cells, control 4.3 ± 0.2(4); PIA treated, 4.2 ± 0.5(4): histamine (0.5mM) plus Ro 20-1724 (0.1mM), control 35.2 ± 2.1(4); PIA treated 34.0 ± 1.7(4)]. Therefore it is suggested that rat parietal cells do not possess A_1 adenosine receptors. These findings are in agreement with those of Puurunen *et al.* (1987b).

A somatostatin antagonist also had no effect on the difference between the response of control and pertussis toxin-treated cells to histamine, which suggests that somatostatin was not responsible for the observed effect (Table 3.9). Parietal cells were stimulated by histamine (3μM) with IBMX (0.1mM), which normally induces the same level of stimulation as 0.1mM-histamine alone, and again the effect of pertussis toxin was still apparent (Table 3.9).This result suggests that it was not IBMX per se which blocked the effect of pertussis toxin, but that no effect of pertussis toxin was detectable when the cells were maximally stimulated by 0.5 mM-histamine with 0.1mM-IBMX. The effect of pertussis toxin on histamine stimulation could be due to the removal of an inhibitory tone. A stimulatory action of pertussis toxin on the response to agonists has been observed in other types of cells (Garcia-Sainz *et al.*, 1985; Katada *et al.*, 1982).

3.3.2 The effect of preincubation with pertussis toxin on control and histamine-stimulated cyclic AMP content of enriched parietal cell preparations.

Histamine (0.5mM) plus IBMX (0.1mM) significantly increased the cyclic AMP content of purified parietal cells pretreated with and without pertussis toxin [stimulated cyclic AMP levels pmol cyclic AMP/10^6cells (mean ± S.E.M., with the number of batches of cells in parentheses using cells containing > 80% parietal cells): without pertussis toxin pretreatment, control 1.75 ± 0.35(5); histamine plus IBMX 4.39 ± 0.82(5). $P<0.01$ by paired *t*-test: with pertussis toxin pretreatment, control 1.86 ± 0.20(3); histamine plus IBMX 5.34 ± 0.41(3). $P>0.02$ by paired *t*-test]. There were no significant differences between cells preincubated with and without pertussis toxin for basal and histamine-stimulated cyclic AMP contents.

3.3.3 Effect of preincubation with pertussis toxin on the inhibitory action of PGE_2.

Aminopyrine accumulation stimulated by a near-maximally effective concentration of histamine (0.5mM) with IBMX (0.1mM) was inhibited in a concentration dependent fashion by PGE_2 (Table 3.10 and Fig.3.8). The half-maximal inhibitory concentration was 24 ± 2nM, as determined by the FIT program (Barlow, 1983). This was lower than the values of 70nM found by Schepp *et al.* (1983b) using a similar preparation and 75nM stated by Skoglund *et al.* (1980) using a canine enriched parietal cell preparation. The maximal effect of PGE_2 was to inhibit the

Table 3.9

Effect of a somatostatin antagonist and 3μM-histamine with IBMX (0.1mM) on aminopyrine accumulation ratios in control and pertussis toxin (100ng/ml) preincubated parietal cells.

Incubation conditions	APR Control	APR Pertussis toxin
Histamine (0.5mM)	1.8 ± 0.1(4)	5.9 ± 0.2(4)
Histamine (0.5mM) + somatostatin antagonist (10^{-8}M)	1.9 ± 0.1(4)	5.6 (5.7, 5.6)
Histamine (3μM) + IBMX (0.1mM)	1.9 ± 0.1(4)	6.0 ± 0.2(4)

The somatostatin antagonist was cyclo[7-aminoheptanoyl-Phe-D-Tryp-Lys-Thr (Bzl)]. Control and pertussis toxin preincubations were for 2 hours. Control cells were preincubated in medium B'. The aminopyrine accumulation ratios are presented as means ± S.E.M. with the number of cell samples in parentheses, or means with both values in parentheses. Results are from a single batch of cells containing 21% parietal cells.

Table 3.10

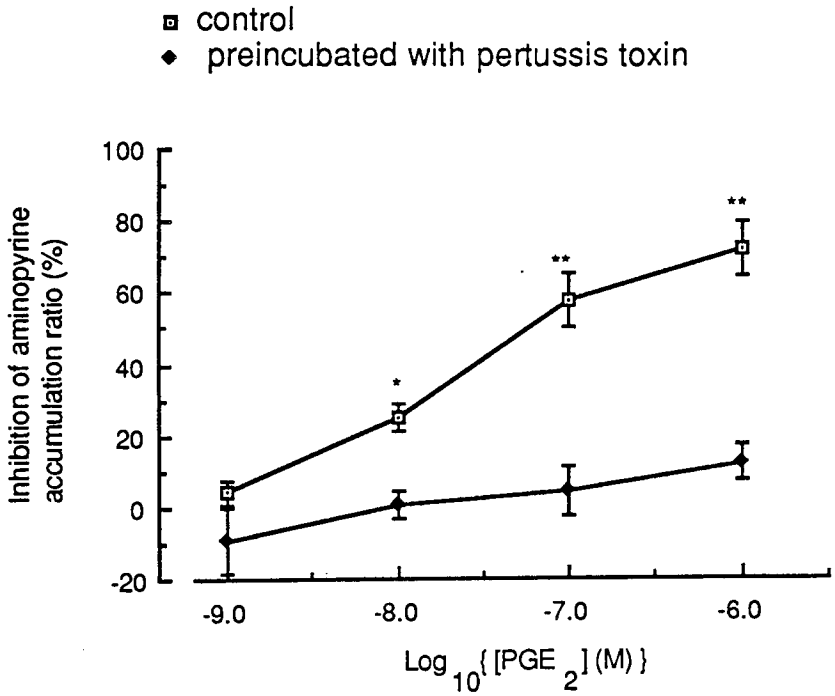
The effect of pertussis toxin (100ng/ml) preincubation (2 hours) on the inhibitory action of PGE_2 on aminopyrine accumulation stimulated by histamine (0.5mM) and IBMX (0.1mM).

Concentration of PGE_2 (M)	APR Control	APR Pertussis toxin
0	153.6 ± 34.0	179.9 ± 44.3
10^{-9}	145.0 ± 29.3	184.8 ± 37.6
10^{-8}	117.5 ± 30.1	181.4 ± 49.3
10^{-7}	64.2 ± 21.9	165.6 ± 39.2
10^{-6}	44.2 ± 20.4	154.1.± 38.3

Results from 4 experiments using batches of cells containing 21.0 ± 1.1% parietal cells are expressed as means ± S.E.M.

Figure 3.8

Effect of preincubation with pertussis toxin (100ng/ml) for 2 hours on the inhibitory action of PGE_2 on the aminopyrine accumulation ratio stimulated by histamine (0.5mM) and IBMX(0.1mM).



Data from Table 3.10 have been normalised and expressed as % inhibition by PGE_2 of the aminopyrine accumulation ratio obtained with histamine and IBMX alone. The effect of preincubation with pertussis toxin has been analysed by a paired *t*-test. ** $P<0.01$; * $P<0.05$. See Appendix A.5.3 for statistical analysis.

histamine-stimulated aminopyrine accumulation ratio by 76 ± 1%, which was similar to the 69% inhibition obtained by Schepp *et al.* (1983b). PGE_2 was shown to have no effect on isolated rat parietal cell basal secretion (Schepp *et al.*, 1983a). Preincubation with pertussis toxin significantly reduced the inhibitory action of 10^{-8}, 10^{-7} and 10^{-6}M-PGE_2. Pertussis toxin decreased the inhibitory action of 10^{-6}M-PGE_2 by 83%. In fact the dose-related inhibitory effect of PGE_2 in cells preincubated in pertussis toxin was abolished (F ratio for the effect of prostaglandin concentration by ANOVAR = 2.36, which does not reach significance).

These results which indicate that pretreatment with pertussis toxin prevented the inhibitory action of PGE_2 are in agreement with the preliminary study by Rosenfeld (1986), who used a single concentration of PGE_2 (300nM), and have been confirmed by studies using canine isolated parietal cells (Chen *et al.*, 1988a). PGE_2 inhibits adenylate cyclase in parietal cells (Schepp *et al.*, 1983a), and recently the inhibitory action of the PGE_2 analogue enprostil on cyclic AMP content was shown to be blocked by pretreatment with pertussis toxin (Chen *et al.*, 1988a). Consequently it is probable that a G_i protein is involved in the receptor-mediated inhibition of adenylate cyclase by PGE_2 in the rat stomach.

3.3.4 Effect of preincubation with pertussis toxin on the inhibitory action of somatostatin.

Many studies involving somatostatin have been carried out in the presence of the sulphydryl reagent dithiothreitol (DTT) (Chew, 1983b; Schepp *et al.*, 1983a). The possibility that its presence affects the action of somatostatin on histamine plus IBMX stimulated aminopyrine accumulation was investigated (Table 3.11) The presence of DTT (0.5mM) did not enhance the inhibitory effect of somatostatin. DTT was not used in subsequent experiments. As both somatostatin-14 and -28 have similar potencies on parietal cell secretory activity stimulated by histamine (section 3.1.2.3) somatostatin-14 was used in all experiments.

It has been observed that peptides may be degraded rapidly during transit through the interstitial space (Bunnet *et al.*, 1983a). Skoglund *et al.* (1982) also observed that somatostatin was degraded by canine isolated parietal cell preparations during incubation. Therefore the action of somatostatin may be diminished due to the presence of protease enzymes. However the presence of leupeptin (0.5μg/ml) and pepstatin (0.7μg/ml) in the incubation medium did not increase the inhibitory action of somatostatin (Table 3.11). Consequently leupeptin and pepstatin were not used in subsequent experiments.

Somatostatin inhibited the aminopyrine accumulation ratio stimulated by a near-maximally effective concentration of histamine (0.5mM) with IBMX (0.1mM)

Table 3.11

The effect of dithiothreitol(DTT), leupeptin and pepstatin on the inhibition of histamine and IBMX-stimulated aminopyrine accumulation by somatostatin.

Incubation conditions	APR 1.5 hours preincubation in B', then 0.5 hours with DTT(0.5mM)	APR 2 hours preincubation in B' only	% inhibition by 10^{-7}M-somatostatin
Histamine(0.5mM)+IBMX (0.1mM)+DTT(0.5mM)	34.5 ± 1.3 (4)		
			25.0
Histamine(0.5mM)+IBMX (0.1mM)+DTT(0.5mM)+ Somatostatin(10^{-7}M)	25.9 ± 1.0 (4)		
Histamine(0.5mM)+IBMX (0.1mM)		26.1 ± 0.9 (4)	
			27.1
Histamine(0.5mM)+IBMX (0.1mM)+ Somatostatin (10^{-7}M)		19.0 ± 0.6 (4)	

Incubation conditions	APR 1.5 hours preincubation in B', then 0.5 hours with DTT(0.5mM)	APR 2 hours preincubation in B' only	% inhibition by 10^{-7}M-somatostatin
Histamine(0.5mM)+IBMX (0.1mM)+DTT(0.5mM)		26.0 ± 1.9 (4)	17.6
Histamine(0.5mM)+IBMX (0.1mM)+DTT(0.5mM)+ Somatostatin(10^{-7}M)		21.4 ± 0.4 (4)	
Histamine(0.5mM)+IBMX (0.1mM)+leupeptin(0.5µg/ml)+ pepstatin(0.7µg/ml)		26.8(26.5,27.2)	15.8
Histamine(0.5mM)+IBMX (0.1mM)+leupeptin(0.5µg/ml)+ pepstatin(0.7µg/ml)+ somatostatin(10^{-7}M)		22.6(25.2,19.9)	

Table 3.11 continued.
Cells were either preincubated in medium B' for 2 hours, or preincubated in medium B' for 1.5 hours then 0.5 hours in medium B' plus DTT. The aminopyrine accumulation ratios (APR) are presented as either means ± S.E.M. with the number of cell samples in parentheses, or as means with both values in parentheses for a single batch of cells containing 20 % parietal cells. Inhibition of aminopyrine accumulation is expressed as a percentage of the mean value in the absence of somatostatin.

(Table 3.12 and Fig. 3.9). The half-maximal inhibitory concentration was approximately 20nM which is similar to 32nM as found by Chew (1983b) in rabbit isolated glands in the presence of DTT. Of the concentrations used maximal inhibition of aminopyrine accumulation by somatostatin occurred at 10^{-6}M and resulted in 28.1 ± 5.2% inhibition. This was very similar to the level of inhibition found by Park *et al.* (1987) of just over 30% but slightly lower than that of 39 or 44% as found by Schepp *et al.* (1983a and b). A decrease in the inhibitory action of somatostatin was seen at the highest concentration. Similar results showing a decrease in the effect of somatostatin at high concentrations have been observed (Nylander *et al.*, 1985; Park *et al.*, 1987). Somatostatin was shown to have no effect on basal secretion in rabbit enriched parietal cells (Chew, 1983b) and isolated rat parietal cells (Schepp *et al.*, 1983a). Preincubation with pertussis toxin for two hours significantly inhibited the action of somatostatin at 10^{-7} ($P<0.05$) and 10^{-6}M ($P<0.01$). Pertussis toxin decreased the inhibitory action of 10^{-6}M-somatostatin by 72.5%.

This action of pertussis toxin has been confirmed by Park *et al.* (1987) using a single concentration of histamine (10^{-4}M) and somatostatin (10^{-7}M). Pertussis toxin sensitive G-proteins are known to be involved with inhibition of cyclic AMP and protein kinase C-dependent mechanisms by somatostatin in pituitary cells (Koch *et al.*, 1985; Yajima *et al.*, 1986). The abolition of the histamine-stimulated aminopyrine accumulation by somatostatin in rat parietal cells preincubated with pertussis toxin was virtually complete. However, the result does not preclude the possibility of a second site of action of somatostatin downstream from adenylate cyclase, which is pertussis toxin insensitive, such as seems to exist in canine parietal cells (Park *et al.*, 1987). It is concluded that somatostatin acts on parietal cells to inhibit aminopyrine accumulation stimulated by histamine, at least in part, by inhibiting adenylate cyclase activity via a pertussis toxin sensitive G-protein.

Pertussis toxin pretreatment of isolated luminally perfused mouse stomach caused a two-fold increase in histamine-stimulated acid secretion and blocked the inhibitory effect of somatostatin on basal and histamine-stimulated acid secretion. Pentagastrin stimulation was not affected by pertussis toxin. Secretagogue stimulated acid secretion was accompanied by a parallel increase in somatostatin secretion which was determined by luminal acidity. These results obtained with a more intact preparation suggest that gastric somatostatin is a true paracrine regulator of acid secretion (Schubert *et al.*, 1989), and that a G_i-like protein is involved in its action.

3.3.5 The effect of incubation with IBMX on the inhibitory actions of PGE_2 and EGF.

Table 3.12

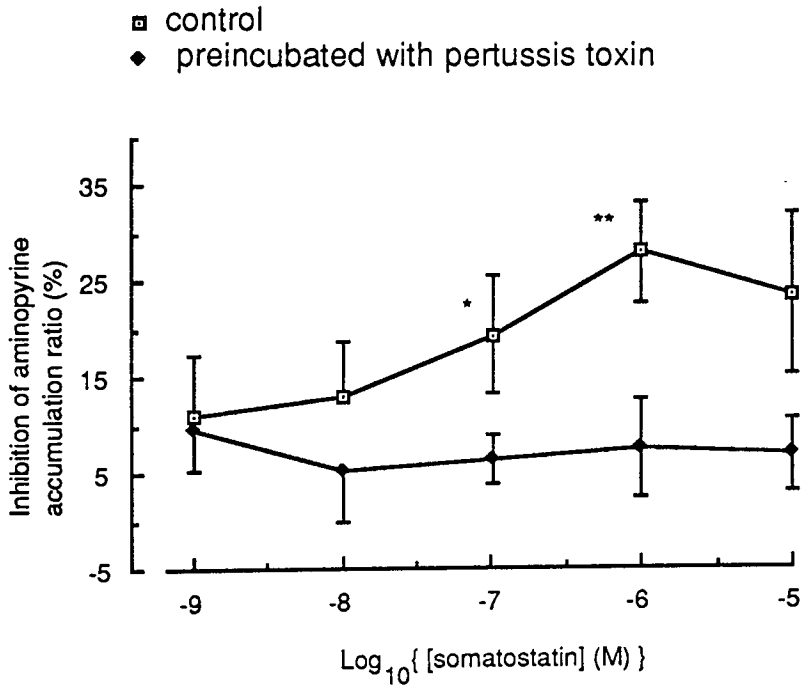
The effect of preincubation with pertussis toxin (100ng/ml) for 2 hours on the inhibitory action of somatostatin on aminopyrine accumulation stimulated by histamine (0.5mM) and IBMX (0.1mM).

Concentration of somatostatin (M)	APR Control	APR Pertussis toxin
0	70.0 ± 25.8	77.4 ± 25.6
10^{-9}	68.7 ± 27.6	71.8 ± 25.1
10^{-8}	65.6 ± 26.4	78.1 ± 28.4
10^{-7}	62.2 ± 24.9	74.1 ± 25.0
10^{-6}	54.9 ± 22.4	76.1 ± 28.1
10^{-5}	38.6 ± 12.6	50.8 ± 14.3

Results from 4 to 5 experiments containing 19.8 ± 0.6% parietal cells are expressed as means ± S.E.M.

Figure 3.9

Effect of preincubation with pertussis toxin (100ng/ml) for 2 hours on the inhibitory action of somatostatin on the aminopyrine accumulation ratio stimulated by histamine (0.5mM) and IBMX(0.1mM).



Data from table 3.13 have been normalised and expressed as % inhibition by somatostatin of the aminopyrine accumulation ratio obtained with histamine and IBMX alone. The effect of preincubation with pertussis toxin has been analysed by a paired *t*-test. ** $P<0.01$; * $P<0.05$.

The dose-response curve for the inhibitory action of PGE_2 on aminopyrine accumulation stimulated by histamine was shifted to the right when 0.1mM-IBMX was present (Fig. 3.10). However, the effect of IBMX on EGF inhibition was different, totally abolishing the inhibitory action of EGF even at near maximally effective concentrations of EGF (Fig. 3.11, data from G.P.Shaw). This effect could not be overcome by raising the concentration of EGF. IBMX also prevented the inhibitory effects of EGF but not PGE_2 on cyclic AMP content of rat isolated parietal cells (Hatt and Hanson, 1988).

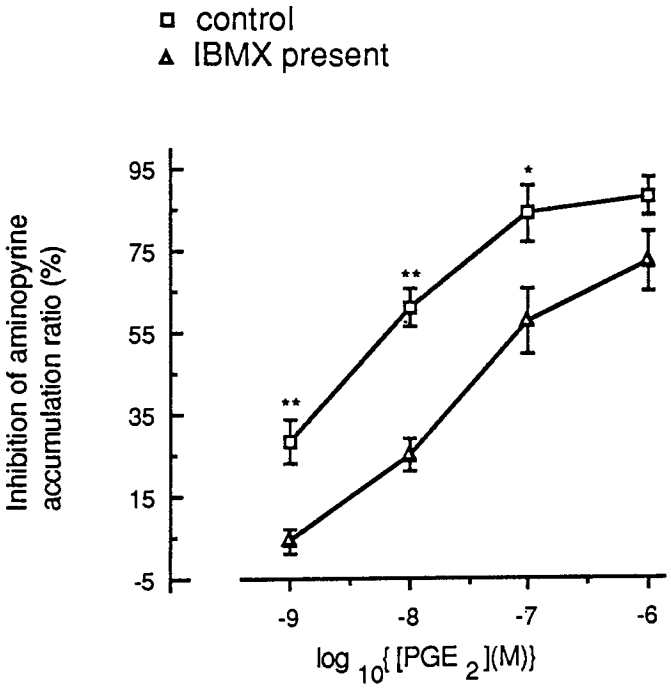
3.3.6 The effect of preincubation with pertussis toxin on the inhibitory action of EGF.

As IBMX blocked the action of EGF these experiments were performed with cells stimulated by histamine alone. Aminopyrine accumulation stimulated by a near maximally effective concentration of histamine (0.5mM) was inhibited by EGF (Table 3.13 and Fig. 3.12). The half-maximal inhibitory concentration of EGF was approximately 4nM, in the absence of pertussis toxin. This is similar to the value found by Hatt and Hanson (1988) of 3nM and that found by Dembinski *et al.* (1986), using a histamine (10^{-4}M) stimulated rabbit gland preparation, of about 5nM. Without the 2 hour preincubation used here the half-maximally effective concentration was 19nM (Shaw *et al.*, 1987). The 2 hour preincubation period may have permitted damaged receptors to be replaced. To further facilitate the recovery of cells from possible receptor damage they were preincubated in the presence of insulin, hydrocortisone and foetal calf serum (Table 3.14). These additions were used by Soll *et al.* (1986) to improve the secretory response of canine parietal cells to secretagogues. However, no increase in the percentage inhibition obtained with a maximally effective concentration of EGF was observed. So subsequent experiments were performed in the absence of insulin, hydrocortisone and foetal calf serum. EGF inhibited the histamine-stimulated aminopyrine accumulation ratio by 49% at 200nM (Table 3.13 and Fig. 3.12) which was slightly higher than that found by Shaw *et al.* (1987) of 38% and similar to that found by Hatt and Hanson (1988) of about 43%. EGF has been found to have no effect on basal rat parietal cell aminopyrine accumulation (Shaw *et al.*, 1987; Hatt and Hanson, 1988). Preincubation with pertussis toxin significantly reduced the action of EGF at 20 and 200nM-EGF. Pertussis toxin reduced the inhibitory action of 200nM-EGF by 70%.

One problem of interpreting the effects of pertussis toxin on the action of EGF was that pertussis toxin enhanced the level of the aminopyrine accumulation ratio stimulated by histamine (Table 3.7 and Table 3.13). Consequently it was important to determine whether the level of the aminopyrine accumulation ratio stimulated by histamine (x) affected the percentage inhibition by EGF (y) of this value. Data for 20

Figure 3.10

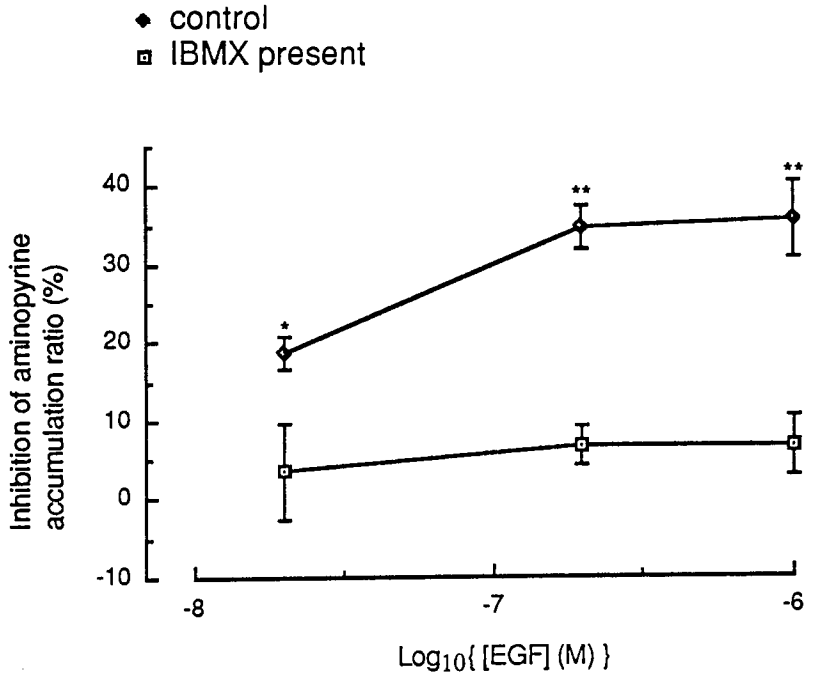
The effect of IBMX (0.1mM) on the inhibitory action of PGE_2 on the aminopyrine accumulation ratio stimulated by histamine (0.5mM).



Data from table 3.10 and 3.16 have been normalised and expressed as percentage inhibition by PGE_2 of the aminopyrine accumulation ratio obtained with histamine without (□) and with (▲) 0.1mM-IBMX. The effect of IBMX has been analysed by an unpaired *t*-test. ** $P<0.01$; * $P<0.05$.

Figure 3.11

The effect of IBMX (0.1mM) on the inhibitory action of EGF on the aminopyrine accumulation ratio stimulated by histamine (0.5mM).



Results, from 4 to 5 separate batches of cells, are from work by G.P.Shaw. Data have ben normalised to give percentage inhibition by EGF of the aminopyrine accumulation ratio and are expressed as means ± S.E.M. The effect of IBMX has been analysed by an unpaired *t*-test . ** $P<0.01$;* $P<0.05$. Stimulation of the aminopyrine accumulation ratios above basal in the absence of EGF were 123 ± 18 and 4.6 ± 0.99 in the presence and absence of IBMX, respectively.

Table 3.13

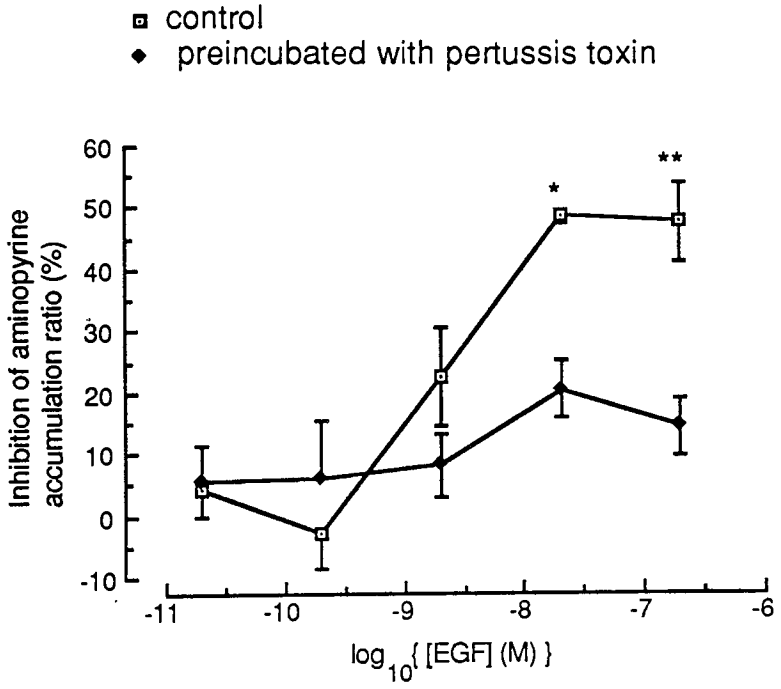
The effect of preincubation for 2 hours with pertussis toxin (100ng/ml) on the inhibitory action of EGF on aminopyrine accumulation stimulated by histamine (0.5mM).

Concentration of EGF (nM)	APR Control	APR Pertussis toxin
0	9.7 ± 3.3	26.8 ± 8.1*
0.02	9.0 ± 0.4	25.0 ± 3.7
0.2	9.7 ± 0.4	24.6 ± 3.4
2.0	5.6 ± 1.4	18.5 ± 5.5
20.0	4.8 ± 0.4	21.0 ± 2.9
200.0	3.6 ± 0.8	17.3 ± 5.4

Results from 4 experiments using cell batches containing 19.3 ± 0.5% parietal cells are expressed as means ± S.E.M. Stimulation of aminopyrine accumulation ratio above control by histamine in the presence of pertussis toxin has been assessed by a paired *t*-test. * $P>0.025$.

Figure 3.12

Effect of preincubation with pertussis toxin (100ng/ml) for 2 hours on the inhibitory action of EGF on the aminopyrine accumulation ratio stimulated by histamine (0.5mM) ~~and IBMX(0.1mM).~~



Data from table 3.13 have been normalised to percentage inhibition by EGF of the aminopyrine accumulation ratio obtained with histamine alone. The effect of preincubation with pertussis toxin has been analysed by a paired *t*-test. * $P<0.01$; ** $P<0.05$.

Table 3.14

The effect of preincubation medium on the secretory response of rat isolated parietal cells in response to histamine (0.5mM) in the absence and presence of EGF (200nM).

Incubation medium	APR Histamine (0.5mM)	APR Histamine (0.5mM) + EGF (200nM)	% inhibition by EGF (200nM)
B'	13.3 ± 0.3(4)	9.1 ± 0.3(4)	31.6
B' + additions*	9.5 ± 0.1(4)	6.6 ± 0.2(4)	30.5

Aminopyrine accumulation ratios are presented as means ± S.E.M., with the number of cell samples in parentheses, from a single batch of cells containing 20% parietal cells.

* additions = Insulin (8µg/ml), hydrocortisone (10nM) and foetal calf serum (5% $^{v}/_{v}$).

and 200nM-EGF from 9 and 10 experiments respectively, were analysed by linear regression and showed no such relationship (20nM-EGF, y = 55 - 0.71x; 200nM-EGF, y = 55 + 0.03x, slope not different from zero in both cases). It was therefore unlikely that the pertussis toxin induced rise was responsible for its prevention of the inhibitory action of EGF as the inhibitory effect of EGF was unrelated to the level of aminopyrine accumulation stimulated by histamine.

A G-protein appears to mediate the inhibitory action of EGF in rat parietal cells. Pertussis toxin has been found to block the action of EGF on phosphoinositide metabolism in other cell types. However, as in many cases regarding the signal transduction pathway involving phospholipase C, pertussis toxin has an inconsistent effect. Thus, the EGF-stimulated rise in intracellular inositol trisphosphate and free calcium was blocked by pertussis toxin in rat hepatocytes (Johnson and Garrison, 1987) but not A231 cells (Tilly *et al.*, 1988). Pertussis toxin also has an inconsistent effects on the mitogenic properties of EGF, blocking the growth promoting effects of EGF on a human breast cancer cell line (Church and Buick, 1988) but not on chinese hamster lung fibroblasts (Chambard *et al.*,1987).

3.3.7 Possible interactions between PGE_2 and EGF.

The fact that IBMX blocks the action of EGF but not PGE_2 (this work; Shaw *et al.*, 1987) suggest that these two agents might operate via different mechanisms. The possibility that these two inhibitors could interact synergistically was studied by determining the inhibitory effect of PGE_2 in the presence and absence of a fixed concentration of EGF (Table 3.15 and Fig. 3.13), and the effect of EGF in the presence snd absence of a fixed concentration of PGE_2 (Table 3.16 and Fig. 3.14). Cells stimulated with histamine (0.5mM) showed no significant effect of EGF on the inhibitory action of PGE_2, or of PGE_2 on the inhibitory action of EGF. Therefore there is no evidence for any synergistic interaction.

3.3.8 EGF stimulated PGE_2 secretion.

3.3.8.1 Expression of PGE_2 release.

The release of PGE_2 from enriched parietal cells was calculated by subtracting the basal level of PGE_2 at time 0 from that obtained after 30 minutes. The basal release over 30 minutes varied over the range of 3.47 to 13.49pg/10^6cells with a mean value of 7.37 and a S.E.M. of 1.78 (data from 5 batches of cells with 81.6 ± 0.51% parietal cells). This large variation (coefficient of variation 53.97) in basal prostaglandin release by rat isolated parietal cells had been found previously (Hatt and Hanson, 1988). Mechanical disruption and the proportion of cells which have been ruptured may be responsible for part of the variation (Ahlquist *et al.*, 1983). However, Hatt and

Table 3.15

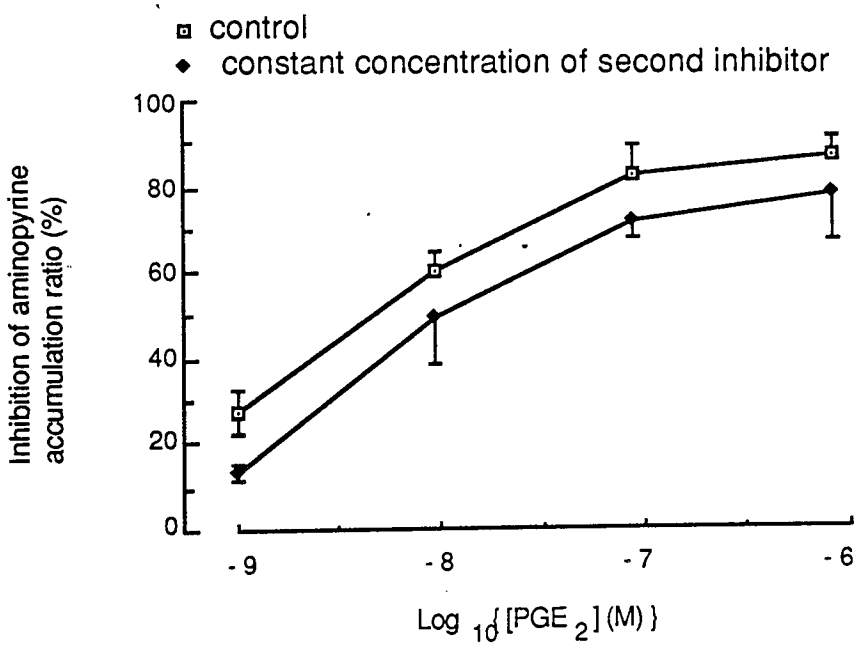
The effect on the inhibitory action of PGE_2 on histamine (0.5mM) stimulated aminopyrine accumulation in the presence and absence of a constant concentration of EGF (1nM).

Concentration of PGE_2 (M)	APR Control	APR EGF (1nM)
0	4.6 ± 2.0	3.7 ± 2.0*
10^{-9}	3.6 ± 1.8	3.2 ± 1.7
10^{-8}	2.0 ± 1.2	2.0 ± 1.3
10^{-7}	0.9 ± 0.6	1.0 ± 0.6
10^{-6}	0.6 ± 0.4	0.6 ± 0.3

Results from 4 batches of cells containing 20.3 ± 1.0% parietal cells are expressed as means ± S.E.M. Stimulation of the aminopyrine accumulation ratio above control by histamine in the presence and absence of EGF has been analysed by a paired *t*-test. * $P<0.01$. There was no effect of EGF (1nM) on basal secretion (aminopyrine accumulation ratio 1.6 ± 0.2 and 1.6 ± 0.1 for basal and EGF (1nM) respectively).

Figure 3.13

Effect of a constant concentration of EGF (1nM) on the inhibitory action of PGE_2 on the aminopyrine accumulation ratio stimulated by histamine.



Data from table 3.15 have been normalised and expressed as percentage inhibition by PGE_2 of the aminopyrine accumulation ratio obtained with histamine without (▫) and with (◆) 1nM-EGF. There was no difference between the inhibitory effect of PGE_2 in the presence and absence of EGF (paired *t*-test).

Table 3.16

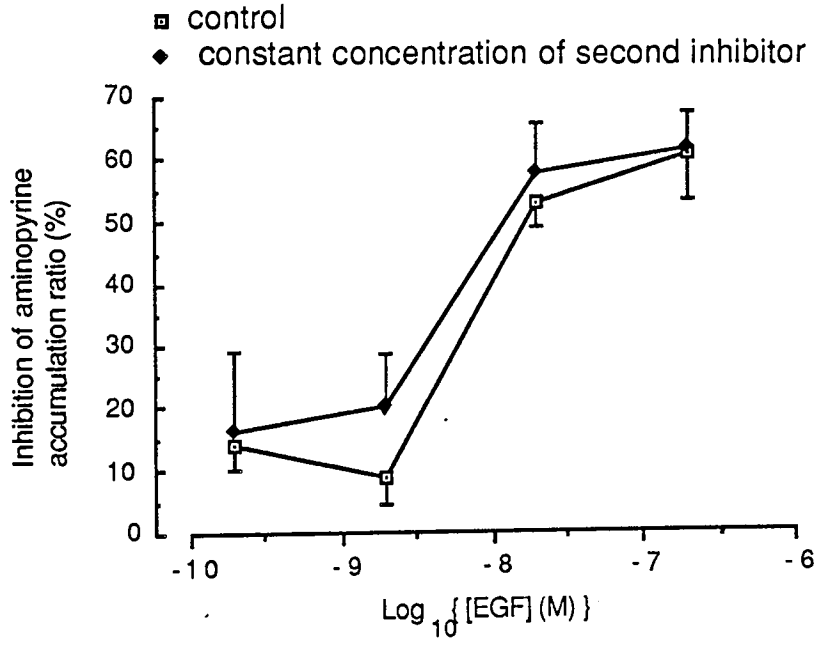
The effect on the inhibitory action of EGF on histamine (0.5mM) stimulated aminopyrine accumulation in the presence and absence of a constant concentration of PGE_2 (2nM).

Concentration of EGF (nM)	APR Control	APR PGE_2 (2nM)
0	3.5 ± 0.8	2.0 ± 0.4*
0.2	2.9 ± 1.0	1.8 ± 0.8
2.0	2.9 ± 0.9	1.6 ± 0.6
20.0	1.6 ± 0.5	0.8 ± 0.2
200.0	1.3 ± 0.5	0.7 ± 0.2

Results from 5 batches of cells containing 20.4 ± 0.6% parietal cells are expressed as means ± S.E.M. Stimulation of the aminopyrine accumulation ratio above control by histamine in the presence and absence of PGE_2 has been analysed by a paired *t*-test. * $P<0.05$. There was no effect of PGE_2 (2nM) on basal secretion (aminopyrine accumulation ratio 2.0 ± 0.5 and 2.0 ± 0.4 for basal and PGE_2 (2nM) respectively).

Figure 3.14

Effect of a constant concentration of PGE_2 (2nM) on the inhibitory action of EGF on the aminopyrine accumulation ratio stimulated by histamine.



Data from table 3.16 have been normalised and expressed as percentage inhibition by EGF of the aminopyrine accumulation ratio obtained with histamine without (▫) and with (◆) 2nM-PGE_2. There was no difference between the inhibitory effect of EGF in the presence and absence of PGE_2 (paired *t*-test).

Hanson (unpublished data) showed that there was no correlation between basal PGE_2 production and the percentage of cells able to exclude trypan blue.

3.3.8.2 Effect of EGF on PGE_2 release.

EGF significantly stimulated PGE_2 production ($P<0.001$, two-way ANOVAR) in enriched parietal cells over a 30 minute incubation period (Table 3.17). The stimulation of PGE_2 production above basal was calculated, by subtracting basal PGE_2 release (level of PGE_2 after 30 minutes incubation with no EGF present) (Fig. 3.15). The level of stimulation of prostaglandin release was similar to that found by Hatt and Hanson (1988). Chiba *et al.* (1982) also found that EGF stimulated prostaglandin production in an isolated stomach preparation.

Preincubation with pertussis toxin for 2 hours significantly inhibited the stimulatory action of EGF at 2 and 20nM, $P<0.02$ and $P<0.05$ respectively. Pertussis toxin decreased the stimulatory action of EGF (200nM) by 76%. Therefore it appears that EGF stimulates PGE_2 production via the activation of a pertussis toxin sensitive G-protein.

Increased prostaglandin production probably results from an increased supply of arachidonic acid. This could arise from the activation of phospholipase C or phospholipase A_2 (see section 3.1.1.1).

As mentioned previously, carbachol activates phospholipase C and phosphatidlyinositol 4,5-bisphosphate turnover and stimulates PGE_2 production. It is unlikely that EGF stimulated prostaglandin production by increased phosphatidylinositol 4,5-bisphosphate turnover via raised phospholipase C activity. This is because EGF like carbachol would then be expected to stimulate acid secretion.

It is possible that EGF stimulates the turnover of another phospholipid via phospholipase C, thus generating diacylglycerol which may be converted to prostaglandins without inositol 1,4,5-trisphosphate production and Ca^{2+} mobilisation (Pelech and Vance, 1989). However this is unlikely as pertussis toxin is typically without effect on "G_p" in non blood-derived cell types. Thus it seems more likely that EGF stimulates phospholipase A_2 activity via a pertussis toxin sensitive G-protein. As mentioned previously (section 3.1.1.1), phospholipase A_2 has been shown to be linked to a pertussis toxin sensitive G-protein in rat mesangial cells (Wang *et al.*, 1988).

Table 3.17

The effect of preincubation with pertussis toxin (100ng/ml) for 2 hours on the stimulatory action of EGF on PGE_2 release.

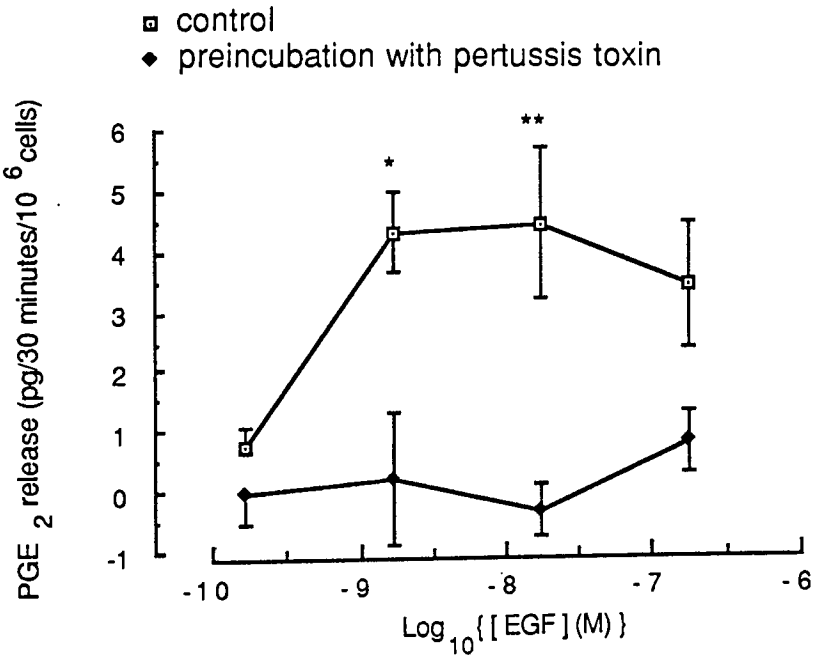
Concentration of EGF (1nM)	PGE_2 release* Control	PGE_2 release* Pertussis toxin
0	16.04 ± 3.71	12.88 ± 2.56
0.2	16.39 ± 3.39	13.06 ± 2.97
2.0	19.95 ± 3.88	13.27 ± 3.04
20.0	20.09 ± 4.44	12.76 ± 2.47
200.0	19.66 ± 3.79	14.56 ± 3.00

Result from 5 batches of cells containing 82.4 ± 0.5% parietal cells are stimulated presented as means ± S.E.M. PGE_2 production was significantly ~~inhibited~~ by EGF ($P<0.001$, two-way ANOVAR). See Appendix A.5.1 for statistical analysis.

*PGE_2 release = pg/10^6cells/30 minutes.

Figure 3.15

The effect of preincubation with pertussis toxin (100ng/ml) for 2 hours on the stimulatory action of EGF on PGE_2 release.



Data from table 3.17 have been recalculated as PGE_2 release above basal and are presented as means ± S.E.M. The effect of pertussis toxin has been analysed by a paired t-test. * $P<0.02$; ** $P<0.05$. Basal release over 30 minutes was 7.37 ± 1.78 and 6.92 ± 1.40 pg/10^6cells/30 minutes for control and pertussis toxin respectively. The action of pertussis toxin on basal release was not significant (paired t-test).

3.4 GENERAL DISCUSSION.

All the inhibitors of histamine (0.5mM) plus IBMX (0.1mM) stimulated rat parietal cells tested are blocked by pertussis toxin. This may bring into question the specificity of pertussis toxin action. However, the stimulation of the aminopyrine accumulation ratio by histamine and IBMX remained unaffected by pertussis toxin, as did the histamine plus IBMX stimulated cyclic AMP concentration. Basal aminopyrine accumulation and basal cyclic AMP concentration are also not affected by pertussis toxin. As will become evident in chapter 5, pertussis toxin has only a small effect on the inhibitory action of 12-*O*-tetradecanoylphorbol 13-acetate (a potent activator of protein kinase C). Thus pertussis toxin does not prevent the action of all inhibitors of secretory activity and their is reason to believe that the effects found here are specific ones.

There are many members of the "G_i-like" family of G-proteins which are substrates for pertussis toxin as mentioned in section 3.1.5.2. Thus it is possible that pertussis toxin is acting on different members of this family to mediate its effects on the inhibitors tested. EGF action is blocked by IBMX but PGE_2 action is not. This suggests that the action of EGF differs from PGE_2 and also that PGE_2 could not mediate the action of EGF. If EGF activates the same G-protein as PGE_2 in the same way then it should work in the presence of IBMX. As this does not happen, EGF either activates a different G-protein or activates the same G-protein in a different way. If the latter is the case then the lack of synergism suggests no interaction between the two sites on the G-protein.

The present evidence suggests the target for PGE_2 is adenylate cyclase and for EGF cyclic AMP phosphodiesterase. The latter suggestion is based on the observation that EGF inhibits the histamine-stimulated cyclic AMP content of rat parietal cells but does not inhibit adenylate cyclase (Hanson and Hatt, 1989), and that the phosphodiesterase inhibitor, IBMX, blocks the action of EGF. PGE_2 appears to inhibit adenylate cyclase via a $G_{i\alpha}$. EGF could activate a cyclic AMP phosphodiesterase via the receptor-mediated phosphorylation of a member of the G_i family on a tyrosine residue. The possible mechanism by which EGF activates a cyclic AMP phosphodiesterase may be similar to the mechanism by which the insulin receptor stimulates a cyclic AMP phosphodiesterase (Elks *et al.*, 1983). It remains unclear whether the same G-protein could activate phospholipase A_2 and inhibit cyclic AMP phosphodiesterase.

3.5 SUMMARY.

1. PGE_2 inhibited secretory activity stimulated by histamine plus IBMX in parietal cell preparations with a half-maximally effective concentration of 24nM. Preincubation with pertussis toxin reduced the inhibitory action of a near-maximally effective concentration of PGE_2 on histamine-stimulated aminopyrine accumulation by 83%. If this result is put alongside the finding of others it seems likely that PGE_2 mediates its inhibitory action via a "G_i-like" protein.

2. Somatostatin inhibited secretory activity of isolated parietal cell preparations stimulated by histamine plus IBMX with a half-maximally effective concentration of approximately 20nM. The inhibitory action of a near-maximally effective concentration of somatostatin on histamine plus IBMX stimulated secretory activity was reduced by 72% by preincubation with pertussis toxin. Therefore somatostatin appears to mediate its action in rat parietal cells, at least in part, by the activation of an inhibitory "G_i-like" protein.

3. EGF inhibited aminopyrine accumulation stimulated by histamine, but not by histamine plus IBMX, in rat parietal cells with a half-maximally effective concentration of about 4nM. Preincubation with pertussis toxin decreased the inhibitory action of a near-maximally effective concentration of EGF by 70%. Consequently EGF seems to mediate its effect on parietal cells by the activation of an inhibitory G-protein. EGF may activate the same G-protein as PGE_2 and/or somatostatin but in a different way, or operate through a different G-protein.

4. EGF stimulated PGE_2 production by parietal cells or cells that co-purified with parietal cells on a Percoll gradient. It is possible that the effect of EGF on prostaglandin production may be via a G-protein mediated activation of phospholipase A_2.

Chapter 4

EFFECT OF EPIDERMAL GROWTH FACTOR ON ACID AND PEPSINOGEN SECRETION BY RAT ISOLATED ~~PARIETAL~~ CELLS STIMULATED WITH FORSKOLIN.

4.1 INTRODUCTION.

Epidermal growth factor (EGF) inhibits gastric acid secretion both *in vivo* (Konturek *et al.*, 1984) and *in vitro* (Shaw *et al.*, 1987). EGF has also been observed to inhibit pepsinogen secretion *in vivo* (Konturek *et al.*, 1981b). EGF has been reported to inhibit pepsinogen secretion *in vitro* in only one brief report (Abstract) on rabbit fundic mucosa fragments (Miyamoto *et al.*, 1987). The exact mechanism by which EGF inhibits acid secretion is not known but it is thought to involve a decrease in cyclic AMP content (Hatt and Hanson, 1988). It is the intention of this work to investigate further the mechanism by which EGF inhibits acid secretion, and to assess for the first time whether EGF could inhibit pepsinogen secretion by rat chief cells *in vitro*. To do this the action of EGF on parietal and chief cells stimulated by forskolin was tested. An advantage of using forskolin was that it provided a common post-receptor stimulus to both parietal and chief cells (see section 4.1.3). Furthermore if an agent inhibits secretory activity stimulated by forskolin this suggests that a site of action exists downstream in the secretory pathway from membrane receptors linked to adenylate cyclase.

Forskolin was originally isolated from the Indian plant *Coleus forskohlii*. It is a diterpene (Fig. 4.1) which activates adenylate cyclase in cellular homogenates, increases the intracellular cyclic AMP content of intact cells and therefore activates cyclic AMP-dependent protein kinase in fundic mucosal glands (Seamon and Daly, 1981a and b; Chew, 1983a). The activation of forskolin is rapid, and reversible and due to the direct activation of the catalytic moiety (Seamon and Daly, 1981a and b; Seamon and Daly, 1986). Increases in cyclic AMP stimulated by forskolin can be inhibited by hormones acting via G_i. In fact the presence and state of G_s also affects the degree of stimulation by forskolin (Seamon and Daly, 1986). Forskolin is believed to have no direct action on, for example, guanylate cyclase, cyclic nucleotide phosphodiesterase, cyclic AMP-dependent protein kinase or Na^+/K^+-ATPase (Seamon *et al.*, 1981a).

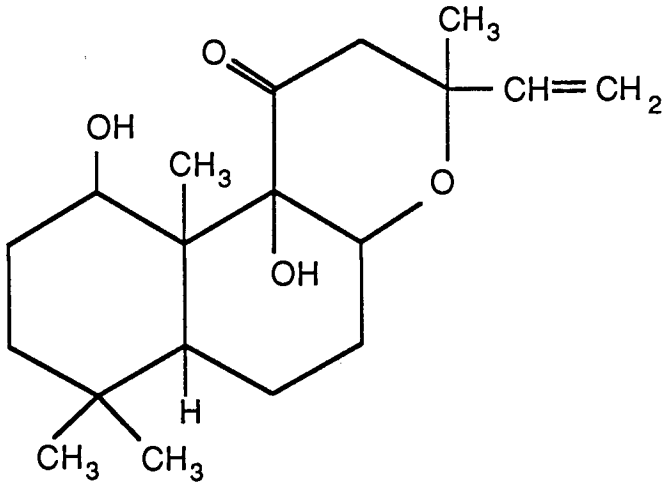
Forskolin has activated adenylate cyclase and increased intracellular cyclic AMP, pepsinogen and acid secretion in rabbit gastric glands (Hersey *et al.*, 1983; Chew, 1983).

4.1.2 EGF and acid secretion.

EGF and its actions have been reviewed in section 3.1.3. Histamine-stimulated acid secretion is inhibited by EGF which may mediate its action by reducing cyclic AMP levels through the activation of a cyclic AMP phosphodiesterase (Hatt and Hanson, 1988). Therefore EGF would be expected to inhibit parietal cells stimulated with forskolin. Although Reichstein *et al.* (1984) observed that EGF inhibited

Figure 4.1

The structure of forskolin.



forskolin-stimulation in rabbit glands, another communication on dog parietal cells (Chen *et al.*, 1984) and a full paper on rabbit parietal cells (Lewis *et al.*, 1990) have suggested that EGF has no effect against forskolin-stimulation. The aim of this work was to examine the situation in rat parietal cells.

4.1.3 Pepsinogen secretion.

Stimulation of pepsinogen secretion is discuss in section 1.3.2. It appears that histamine does not have a direct stimulatory action on pepsinogen secretion. Consequently a different stimulator is required to compare the inhibitory effects, if any, of EGF on both acid and pepsinogen secretion.

Forskolin stimulates pepsinogen secretion in rabbit gastric glands (Chew, 1983; Hersey *et al.*, 1983) and in cultured enriched chief cell preparations (Sanders *et al.*, 1983). Chief cell activity stimulated by forskolin was not affected by atropine, propanolol and dbcGMP in rabbit gastric glands (Hersey *et al.*, 1983). More recently forskolin-stimulated pepsinogen secretion was found to be inhibited by a somatostatin analogue in a cultured chief cell preparation (Chen *et al.*, 1988b). EGF also inhibited pepsinogen secretion *in vivo* (Konturek *et al.*, 1981b) but the mechanism of inhibition may not be a direct effect, as is the case with the study on rabbit fundic fragments (Miyamoto *et al.*, 1987). The aim of this work was to determine if the action of EGF on forskolin-stimulated chief cell activity, if any, was analogous to that on parietal cell activity.

4.2 METHODOLOGY FOR THE DETERMINATION OF PEPSINOGEN RELEASE FROM CHIEF CELLS.

Pepsinogen release has often been determined by the spectrophotometric assay of the cleavage of acid-denatured haemoglobin by pepsin (Anson and Mirsky, 1932; Koelz *et al.*, 1982). However, more recent assays utilising radiochemical detection of proteolysis have been developed which have increased sensitivity (Kasbekar *et al.*, 1983; Raufman *et al.*, 1984; Raufman *et al.*, 1986). A radiochemical and a specrophotometric assay of pepsinogen were compared to establish the most suitable assay for the measurement of secretion of pepsinogen from rat chief cells.

4.2.1 Pepsinogen assay using ^{125}I-BSA as substrate.

4.2.1.1 The manufacture of ^{125}I-BSA.

Two gamma-emitting iodine radioisotopes are available, ^{125}I and ^{131}I. ^{125}I can be obtained at an isotopic abundance approaching 100% whereas ^{131}I is available at approximately 20%. Consequently ^{125}I produces products with a higher specific activity and is therefore preferentially used. ^{125}I also has the advantages of a longer half-life, 60 days compared to 8 days, and a higher counting efficiency in most gamma counters. It is important not to over-iodinate the BSA as the probability of damage due to irradiation will be increased. Raufman *et al.* (1986) manufactured ^{125}I-BSA with a specific activity of 124μCi/μg which was reported to be stable for at least 4 months.

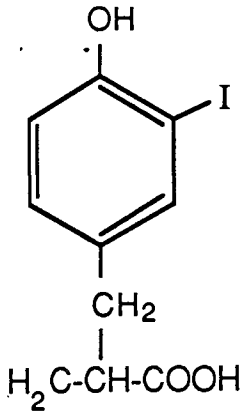
Radioiodine is readily substituted into tyrosine residues of proteins. A smaller proportion of the iodine may also react with histidine (Covelli and Wolfe, 1966), tryptophan (Koshland *et al.*, 1963) and sulphydryl groups (Jovanovic *et al.*, 1972).

In common with Raufman *et al.* (1986) the production of ^{125}I-BSA was performed according to the chloramine-T method of Hunter and Greenwood (1962). Chloramine-T breaks down in aqueous solution to form hypochlorous acid which is a mild oxidising agent. In the presence of chloramine-T and under mildly alkaline conditions at pH 7.5, Na^{125}I is oxidised to form cationic iodine ($^{125+}$I). At this pH a small proportion of tyrosine molecules will be ionised as the pKa of the phenolic side chain of tyrosine is greater than 10. It is through these ionised groups that the reaction probably proceeds, iodinating the phenolic ring in the ortho position (Fig. 4.2). The amount of chloramine-T required for optimum iodination was independent of the iodine concentration (Greenwood and Hunter, 1963). Addition of sodium metabisulphite reduces surplus chloramine-T and free iodine to iodide. The amount of sodium metabisulphite is limited to approximately twice that of chloramine-T.

4.2.1.2 Reagents required for iodination.

Figure 4.2

Diagram showing ortho-monoiodotyrosine.



1. Phosphate buffer (0.5M) pH 7.4: For 250ml of solution 10.8954g of Na_2HPO_4 and 7.5275g of NaH_2PO_4 were weighed out and dissolved in about 220ml of double-distilled water at room temperature. The pH was checked and altered to exactly 7.4, by adding either acid (HCl) or base (NaOH), if necessary. The solution was then made up to 250ml using a volumetric flask.

2. Phosphate buffer (0.05M) pH 7.4: For 250ml of solution 1.0895g of Na_2HPO_4 and 0.7527g of NaH_2PO_4 were weighed out and treated as above for 0.5M-phosphate buffer. Alternatively a volume of 0.5M-phosphate buffer was diluted 1:10.

3. BSA (0.5mg/ml): 5mg of BSA was dissolved in 10ml of 0.05M phosphate buffer and adjusted to pH 7.4. This was prepared freshly on the day of iodination.

4. Chloramine-T and sodium metabisulphite: 5mg of chloramine-T and 12mg of sodium metabisulphite were weighed out into separate polypropylene LP3 tubes (Luckham) which were then wrapped in foil to prevent the deleterious effects of light (DeMeyts, 1976). The chorlamine-T was dissolved in 2ml of 0.05M-phosphate buffer, pH 7.4, to provide a concentration of 2.5mg/ml. The sodium metabisulphite was dissolved in 2ml of 0.05M-phosphate buffer, pH7.4, to provide a concentration of 6mg/ml.

5. Tricarboxylic acid (TCA) (10%($^w/_v$)): 10g of TCA was made up to 100ml with double-distilled water in a volumetric flask.

6. 10µl of $Na^{125}I$ was supplied by Amersham in a microvial.

4.2.1.3 Radioiodination of BSA.

Iodination using $Na^{125}I$ was carried out one or two days after batch synthesis. The iodination took place in the conical glass vial supplied by Amersham. Automatic pipettes (Gilson) with disposable tips were used to add solutions and therefore uncapping and capping of the reaction vessel was required several times during the iodination procedure. The reaction was conducted in a fume cupboard behind lead shielding.The microvial was briefly centrifuged to ensure the $Na^{125}I$ solution was at the bottom of the vial. Then the following additions were made to the reaction vial.

90µl of phosphate buffer (0.5M).
10µl of BSA (0.5mg/ml) in phosphate buffer (0.05M) (5µg BSA).
20µl of chloramine-T (2.5mg/ml) in phosphate buffer (0.05M) (50µg chloramine-T).

The contents were mixed gently by inverting twice. 30 seconds after the addition of chloramine-T, 20µl of sodium metabisulphite (6mg/ml) (120µg) in

phosphate buffer (0.05M) was introduced into the reaction vial which was subsequently inverted twice.

A Centricon-30 microconcentrator (cut-off 30,000) was prepared by centrifugation, for 15 minutes using a MSE MISTRAL 4L at 2000rpm, of 2ml of phosphate buffer (0.05M) containing BSA (0.5mg/ml). The concentrated BSA solution was removed from the membrane filter by inversion and centrifugation.

The solution in the reaction vial was transferred into the prepared Centricon-30. 150µl of BSA solution was used to rinse out the reaction vial and then added to the Centricon-30 and the solution gently mixed using a Gilson pipette. Two 5µl samples of the solution were taken for the assessment of the percentage of ^{125}I incorporated into BSA and the specific activity.

The Centricon-30 was then centrifuged until approximately 100µl of solution was left (15 minutes at 2000rpm in a MSE MISTRAL 4L). 1.9ml of phosphate buffer (0.05M) was then added and the centrifugation repeated. The iodinated BSA was washed off the membrane and resuspended in a polypropylene counting vial using 4.9ml of phosphate buffer (0.05M). Two 5µl samples from this stock solution were used to assess the percentage of undamaged iodinated BSA (4.2.1.4). Then 10µl samples of the stock solution were dispensed into 1.5ml microfuge tubes and stored at -20°C. For use the tracer was allowed to thaw at room temperature. After thawing the ^{125}I-BSA was discarded unless the stock was repurified.

4.2.1.4 Assessment of the percentage incorporation of ^{125}I and the specific activity of ^{125}I-BSA.

The 5µl aliquots, taken from the solution in the Centricon-30 just after the iodination reaction, were capped, and the radioactivity counted. Subsequently 250µl of BSA (0.5mg/ml) in phosphate buffer (0.05M) and 250µl of TCA (10% ($^{w}/_{v}$)) were added to each 5 µl aliquot. The tubes were then left on ice for 30 minutes. The BSA was precipitated by the TCA solution. After 30 minutes the tubes were centrifuged for 10 minutes (3000rpm in a MSE Chilspin) and the supernatant containing radioactive iodine removed by aspiration. The pellet was washed with resuspension in TCA (10% ($^{w}/_{v}$)). Thereafter the supernatant was once more aspirated and the ^{125}I associated with the pellet counted in a capped LP3 tube. Thus the percentage incorporation of ^{125}I into BSA was calculated as follows:

$$\frac{\text{Radioactivity in the precipitate}}{\text{Total radioactivity in the 5 µl sample before purification}} \times 100$$

Since the percentage incorporation of ^{125}I into BSA and the total amount of ^{125}I and BSA in the reaction mixture were known, the specific activity could be calçulated as follows:

$$\text{Specific Activity } (\mu Ci/\mu g) = \frac{\left(\frac{\%\ \text{incorporation}}{100}\right) \times \mu Ci\ ^{125}I \text{ present at the start}}{\text{Amount of BSA present } (\mu g)}$$

4.2.1.5 Determination of iodination damage to ^{125}I-BSA.

The percentage of undamaged purified ^{125}I-BSA was determined by counting the radioactivity in two 5µl samples taken from the ^{125}I-BSA stock solution. Each ^{125}I-BSA sample was precipitated, washed and then precipitated as in section 4.2.1.4. Finally the radioactivity in the pellet was counted and the percentage of undamaged material was calculated as follows:

$$\frac{\text{Radioactivity in the pellet}}{\text{Total radioactivity in the 5µl purified sample}} \times 100$$

4.2.1.6 ^{125}I-BSA repurification.

The Centricon-30 was prepared as described previously (4.2.1.3). Stock ^{125}I-BSA was diluted to a total volume of 2ml with phosphate buffer 0.05M. Two 5µl samples were taken for the determination of percentage of undamaged material (see section 4.2.1.5). The Centricon-30 was then centrifuged until about 100µl was left (15 minutes at 2000rpm). 100µl of phosphate buffer (0.05M) was added to and mixed with the ^{125}I-BSA solution using a Gilson pipette. The ^{125}I-BSA solution was transfered to a polypropylene vial. This solution was diluted by a known amount and two 5µl samples were taken to determine the percentage of undamaged material and percentage recovery.

The percentage recovery was calculated as follows.

$$\%\ \text{recovery} = \frac{\text{Total counts after purification}}{\text{Total counts before purification}} \times 100$$

4.2.1.7 Assay of pepsinogen using ^{125}I-BSA.

Pepsin stock solutions were prepared by dissolving pepsinogen in 0.005M-acetate buffer pH 4.0. Pepsin standards were then prepared by serial dilution of the stock solution in double-distilled water.

The pepsinogen assay was based on that used by Raufman *et al.* (1986). The substrate (^{125}I-BSA) was diluted in double-distilled water so that 100µl of solution contained about 10,000dpm. 100µl samples of pepsinogen standards or unknown samples were added to microfuge tubes followed by 400µl of ice-cold glycine buffer (50mM) pH 3.0. Then 100µl of the diluted substrate was added and the resulting solution incubated in an oven for 60 minutes at 37°C. Enzyme activity was then terminated by the addition of 200µl of BSA (10mg/ml) in double-distilled water and 400µl of TCA (4.5%($^{w}/_{v}$)). After 10 minutes on ice, the tubes were centrifuged for 2 minutes at 12,000rpm (Beckman Microfuge). Radioactivity in a 500µl supernatant sample was then determined.

In summary, as a consequence of peptic activity the supernatant contained digested ^{125}I-BSA. The amount of digested ^{125}I-BSA was determined by γ-counting. The number of counts were related to the amount of pepsin present.

4.2.2 Results and discussion for pepsinogen assay using ^{125}I-BSA as substrate.

The percentage incorporation of ^{125}I was 61.3, which was in the same range as that obtained by Hunter and Greenwood (1962) of 60-75 %. Iodinated BSA stock had a specific activity of 7.66µCi/µg and was lower than 124µCi/µg as found by Raufman *et al.* (1986). However, this was because the labelled BSA (5µg) was diluted with carrier unlabelled BSA (75µg). Without dilution of the labelled BSA the specific activity in this stock would be 123µCi/µg. The percentage of undamaged ^{125}I-BSA as determined by TCA precipitation was 98.2 and close to values obtained by other workers using a similar method of iodination (Freychet *et al.*, 1971; Gavin *et al.*, 1972). Recovery of the iodinated BSA added to the Centricon-30 was 66%.

Six days after manufacturing the ^{125}I-BSA, the assay blank (a control without any pepsinogen) was 36.8% of the total counts (counts (mean dpm with the number of determinations in parentheses): total counts 5065(4); water blank 1866(2)). A day later the blank rose further to 41.6% of the total counts (counts (mean dpm with the number of determinations in parentheses): total counts 10843(2); water blank 4515(2)). This suggests that the percentage of damaged ^{125}I-BSA was increasing.

As suspected the percentage of undamaged ^{125}I-BSA was increasing over time (Table 4.1 and Fig. 4.3). Therefore repurification of the stock solution was required to reduce the high background counts. Repurification increased the percentage of undamaged material from for, example, 68.3 to 96.2 (counts (mean dpm with the number of determinations in parentheses): before purification, pellet 12146.5(2); total 177970(2): after purification, pellet 29614.5(2); total 30779.5(2)) with 39.0% recovery. The percentage recovery was probably lower than that attained during

Table 4.1

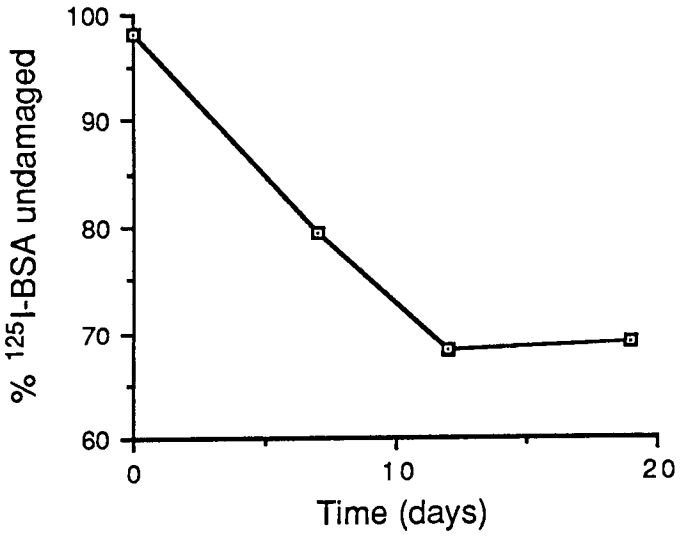
Effect of time on the percentage of undamaged tracer (raw data).

Time [after manufacture] (days)	Pellet radioactivity after precipitation (dpm)	Total radioactivity before precipitaton (dpm)
0	723552.5 (2)	736764.5 (2)
7	4529.5 (2)	5699.5 (2)
12	12146.5 (2)	17797.0 (2)
19	28761.0 (2)	41599.5 (2)

Dpm are presented as means with the number of determinations in parentheses. The percentage of undamaged material was calculated as described in section 4.2.1.5 and is plotted in Fig 4.3.

Figure 4.3

Effect of time on the percentage of undamaged tracer.



The percentage undamaged has been calculated from data in Table 4.1.

manufacture because no carrier BSA was added. After repurification the water blank was only 7.3% of total counts (counts (mean dpm with the number of determinations in parentheses): total 8574.5(2); water blank 624.5(2)). Subsequent to repurification the percentage of undamaged ^{125}I-BSA increased rapidly necessitating repurification on the day of each assay.

The cause of damage to the BSA molecule is probably due to irradiative breakdown. The reason for the contrast with Raufman *et al.* (1986) who reported that their ^{125}I-BSA was stable for 4 months is unclear.

Increasing the concentration of pepsin in the standards (section 4.2.1.7) raised the level of ^{125}I-BSA proteolysis as expected (Fig. 4.4).

4.2.3 Pepsinogen assay using acidified haemoglobin as substrate.

4.2.3.1 Pepsinogen release from cell suspensions.

Rat isolated fundic cell suspensions were prepared, as in section 2.1.3, and incubated in a shaking waterbath (120cycles/minute) at 37°C in capped vials with 95%O2 and 5%CO_2 as the gas phase. Medium B' was replaced with oxygenated Krebs-Ringer bicarbonate buffer pH 7.35 (KRB see Appendix A.2 for composition) with 0.1% ($^{w}/_{v}$) BSA, 10mM-glucose and 1mM-glutamine. Use of medium B' gave too high a background in the haemoglobin pepsinogen assay (see 4.4.2.2). Cell suspensions were incubated for 30 minutes, then a 1ml aliquot was taken and centrifuged (30s at 10,000g in a Beckman Microfuge). The supernatant was separated from the pelleted cells and usually stored overnight at 4°C before assay. For the determination of cellular pepsinogen the pellet was resuspended in 1ml of KRB and sonicated on ice at 5 Watts for 5x5 second bursts with 5 seconds between each sonication. The solution was then diluted typically 1:80 and 1:160 with KRB before storage at 4°C and assay.

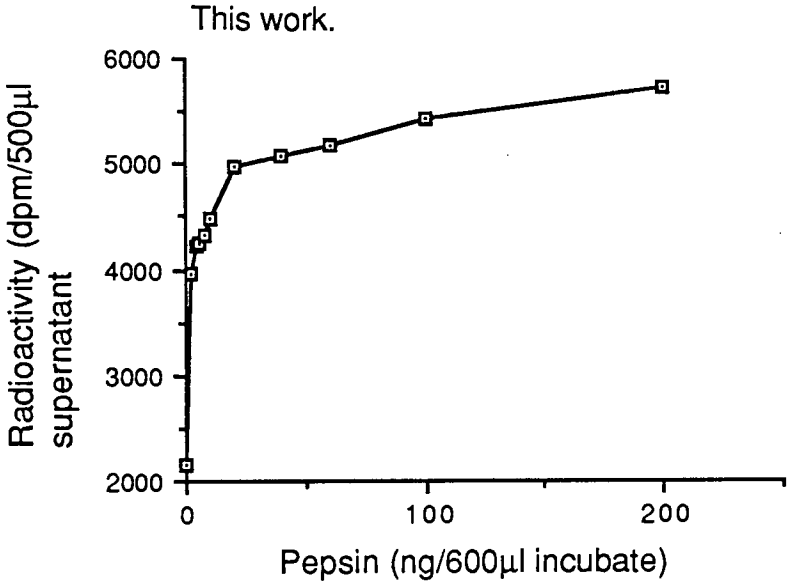
4.2.3.2 Measurement of pepsinogen release using haemoglobin as the substrate.

Pepsin solutions were prepared as in section 4.2.1.7. Alternatively, pepsinogen stock solutions were prepared by dissolving pepsinogen in saline.

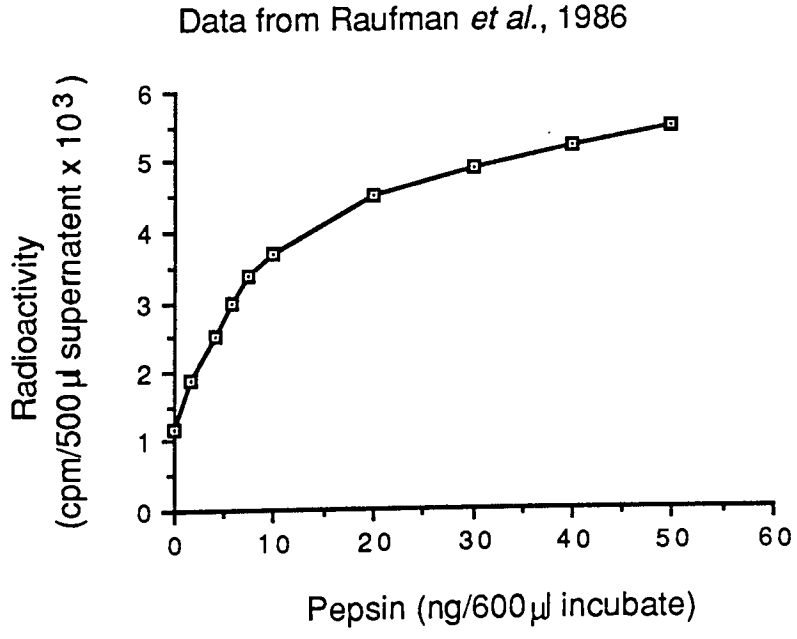
The pepsinogen assay was a modification of that used by Anson and Mirsky (1932). Modifications were based on those by Hersey *et al.* (1983) and Raufman *et al.* (1984). To microfuge tubes containing 100µl of double-distilled water, 100µl of supernatant or diluted sonicated cell pellet were added. Then 200µl of 50mM-glycine containing 2%($^{w}/_{v}$) bovine haemoglobin in 0.1M-HCL (pH 1.8) was added to each

Figure 4.4

Comparison between this work and that of Raufman *et al.* (1986) of the effect of increasing pepsinogen concentrations on the hydrolysis of ^{125}I-BSA.



Each value was determined in duplicate and the results presented as means. Total counts added per sample were 5945 dpm (mean of 2 samples).



In each experiment each value was determined in duplicate, and results given are means from 3 experiments.

tube and briefly mixed before incubation for 10 minutes at 37°C. The action of pepsin on haemoglobin was stopped by adding 1ml of 4.2%($^{w}/_{v}$) trichloroacetic acid (TCA), mixing, and centrifuging at 10,000g for 1 minute (Beckman Microfuge). Subsequently 1ml of the supernatant from each microfuge tube was removed and placed into a LP3 tube. To this 1ml of 0.5M-NaOH was added and then 280µl of Folin and Ciocalteau phenol reagent (1 volume of Folin and Ciocalteau regent was diluted into 2 volumes of double-distilled water). After the addition of Folin and Ciocalteau phenol reagent the sample was immediately vortex-mixed and then left for at least 10 minutes before reading the absorbance at 750nm. The activity of the sample was assessed by using a standard curve made up of at least 6 different concentrations of porcine pepsinogen. The conversion of pepsinogen to pepsin in the acid haemoglobin solution was essentially instantaneous (Koelz *et al.*, 1982).

In summary, the supernatant contained peptides derived from the digestion of haemoglobin which were estimated by the blue colour they produced with Folin and Ciocalteau phenol regent. The intensity of colour measured at 750nm reflected the amount of pepsin present, which was determined by comparison with a standard curve.

4.2.4 Results and discussion for pepsinogen assay using acidified haemoglobin as substrate.

As anticipated raising the concentration of pepsin in the standards (section 4.2.1.7) increased the proteolysis of haemoglobin (Fig. 4.5). Medium B', the usual incubation medium, increased the blank absorbance at 750nm (Fig. 4.6A). An alternative incubation medium, KRB plus 0.1%($^{w}/_{v}$)-BSA, 10mM-glucose and 1mM-glutamine, was tested and did not do this (Fig. 4.6B). Pepsinogen was preferred to pepsin as a standard because pepsinogen was present in the samples of incubation medium, and because pepsin unlike pepsinogen was inactivated if made up in incubation medium (Table 4.2).

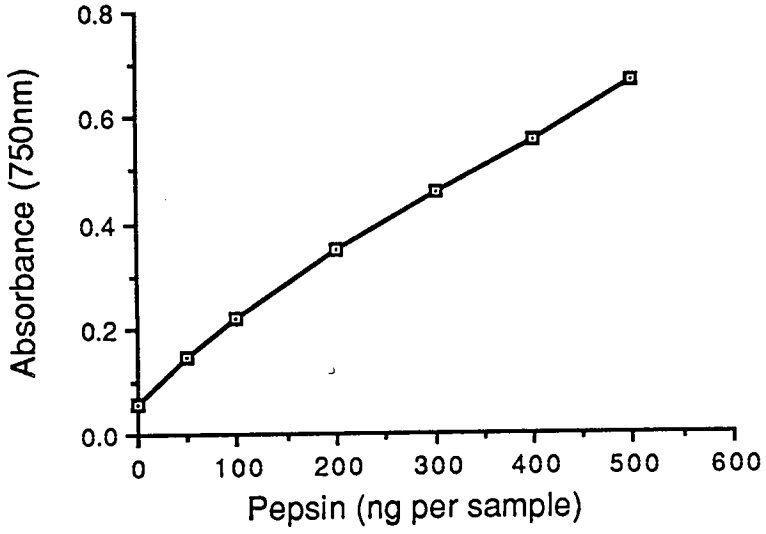
A typical pepsinogen standard curve is shown in Fig 4.7. The background absorbance increased gradually from the time of manufacture of the haemoglobin substrate (data not shown). Consequently, the haemoglobin substrate was prepared freshly every 3 weeks and stored at 4°C.

4.2.5 Discussion and evaluation of pepsinogen assays.

The most technically easy and rapid pepsinogen assay was the acid-denatured haemoglobin assay. The solutions required were easily prepared and the manipulations were straightforward. However, the ^{125}I-BSA assay was more problematic requiring the manufacture and particularly the repurification of ^{125}I-BSA. The radiochemical

Figure 4.5

Effect of increasing sample pepsinconcentration on the hydrolysis of acid-denatured haemoglobin.

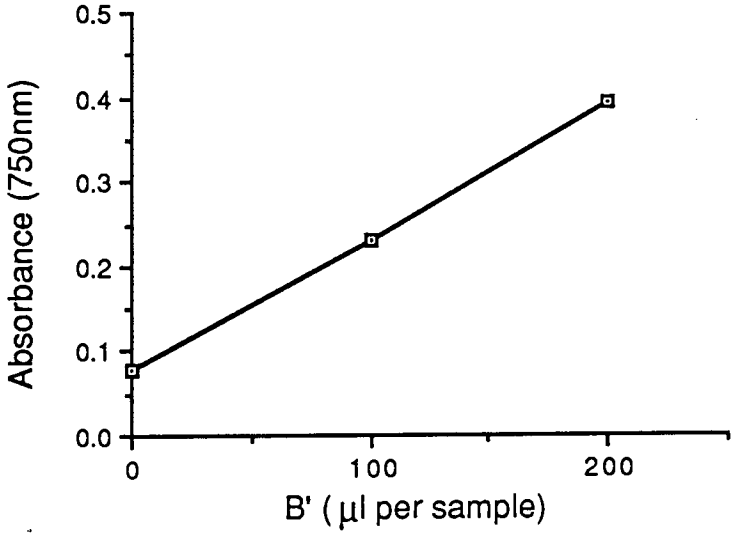


Each value was determined in duplicate and the results presented as means. Pepsin samples were prepared by dissolving the initial stock in 0.005M-acetate pH 4.0, followed by serial dilution in water.

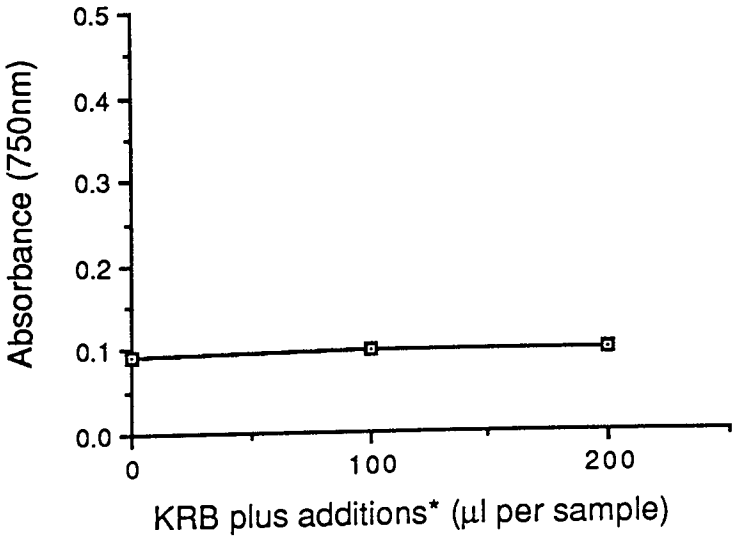
Figure 4.6

Effect of the amout of medium B' (Fig. A) and KRB plus additions (Fig. B) on absorbance (750nm).

A



B



The total sample volume was 200µl. Each value was determined in duplicate and the results are presented as means.

*Additions are 0.1%($^{w}/_{v}$)-BSA, 10mM-glucose and 1mM-glutamine.

Table 4.2

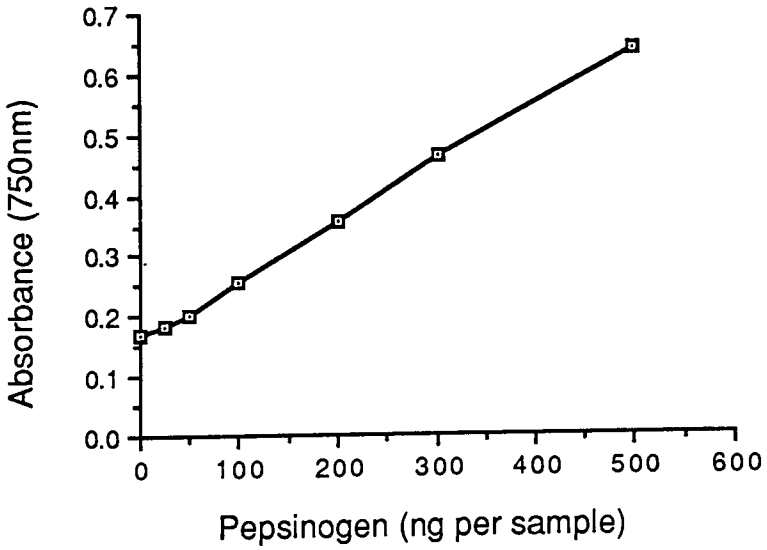
Effect of pH changes on the ability of pepsin (1µg/sample) to catalyse the hydrolysis of acid -denatured haemoglobin.

Solvent for pepsin	Subsequent additions	Absorbance(750nm) Blank	Absorbance(750nm) Pepsin (1µg)
KRB+HCl	None	0.137 (2)	0.698 (2)
KRB	HCl	0.146 (2)	0.137 (2)
$NaHCO_3$ (25mM)	HCl	0.137 (2)	0.121 (2)

HCl (0.4 N) when added was 10% $^{v}/_{v}$. Absorbances are presented as means with the number of determinations in parentheses.

Figure 4.7

Effect of increasing sample pepsinogen concentration on the hydrolysis of acid denatured haemoglobin.



Each value was determined in duplicate and the results presented as means. Pepsinogen standards were prepared by dissolving and serially diluting in saline (0.9% ($^{w}/_{v}$)). The pepsinogen samples were prepared by adding 5µl of the stock pepsinogen standards to 195µl of KRB plus BSA (0.1%($^{w}/_{v}$)) plus substrates (glucose (10mM) and glutamine (1mM)).

method was more sensitive than the acid-denatured haemoglobin method but this sensitivity was exhibited over a narrow range of enzyme concentration. The standard curve for the radiochemical method was clearly non-linear and this appears to be due to exhaustion of substrate (Fig. 4.4). The acid-denatured haemoglobin method, when combined with the Folin and Ciocalteau method for the detection of digested haemoglobin, was found to be sensitive enough for detecting pepsinogen release by the cell preparation. Therefore sample pepsinogen levels were routinely determined by using the acid denatured haemoglobin assay with pepsinogen as standard (section 4.2.3.2).

4.3 OTHER METHODOLOGY.

4.3.1 Preparation of reagents.

Cimetidine was dissolved and diluted in incubation media to provide a concentration of 10μM. Cell suspensions were resuspended and incubated in this medium where appropriate. Forskolin was dissolved and diluted in absolute ethanol, and a small volume (2μl) was added to incubation vials. Ethanol was added to the control vials also providing a final concentration of 0.125%($^{v}/_{v}$) in all vials. Pertussis toxin, IBMX and EGF were prepared as in section 3.2.1.

4.3.2 Effect of EGF on forskolin and forskolin plus IBMX (0.1mM) stimulated aminopyrine accumulation in crude preparations of parietal cells.

Suspensions consisting of approximately 20% parietal cells were isolated as described in section 2.1.3. After isolation the cells were resuspended in medium B' with or without cimetidine (10μM). Cell suspensions were diluted to a final concentration of 2-6x10^6cells/ml. Aliquots of the cell suspension (1.5ml) were added to incubation vials, containing secretagogue and inhibitor where appropriate, and the airspace above the cells gassed for 5 seconds with 95%O_2/5%CO_2 before capping. Treatment vials were then incubated at 37°C for 30 minutes with shaking at 120 cycles/minute. Estimation of the aminopyrine accumulation ratio was determined as in section 2.3.

4.3.3 Action of pertussis toxin on the inhibitory effect of EGF on forskolin-stimulated aminopyrine accumulation in crude suspensions of rat parietal cells.

Parietal cell suspensions were prepared as in section 2.1.3. The cell suspensions were split and preincubated, one portion with pertussis toxin (100ng/ml) and the other with pertussis toxin carrier solution alone. Cell suspensions were then washed and incubated as previously described in section 3.2.2. Aminopyrine accumulation was estimated as in section 2.3.

4.3.4 Analysis and presentation of data.

The variation in aminopyrine accumulation between cell batches was overcome by normalising the data for each experiment to the value obtained with 50μM-forskolin which was set at 100. Before normalisation the basal (unstimulated) aminopyrine accumulation ratio was subtracted from the stimulated value.

4.4 RESULTS AND DISCUSSION.

4.4.1 Results and discussion for acid secretion.

4.4.1.1 The effect of EGF on the aminopyrine accumulation ratio stimulated by forskolin alone.

Forskolin produced a dose-related increase in secretory activity which gave an aminopyrine accumulation ratio of 37.9 ± 10.3 (mean ± S.E.M. from 5 batches of cells) at 50μM-forskolin (Table 4.3). The presence of 1.6, 5 and 16μM-forskolin produced a response 13.3, 33.5 and 58.0% of that achieved by 50μM-forskolin. Forskolin-stimulated secretory activity was significantly inhibited by EGF (200nM) when the concentration of forskolin was 1.6 and 5μM (Table 4.3 and Fig. 4.8). The percentage inhibition by EGF of the aminopyrine accumulation ratio decreased as the concentration of forskolin increased (Fig. 4.9). These results contradict those found by Lewis *et al.* (1990) using a rabbit parietal cell preparation and Chen *et al.* (1984) using a canine parietal cell preparation, but are in agreement with those found by Reichstein *et al.* (1984) using a rabbit gland preparation.

The action of EGF against forskolin-stimulated secretion resembles that against histamine-stimulation. Thus the aminopyrine accumulation ratio attained with cells incubated with 1.6μM-forskolin (6.4 ± 2.6 see Table 4.3) was similar to that of cells incubated with 0.5mM-histamine (7.5 ± 1.7 see Table 3.7), and the percentage inhibition by EGF (200nM) was also similar (45% inhibition against 1.6μM-forskolin and 49% inhibition against 0.5mM-histamine (section 3.3.6)).

As EGF only inhibited secretory activity induced by low concentrations of forskolin it is conceivable that the inhibitory action of EGF was against endogenous histamine and not forskolin. In fact forskolin at submaximal concentrations potentiated the stimulatory action of endogenous histamine on parietal cell secretory activity in rabbit glands (Chew, 1983).

4.4.1.2 The effect of EGF on parietal cell secretory activity stimulated by forskolin in the presence of cimetidine.

To eliminate the possible effects of endogenous histamine 10μM-cimetidine was included in the incubation medium. This concentration of cimetidine was chosen because histamine released by gland preparations does not reach an effective concentration greater than 10^{-6}M and this is completely suppressed by 10μM-cimetidine (Chew, 1980).

Forskolin in the presence of cimetidine (10μM) stimulated aminopyrine accumulation in a concentration-dependent fashion which gave an aminopyrine

Table 4.3

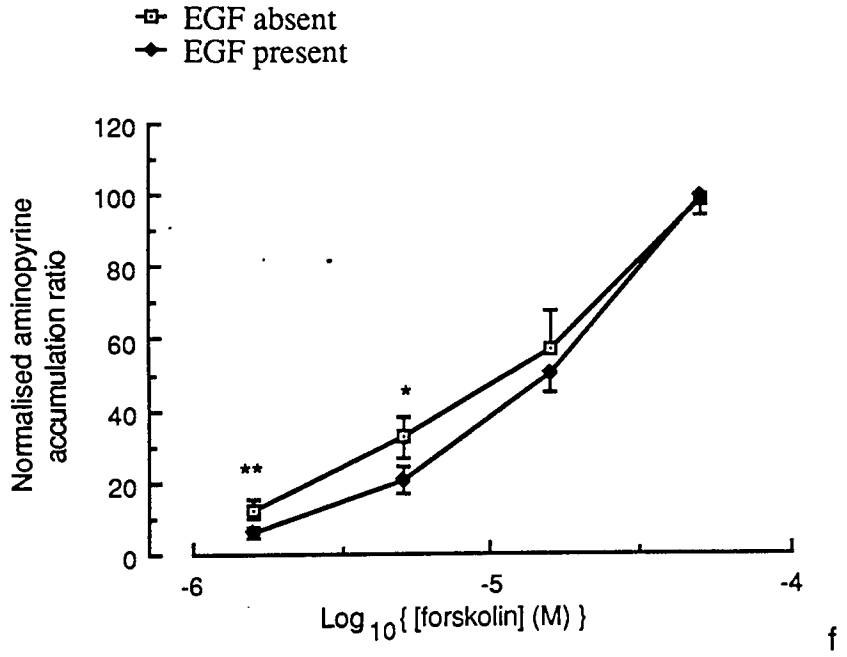
The action of EGF (200nM) on the aminopyrine accumulation ratio (APR) stimulated by different concentrations of forskolin.

Forskolin(μM)	APR Control	APR EGF
1.6	6.4 ± 2.6	3.6 ± 1.6
5	17.0 ± 7.4	11.3 ± 5.1
16	27.8 ± 9.1	25.5 ± 8.3
50	37.9 ± 10.3	36.9 ± 8.7

Results are from 4 to 5 batches of cells containing 18.4 ± 0.2% parietal cells, and are expressed as means ± S.E.M. The basal value (unstimulated) was 2.2 ± 0.4. There was no significant effect of EGF on the forskolin-stimulated aminopyrine accumulation ratio (paired *t*-test). The lack of a significant effect of EGF at low concentrations of forskolin seems to be a consequence of variations in responsiveness between batches of cells. A significant effect becomes apparent on normalisation (Fig. 4.14).

Figure 4.8

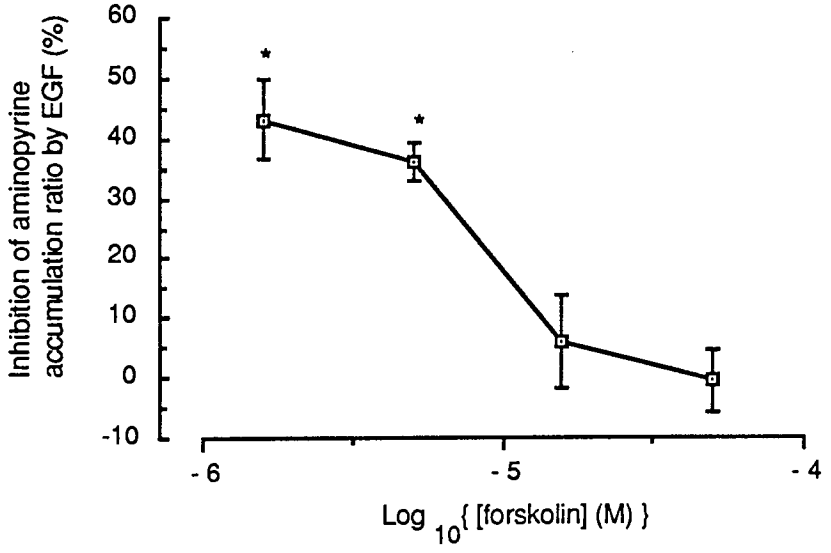
Effect of EGF (200nM) on the aminopyrine accumulation ratio stimulated by different concentrations of forskolin.



Data from Table 4.3 have been normalised to the result for 50μM-forskolin alone, which has been set at 100. The effect of EGF has been analysed by a paired *t*-test. $^{**}P<0.05$, $^{*}P<0.01$.

Figure 4.9

The inhibitory effect of EGF, on gastric cells stimulated by forskolin, expressed as a percentage of the appropriate uninhibited aminopyrine accumulation ratio.



Data from Table 4.3 have been used to calculate the percentage inhibition. The effect of EGF has been analysed for significant difference from zero by a *t*-test. **P*<0.01.

accumulation ratio of 57.1 ± 8.6 (mean ± S.E.M. from 4 batches of cells) at 50μM-forskolin (Table 4.4). The presence of 1.6, 5 and 16μM-forskolin produced a response 6.6, 23.8 and 28.5% of that achieved by 50μM-forskolin. EGF (200nM) significantly inhibited forskolin-stimulated secretory activity at 1.6 and 5μM (Table 4.4 and Fig. 4.10). As without cimetidine, the percentage inhibition by EGF of the aminopyrine accumulation ratio declined as the concentration of forskolin increased (Fig. 4.11).

EGF was not producing a constant absolute decrease in the aminopyrine accumulation ratio which became proportionally less as the level of stimulation increased in response to higher forskolin concentrations. Thus, at 5μM-forskolin the absolute reduction in the aminopyrine accumulation ratio effected by EGF was 2.1 times greater than in cells treated with 1.6μM-forskolin ($P<0.025$ by paired *t*-test, data pooled from experiments with and without cimetidine). There was no significant difference between the action of EGF (200nM) against forskolin in the presence and absence of cimetidine (10μM) (unpaired *t*-test). In this context it should be noted that in rat parietal cells IBMX alone does not stimulate aminopyrine accumulation (Shaw *et al.*, 1987). An effect of IBMX would be expected in the presence of significant endogenous histamine. Thus it appears that the effect of EGF is a genuine inhibition of forskolin-stimulation and not an effect on the action of any endogenous histamine.

Stimulation by forskolin (5μM) of secretory activity with cimetidine (10μM) present was significantly inhibited by EGF (200nM) in enriched parietal cells isolated from the rat (aminopyrine accumulation ratio (means ± S.E.M., with the number of cell samples in parentheses using one batch of cells containing 85% parietal cells): without EGF 3.9 ± 0.4(5); with EGF 2.3 ± 0.5(5). $P<0.05$ by unpaired *t*-test). This suggests that the action of EGF is a direct one and not via an action on another cell type.

4.4.1.3 The effect of pertussis toxin on the inhibitory action of EGF on aminopyrine accumulation stimulated by different concentrations of forskolin in the presence of cimetidine.

In the presence of pertussis toxin (100ng/ml) and cimetidine (10μM) forskolin produced a dose-dependent increase in aminopyrine accumulation. Pertussis toxin had little effect on the dose response curve to forskolin. The response to 50μM-forskolin resulted in an aminopyrine accumulation ratio of 53.3 ± 11.1 and 57.5 ± 14.0 (mean ± S.E.M. from 4 batches of cells) in the absence and presence of pertussis toxin respectively. Forskolin at 0.5, 1.6 and 5μM produced a response 4.5, 11.4 and 32% of that attained by 50μM-forskolin (pertussis toxin present in all cases). Secretory

Table 4.4

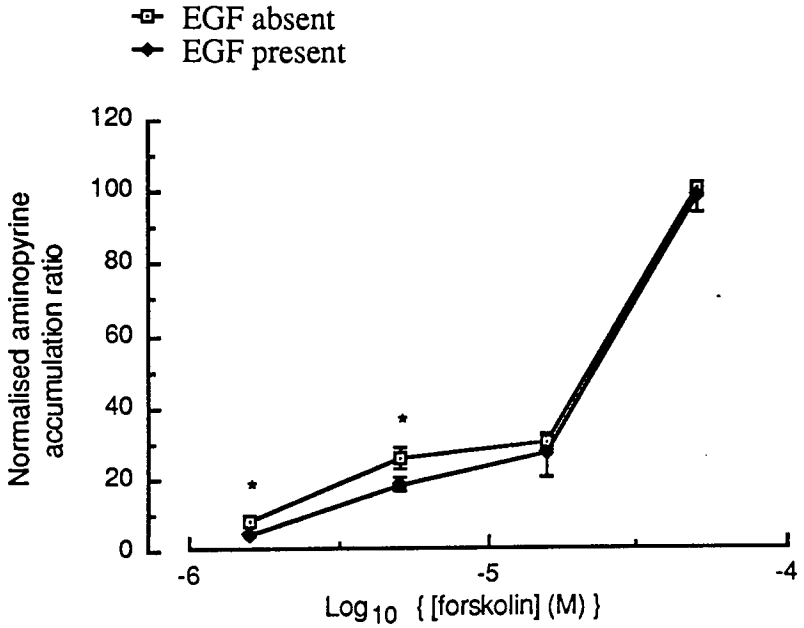
Action of EGF (200nM) on the aminopyrine accumulation ratio (APR) stimulated by concentrations of forskolin in the presence of cimetidine (10μM).

Forskolin(μM)	APR Control	APR EGF
1.6	3.8 ± 0.6	1.9 ± 0.3*
5	13.6 ± 2.4	9.4 ± 1.1*
16	16.3 ± 2.0	13.4 ± 2.2
50	57.1 ± 8.6	53.4 ± 7.1

Results from 4 experiments, on suspensions containing 19.0 ± 0.4% parietal cells, are expressed as means ± S.E.M. The basal (unstimulated) aminopyrine accumulation ratio was 2.6 ± 0.4. The significance of effects of EGF have been analysed by a paired *t*-test. *$P<0.05$.

Figure 4.10

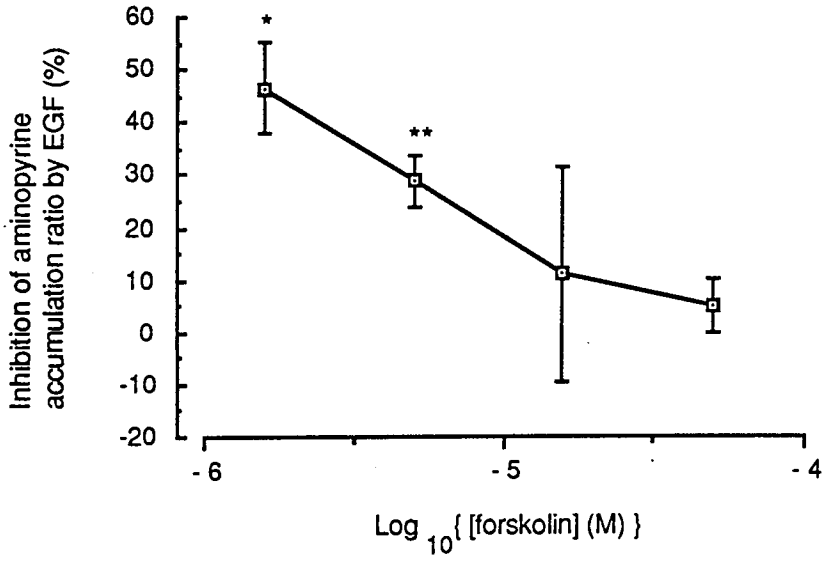
Effect of EGF (200nM) on the aminopyrine accumulation ratio stimulated by increasing concentrations of forskolin in the presence of 10μM-cimetidine.



Data from Table 4.4 have been normalised to the result for 50μM-forskolin, which has been set at 100. The effect of EGF has been analysed by a paired *t*-test. **P*<0.05.

Figure 4.11

The inhibitory effect of EGF, on gastric cells stimulated by forskolin in the presence of cimetidine (10μM), expressed as a percentage of the appropriate uninhibited aminopyrine accumulation ratio.



Data from Table 4.4 have been used to calculate the percentage inhibition. The effect of EGF has been analysed for significant difference from zero by a *t*-test. **P*<0.05, ***P*<0.01.

activity stimulated by forskolin, in the presence of pertussis toxin, was not inhibited at any concentration by EGF (200nM) (Table 4.5 and Fig. 4.12 plus 4.13).

Inhibition by EGF of parietal cell secretory activity stimulated by both histamine and forskolin was blocked by pertussis toxin. This suggests that a G-protein from the "G_i-like" family is involved in the action of EGF in both cases.

4.4.1.4 The action of IBMX on the inhibitory effect EGF has on forskolin-stimulated aminopyrine accumulation.

The presence of IBMX (0.1mM) shifted the dose-response curve for forskolin-stimulation of the aminopyrine accumulation ratio to the left. The potency and probably the efficacy of forskolin were increased by IBMX (Fig. 4.14). The presence of 0.16, 0.5 and 1.6μM-forskolin with IBMX resulted in a response 0.7, 13.4 and 73.8% of that produced by 50μM-forskolin with IBMX. In the presence of IBMX, forskolin-stimulated secretory activity was not inhibited at any concentration by EGF (200nM) (Table 4.6 and Fig. 4.15). Basal aminopyrine accumulation was not affected by EGF (Table 4.6).

In the absence of IBMX, EGF inhibited 1.6 and 5μM-forskolin-stimulation (Fig. 4.8 and 4.10) which was at a level of secretory activity up to 35% of that obtained with 50μM-forskolin (section 4.4.1.1 and 4.4.1.2). At the same level of secretory activity, when IBMX was present, the inhibitory effect of EGF was either abolished or reduced (Fig. 4.15 and 4.16).

4.4.1.5 The action of EGF on acid secretion.

The action of EGF against secretory activity stimulated by forskolin resembles that of EGF against histamine-stimulation. The reasons for this are as follows. Preincubation with pertussis toxin blocked the effect of EGF in cells stimulated with histamine (section 3.3.6) or with forskolin. IBMX reduced or prevented the action of EGF when added to cells incubated with either histamine or forskolin. The aminopyrine accumulation ratio obtained with cells stimulated with 1.6μM-forskolin and 0.5mM-histamine were similar.

EGF action against aminopyrine accumulation stimulated by forskolin seems to be a direct one as it takes place against a purified parietal cell preparation. It is unlikely that PGE_2 or somatostatin mediate the action of EGF as IBMX blocks the inhibitory effects of EGF but not PGE_2 or somatostatin (section 3.3.5 and 3.3.4).

The action of pertussis toxin indicates that a G-protein is probably involved in the inhibitory action of EGF. The above findings make it unlikely that the antisecretory effect of EGF is mediated by a direct action on the histamine H_2 receptor.

Table 4.5

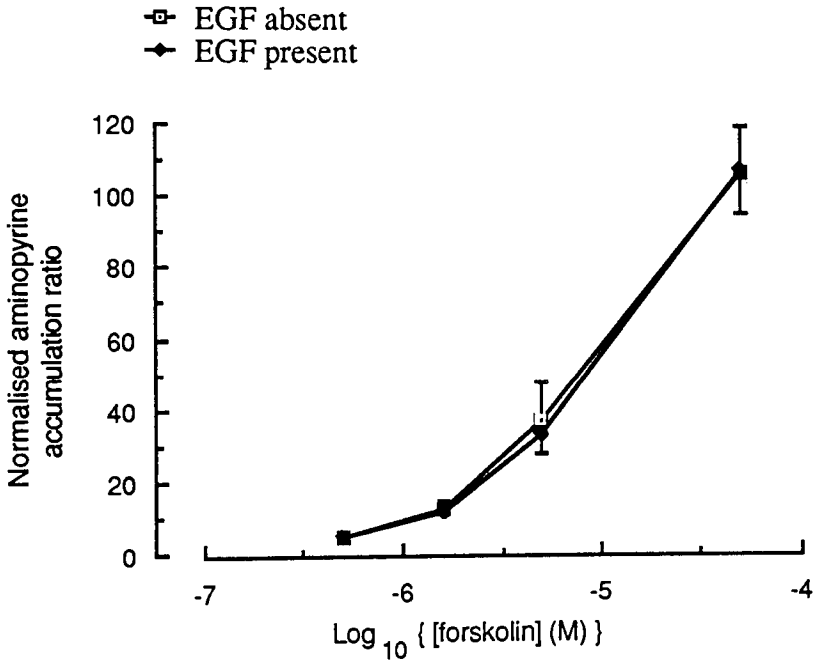
Effect of pertussis toxin (100ng/ml) on the inhibitory action of EGF (200nM) on the aminopyrine accumulation ratio (APR) stimulated by different concentrations of forskolin in the presence of cimetidine (10μM).

Forskolin(μM)	APR Control	APR EGF
0.5	2.6 ± 0.6	2.3 ± 0.6
1.6	6.6 ± 1.4	6.5 ± 1.8
5	18.4 ± 4.5	17.0 ± 3.1
50	57.5 ± 14.0	58.5 ± 15.4

Data from 4 experiments using cell suspensions containing 19.0 ± 0.6% parietal cells are expressed as means ± S.E.M. Forskolin (50μM) resulted in an aminopyrine accumulation ratio of 53.3 ± 11.1 in the absence of pertussis toxin. The basal (unstimulated) value was 1.7 ± 0.2 and 1.2 ± 0.2 without and with pertussis toxin respectively. Data were analysed by an unpaired *t*-test. EGF had no significant effect on the aminopyrine accumulation ratio stimulated by any concentration of forskolin. There was no significant difference between basal release with and without pertussis toxin.

Figure 4.12

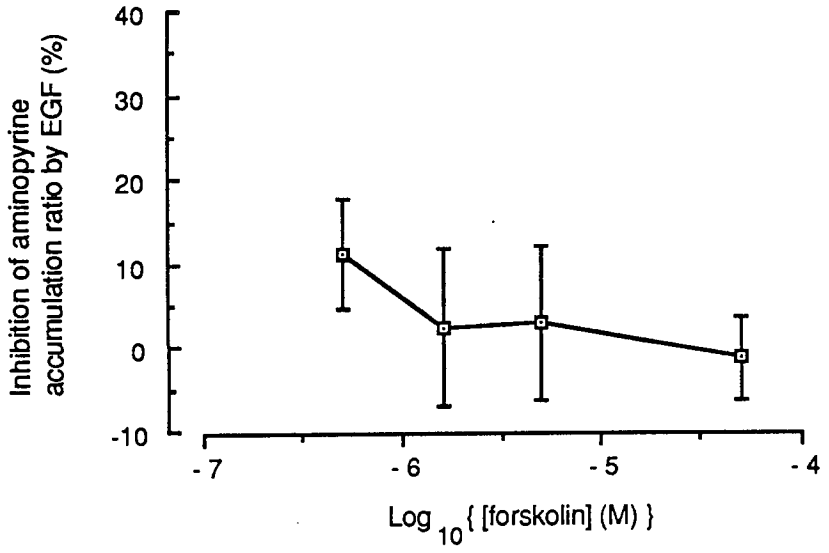
Effect of EGF (200nM) on the aminopyrine accumulation ratio stimulated by different concentrations of forskolin in the presence of pertussis toxin (100ng/ml) and cimetidine (10μM).



Data from Table 4.5 have been normalised to the result with 50μM-forskolin, which has been set at 100. EGF had no significant effect on the normalised aminopyrine accumulation ratio stimulated by any concentration of forskolin (paired *t*-test).

Figure 4.13

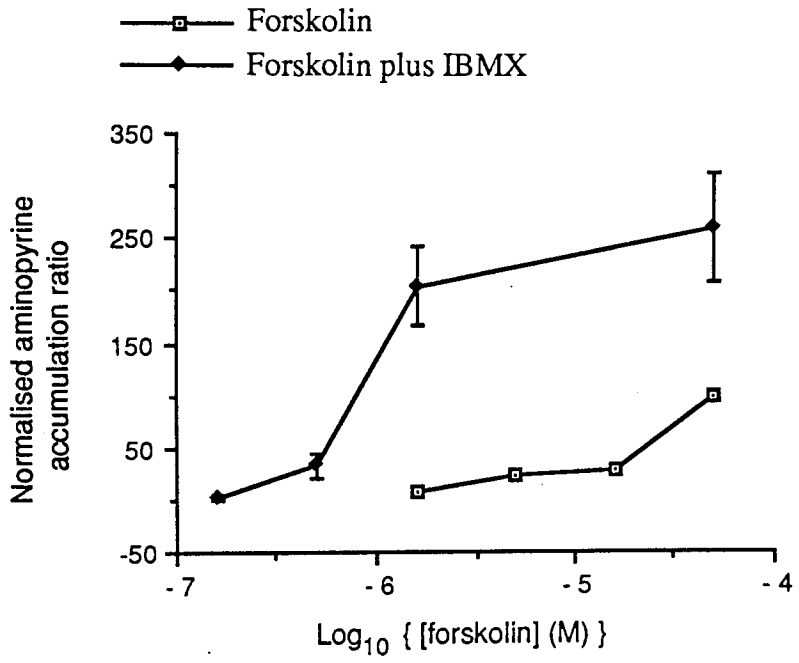
The inhibitory effect of EGF, on gastric cells stimulated by forskolin in the presence of pertussis toxin (100ng/ml) and cimetidine (10μM), expressed as a percentage of the appropriate uninhibited aminopyrine accumulation ratio.



Data from Table 4.5 have been used to calculate the percentage inhibition. All results were not significantly different from zero (*t*-test).

Figure 4.14

Effect of IBMX (0.1mM) on the aminopyrine accumulation ratio stimulated by different concentrations of forskolin in the presence of 10μM-cimetidine.



Data from Table 4.4 and 4.6 have been normalised to the result for 50μM-forskolin alone, which has been set at 100.

Table 4.6

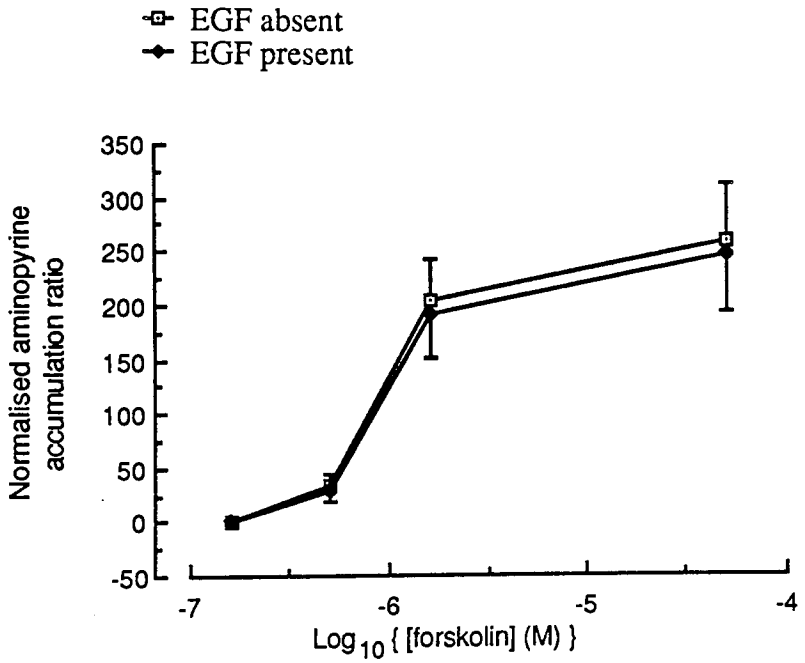
The action of EGF (200nM) on the aminopyrine accumulation ratio (APR) stimulated by different concentrations of forskolin in the presence of IBMX (0.1mM) and cimetidine (10μM).

Forskolin(μM)	APR Control	APR EGF
1.6	0.7 ± 0.4	0.4 ± 0.3
5	12.8 ± 6.7	11.4 ± 6.3
16	70.4 ± 30.4	63.0 ± 24.0
50	95.4 ± 31.9	88.4 ± 27.7

Results from 4 experiments using cell suspensions containing 19.7 ± 1.0% parietal cells are expressed as means ± S.E.M. With these cell suspensions, forskolin (50μM) with cimetidine (10μM) resulted in an aminopyrine accumulation ratio of 38.7 ± 9.5 (no IBMX present). The basal (unstimulated) value without IBMX was 1.9 ± 0.3 and basal value with IBMX was 1.9 ± 0.33. Data were analysed by a paired *t*-test. EGF had no significant effect on the aminopyrine accumulation ratio stimulated by any concentration of forskolin. There was no significant difference between basal values.

Figure 4.15

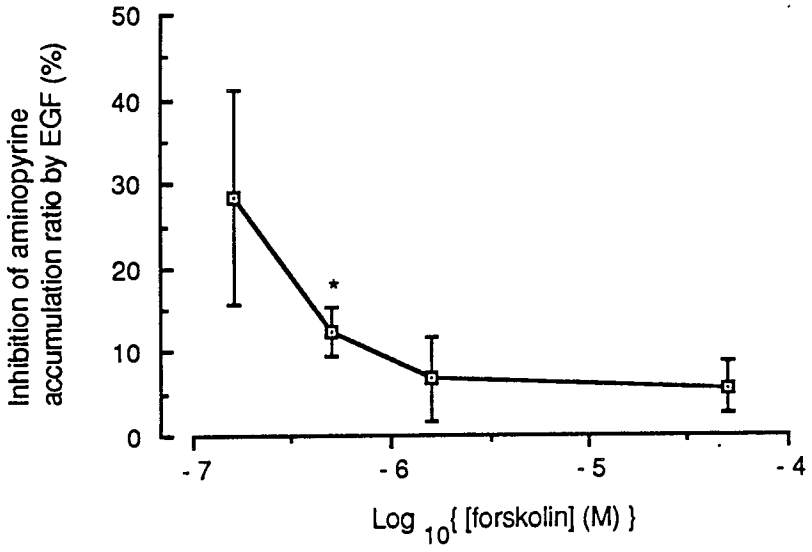
Effect of EGF (200nM) on the aminopyrine accumulation ratio stimulated by different concentrations of forskolin in the presence of IBMX (0.1mM) and cimetidine (10μM).



Data from Table 4.6 have been normalised to the result at 50μM-forskolin alone (no IBMX present), which has been set at 100. EGF had no significant effect on the normalised aminopyrine accumulation ratio stimulated by any concentration of forskolin (paired *t*-test).

Figure 4.16

The inhibitory effect of EGF, on gastric cells stimulated by forskolin in the presence of IBMX (0.1mM) and cimetidine (10μM), expressed as a percentage of the appropriate uninhibited aminopyrine accumulation ratio.



Data from Table 4.6 have been used to calculate the percentage inhibition. The effect of EGF has been analysed for significant difference from zero by a *t*-test. $^{*}P<0.05$.

Recently the action of EGF on adenylate cyclase was tested. This was conducted using an enriched parietal cell preparation containing over 80% parietal cells which was sonicated (Schepp *et al.*, 1983a) and adenylate cyclase activity stimulated by forskolin measured in the presence and absence of EGF. A concentration of forskolin (5μM) against which EGF was active in intact cells stimulated adenylate cyclase by 81 ± 17 pmole cyclic AMP formed/10^6cells per 10 minutes. The effect of EGF, expressed as a percentage of the above control result was 99 ± 1 and thus insignificant (paired t-test) (data from J. F. Hatt). This result makes a direct interaction between the EGF receptor and the adenylate cyclase via a G-protein unlikely, and adds some indirect support to the suggestion that EGF may affect the cyclic AMP content of parietal cells via modulation of cyclic AMP hydrolysis rather than its production.

Measurement of cyclic AMP levels in an enriched parietal cell preparation stimulated by forskolin revealed that EGF apparently had no effect (Table 4.7 data by J. F. Hatt). One interpretation is that the site of action of EGF is distal to cyclic AMP production and breakdown. However, EGF has consistently been shown to have no effect on cells stimulated by dbcAMP (Shaw *et al.*, 1987; Reichstein *et al.*,1984). Thus EGF action appears to be upstream from cyclic AMP action. To explain this anomaly it is possible that EGF has a stimulatory effect on the cyclic AMP content of the other non-parietal cells (~17%) and thus masks the decrease in cyclic AMP present in the parietal cells. Alternatively EGF (200nM) has been shown to cause a significant reduction in cyclic AMP accumulation stimulated by 0.5mM-histamine, but the actual decrease was small and with forskolin-stimulation may not have been picked up (cyclic AMP accumulation (pmole/10^6cells/30 minutes) from 4 batches of cells expressed as means ± S.E.M. Histamine (0.5mM) 1.15 ± 0.27: histamine (0.5mM) plus EGF (200nM) 0.96 ± 0.23. $P<0.05$ by Dunnett's test) (Data from Hatt and Hanson, 1988).

EGF inhibits parietal cell secretory activity stimulated by low concentrations of forskolin only. The reason for this is not known but there are two possibilities. Firstly, at high concentrations of forskolin the putative cyclic AMP phosphodiesterase (low Km) plays only a minor contribution in total cyclic AMP breakdown due to high cyclic AMP concentrations causing high Km enzymes to be recruited. A second possibility is that both EGF and high intracellular cyclic AMP activate the same cyclic AMP phosphodiesterase. DbcAMP has been shown to activate a dense vesicle cyclic AMP phosphodiesterase in rat hepatocytes (Heyworth *et al.*, 1983).

4.4.2 Pepsinogen secretion.

4.4.2.1 Cellular pepsinogen.

Table 4.7

Effect of EGF (200nM) on cyclic AMP accumulation in a parietal cell enriched preparation stimulated by different concentrations of forskolin in the presence of cimetidine (10μM).

Forskolin (μM)	cyclic AMP accumulation Control	cyclic AMP accumulation EGF
0	2.10 ± 0.37	2.24 ± 0.45
1.6	5.07 ± 1.28	5.10 ± 1.29
5	8.93 ± 2.16	9.17 ± 2.18
16	11.79 ± 2.47	11.71 ± 2.68
50	24.20 ± 5.44	25.45 ± 5.99

Data are from 4 separate experiments using cell preparations containing 83.1 ± 2.4% parietal cells and are expressed as means ± S.E.M. Cyclic AMP accumulation values are pmoles/10^6cells/30 minutes. Data of J. F. Hatt.

The chief cell content of the cell preparation was not known but was below ~50% as the parietal cell content was ~20% and the mucous cell content was ~30% (A.Keates oral communication). The pepsinogen content of the crude cell suspension was 195.7 ± 24.2μg/10^6cells (mean ± S.E.M. from 7 batches of cells) which was higher than that found by other workers (Table 4.8). Serial dilution of the cellular extract (1:10 up to 1:80) gave a progressively higher calculated intracellular pepsinogen content (Table 4.9). At a dilution of 1:80 and 1:160 the calculated intracellular pepsinogen content was similar (Table 4.9) and this dilution was therefore used routinely to estimate intracellular pepsinogen levels. Lack of a recognition of this effect may have resulted in the lower results obtained by other workers, although species difference could also play a part in some cases. The reason for this effect of sample dilution is not known, but may be due to the presence of an intracellular inhibitor of pepsinogen activation or pepsin activity.

4.4.2.2 Pepsinogen secretion.

Results (section 4.4.1.1 and 4.4.1.2) suggest that endogenous histamine has no effect on forskolin-stimulation or the action of EGF in rat isolated fundic cell suspensions. Also, in contrast to acid secretory activity, cimetidine had no effect on pepsinogen secretion stimulated by forskolin in rabbit gastric glands (Chew, 1983a) and histamine does not apparently induce pepsinogen secretion in the rat (see section 4.1.3). Therefore, cimetidine was omitted from the incubation medium.

Forskolin caused a dose-related increase in pepsinogen secretion which was 2106 ± 450ng pepsinogen/10^6cells/30 minutes (mean ± S.E.M. from 7 batches of cells) in response to 50μM-forskolin (Table 4.10). The S.E.M. in Table 4.11 were large, particularly at low concentrations of forskolin. This was due to a small stimulation against a high background rate of secretion, however analysis by normalisation and paired *t*-test reduced this problem (compare Table 4.10 and Fig. 4.17). The presence of 0.05, 0.16, 0.5 and 5μM-forskolin resulted in a response 17, 12 and 22 and 78% of that attained by 50μM-forskolin. EGF (200nM) did not inhibit forskolin-stimulated pepsinogen secretion at any level of the secretory response (Table 4.10 and Fig. 4.17). EGF caused a rise in secretory activity which reached significance at 5μM-forskolin (Fig. 4.17). EGF had no effect on pepsinogen secretion in the absence of forskolin (Table 4.10).

These findings are in contrast with the abstract by Miyamoto *et al.* (1987) and the *in vivo* findings by Konturek *et al.* (1981b). However, as the above were carried out *in vivo* or with mucosal fragments the possibility that EGF was acting indirectly by causing the release of an endogenous inhibitor of pepsinogen secretion cannot be eliminated. It is possible that EGF may inhibit pepsinogen secretion *in vivo* by

Table 4.8

A comparison of the cellular pepsinogen content in isolated cell preparations.

Species	Preparation	Pepsinogen $\mu g/10^6$cells	Reference
Rat	Isolated stomach cell	196 ± 24	This work
Rat	Isolated stomach cell	22.0 ± 2.0	Fatemi *et al.* (1982)
Guinea pig	Isolated stomach cell	6.1 ± 0.9	Raufman *et al.* (1984)
Dog	Enriched isolated chief cells	23	Wollin *et al.* (1979)
Rat	Enriched isolated chief cells	72.0 ± 7.0	Fatemi *et al.* (1982)
Guinea pig	Enriched isolated chief cells	54.2 ± 9.3	Raufman *et al.* (1984)

Table 4.9

The effect of dilution of a cellular extract on the calculated pepsinogen content.

Dilution of sample	Cellular pepsinogen ($\mu g/10^6$cells)
1:10	36.1
1:20	56.9
1:40	73.8
1:80	97.0
1:160	107.7

Table 4.10

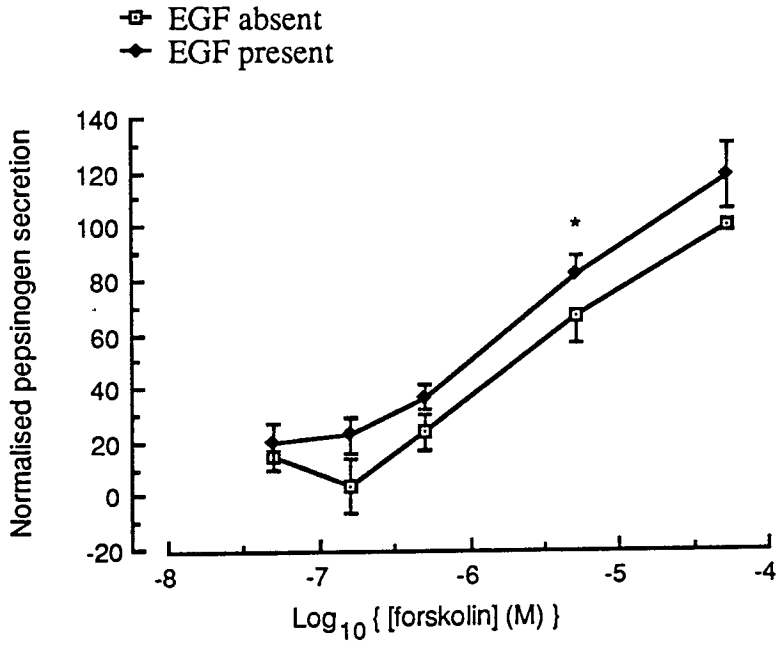
Action of EGF (200nM) on pepsinogen secretion stimulated by concentrations of forskolin.

Forskolin	Pepsinogen secretion ($ng/10^6$cells/30minutes) Control	Pepsinogen secretion ($ng/10^6$cells/30minutes) EGF
0.05	353.0 ± 202.6	469.5 ± 257.1
0.16	250.3 ± 228.7	601.8 ± 366.5
0.5	456.3 ± 145.4	699.9 ± 119.0
5	1647.83± 427.9	1953.7 ± 432.6
50	2106.1 ± 449.8	2376.0 ± 508.5

Results from 6 or 7 batches containing 19.3 ± 0.7% parietal cells were expressed as means ± S.E.M. Basal (unstimulated) pepsinogen secretion was 2590.5 ± 232.9 $ng/10^6$cells/30minutes and 2774.5 ± 346.1 $ng/10^6$cells/30minutes for basal without EGF and basal with EGF respectively. Results were analysed by a paired *t*-test. $^*P<0.001$. There was no significant difference between basal values.

Figure 4.17

Effect of EGF (200nM) on pepsinogen secretion stimulated by different concentrations of forskolin.



Data from Table 4.10 have been normalised to the result obtained with 50μM-forskolin alone, which has been set at 100. The effect of EGF has been analysed by a paired *t*-test. **P*<0.01.

inhibiting acid secretion which has been shown to stimulate pepsinogen secretion *in vivo* (Johnson, 1972). This indirect effect is unlikely to occur in an isolated cell preparation, as the mechanism may be a 'wash out' of pepsinogen from the gastric glands by the secreted acid.

In contrast to acid secretion, EGF appears to have no inhibitory effect on forskolin-stimulated pepsinogen secretion at least in rat ~~parietal~~ cells.

4.5 GENERAL DISCUSSION.

The results suggest that EGF has a direct inhibitory effect on the parietal cell. There is much evidence which indicates that EGF receptors are present on the parietal cell membrane, and it is via these receptors that EGF may mediate its action. Thus immunocytochemical techniques suggest that EGF receptors are present on the vascular facing basolateral membrane of parietal cells in man (Mori *et al.*, 1987). Two distinct binding sites for EGF have been detected, using ^{125}I-EGF, on gastric mucosal cells from the rabbit (Olender and Reid, 1988). Specific binding of ^{125}I-EGF was also found to take place on enriched parietal cell preparations from the dog (Chen *et al.*, 1984). Transforming growth factor α (TGFα) has a structural homology with EGF and shares the EGF cell-surface membrane receptor. The expression of TGFα/EGF receptor mRNA of a parietal cell enriched and crude isolated cell preparation from the guinea-pig stomach were compared. It was found that the parietal cell enriched preparation exhibited a more intense expression (7.8-fold increase compared to the crude preparation) (Beauchamp *et al.*, 1989). The evidence for EGF receptors on chief cells is less strong. However, Mori et *al.* (1987) found a weak immunocytochemical response to the EGF receptor on chief cells, and Beauchamp *et al.* (1989) found that an enriched chief cell fraction displayed a stronger expression of TGFα/EGF receptor mRNA than a crude cell preparation (2.7-fold increase). These findings are consistent with the possibility of direct actions of EGF on parietal and possibly chief cells. In this work a direct action on parietal cells is supported by the use of a cell suspension where any mediators released from other cells will be greatly diluted in the medium. Furthermore, the characteristics of the inhibitory action of EGF rule out the involvement of somatostatin or prostaglandins. By contrast no major action on chief-cell pepsinogen secretion was detectable.

The finding that EGF inhibits forskolin-stimulated aminopyrine accumulation at low levels of stimulation by low concentrations of forskolin may go some way to explain the contradiction in the literature. Thus an inhibitory effect of EGF will not be present at high concentrations of forskolin. For this reason it may be that Chen *et al.* (1984) detected no effect of EGF on forskolin-stimulation. The reduction in aminopyrine accumulation elicited by EGF on forskolin-stimulation is in absolute terms small. Therefore unless the system used to detect changes is very sensitive it may not detect the inhibitory action of EGF. For this reason it may be that Lewis *et al.* (1990) found that forskolin, even at low concentrations (0.1-10μM) was not inhibited by 100nM-EGF in an isolated parietal cell preparation from the rabbit. Another possibility is that the difference observed was due to a species difference. In contrast to the findings by Lewis *et al.* (1990), Reichstein *et al.* (1984) found that EGF (160nM) inhibited aminopyrine accumulation stimulated by forskolin (10μM) in a rabbit gland preparation.

4.6 SUMMARY.

1. EGF(200nM) diminished the aminopyrine accumulation ratio in rat fundic cell suspensions, containing 18-23% parietal cells and 85% parietal cells, which were stimulated by submaximal concentrations of forskolin. This effect was not due to an action against the effects of endogenous histamine.

2. Inclusion of the phosphodiesterase inhibitor IBMX abolished or reduced the inhibitory effect of EGF on forskolin-stimulated aminopyrine accumulation. This effect was probably not related to the enhancement of the stimulatory effects of forskolin by IBMX.

3. Pertussis toxin blocked the inhibitory action of EGF on parietal cell activity stimulated by forskolin. Thus the intracellular mechanism by which EGF mediates its inhibitory action probably involves a G-protein.

4. The existence of an action of EGF against forskolin-stimulated secretory activity, suggests that EGF is able to exert an inhibitory action in the parietal cell downstream in the secretory pathway from the histamine receptor.

5. Two assays for the measurement of pepsinogen from fundic cell suspensions have been developed. One used ^{125}I-BSA and the other acid-denatured haemoglobin as substrate. The method using haemoglobin was the more appropriate. This being a quick, reasonably sensitive, reliable and technically easy assay.

6. Pepsinogen secretion stimulated by forskolin was not inhibited, at any concentration, by EGF.

Chapter 5

ASPECTS OF THE INHIBITION OF ACID SECRETORY ACTIVITY INDUCED BY ACTIVATION OF PROTEIN KINASE C.

5.1 INTRODUCTION.

Inhibition of the secretory activity of histamine-stimulated parietal cells by potential inhibitors such as PGE_2, somatostatin and EGF involves pertussis toxin sensitive G-proteins (see chapter 3). These inhibitors mediate their action in parietal cells by lowering intracellular cyclic AMP (Schepp *et al.*, 1983b; Hatt and Hanson, 1988). A family of calcium-sensitive phospholipid-dependent protein kinases (protein kinase C) are involved in the regulation of cellular activity in many cell-types (Nishizuka, 1986). Tumour-promoting phorbol esters such as 12-O-tetradecanoylphorbol 13-acetate (TPA) activate protein kinase C (Castagna *et al.*, 1982). TPA, presumably through the activation of protein kinase C (Anderson and Hanson, 1985), inhibits histamine-stimulated secretory activity in the parietal cell (Anderson and Hanson, 1984) and cyclic AMP accumulation (Hatt and Hanson, 1989). It is of interest to determine whether a G-protein sensitive to pertussis toxin mediates this action of TPA.

Protein kinase C appears to act as a receptor for TPA (Niedel *et al.*, 1983; Kikkawa *et al.*, 1983) and prolonged incubation with TPA has resulted in down-regulation of protein kinase C in many cell lines (Adams and Gullick, 1989). The possibility that prolonged preincubation with TPA down-regulates protein kinase C in parietal cells is also of interest, because it would provide a tool for investigating whether the action of inhibitors of acid secretion involved protein kinase C.

A brief review of the major properties and action of protein kinase C is set out below.

5.1.1 Protein kinase C.

5.1.1.1 Isoforms of protein kinase C

Protein kinase C is believed to play a major role in the regulation of a wide variety of cellular processes, such as secretion, membrane receptor function and cell differentiation (Nishizuka, 1986; Berridge, 1987). The molecular diversity of this family of enzymes, termed protein kinase C, was established following the analysis of several complementary DNA clones for protein kinase C and isolation of multiple protein kinase C isoenzymes (Nishizuka, 1988).

Protein kinase C has been separated into three fractions designated types I, II and III by hydroxyapatite chromatography. Types I, II and III protein kinase C have been identified as products of γ, β and α genes respectively (Kikkawa *et al.*, 1987). Due to a difference in the nucleotide sequence for the carboxy-terminus region the ß sequence may be further divided into $ß_I$ and $ß_{II}$. It has been proposed that this

difference is due to alternate splicing of a single gene (Ono *et al.*, 1986). More recently, three further subspecies of protein kinase C have been isolated from the rat brain (δ, ε and ζ). The structure of these subspecies is closely related to, but distinct from the other subspecies (α, $ß_I$, $ß_{II}$ and γ) (Ono *et al.*, 1988). A schematic representation of the structure of protein kinase C is shown in Fig. 5.1.

It has been suggested that some subspecies of protein kinase C are expressed specifically in certain tissue and cells (Table 5.1) (Nishizula, 1988; Ono *et al.*, 1987). Many tissues such as heart, lung and platelets contain several unidentified subspecies of protein kinase C (Pelosin *et al.*, 1987). The different subspecies of protein kinase C exhibit different rates of phosphorylation of substrates such as the EGF receptor from A431 cells (Ido *et al.*, 1987). Ono *et al.* (1987) speculated that the different tissue distribution of the isoforms of protein kinase C suggested different roles for each subspecies, and that if such closely related genes were expressed in a single cell type the distinct enzyme molecules could co-exist and play distinct roles.

It is not certain which subspecies of protein kinase C are present in the rat parietal cell. Consequently the general term protein kinase C will be used although it is not a single enzyme.

5.1.2 Regulation of protein kinase C activity.

5.1.2.1 Activation of isolated protein kinase C.

Protein kinase C activity is modulated by phospholipid, Ca^{2+}, diacylglycerol, certain phorbol esters and arachidonic acid. Activators of protein kinase C such as diacylglycerol and phorbol esters can raise the affinity of protein kinase C for Ca^{2+} and phospholipid (Kishimoto *et al.*, 1980). Thus protein kinase C under certain conditions may be essentially Ca^{2+}-independent.

Diacylglycerol with the 1, 2-*sn* configuration (particularly with one or two unsaturated fatty acids) is effective in activating protein kinase C by enhancing its affinity for Ca^{2+} and phospholipid (Mori *et al.*, 1982). The two sterioisomers 2, 3-*sn*-diacylglycerol, produced by lipoprotein lipase and heparin-releasable lipase, and 1, 3-*sn*-diacylglycerol have no effect on protein kinase C (Boni and Rando, 1985). Therefore a highly specific lipid protein interaction is required for enzyme activation.

Phosphatidylserine seems to be the most effective phospholipid activator of protein kinase C. Phosphatidylethanolamine can enhance this effect and phosphatidylcholine or sphingomyelin can reduce it (Kaibuchi *et al.*, 1981).

Although not carcinogenic themselves phorbol esters enhance the formation of tumours by carcinogenic substances and are thus refered to as tumour promoters. 12-

Figure 5.1

Diagrammatic representation of protein kinase C (Nishizuka, 1988).

The 4 conserved regions of α, β and γ protein kinase C subspecies are C_1 to C_4. δ, ε and ζ-subspecies of protein kinase C lack the second conserved region (C_2). β_I and β_{II}-subspecies differ from each other only in ~50 amino acid residues at the carboxy-terminal end region. The calcium, diacylglycerol and phospholipid binding regions have not been identified with certainty but must be in the C_1 and C_2 regions. Proteolysis by calpain in region A yields a 50-kDa active fragment termed protein kinase M which is not dependent on Ca^{2+}, phospholipid or diacylglycerol for activity (Parker *et al.*, 1986; Young *et al.*, 1988).

Table 5.1

Subspecies of protein kinase C from mammalian tissues (Nishizuka, 1988).

O-tetradecanoylphorbol 13-acetate (TPA) has a structural moiety which is similar to diacylglycerol (Fig. 5.2) and readily intercalates into biological membranes. Thus it is able to emulate the action of diacylglycerol. TPA is the most effective phorbol ester in activating protein kinase C and promoting tumours. In fact there appears to be a direct correlation between the ability of phorbol esters to promote tumours and to activate partially purified protein kinase C (Castagna *et al.*, 1982). TPA reduces the Ca^{2+} requirement for activation of protein kinase C in the presence of phospholipid and so protein kinase C may be activated at resting physiological Ca^{2+} levels (Castagna *et al.*, 1982; Kishimoto *et al.*, 1980).

Part of the effect of TPA is probably due to the fatty acyl groups at positions 12 and 13 as phorbol does not activate protein kinase C. Other parts of the molecule are also important as phorbol 12, 13-didecanoate is an activator of protein kinase C but 4α-phorbol 12,13-didecanoate is totally inactive (Castagna *et al.*, 1982). It is important to note that diacylglycerol is rapidly metabolised and thus activates protein kinase C transiently, whereas TPA and other phorbol esters are hardly degraded and permanently activate protein kinase C (Nishizuka, 1986).

5.1.2.2 Activation of protein kinase C in whole cells.

In most tissue the inactive form of protein kinase C is found mainly in the cytosol. Upon stimulation by hormones or phorbol esters protein kinase C is usually translocated to the membrane in a Ca^{2+}-dependent fashion. Protein kinase C becomes activated when associated with the membrane probably because it comes into contact with phospholipids (Fig. 5.3). This has been shown to occur, for example, with carbachol (Park *et al.*, 1987), gastrin and TPA in canine parietal cells (Chiba *et al.*, 1989a).

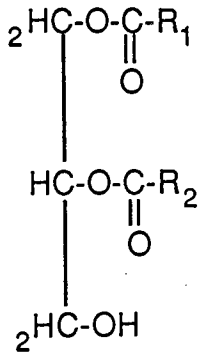
Under physiological conditions protein kinase C is probably activated by 1,2-*sn*-diacylglycerol. This is normally nearly absent from the membranes but is transiently produced as a result of hormonal activation of phospholipase C which leads to the breakdown of phosphoinositides (reviewed by Berridge, 1984; see Fig. 5.4).

5.1.2.3 Down-regulation of protein kinase C.

Early studies indicated that chronic exposure to some phorbol esters lead to the loss or down-regulation of phorbol ester binding sites (Philips and Jaken, 1983). It has been demonstrated more recently that the loss of immunoreactive protein kinase C and extractable protein kinase C activity precisely parallels the phorbol ester induced down-regulation of binding and responsiveness in Swiss 3T3 cells (Stable *et al.*, 1987). Thus protein kinase C appears to be the phorbol ester binding site and mediator of the effects of phorbol esters.

Figure 5.2

A comparison of the structure of TPA (bottom) containing a diacylglycerol-like moiety (dotted area) and diacylglycerol (top). R_1 and R_2 represent hydrocarbon chains of fatty acid.



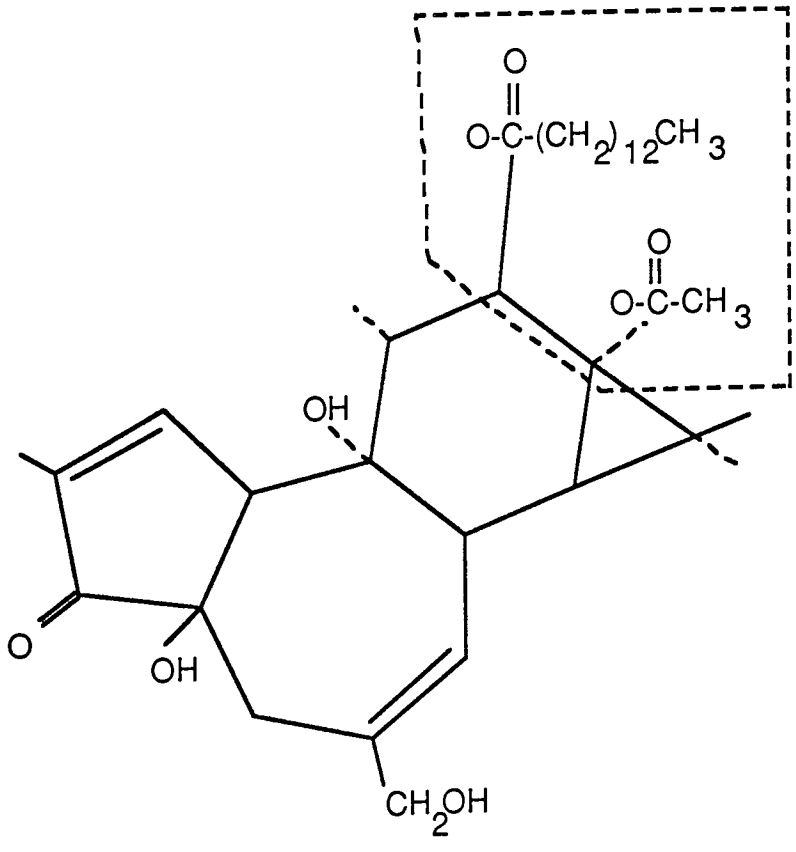
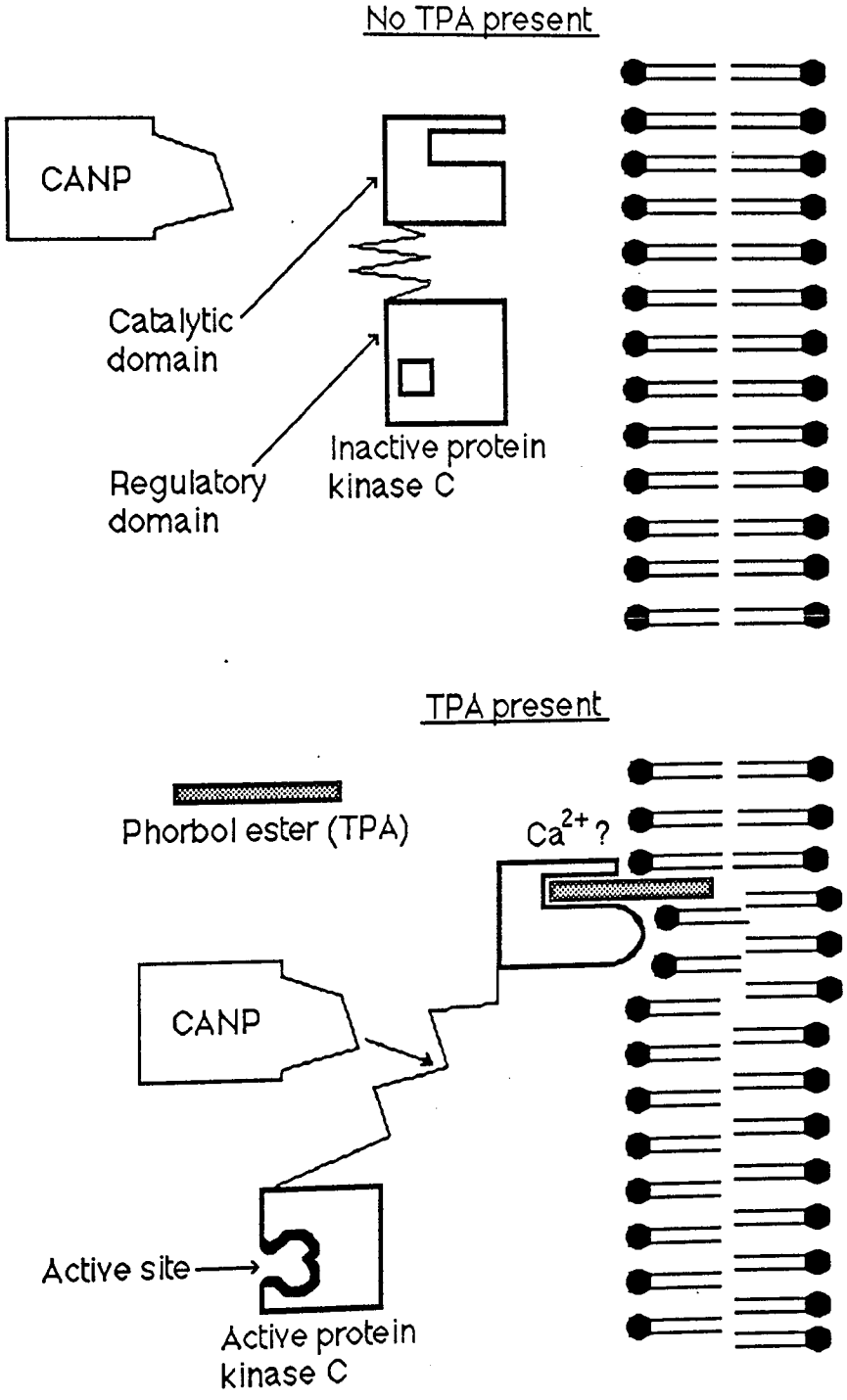


Figure 5.3

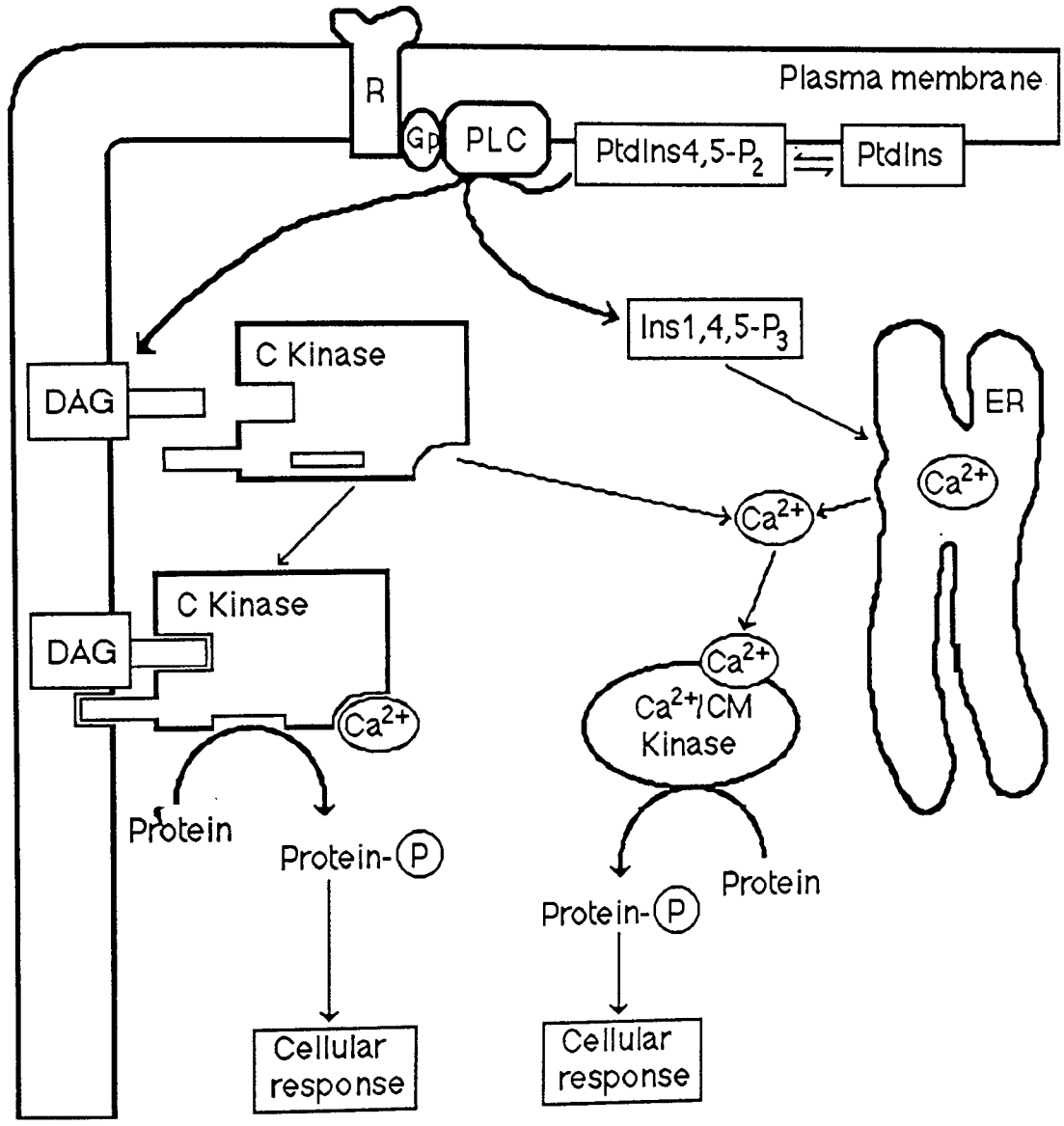
Schematic representation of the activation of protein kinase C.



The phorbol ester TPA probably activates protein kinase C by causing the enzyme to be intercalated into the cell membrane were it comes into contact with phosholipids. The conformational change induced by activation exposes the hinge region between the regulatory and catalytic domains. This region then becomes susceptible to proteolytic attack by the calcium-activated neutral protease (CANP).

Figure 5.4

A simplified diagrammatic representation of the possible role of diacylglycerol and inositol 1,4,5-trisphosphate as second messengers.



Key:

R, receptor
G_p, G-protein
PLC, phospholipase C
PtdIns, phosphatidylinositol
PtdIns4,5-P_2, phosphatidylinositol 4,5-bisphosphate
Ins 1,4,5-P_3, Inositol 1,4,5-trisphosphate
DAG, diacylglycerol
ER, endoplasmic reticulum
C-kinase, protein kinase C
Ca^{2+}/CM Kinase, calmodulin-dependent protein kinase

Interaction of the agonist with the receptor on the cell surface stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate induces the liberation of bound Ca^{2+} from the endoplasmic reticulum, probably by interacting with its own receptor (Streb *et al.*, 1983). The Ca^{2+} may activate calmodulin-dependent protein kinase and other calcium-dependent systems. Diacylglycerol is involved in the activation of protein kinase C. The subsequent phosphorylation of proteins by these two kinases may elicit the biological response to the agonists. In rat parietal cells protein kinase C activity, as stimulated by TPA inhibits acid secretion stimulated by histamine and can feedback on the muscarinic cholinergic receptor (Puurunen *et al.*, 1987 see section 5.1.3).

Continuation of Fig. 5.4.

The above depletion of protein kinase C has been shown to be caused by an increased rate of degradation of protein kinase C. This occurs without any change in mRNA for protein kinase C or in the rates of polypeptide synthesis (Young *et al.*, 1987).The membrane associated form of protein kinase C is susceptible to Ca^{2+}-activated neutral proteinase (Melloni *et al.*, 1986) and so degradation in the presence of phorbol esters is probably due to the membrane association of the kinase induced by these agents. An IgG_{2a} monoclonal antibody which specifically binds protein kinase C in the hinge region, between the catalytic and regulatory domains, inhibited phorbol ester induced down-regulation. Antibody binding was also lost following limited proteolysis suggesting that the epitope was destroyed. These findings are consistent with the agonist induced production of protein kinase M and it is by this mechanism that down-regulation is believed to occur (Young *et al.*, 1988). The rates of down regulation of different isoforms of protein kinase C may vary (Cooper *et al.*, 1989).

5.1.3 The effects of activation of protein kinase C in parietal cells.

The effect of TPA on acid secretion has been reviewed recently by Hatt and Hanson (1989). TPA has variable actions on parietal cell secretory activity which seems to depend on the mode of stimulation and the species from which the cells are taken.

Modulation of parietal cell secretory activity by TPA is probably due to activation of protein kinase C (Anderson and Hanson, 1985). Protein kinase C has been detected in rat (Anderson and Hanson, 1985), rabbit (Chew, 1985), guinea-pig (Biel *et al.*, 1987) and dog (Chiba *et al.*, 1989a) parietal cells. TPA increased the membrane association and activity of protein kinase C in canine parietal cells (Park *et al.*, 1987; Chiba *et al.*, 1989a). Therefore it is believed that TPA activates parietal cell protein kinase C.

5.1.3.1 Stimulatory action of TPA in parietal cells.

Under certain conditions TPA has a stimulatory effect on the secretory activity of parietal cells (Table 5.2). A stimulatory action of TPA on basal aminopyrine accumulation has been observed in some cases but not in rats (Table 5.2). The further stimulation by TPA of rabbit glands stimulated by dbcAMP may be related to the elevation of secretory response to dbcAMP observed in the presence of carbachol (Soll, 1982; Pfeiffer *et al.*, 1987). Low concentrations of TPA, up to 10nM, elevated the secretory activity of rat parietal cells stimulated by 100mM-K^+. The stimulatory action of 100mM-K^+, which is thought to act near to the site of the K^+/H^+-ATPase, does not involve the conversion of tubulovesicles to secretory canaliculi. The stimulatory effect of TPA on this response does not involve cyclic AMP (Hatt and

Table 5.2

Stimulatory action of TPA on parietal cell secretory activity.

Species	Preparation	Secretagogue	Action of TPA	Reference
Rabbit	Gastric gland	None Carbachol Forskolin DbcAMP	Stimulation Stimulation* Stimulation* Stimulation	Brown and Chew (1987)
Dog	Enriched isolated cell	None	Stimulation	Chiba *et al.* (1989)
Rat	Enriched isolated cell	Carbachol	Stimulation (at 1-5nM)	Pfeiffer and Noelke (1988)
Rat	Isolated cells crude and enriched preparations	100mM-K^+	Stimulation (up to 10nM)	Hatt and Hanson (1989)

Key * = Transient stimulation.

Hanson, 1989). TPA has also further stimulated aminopyrine uptake in parietal cells stimulated with carbachol under one particular condition (Table 5.2).

5.1.3.2 Inhibitory action of TPA in parietal cells.

TPA has an inhibitory action on parietal cell secretory activity stimulated by various secretagogues (Table 5.3). Aminopyrine accumulation stimulated by carbachol was inhibited by TPA in rat parietal cells (Anderson and Hanson, 1984) and in rabbit gastric glands (Brown and Chew, 1987; Muallem *et al.*, 1986). TPA also decreased carbachol-stimulated inositol trisphosphate production in an enriched rat parietal cell preparation (Puurunen *et al.*, 1987a). Chiba *et al.* (1989a) indicated that binding of carbachol and gastrin to receptors present on canine parietal cells may be substantially decreased by preincubation with TPA for 1 hour. Incubation with TPA had no effect on binding affinity and a reduction in receptor number was therefore suggested.

Aminopyrine accumulation stimulated by dbcAMP was inhibited by TPA in rat parietal cells (Anderson and Hanson, 1984; see Table 5.3). The potency of TPA as an inhibitor of secretory activity stimulated by dbcAMP was similar to that obtained when cells were stimulated by histamine. Biel *et al.* (1987) observed a similar effect of TPA on guinea-pig parietal cells. In contrast to this TPA enhanced the action of submaximally effective concentrations of dbcAMP in rabbit gastric glands. These findings may be construed to suggest that activated protein kinase C inhibits rat parietal cells at a site which is located after cyclic AMP production and hydrolysis but before the K^+/H^+-ATPase. In rabbit parietal cells this site seems to be missing (Hanson and Hatt, 1989).

Protein kinase C activation by TPA results in a potent inhibition of secretory activity stimulated by histamine and IBMX in rat parietal cells (Anderson and Hanson, 1984 and 1985). It is likely that TPA exerts its action by a direct action on the parietal cell rather than on another cell type. The effect of TPA on aminopyrine accumulation stimulated by histamine produced a curve which fitted closely to a classical dose-response curve, and there was no relationship between cell concentration and the action of TPA (Anderson and Hanson, 1985). This supports the theory that TPA has a direct action. TPA stimulates somatostatin release from the perfused rat stomach (Yamatani *et al.*, 1985) and from D-cells in culture (Sugano *et al.*, 1986). PGE_2 release is also stimulated by TPA in human gastric cells (Schepp *et al.*, 1987). However it is unlikely that TPA mediates its inhibitory action, on histamine-stimulated rat parietal cells, by somatostatin or PGE_2. This is because inhibition of prostaglandin production did not prevent the action of TPA on histamine-stimulated aminopyrine accumulation (Anderson and Hanson, 1984) or cyclic AMP production (Hatt and Hanson, 1989) in the rat. A somatostatin antagonist, cyclo(7-aminoheptanoyl-Phe-D-

Table 5.3

Inhibitory action of TPA on parietal cell secretory activity.

Species	Preparation	Secretagogue	Action of TPA	Reference
Rabbit	Gastric glands	Carbachol Forskolin Histamine Cholera toxin	Inhibition* Inhibition* Inhibition Inhibition	Brown and Chew (1987)
Dog	Enriched isolated cell	Carbachol Gastrin	Inhibition Inhibition	Chiba *et al.* (1989)
Rat	Enriched isolated cell	Carbachol DbcAMP	Inhibition at 20-100nM-TPA Inhibition	Pfeiffer and Noelke (1988)
Rat	Enriched isolated cell	None Histamine DbcAMP	No effect Inhibition Inhibition	Ostrowiski and Bromsztyk (1989)
Rat	Isolated cell	None Histamine+ IBMX Carbachol DbcAMP	No effect Inhibition Inhibition Inhibition	Anderson and Hanson (1984)

Key: * = Inhibition following transient stimulation.

Tryp-Lys-Thr(Bzl)), also did not inhibit the effect of TPA on histamine-stimulated cyclic AMP. Furthermore somatostatin and PGE_2, in contrast with TPA, do not appear to inhibit dbcAMP-stimulated aminopyrine accumulation in parietal cells.

TPA, as well as acting at a site downstream from the production and breakdown of cyclic AMP, appears to act at a site near to adenylate cyclase. Increases in cyclic AMP generated by histamine in parietal cells are inhibited by TPA (half maximal effective concentration of 3nM) (Hatt and Hanson, 1989). Preincubation with TPA also inhibited histamine-stimulated adenylate cyclase activity in rat parietal cells (Hanson and Hatt, 1989).

The aim of the work descibed in this chapter was to determine whether a G-protein sensitive to pertussis toxin was involved in the inhibitory action of TPA on secretory activity stimulated by histamine in rat parietal cells. Another objective of this chapter was to determine if long-term preincubation with TPA could abolish the inhibitory action of TPA on histamine-stimulated rat parietal cells, and whether this effect could be coupled with any detectable alteration in immunodetectable protein kinase C. As mentioned previously, if protein kinase C were to prove susceptible to down-regulation then this procedure would be extremely useful in identifying agents which required functional protein kinase C for activity.

5.2 METHODOLOGY.

5.3.2 Preparation of reagents and media.

Pertussis toxin, histamine and IBMX were prepared as in section 3.2.1. TPA was dissolved in dry DMSO to a concentration of 16mM and stored at -20°C. For each experiment the TPA stock was thawed at room temperature and serially diluted in DMSO to the required concentrations. A small volume (2µl) was added to each vial. DMSO was also added to control vials so that the final concentration was 0.125% ($^{v}/_{v}$) in all vials.

In this section the media were either medium B' (Table 2.1), with specified additions where appropriate, or a mixture of Ham's F-12 and DMEM (pH 7.4) with BSA (2mg/ml).

Insulin (3mg) when added to the medium was dissolved in 1ml of 0.01M-HCl and then diluted by the addition of 4ml of medium B'. Then 133µl of this solution was added to each 10ml of incubation medium for a final concentration of 8µg/ml. Other additions included foetal calf serum (0.5ml per 10ml of medium) and hydrocortisone (10nM) which was dissolved in the medium.

5.2.2 The effect of pertussis toxin on the inhibitory action of TPA on aminopyrine accumulation stimulated by histamine plus IBMX.

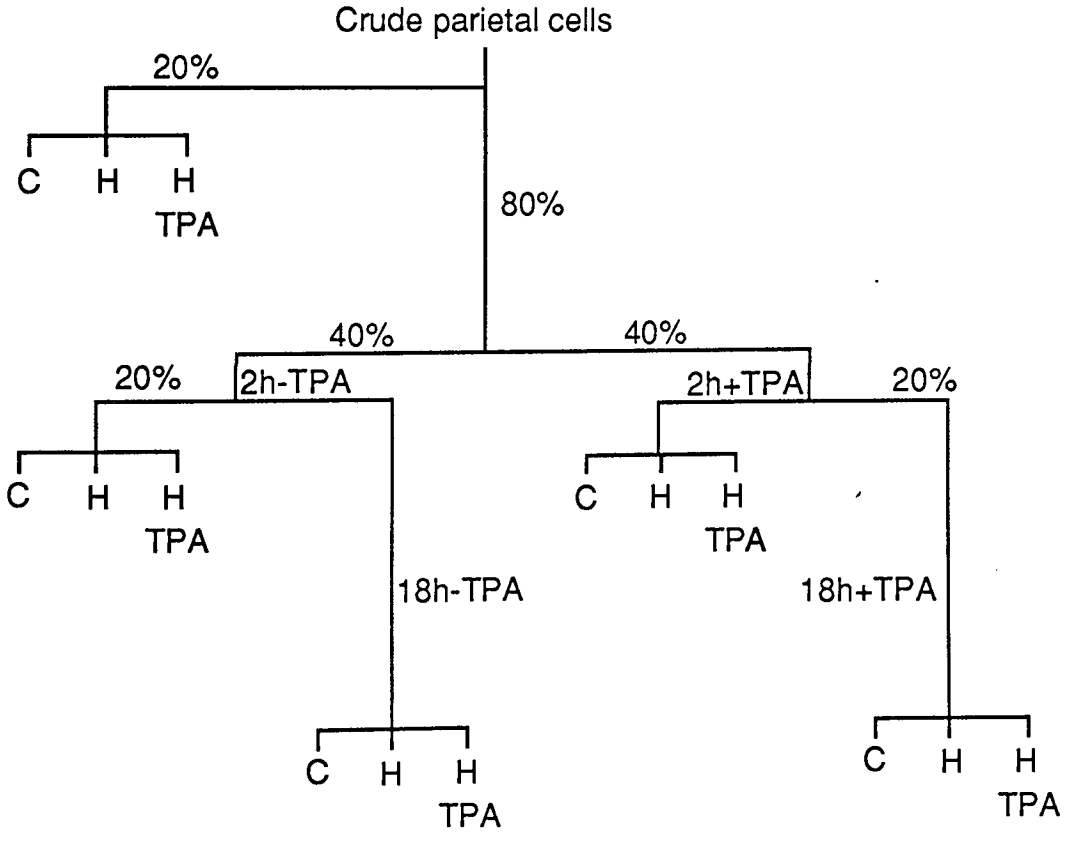
Cell suspensions containing approximately 20% parietal cells were preincubated with pertussis toxin and then incubated with secretagogues (histamine plus IBMX) and the inhibitor (TPA) as described in section 3.2.2. The aminopyrine accumulation ratio was estimated as in section 2.3.

5.2.3 The effect of the time of preincubation with TPA (100nM) on aminopyrine accumulation stimulated by histamine plus IBMX and the subsequent inhibitory action of TPA (100nM) on this response.

The experimental protocol is illustrated in Fig. 5.5. A pool of cells containing approximately 20% parietal cells was split into two portions. One portion (20% of the cells) was washed an extra two times in medium B' (centrifugation for 5 minutes at 175g and 5°C), resuspended in medium B' at a final concentration of 2-5x10^6 cells/ml and aliquots (1.5ml) incubated with and without histamine (0.5mM) plus IBMX (0.1mM) for 30 minutes for the determination of the aminopyrine accumulation ratio (section 2.3).

Figure 5.5

Illustration of the experimental protocol for the determination of the effect of time of preincubation with TPA (100nM) on aminopyrine accumulation stimulated by histamine (0.5mM) plus IBMX (0.1mM), and the subsequent inhibitory action of TPA (100nM) on this response.



Key:- C, control; H, histamine (0.5mM) plus IBMX (0.1mM); TPA, 12-*O*-tetradecanoylphorbol 13-acetate (100nM).

The other portion of cells (80% of the cells) was resuspended in medium B' with gentamicin (50μg/ml), insulin (8μg/ml), hydrocortisone (10nM) and foetal calf serum (5%($^{v}/_{v}$)) and split into two, one with and the other without TPA (100nM). These cells were preincubated at 37°C in a shaking waterbath (76 cycles/minute) for 2 hours and were constantly gassed with a slow stream of 95%O_2/5%CO_2.

After 2 hours half the cell pool from each treatment (with and without TPA) was removed, washed twice in medium B' and incubated as above both with and without TPA for 30 minutes for the determination of the aminopyrine accumulation ratio.

The remaining cells were diluted with preincubation medium with and without TPA (100nM) to ensure there was a sufficient volume of medium in each flask and then incubated as above for a further 18 hours. After a total of 20 hours incubation the cells were washed twice in medium B', incubated as above with and without TPA for the determination of the aminopyrine accumulation ratio.

5.2.4 The effect of preincubation with TPA (1μM) on the aminopyrine accumulation ratio stimulated by histamine plus IBMX and the subsequent action of TPA (100nM) on this response.

The experimental protocol was the same as in section 5.2.3 except that the parietal cell response to histamine plus IBMX before preincubation was omitted and preincubation was with 1μM-TPA not 100nM-TPA. Preincubation with TPA (1μM) was for 2 and 24 hours.

5.2.5 The effect of culture on matrigel with TPA (1μM) for 24 hours on the content of protein kinase C in an enriched parietal cell fraction.

A purified parietal cell preparation containing 82% parietal cells was prepared from rats as in section 2.2.2. Subsequent to isolation, the cells were incubated in medium B' with gentamicin (50μg/ml) and amphotericin B (25μg/ml) for 10 minutes. Then the cells were washed 3 times under sterile conditions in a 1:1 mixture of Ham's F-12 and Dulbecco's Minimal Essential Medium (DMEM) (pH 7.4) containing BSA (2mg/ml), gentamicin (50μg/ml), EGF (8nM), hydrocortisone (10nM), insulin (800nM) and transferrin (5μg/ml). For culture, cells were resuspended in the above medium both with and without TPA (1μM) at a density of $1x10^5$ cells/ml and plated onto 50mm tissue culture dishes which had been precoated with Matrigel. Dishes were coated with 0.5ml of Matrigel that had been diluted 1:7 with ice cold-sterile water and allowed to dry overnight in a tissue culture hood. Cell suspensions (4ml per dish)

were incubated in a humidified air incubator in air atmosphere. Bicarbonate was not added to the tissue culture medium when it was reconstituted from powder as Chew *et al.* (1989), from whom this protocol was derived, suggested that 20-25mM-bicarbonate was toxic to parietal cells in culture and omission of bicarbonate had no adverse effects on enriched parietal cells.

After overnight culture the unattached cells were removed and washed in medium B'. The adherent cells were collected by trypsinisation using 1.385ml of trypsin solution (0.05%($^{w}/_{v}$)-trypsin with 0.02%($^{w}/_{v}$)-EDTA in Ca^{2+} and Mg^{2+}-free phosphate buffered saline (Flow Laboritories)). Dishes containing the trypsin solution were left at 37°C for 2 minutes and then agitated for 8 minutes using a Luckhams shaker at room temperature. The dish was washed twice with medium B' plus STI (0.1mg/ml). The adherent cells were washed in medium B'. Just over half the cells were attached so the attached and unattached cells were pooled, so that sufficient cells were available for the experiment, and centrifuged for 5 minutes at 175g and 5°C. Supernatants were discarded and the cell pellets resuspended in ice-cold Eagle's Minimum Essential Medium (EMEM) with HEPES (20mM) and bicarbonate (25mM) (pH 7.4) to a concentration of $5x10^5$cells/ml.

Aliquots of $5x10^5$cells were placed into microfuge tubes and centrifuged for 15 seconds at 12,000g. The supernatant was then discarded and the remaining cells prepared for SDS-polyacrylamide gel electrophoresis as described in section 5.2.6.2.

5.2.6 Immunodetection of cellular protein kinase C.

5.2.6.1 Preparation of cell samples for electrophoresis.

Cells pelleted in microfuge tubes ($5x10^5$cells per tube) were resuspended in boiling sample buffer (Table 5.4) which had been diluted 1:1 with double-distilled water. Samples were sonicated for 6x5 second bursts at 15W with 5 second intervals between each burst (Soniprep Sonicator). Then the sample was heated in a beaker of boiling water for 5 minutes before being allowed to cool to room temperature. The sample was then stored at -20°C until electrophoresis was performed.

5.2.6.2 Electrophoresis.

Samples were resolved on 8%-SDS-polyacrylamide gels which were prepared one day before use. The ingredients for 20ml of separating gel, sufficient for one gel, are shown in Table 5.5. The ingredients were stirred and 7.5µl of tetramethylethylenediamine added. This solution was introduced between two glass plates followed by a 1cm overlay of a 1:1 mixture of double-distilled water and methanol. While the separating gel was setting the main constituents of the stacking

Table 5.4

Preparation of sample buffer (4ml) for electrophoresis.

Reagent	Volume or weight	Final concentration
Tris (0.5M) (pH 6.8)	1.0ml	0.125M
SDS (10% ($^{w}/_{v}$))	1.6ml	4% ($^{w}/_{v}$)
Double-distilled water	0.4ml	
Glycerol	0.8ml	20% ($^{w}/_{v}$)
DTT	123mg	0.2M
Bromophenol blue (0.5mg/ml)	0.2ml	25µg/ml

Table 5.5

The composition of the separation and stacking gels used in electrophoresis.

Stock reagent	8% separating gel (20ml)		3% stacking gel (10ml)	
	Volume (ml)	Final concentration	Volume (ml)	Final concentration
Tris (1.5M) (pH 8.8)	5.0ml	0.375M		
Tris (0.5M) (pH 6.8)			2.5	0.125M
SDS (10% ($^{w}/_{v}$))	0.2	0.1% ($^{w}/_{v}$)	0.1	0.1% ($^{w}/_{v}$)
Acrylamide (30% ($^{w}/_{v}$)) plus Bis (0.8% ($^{w}/_{v}$))	5.34	8% ($^{w}/_{v}$)	1.0	3% ($^{w}/_{v}$)
Double-distilled water	9.36		6.35	
Fresh ammonium persulphate (0.1g/ml)	0.10	0.5mg/ml	0.05	0.5mg/ml

gel were prepared and mixed (Table 5.5). Once the separating gel was set, the overlay was removed. Tetramethylethylenediamine (5µl) was then added to the stacking gel solution, briefly mixed and then introduced above the separating gel. A comb was inserted to produce the loading wells, followed by an overlay of the 1:1 mixture of double-distilled water and methanol. Once polymerisation was complete the comb and overlay were removed. Then the stacking gel was overlayed with Tris (0.125M) containing SDS (0.1%($^{v}/_{v}$)) pH 6.8. The gel was covered with parafilm to prevent evaporation and left overnight. Before loading, the wells of the stacking gel were emptied and refilled with cold running buffer and the upper reservoir attached to the top of the gel plates. Running buffer was composed of Tris (25mM), glycine (0.2M) and SDS (0.1%($^{w}/_{v}$))

A positive control of mouse immunoglobin, supplied in a blotting detection kit from Amersham, was diluted 1:100 into sample buffer (itself diluted 1:1 with water) and then boiled for 2 minutes before loading onto the gel. Rainbow molecular weight markers (Mr 14,300-200,000) (10µl) were diluted with sample buffer (10µl) and boiled for 1 minute before loading onto the gel.

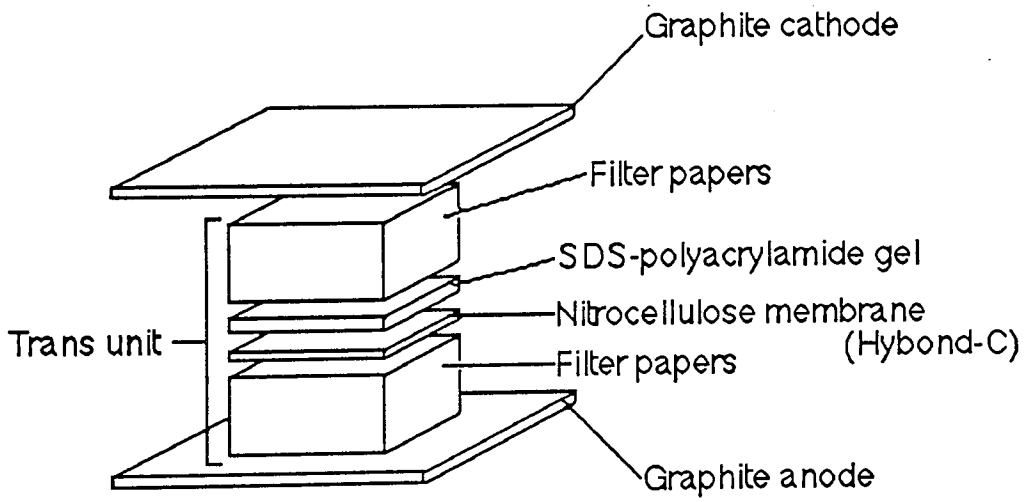
Cell samples were allowed to thaw at room temperature, mixed and centrifuged at 10,000g for 5 seconds to remove debris. Then 50µl of each sample was loaded into a separate well. The Rainbow molecular weight markers and the positive control mouse immunoglobulin were loaded adjacent to the samples. Then the gel with upper reservoir was loaded into the main tank containing running buffer. Running buffer was poured into the upper reservoir until the electrode was covered. Electrophoresis was carried out at 10°C and at a constant current of 25mA with a maximum voltage of 500V. The bromophenol blue tracking dye normally reached the bottom of the gel after 3-4 hours.

5.2.6.3 Western blotting.

Proteins separated on the polyacrylamide gels were electrophoretically transferred (blotted) onto nitrocellulose membranes (Hybond C). Trans units were constructed of filterpapers and immobilising membrane which were cut to the same size as the gel and soaked in electrode solution [composed of Tris (48mM), glycine (39mM), SDS (0.0375%($^{w}/_{v}$)) and methanol (20%($^{v}/_{v}$))] (Fig. 5.6). The surface of the graphite anode plate was soaked with double-distilled water and 9 filterpapers soaked in electrode solution were placed onto the surface of this electrode. The nitrocellulose membrane, presoaked in electrode solution, was carefully placed on top of the filterpaper and the gel on top of this. Care was taken to ensure that all airbubbles between the gel and immobilising membrane were expelled. The trans unit was completed after 6 more filter papers were soaked in electrode solution and placed on

Figure 5.6

Diagrammatic representation of the composition of a trans-unit. (LKB-semi-dry blotting system).



top of the gel. The graphite cathode plate, which was soaked with double-distilled water, was then placed on top of the trans unit and the current set at 0.8mA/cm^2. After 1 hour substantial transfer of coloured marker proteins onto the nitrocellulose membrane was evident.

5.2.7.4 Immunodetection.

Reagents were prepared as instructed in the booklet supplied with the detection kit. To prevent non-specific binding of the detection reagents to the nitrocellulose membrane the membrane was blocked overnight by immersion in Tris buffered saline (pH 7.5) with Tween-20 (0.2%($^v/_v$)) (TBS-T) containing 5 or 10% dried de-fatted milk at 4^{o}C. Then the nitrocellulose membrane was washed 2 times in TBS-T for 1 minute and then 3 times in TBS-T for 5 minutes, at room temperature with shaking. All subsequent incubations were at room temperature with shaking. The membrane was then incubated for 1 hour with mouse monoclonal anti-protein kinase C (Amersham), which was diluted in Tris buffered saline (pH 7.5) (TBS). Only the α and β (β_1 and β_2) species of protein kinase C are recognised by this antibody. A region in the hinge domain, close to the site of trypsin cleavage, is recognised by this antibody.

After incubation with the primary antibody the membrane was washed and then incubated for 30 minutes with the second antibody which was also diluted in TBS. This was an affinity purified biotinylated anti-mouse IgG (polyclonal). Then the membrane was washed once again and incubated for 30 minutes with a streptavidin-alkaline phosphatase conjugate. Streptavidin is a cell wall protein from *Streptomyces avidinii* which has very high affinity for biotin. Thus the streptavidin-alkaline phosphatase conjugate binds to the secondary antibody. The membrane was then washed once more and incubated with the substrates for alkaline phosphatase, nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in diethanolamine buffer. After 15 to 30 minutes the alkaline phosphatase generated a high contrast purple colour by acting upon the substrates provided. Once development had occurred, the membrane was washed as before only in double-distilled water. The membrane was then dried between sheets of filterpaper and stored.

5.3 RESULTS AND DISCUSSION.

5.3.1 Effect of preincubation with pertussis toxin on the action of TPA.

Parietal cell secretory activity stimulated by histamine (0.5mM) plus IBMX (0.1mM) was inhibited by TPA (Fig 5.7). This result is in agreement with data obtained previously (Anderson and Hanson, 1984; Ostrowski and Bromsztyk, 1989). Near-maximal inhibition occured at 10^{-7}M-TPA and resulted in 87 ± 3% inhibition. The half-maximal inhibitory concentration of TPA was 7 ± 4nM which was slightly higher than that of 3.3nM found by Anderson and Hanson (1985).

Preincubation with pertussis toxin significantly reduced the inhibitory action of 10^{-8} and 10^{-7}M-TPA on aminopyrine accumulation (Table 5.6 Fig. 5.7). The reduction being 25% at 10^{-8}M-TPA and 12% at 10^{-7}M-TPA. This small inhibitory effect of pertussis toxin on the action of TPA was not observed in rabbit gastric glands (Brown and Chew, 1987). Brown and Chew (1987) preincubated their glands with TPA (100nM) for 30 minutes before a 45 minute challenge with histamine. In this work cell suspensions were added to histamine and TPA simultaneously and then incubated for 30 minutes. The lack of agreement could be a consequence of differences in experimental protocol.

By contrast with the effects of pertussis toxin on the inhibitory action of PGE_2, somatostatin and EGF (sections 3.3.3, 3.3.4 and 3.3.6) TPA still had a significant inhibitory action on the aminopyrine accumulation ratio in rat parietal cells that had been preincubated with pertussis toxin ($P<0.01$, by ANOVAR). Therefore, the inhibitory action of TPA may involve a G-protein sensitive to pertussis toxin, but this putative mechanism seems to be responsible for only a small part of the inhibitory effect.

As mentioned before in rat parietal cells it has been suggested that TPA mediates its inhibitory action on two sites, on cyclic AMP content and at another site downstream from adenylate cyclase (Hatt and Hanson, 1989). One possibility is that preincubation with pertussis toxin may block the action of TPA on cyclic AMP levels but, because the action of TPA on the second site was unaffected, only a small reduction of the inhibitory effect of TPA was observed. However, there was no evidence that preincubation with pertussis toxin substantially prevented the inhibitory effect of TPA on stimulation by histamine of either cyclic AMP content (in intact cells) or adenylate cyclase (in a membrane preparation) (Hatt, J.F. and Hanson, P.J., unpublished work).

Table 5.6

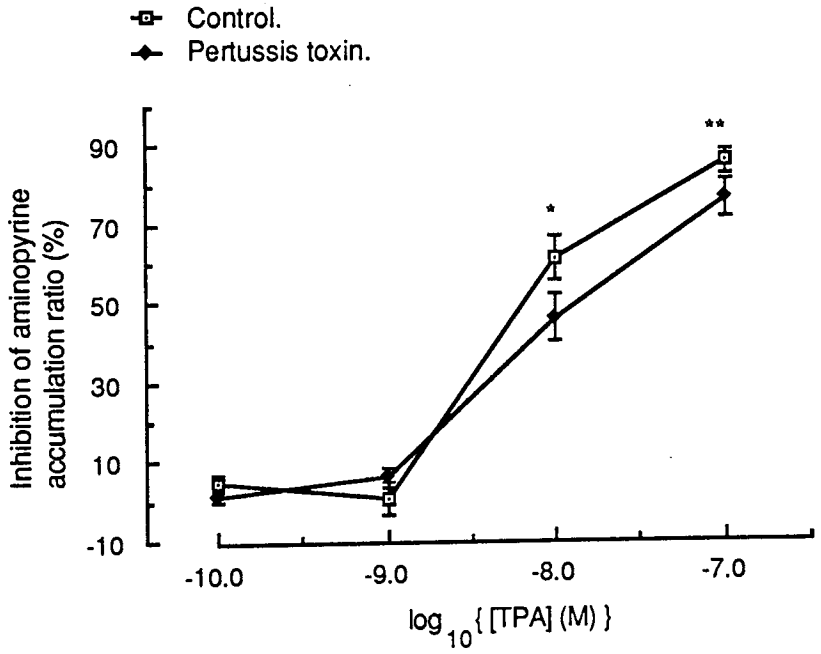
The effect of preincubation for 2 hours with pertussis toxin (100ng/ml) on the inhibitory action of TPA on the aminopyrine accumulation ratio (APR) stimulated by histamine (0.5mM) and IBMX (0.1mM).

Concentration of TPA(M)	APR Control	APR Pertussis toxin
0	144.0 ± 19.8	152.9 ± 27.2
10^{-10}	136.4 ± 16.9	149.7 ± 25.7
10^{-9}	144.9 ± 23.7	142.2 ± 24.3
10^{-8}	54.2 ± 8.5	83.2 ± 18.8
10^{-7}	21.2 ± 7.9	39.5 ± 15.4

Results from 5 experiments using batches of cells containing 21.5 ± 0.8 % parietal cells are expressed as means ± S.E.M. There was a significant inhibitory effect of TPA in the presence of pertussis toxin against histamine plus IBMX stimulated parietal cells ($P<0.01$ by ANOVAR).

Figure 5.7

Effect of preincubation with pertussis toxin (100ng/ml) for 2 hours on the inhibitory action of TPA on aminopyrine accumulation stimulated by histamine (0.5mM) and IBMX (0.1mM).



Data from Table 5.7 have been normalised and expressed as percentage inhibition by TPA of the aminopyrine accumulation ratio. The effect of preincubation with pertussis toxin has been analysed by a paired *t* -test. $^{**}P<0.01$; $^{*}P<0.05$.

Protein kinase C appears to be able to phosphorylate G-proteins in their inactive form (Sagi-Eisenberg, 1989). This has been suggested to be the case with G_i and the effect of phosphorylation was suggested to be the attenuation of the inhibitory action of G_i on adenylate cyclase (Houslay *et al.*, 1990). The above data suggests that if protein kinase C induced phosphorylation of G-proteins occurs it results in the inhibition of adenylate cyclase and does not the prevent any negative action of G_i on adenylate cyclase. Thus in rat parietal cells TPA may induce the phosphorylation of an inhibitory G-protein and activate it or alternatively induce the phosphorylation of a stimulatory G-protein and inhibit its effects on adenylate cyclase.

5.3.2 Down-regulation of protein kinase C.

5.3.2.1 The effect of preincubation time with TPA (100nM) on the aminopyrine accumulation ratio stimulated by histamine plus IBMX and the subsequent inhibitory action of TPA (100nM) on this response.

Parietal cell secretory activity was stimulated by histamine (0.5mM) plus IBMX (0.1mM) in the presence and absence of TPA at 0, 2 and 20 hours (Table 5.7). The histamine-stimulated aminopyrine accumulation ratio was raised from 41.2 ± 0.78 to 71.5 ± 5.6 after 2 hours preincubation. Preincubation of parietal cells for 2 hours also appears to reduce the half-maximally effective concentration of EGF (section 3.3.6). This may be because the cells were allowed to recover from the isolation procedure. After 20 hours the aminopyrine accumulation ratio stimulated by histamine decreased to 11.9 ± 0.53. The reason for this was probably the drop in parietal cell viability, as estimated by the exclusion of trypan blue, from 90% at 2 hours to 45% at 20 hours.

Preincubation with TPA for 2 hours, followed by 2 washes in medium B', reduced aminopyrine accumulation stimulated by histamine to below basal levels. Thus it appears that the effects of TPA were not removed by washing the cells. A similar effect of TPA was observed by Brown and Chew (1987) where preincubation with TPA for 3 minutes followed by washing did not remove the action of TPA on rabbit glands stimulated by histamine. Incubation with histamine and TPA also resulted in a similar aminopyrine accumulation ratio which was below basal levels. Clearly no down-regulation of the effects of TPA was observed after 2 hours.

Although the aminopyrine accumulation ratios were lower after 20 hours preincubation with TPA, results were similar to those obtained after 2 hours of preincubation. Thus preincubation with TPA decreased secretory activity stimulated by histamine to below basal levels whether or not TPA was present during the incubation with aminopyrine. The critical result is the lack of any stimulatory effect of histamine

Table 5.7

Effect of preincubation time with TPA (100nM) on the histamine (0.5mM) plus IBMX (0.1mM) stimulated aminopyrine accumulation ratio (APR) and the subsequent inhibitory action of TPA on this response.

Preincubation time (hours)	Pretreatment	APR Basal	APR Histamine + IBMX	APR Histamine + IBMX + TPA (100nM)
0	Control	2.6 ± 0.2	42.0 ± 0.87	
2	Control	2.5 (2.3, 2.7)	71.6 ± 5.6	2.9 ± 0.3
2	TPA	2.3 (2.4, 2.2)	0.72 ± 0.05	0.89 ± 0.05
20	Control	2.3 (2.4, 2.3)	11.9 ± 0.53	2.9 ± 0.2
20	TPA	3.2 (3.2, 3.2)	0.64 ± 0.26	0.45 ± 0.15

Cells were preincubated in medium B' plus gentamycin (50μg/ml), insulin (8μg/ml), hydrocortisone (10nM) and foetal calf serum (5% ($^{v}/_{v}$)) for 2 or 20 hours both with and without TPA (100nM). Subsequently cell suspensions were incubated for 30 minutes in medium B'. The aminopyrine accumulation ratio are presented as either means ± S.E.M., or as means with values in parentheses. Results are from a single batch of cells containing 22% parietal cells.

plus IBMX after 20 hours exposure to TPA. Removal of TPA from the cells had clearly not been achieved by washing. However, if protein kinase C had been down-regulated then this residual TPA should have been ineffective. These results suggest that substantial down-regulation of protein kinase C by TPA (100nM) did not take place after 2 and 20 hours of exposure.

The rates of down-regulation vary quite considerably between cell types (Adams and Gullick, 1989) and it is possible that down-regulation is taking place in parietal cells but at a slower rate. As cell viability was falling it was not considered feasible to increase greatly the time of preincubation with TPA, so the effects of a higher phorbol ester concentration (1μM-TPA) were examined. Higher phorbol ester concentrations usually increase the rate of down-regulation (Dale *et al.*, 1989).

5.3.2.2 Effect of preincubation with TPA (1μM) on the aminopyrine accumulation ratio stimulated by histamine plus IBMX and the subsequent inhibitory action of TPA (100nM) on this response.

Similar results to the previous section were obtained with cells preincubated with 1μM-TPA for 2 hours. Preincubation with 1μM-TPA decreased the secretory response to histamine with and without TPA (100nM) to below basal levels (Table 5.8).

In this experiment the control, histamine-stimulated aminopyrine accumulation ratio after 24 hours, was much lower than after 20 hours (section 5.3.2) and close to basal levels. However, TPA inhibited this response by 77.6% which was similar to that of 75.6% obtained with 20 hours preincubation (section 5.3.2). Preincubation with 1μM-TPA reduced the aminopyrine accumulation ratio in response to histamine to below basal levels, whether or not TPA was added during incubation with aminopyrine. The parietal cell viability dropped from 93% after 2 hours to 38% after 24 hours preincubation. No substantial down-regulation of protein kinase C by a high concentration of TPA occurred after 24 hours.

In both the experiments described so far parietal cells were maintained in suspension in the presence of gentamicin, but in otherwise non-sterile conditions. Lack of attachment of the parietal cells to a matrix or the conditions of culture were considered as potential contributors to the lack of any apparent down-regulation of protein kinase C. Therefore the down-regulation of protein kinase C was investigated by culturing parietal cells on matrigel as described by Chew *et al.* (1989). Immunological detection of protein kinase C was attempted on these cells as it is a more direct method and may reveal a degree of down-regulation of protein kinase C

Table 5.8

Effect of preincubation with TPA (1μM) on the aminopyrine accumulation ratio (APR) stimulated by histamine (0.5mM) plus IBMX (0.1mM) and the subsequent inhibitory action of TPA (100nM) on this response.

Preincubation time (hours)	Pretreatment	APR Basal	APR Histamine + IBMX	APR Histamine + IBMX + TPA
2	Control	4.9 (4.7, 5.0)	158.2 ± 5.0	9.0 ± 0.45
2	TPA	4.9 (4.7, 5.1)	1.5 ± 0.14	1.3 ± 0.13
24	Control	2.0 (2.1, 2.0)	2.3 ± 0.13	0.52 ± 0.22
24	TPA	2.2 (2.1, 2.3)	-0.20 ± 0.030*	-0.44 ± 0.18*

Cells were either preincubated in medium B' with gentamycin (50μg/ml), insulin (8μg/ml), hydrocortisone (10nM) and foetal calf serum (5% ($^{v}/_{v}$)) for 2 or 20 hours both with and without TPA (1μM).The aminopyrine accumulation ratio are presented as either means ± S.E.M., or as means with values in parentheses. Results are from a single batch of cells containing 23% parietal cells.

* Negative results for APR can be obtained if the intracellular amount of aminopyrine is very small in relation to the amount of aminopyrine outside the pellet and if the latter through error is slightly overestimated.

that would not affect the inhibitory action of TPA. Clearly, enriched parietal cells had to be used for these experiments.

5.3.2.4 Immunological detection of protein kinase C and validation of methodology.

Rat parietal cell protein kinase C was successfully detected by polyacrylamide gel electrophoresis, western blotting and immunodetection. Both mouse IgG bands were visible (lane1) as was a strong band at ~80-kDa which was presumed to be protein kinase C (see Fig. 5.8). Note that background staining was quite strong and in subsequent experiments the nitrocellulose membrane was blocked in 10% dried milk blocking agent, opposed to 5% as used here.

A549 human lung carcinoma cells were cultured in a humidified air (95%)/CO_2 (5%) incubator. Cells which were 75-95% confluent were preincubated with and without TPA (10nM) for 24 hours. These cells were prepared for gel electrophoresis and then the protein kinase C levels were determined as in section 5.2.7. There was a visible difference between cells which had been preincubated with and without TPA on the western blot (Fig. 5.9). Protein kinase C appears to have been down-regulated in cells pretreated with TPA. All experiments with A549 cells were carried out by Tracey D. Bradshaw. Thus down-regulation of protein kinase C was detectable using the above method.

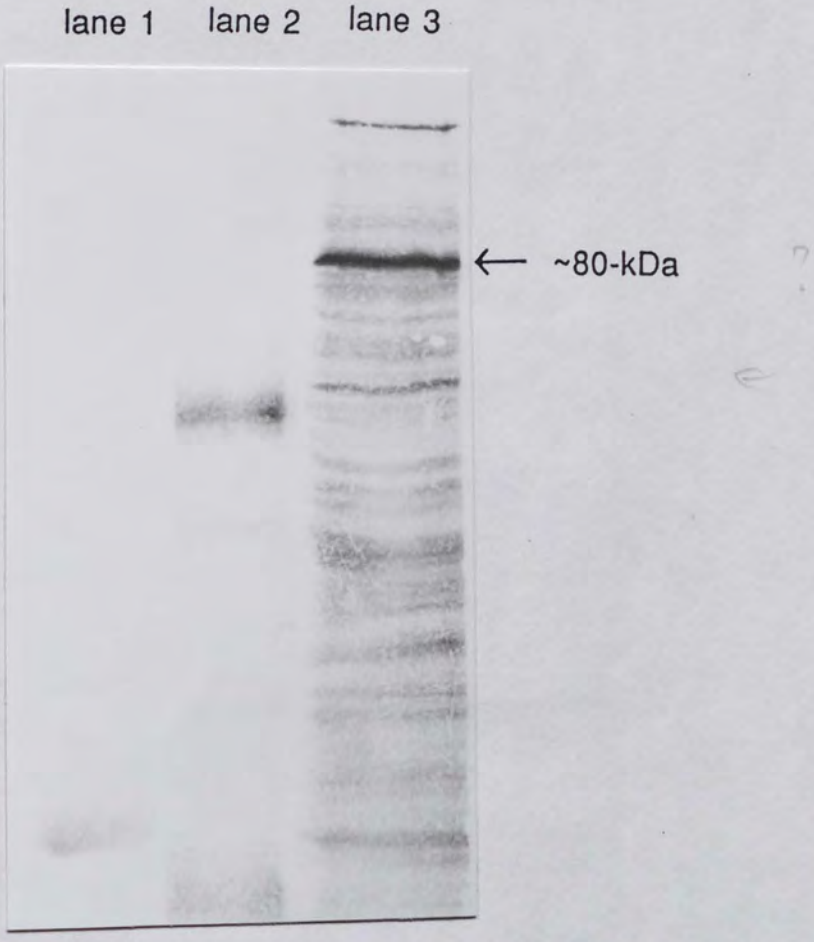
5.3.2.5 Effect of TPA (1μM) on protein kinase C in enriched parietal cells cultured for 24 hours on Matrigel.

The viability of parietal cells dropped from 90% (after purification) to 45% (after overnight incubation). From the position of the rainbow markers a graph of R_f verses log molecular weight was constructed (Fig. 5.10) to determine molecular weights. Two bands with apparent molecular weights of ~79 and 77-kDa were detected from cells incubated with and without TPA (1μM) (Fig. 5.11). The second band with an apparent molecular weight of ~77-kDa may be an unphosphorylated species of protein kinase C. Young *et al.* (1987) found that protein kinase C was initially synthesized as a Mr 76,000 species which is consistent with the value predicted from the amino acid sequence. This form was subsequently converted into a species with an apparent Mr of 79,000, possibly due to a post transcriptional modification. It was suggested that this modification was due to phosphorylation (Young *et al.*, 1987).

There was no detectable difference between the levels of protein kinase C in cells preincubated both with and without TPA (1μM). Clearly the method used was able to detect changes in protein kinase C levels (section 5.3.2.4). Thus this result

Figure 5.8

Detection of protein kinase C in a preparation of enriched parietal cells from the rat.

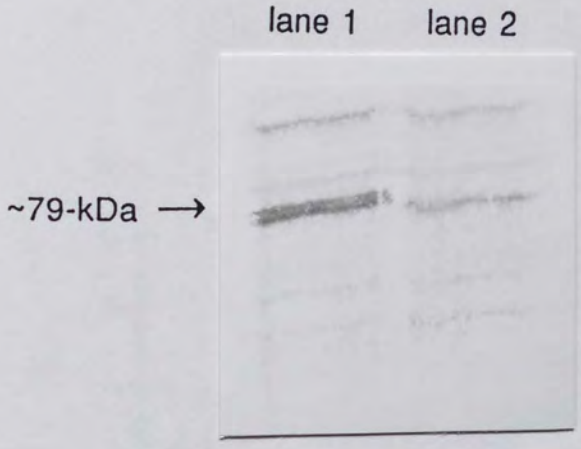


Lane 1 rainbow markers.
Lane 2 mouse IgG.
Lane 3 preparation from rat parietal cells.

Parietal cells were sonicated and then boiled in the presence of hot SDS. Then the whole cellular homogenates were separated on SDS polyacrylamide gel, transferred onto a nitrocellulose membrane which was then used for immunodetection of protein kinase C. The arrow indicates the major immunoreactive form of protein kinase C in these cells. This has an apparent M_r of ~80,000.

Figure 5.9

Photograph of Western blot after immunodetection of A549 human lung carcinoma cells which were preincubated with and without TPA (10nM) for 24 hours (by T.D.Bradshaw).



Lane 1 = control
Lane 2 = preincubation with TPA

Cellular homogenates of A549 cells were prepared, separated, blotted and subjected to immunodetection for protein kinase C as described in Fig. 5.7 and section 5.2.6. The arrow indicates protein kinase C with an apparent Mr of ~79,000. Note that 2 species of protein kinase C appear to be present. See section 5.3.2.5 for an explaination.

Figure 5.10

Graph, showing Rf verses log_{10} molecular weight , used to calculate the apparent molecular weight of unknown protein bands stained on the western blot.

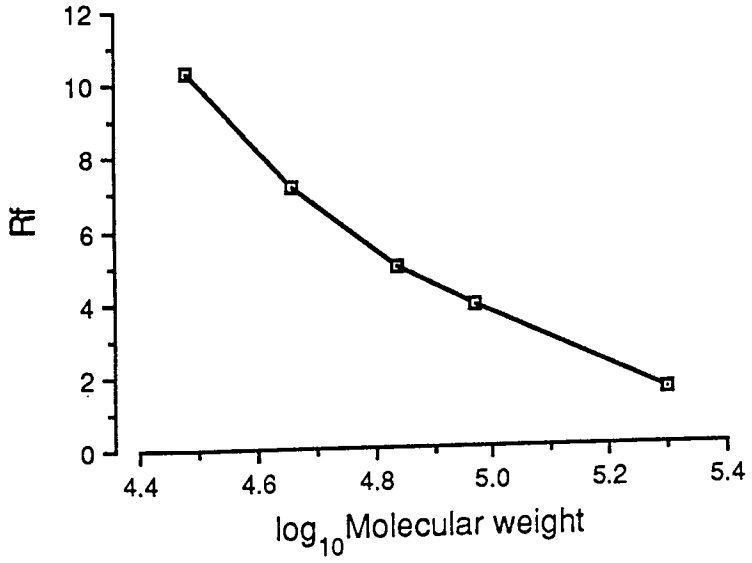
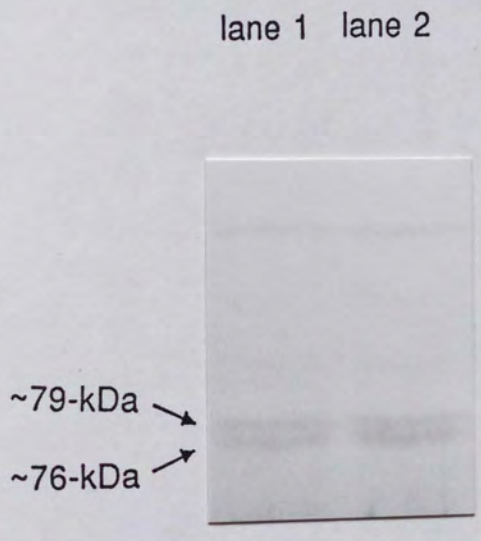


Figure 5.11

Immunodetection of protein kinase C from enriched parietal cells from the rat after 24 hours preincubation with and without 1μM-TPA.



Lane 1 = control.
Lane 2 = TPA pretreatment.

Cells were cultured on Matrigel for 24 hours both with and without TPA (1μM). Cellular homogenates of these cells were prepared, separated, blotted and subjected to immunodetection for protein kinase C as described in Fig. 5.7 and section 5.2.6. The arrows indicate protein kinase C with an apparent Mr of ~79,000 and 76,000.

confirms the suggestion from the aminopyrine accumulation ratio data that detectable down-regulation does not occur in rat parietal cells pretreated with TPA for 20 to 24 hours.

In some cell lines no down-regulation of phorbol ester binding sites as observed (Jaken *et al.*, 1981; Solanki *et al.*, 1981). However, although Western blotting and immunoprecipitation has been carried out in only a few cell lines, they have generally indicated a loss of protein kinase C over a 24 hour period (Stable *et al.*, 1987). Various cell lines have displayed different sensitivites to down-regulation induced by 100nM-TPA (Blackshear, 1988; Adams and Gullick, 1989). Parietal cells are highly differentiated cells and may not be subject to down-regulation. The reason for the lack of down-regulation in parietal cells is not known but it may be due to low levels of Ca^{2+}-activated neutral proteinase. An alternative explanation is that the form of protein kinase C in parietal cells is resistant to cleavage by this enzyme. Although after preincubation for 20-24 hours the parietal cells were still responsive, low viabilities may have prevented down-regulation of protein kinase C in some way.

5.4 SUMMARY.

1. TPA inhibited parietal cell secretory activity stimulated by histamine plus IBMX with a half-maximally effective concentration of 7 ± 4nM.

2. Preincubation with pertussis toxin significantly reduced the inhibitory action of 10^{-7} and 10^{-8}M-TPA. Pertussis toxin reduced the inhibitory action of 10^{-7}M-TPA by 12%. Therefore TPA appears to mediate its inhibitory effect, in only a small part, by a pertussis toxin-sensitive mechanism. This result differentiates TPA from EGF, somatostatin and PGE_2.

3. Preincubation with TPA (100nM-1μM) did not induce detectable down-regulation of protein kinase C in rat parietal cells after 2 and 20 to 24 hours of preincubation.

4. Immunochemical detection of protein kinase C on protein blots suggested that no down-regulation of protein kinase C by 1μM-TPA took place after 24 hours of culture of parietal cells on Matrigel.

Chapter 6

INHIBITION OF RAT ISOLATED PARIETAL CELL SECRETORY ACTIVITY BY CYCLIC GMP.

6.1 INTRODUCTION.

Although the role of cyclic AMP as a second messenger has been widely recognised for some time, little evidence has been found to support a widespread role for cyclic GMP as a second messenger. However, cyclic GMP seems to play a role in signal transduction in a few particular circumstances. Thus the intracellular level of cyclic GMP plays a crucial role in the photosensitive rod cells of vertebrate retina (Kaupp and Koch, 1984) and transduces the interaction of atrial natriuretic peptide with its receptor into a physiological response (Inagami, 1989).

There are conflicting reports on the action of cyclic GMP on acid secretion. Thus it is the intention of this work to clarify the action, if any, of cyclic GMP on aminopyrine accumulation by rat parietal cells. A brief review of cyclic GMP and its effects on gastric acid secretion is set out below.

6.1.1 Synthesis, metabolism and action of cyclic GMP.

Cyclic GMP (Fig. 6.1) is synthesized by guanylate cyclase from GTP. Guanylate cyclase exists in two distinct forms of which one is membrane bound and the other cytosolic. Both forms are present in most tissues. It seems that the two forms are completely different molecules having different properties and being immunologically distinguishable (Morgan, 1989).

6.1.1.1 Soluble guanylate cyclase.

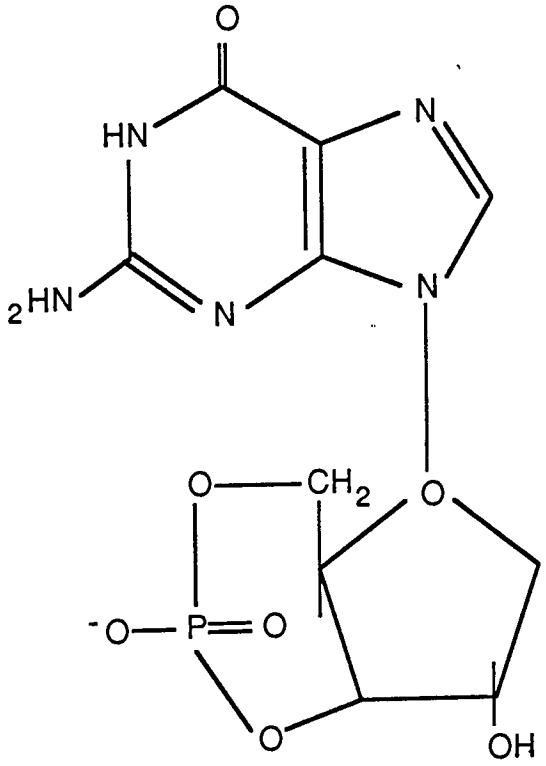
Soluble guanylate cyclase is a large protein Mr 150,000 which is composed of 2 distinct subunits and probably requires both Mg^{2+} and Ca^{2+} for biological activity (Kamisaki *et al.*, 1986). Nitric oxide which is produced by macrophages, endothelial and possibly other cell types has been found to activate guanylate cyclase (Marletta, 1989). However, it has yet to be established that other extracellular ligands can modulate soluble guanylate cyclase activity. Arachidonic acid has been reported to activate soluble guanylate cyclase in some cells and this may be a link between hormone receptors (which initiate the breakdown of phospholipids such as phospholipase C, see section 3.1.1.1) and alterations in intracellular cyclic GMP levels (Morgan, 1989).

6.1.1.2 Particulate guanylate cyclase.

So far only one group of endogenous ligands, atriopeptides, have been found to mediate their action by activation of particulate guanylate cyclase (Inagami, 1989). Atriopeptides promote smooth muscle relaxation, diuresis and excessive sodium excretion (natriuresis) from the kidney. The most active atriopeptide is a 28 amino acid peptide. This peptide termed atrial natriuretic peptide (ANP) is the cleavage product of

Figure 6.1

The structure of cyclic GMP.



pro-atrial natriuretic peptide which is found predominantly in the right atrium of the heart (Inagami, 1989).

Ligand binding experiments have led to the identification of ANP receptors. Two types of ANP receptor have been isolated and are the products of different genes (Inagami, 1989). One receptor (Mr ~120,000), as isolated from rat lung (Kuno *et al.*, 1986) and adrenal cortex (Paul *et al.*, 1987), co-purified with guanylate cyclase activity. This led to the suggestion that both the receptor and the cyclase activity were present in the same protein.

The gene for particulate guanylate cyclase has been isolated from the rat brain (Chinkers *et al.*, 1989). Cultured cells transfected with the cloned gene were shown to possess active guanylate cyclase and the ability to bind ANP in a manner that increased guanylate cyclase activity. Thus it appears that the receptor and the catalytic site reside in the same protein. The gene codes for a large protein (Mr 116,000-Da) with a single transmembrane domain. Approximately 45% of the amino acids are in the extracellular space where the ANP binding site is located and about 55% are intracellular and contain the active site (Chinkers *et al.*, 1989).

6.1.1.3 Metabolism of cyclic GMP.

Cyclic GMP is broken down to GMP by 3':5' cyclic nucleotide phosphodiesterases. It seems that most cells contain multiple forms of phosphodiesterase and that these are differentially compartmentalised and have variable specificities for cyclic GMP and cyclic AMP (Morgan, 1989).

6.1.1.4 Cyclic GMP-binding proteins.

The actions of cyclic GMP are often mediated by cyclic GMP-dependent protein kinase. This enzyme appears to be a soluble protein which is composed of two subunits. Each can bind cyclic GMP and both seem to have catalytic activity. In the retinal rods cyclic GMP appears to have a direct inhibitory effect after binding to a Na^+ channel (Morgan, 1989).

6.1.2 Effect of cyclic GMP on gastric acid secretion.

The major route used to investigate the action of cyclic GMP in acid secretion has been to employ the dibutyryl derivative which probably is less readily hydrolysed and permeates the cell membrane more easily than cyclic GMP itself. In contrast to dbcAMP, dbcGMP (1 to 1000μM) did not increase basal aminopyrine accumulation in the rat.(Heim and Ruoff, 1985). 1mM-dbcGMP also did not raise basal aminopyrine accumulation in the dog (Soll, 1980b). Vagal stimulation and acetylcholine infusion (0.05mg in 1 minute) raised cyclic GMP levels in the fundus of the dog, as determined

by radioimmunoassay. It was suggested that cyclic GMP possesses mediator function for cholinergic H^+ secretion (Eichhorn *et al.*, 1974). This has not been confirmed on isolated cell preparations. Brennen *et al.* (1975) found that 0.1mM-cyclic GMP stimulated acid secretion in a rat stomach preparation. However, as this was not an isolated cell preparation, cyclic GMP may have been acting indirectly to stimulate acid secretory activity. Thus the case for cyclic GMP having a direct stimulatory action on the parietal cell is not strong.

Heim and Ruoff (1985) found that dbcGMP inhibited a crude parietal cell suspension from the rat (21 ± 0.9% parietal cells (mean ± S.E.M.) stimulated by 0.1mM-dbcAMP. 1mM-dbcGMP potently inhibited dbcAMP-stimulated cells by 75% (Fig. 6.2). In contrast to these findings Batzri and Dyer (1981) found that 5mM-dbcGMP had no effect on aminopyrine accumulation stimulated by 1mM-dbcAMP in a guinea-pig gastric cell suspension.

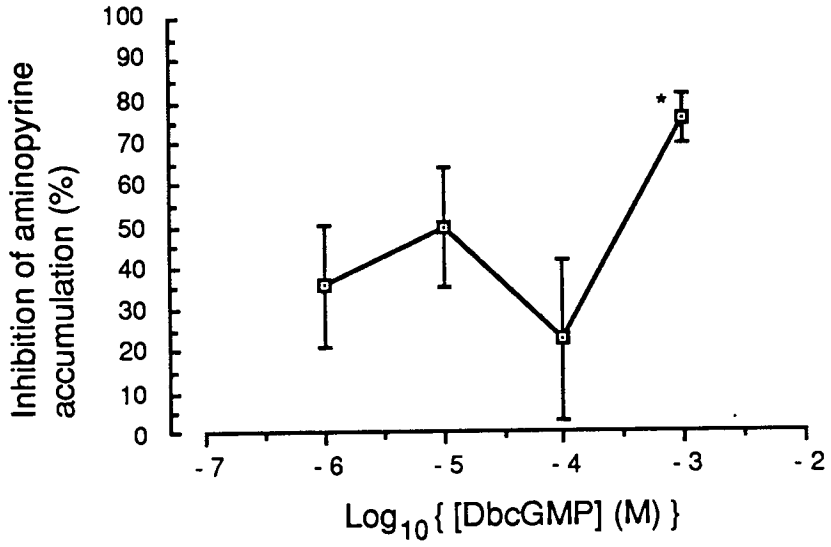
If dbcGMP inhibits dbcAMP stimulated acid secretory activity then dbcGMP might be expected to inhibit aminopyrine accumulation stimulated by histamine. This has not been tested in the rat. However, dbcGMP did not inhibit aminopyrine accumulation stimulated by histamine in gastric cells from the dog (Rutten and Soll, 1981). Also in the rabbit dbcGMP (0.1mM) did not inhibit secretory activity in a gastric gland preparation stimulated by 10^{-6}M-histamine (Hersey *et al.*, 1983).

Various agents are known to raise intracellular cyclic GMP levels. Thus nitrogen compounds and thiols such as hydroxylamine and 2-mercaptoethanol, which are known to stimulate guanylate cyclase, potently increased cellular cyclic GMP levels in a cell suspension enriched with parietal cells (66-70% parietal cells) (Heim and Ruoff, 1985). Histamine (0.1mM) and carbachol (0.1mM) did not raise cyclic GMP levels in a parietal cell enriched preparation from the rat (Heim and Ruoff, 1985). Thus it seems that cyclic GMP is not directly involved in cholinergic stimulated H^+ production in the rat. Carbachol did however raise cyclic GMP in a parietal cell depleted fraction (5% parietal cells) which contained chief and mucous cells (Heim and Ruoff, 1985). The phosphodiesterase inhibitor IBMX (1mM) enhanced the level of both cyclic AMP and cyclic GMP in a parietal cell suspension from the rat. However, 0.1mM-IBMX did not raise cyclic GMP above basal (Heim and Ruoff, 1985).

The investigation of the inhibitory effect of dbcGMP on secretory activity in rat parietal cells as presented by Heim and Ruoff (1985) poses two questions. Firstly is there a dose-relationship for the effect of dbcGMP on dbcAMP? No clear-cut dose-response was actually shown (Fig. 6.2). Secondly, does dbcGMP have an effect when the cells were stimulated by other secretagogues? Only the effects against dbcAMP were investigated. An investigation of the dose-dependency of dbcGMP

Figure 6.2

The inhibitory action of dbcGMP on aminopyrine accumulation stimulated by dbcAMP (0.1mM), as found by Heim and Ruoff (1985).



Data from Heim and Ruoff (1985) have been expressed as percentage inhibition by dbcGMP of aminopyrine accumulation obtained by dbcAMP (0.1mM). Results are from 5 cell preparations containing 26% parietal cells. * $P<0.01$ by ANOVAR.

against aminopyrine accumulation stimulated by dbcAMP, histamine and 100mM-K^+ is presented here. The one unusual action of TPA, which causes it to stand out, is its inhibitory effect against dbcAMP. Other inhibitors such as somatostatin (Nylander *et al.*, 1985), PGE_2 (Soll 1980b) and EGF (Shaw *et al.*, 1987) do not inhibit dbcAMP-stimulated parietal cell secretory activity in the rat. Thus it is possible that cyclic GMP is the mediator of the effects of TPA in parietal cells. The action of dbcGMP on parietal cell secretory activity stimulated by the secretagogues mentioned will be compared to that by TPA.

6.2 METHODOLOGY.

6.2.1 Preparation of reagents.

DbcGMP and dbcAMP were dissolved and diluted in saline (NaCl 0.9% ($^{w}/_{v}$)). Where appropriate a small volume (10µl) was added to the incubation vials. Histamine and IBMX were prepared as in section 3.2.1. The composition of the 100mM-K^+ medium is shown in appendix A.2.2. This solution was stored at 2-8°C. On the day of the experiment a 100ml sample was gassed with 95% O_2/5% CO_2 for 30 minutes at room temperature. Subsequently, bovine serum albumin (1mg/ml), glucose (5mM) and isoleucine (0.1mM) were added and the pH then adjusted to 7.4 using NaOH.

6.2.2 Effect of dbcGMP on the aminopyrine accumulation ratio stimulated by histamine plus IBMX, and dbcAMP.

Parietal cell preparations which consisted of approximately 20% parietal cells were isolated as described in section 2.1.3. After isolation the cells were resuspended to a final concentration of 2-6x10^6 cells/ml in medium B'. Aliquots of the cell suspension (1.5ml) were added to incubation vials containing secretagogues and dbcGMP where appropriate. Treatment vials were incubated and the aminopyrine accumulation ratio estimated as described in section 4.3.2.

6.2.3 The effect of dbcGMP on the aminopyrine accumulation ratio stimulated by 100mM-K^+.

After isolation the cell suspension was washed in 100mM-K^+ medium and then resuspended in 100mM-K^+ medium to a concentration of 2-6x10^6 cells/ml. Otherwise the methodology was the same as in section 6.2.2.

6.2.4 Presentation of data.

To overcome the variation in the aminopyrine accumulation ratio between cell batches, the effect of dbcGMP on the aminopyrine accumulation ratio was expressed by normalising the data to percentage inhibition as described in section 3.2.4.

6.3 RESULTS AND DISCUSSION.

6.3.1 The action of dbcGMP on the aminopyrine accumulation ratio stimulated by dbcAMP.

DbcGMP significantly inhibited the aminopyrine accumulation ratio stimulated by 0.1mM-dbcAMP ($P<0.001$, by two-way ANOVAR) (Table 6.1 and Fig. 6.3). The inhibition by dbcGMP of dbcAMP-stimulated aminopyrine accumulation reached significance at 10^{-2} and 10^{-3}M-dbcGMP. This resulted in a 97.7 ± 0.2 and 38.2 ± 5.7% inhibition respectively. Thus as found by Heim and Ruoff (1985) dbcGMP (1mM) inhibited dbcAMP (0.1mM)-stimulated aminopyrine accumulation by isolated gastric cells from the rat (see section 6.1.2). However, in contrast to Heim and Ruoff (1985) this work indicated that dbcGMP inhibited secretory activity stimulated by dbcAMP in a dose-related fashion (Fig. 6.3).

The curve obtained by this work (Fig 6.3) did not resemble a classical dose-response curve, although it just possibly could represent the bottom part of such a curve. The marked increase in the inhibitory action of 10mM-dbcGMP, compared to 1mM-dbcGMP, was possibly caused by a non-specific effect. The inhibitory action of dbcGMP concentrations below 10^{-2}M were not likely to be non-specific as 1mM-dbcAMP, which has a structural similarity to dbcGMP, does not have an adverse effect on parietal cells. In fact 0.1mM-dbcAMP was a potent stimulator of parietal cell secretory activity (Table 6.1) and 1mM-dbcAMP was found to be more so (data not shown, 0.1mM-dbcAMP was used to enable comparison with Heim and Ruoff (1985)).

At each concentration of dbcGMP tested, Heim and Ruoff (1985) obtained a higher degree of inhibition of dbcAMP-stimulated secretory activity (Fig. 6.3). In fact 1mM-dbcGMP was only inhibited by 38% (this work) compared to 75% obtained by Heim and Ruoff (1985). It is possible that differences in the level of aminopyrine accumulation stimulated by 0.1mM-dbcAMP (if present) may have contributed to the different levels of inhibition observed. Thus, although dbcGMP was found to have an inhibitory effect, the pattern of inhibition found was different to that found by Heim and Ruoff (1985).

It is possible that the intracellular concentration of dbcGMP was far lower than the extracellular dbcGMP concentration. This would then explain why such high concentrations of dbcGMP were required for a marked inhibitory action and why the dose-response curve did not tail off at the highest concentration tested.

Table 6.1

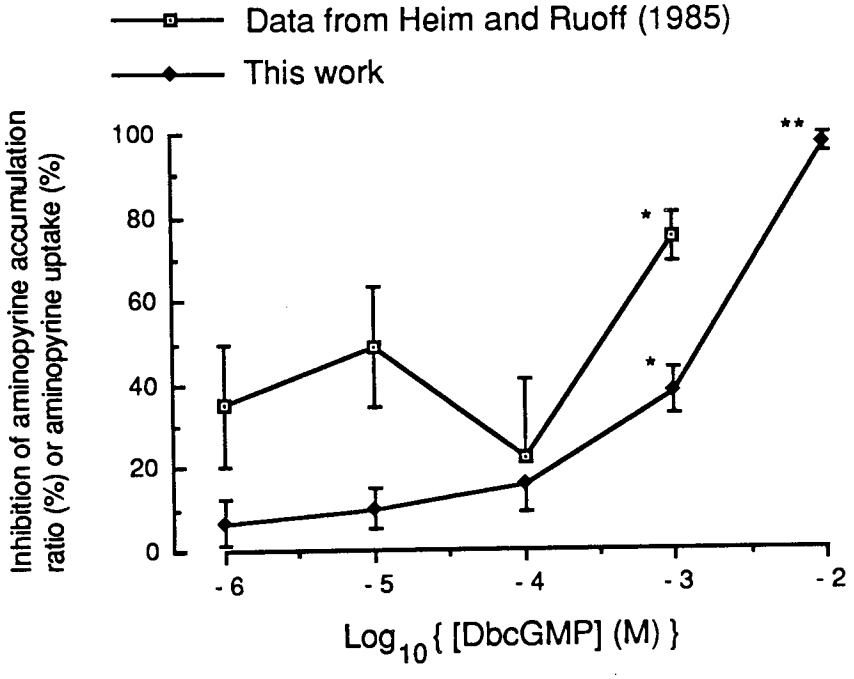
The effect of concentrations of dbcGMP on the aminopyrine accumulation ratio stimulated by dbcAMP (0.1mM).

Concentration of dbcGMP (M)	Aminopyrine Accumulation Ratio
0	17.6 ± 4.5
10^{-6}	16.8 ± 4.7
10^{-5}	16.5 ± 4.6
10^{-4}	16.0 ± 4.7
10^{-3}	10.9 ± 2.8*
10^{-2}	0.6 ± 0.4*

Results from 3 separate batches of cells containing 19.7 ± 0.3% parietal cells are expressed as means ± S.E.M. The results were transformed (log_{10} transformation) and analysed by a Dunnett's test where dbcGMP treatments were compared to the control (no dbcGMP). *$P<0.01$.

Figure 6.3

The action of dbcGMP on secretory activity stimulated by dbcAMP (0.1mM).



Data from Table 6.1 (◆) have been normalised and expressed as percentage inhibition by dbcGMP of the aminopyrine accumulation ratio obtained with dbcAMP. The effect of dbcGMP has been analysed by a Dunnett's test (see Table 6.1). $^{**}P<0.01$; $^{*}P<0.05$.

Data from Heim and Ruoff (1985) (▣) have been expressed as percentage inhibition by dbcGMP of aminopyrine accumulation obtained by dbcAMP (0.1mM). Results are from 5 cell prepatrations containing 26% parietal cells. * $P<0.01$ by ANOVAR.

6.3.2 The action of dbcGMP on the aminopyrine accumulation ratio stimulated by histamine plus IBMX.

Aminopyrine accumulation stimulated by a near-maximally effective concentration of histamine (0.5mM) plus IBMX (0.1mM) was significantly inhibited by dbcGMP ($P<0.001$, two-way ANOVAR) (Table 6.2 and Fig. 6.4). IBMX (0.1mM) did not raise cyclic GMP levels above basal in the rat Heim and Ruoff (1985) (see section 6.1.2).

DbcGMP dose-dependently inhibited histamine plus IBMX-stimulated aminopyrine accumulation and reached significance at 10^{-2} ($P<0.02$, unpaired *t*-test), 10^{-3} and 10^{-4}M-dbcGMP ($P<0.01$ and $P<0.05$ respectively by two-way ANOVAR) (Table 6.2 and Fig. 6.4). This finding contradicts those found by Rutten and Soll (1981) where dbcGMP had no inhibitory effect on histamine stimulated aminopyrine accumulation in gastric cells from dog. DbcGMP (0.1mM) was also found to have no inhibitory effect on an isolated gastric gland preparation from the rabbit (Hersey *et al.*, 1983) (see section 6.1.2). However, this result supports the suggestion by Heim and Ruoff (1985) that cyclic GMP inhibits the stimulatory action of cyclic AMP on acid secretory activity in the rat. The contradictory results may be due to species differences.

The basal aminopyrine accumulation ratio was significantly inhibited by 10^{-3}M-dbcGMP (aminopyrine accumulation ratio (mean ± S.E.M. from 9 separate batches of cells containing 20.6 ± 0.7% parietal cells), basal 1.8 ± 0.2: 10^{-3}M-dbcGMP 1.3 ± 0.1. $P<0.025$ as determined by a paired *t*-test). DbcGMP may be inhibiting the action of an endogenous stimulator of secretory activity present in the incubation media, or alternatively dbcGMP may lower the basal tone of secretory activity. This action of dbcGMP is in contrast to the finding by Heim and Ruoff (1985) where no effect of dbcGMP was seen on the basal secretory activity exhibited by a gastric cell suspension from the rat. However, this effect on basal secretory activity by dbcGMP cannot account for its inhibitory effect on stimulated secretory activity.

The pattern of inhibition of aminopyrine accumulation by dbcGMP was similar when the cells were stimulated by histamine plus IBMX or dbcAMP (Fig. 6.5). Stimulation of aminopyrine accumulation by dbcAMP was inhibited to a greater extent than that by histamine plus IBMX, when the concentration of dbcGMP was 10^{-2}M (Fig. 6.5). The stimulation of the aminopyrine accumulation ratio by histamine (0.5mM) and IBMX (0.1mM) was greater than that by dbcAMP (0.1mM) ($P<0.01$

Table 6.2

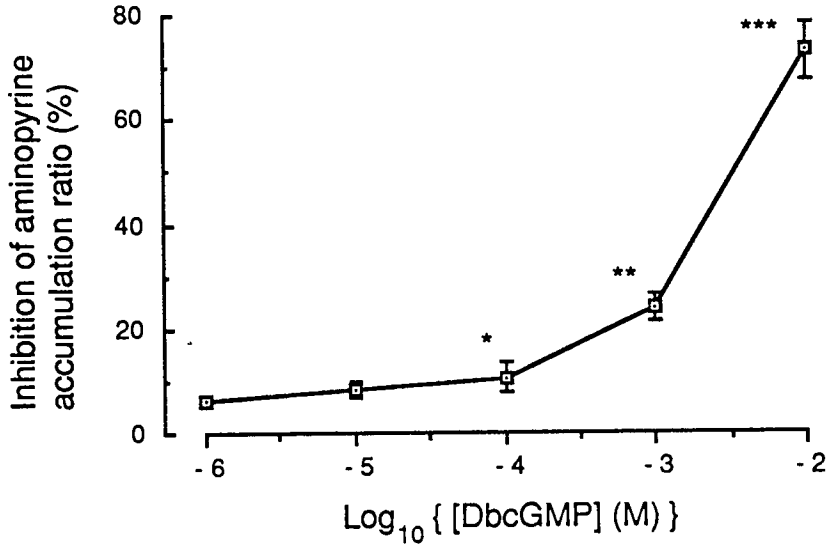
The effect of concentrations of dbcGMP on the aminopyrine accumulation ratio stimulated by histamine (0.5mM) plus IBMX (0.1mM).

Concentration of dbcGMP (M)	Aminopyrine Accumulation Ratio
0	49.1 ± 7.2
10^{-6}	47.4 ± 7.6
10^{-5}	45.1 ± 5.9
10^{-4}	43.5 ± 4.3*
10^{-3}	36.7 ± 4.2**
0	47.8 ± 14.2
10^{-2}	13.3 ± 5.3***

Results from 4 separate batches of cells containing 22.5 ± 1.0% parietal cells are expressed as means ± S.E.M. The results (10^{-6} to 10^{-3}M-dbcGMP) were transformed ($\log_{10}$ transformation) and analysed by a Dunnett's test where dbcGMP treatments were compared to the control (no dbcGMP). *$P<0.05$; **$P<0.01$. The results for 10^{-2}M-dbcGMP were obtained from 3 batches of cells containing 21.7 ± 0.8 parietal cells and have been analysed by an unpaired *t*-test. ***$P<0.02$. See Appendix A.5.2 for statistical analysis.

Figure 6.4

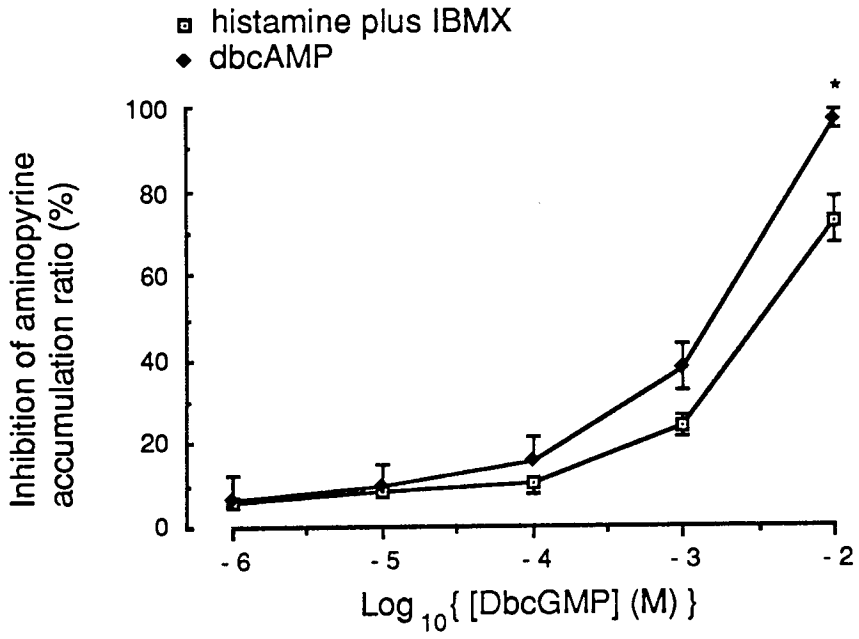
The action of dbcGMP on the aminopyrine accumulation ratio stimulated by histamine (0.5mM) plus IBMX (0.1mM).



Data from Table 6.2 have been normalised and expressed as percentage inhibition by dbcGMP of the aminopyrine accumulation ratio obtained with histamine plus IBMX. The effect of dbcGMP (10^{-6} to 10^{-3}M) has been analysed by a Dunnett's test and by an unpaired *t*-test for 10^{-2}M-dbcGMP (see Table 6.2). **P*<0.05; ***P*<0.01; ****P*<0.02.

Figure 6.5

Comparison of the effect of dbcGMP on the aminopyrine accumulation ratio stimulated by histamine (0.5mM) plus IBMX (0.1mM) and by dbcAMP (0.1mM).



Data from Table 6.1 and 6.2 have been normalised and expressed as percentage inhibition by dbcGMP of the aminopyrine accumulation ratio obtained with histamine plus IBMX (▫) and dbcAMP (◆). Data from the two secretagogues were compared by an unpaired *t*-test. **P*<0.025.

unpaired *t*-test), so it is possible that differences in the level of stimulation of secretion may have contributed to this effect.

6.3.3 The action of dbcGMP on acid secretory activity stimulated by 100mM-K^+.

As mentioned in section 1.3.1.6 the site of action of K^+ is believed to be close to the H^+/K^+-ATPase. Aminopyrine accumulation stimulated by a near-maximally effective concentration of K^+ (100mM) was significantly inhibited by dbcGMP ($P<0.001$, two-way ANOVAR) (Table 6.3 and Fig. 6.6). Gastric cells stimulated by 100mM-K^+ were inhibited by 44.3 ± 8.5% by 10^{-2}M-dbcGMP. DbcGMP inhibited K^+-stimulated aminopyrine accumulation at 10^{-2}, 10^{-4} and 10^{-5}M-dbcGMP. The reason for a loss of an inhibitory effect at 10^{-3}M-dbcGMP (Fig. 6.6) is not known, but it was highly repeatable as evidenced by the small standard errors associated with the normalised data.

The level of inhibition exhibited by 10^{-2} and 10^{-3}M-dbcGMP against 100mM-K^+ stimulation was lower than that against dbcAMP-stimulation, despite the similarity of the aminopyrine accumulation induced by 100mM-K^+ or 0.1mM-dbcAMP (Tables 6.3 and 6.1) (Percentage inhibition of the aminopyrine accumulation ratio by 10^{-2}-dbcGMP (means ± S.E.M. with the number of cell batches in parentheses): 100mM-K^+ 44.3 ± 8.5(5); dbcAMP 97.7 ± 0.2(3)).

Table 6.3

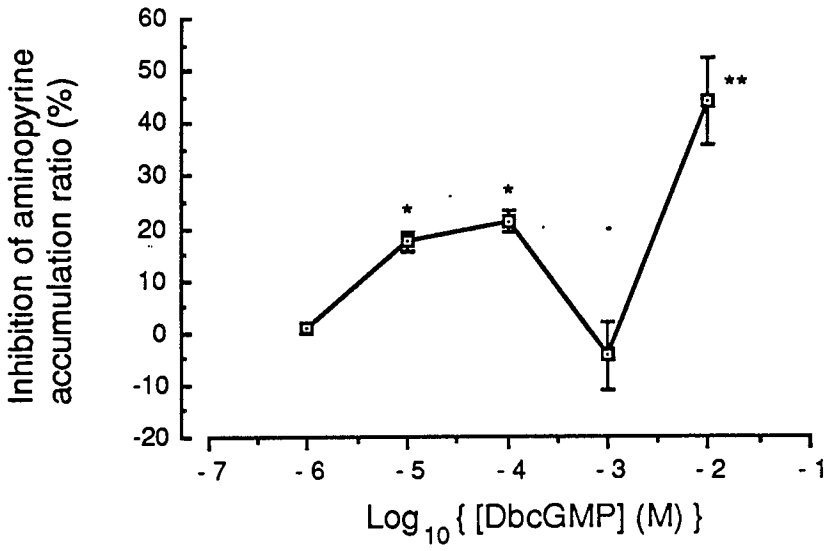
The effect of concentrations of dbcGMP on the aminopyrine accumulation ratio stimulated by K^+ (100mM).

Concentration of dbcGMP (M)	Aminopyrine Accumulation Ratio
0	21.2 ± 4.3
10^{-6}	21.0 ± 4.2
10^{-5}	17.5 ± 3.6*
10^{-4}	16.5 ± 3.4*
10^{-3}	21.9 ± 4.8
10^{-2}	10.7 ± 2.1**

Results from 5 separate batches of cells containing 19.6 ± 0.5% parietal cells are expressed as means ± S.E.M. Transformed results (log_{10} transformation) have been analysed by a Dunnett's test where dbcGMP treatments were compared with control (no dbcGMP). *$P<0.05$; **$P<0.01$.

Figure 6.6

The effect of concentrations of dbcGMP on the aminopyrine accumulation ratio stimulated by K^+ (100mM).



Data from table 6.3 have been normalised and expressed as percentage inhibition by dbcGMP of the aminopyrine accumulation ratio obtained with 100mM-K^+. The effect of dbcGMP has been analysed by a Dunnett's test (see Table 6.3). **P<0.01; *P<0.05 for comparison with aminopyrine accumulation stimulated by 100mM-K^+ and no dbcGMP.

6.4 GENERAL DISCUSSION.

All the stimulators of parietal cell secretory activity tested were inhibited by dbcGMP, as was the basal secretory activity. This brings into question the specificity of dbcGMP action. The effect of dbcGMP did not display the classical dose-response curve over the range of concentrations tested. At the highest concentrations of dbcGMP the dose-response curve did not tail off. Therefore, dbcGMP may indeed be having a non-specific inhibitory effect. Alternatively, higher concentrations of dbcGMP may be required to elicit the classical dose-response curve, or high concentrations of dbcGMP (10^{-2}M) may have a toxic effect. This possibility is supported by the data with K^+ stimulated cells where a bell-shaped dose-response curve is seen over the range 10^{-6} to 10^{-3}M-dbcGMP, but a substantial rise in inhibition then occurs at 10^{-2}M-dbcGMP. Release of lactate dehydrogenase or a trypan blue dye exclusion test before and after incubation with dbcGMP would indicate whether dbcGMP had a disruptive effect on gastric mucosal cell membranes .

Heim and Ruoff (1985) found that dbcGMP had no effect on basal secretory activity in parietal cell suspensions from the rat. In contrast to the rat, gastric mucosal preparations from guinea pig, rabbit and dog did not exhibit an inhibitory response to dbcGMP (section 6.1.2). Thus dbcGMP shows some degree of specificity.

As mentioned in section 6.1.2, TPA unlike many other inhibitors of acid secretion inhibits dbcAMP-stimulated parietal cells. Thus it is possible that protein kinase C mediates its inhibitory action via cyclic GMP and indeed protein kinase C has been suggested to regulate guanylate cyclase activity (Duda and Sharma, 1990).

TPA inhibits histamine (0.5mM) plus IBMX (0.1mM) and dbcAMP (1mM) stimulated rat parietal cell suspensions (Anderson and Hanson, 1984). Low concentrations of TPA (10^{-9} to 10^{-8}M) had a stimulatory action on 100mM-K^+ (Hatt and Hanson, 1989). At higher concentrations of TPA (10^{-8} to 10^{-6}M) this stimulatory action of TPA on cells stimulated by 100mM-K^+ was lost (Hatt and Hanson, 1989). As with TPA, dbcGMP inhibited 0.5mM-histamine- and 0.1mM-dbcAMP-stimulated rat parietal cells. However, in contrast to the action of TPA, low concentrations of dbcGMP were found to inhibit rather than stimulate 100mM-K^+ stimulated parietal cells. Thus this disparity with the K^+ results makes it unlikely that dbcGMP mediates the inhibitory action of TPA in rat parietal cells.

It must be noted that the investigation into the action of dbcGMP was on crude parietal cell suspensions and that the action of dbcGMP may therefore be via the release of an inhibitor of parietal cell secretory activity. This is not probable as any inhibitors released into the incubation medium would be expected to be diluted below

threshold. Finally all of this work assumes that dbcGMP is a satisfactory agent for mimicking changes in intracellular cyclic GMP.

6.5 SUMMARY.

1. Secretory activity stimulated by histamine (0.5mM) plus IBMX (0.1mM), dbcAMP (0.1mM) and 100mM-K^+ was inhibited by dbcGMP in parietal cell preparations from the rat.

2. The inhibitory profile obtained with different concentrations of dbcGMP was similar when histamine or dbcAMP were secretagogues but was different in cells stimulated with 100mM-K^+.

3. The inhibitory effects of 10^{-2}M-dbcGMP are possibly non-specific but it remains to be established whether effects seen at lower concentrations could have physiological relevance.

Chapter 7

GENERAL DISCUSSION

7.1 MODE OF ACTION AND PHYSIOLOGICAL ROLE OF INHIBITORS.

The aim of this chapter is to consider the mode of action and physiological significance of inhibitors of parietal cell secretory activity with particular reference to agents investigated in this thesis.

7.1.1 The physiological role of PGE_2 as an antisecretory agent in the rat stomach.

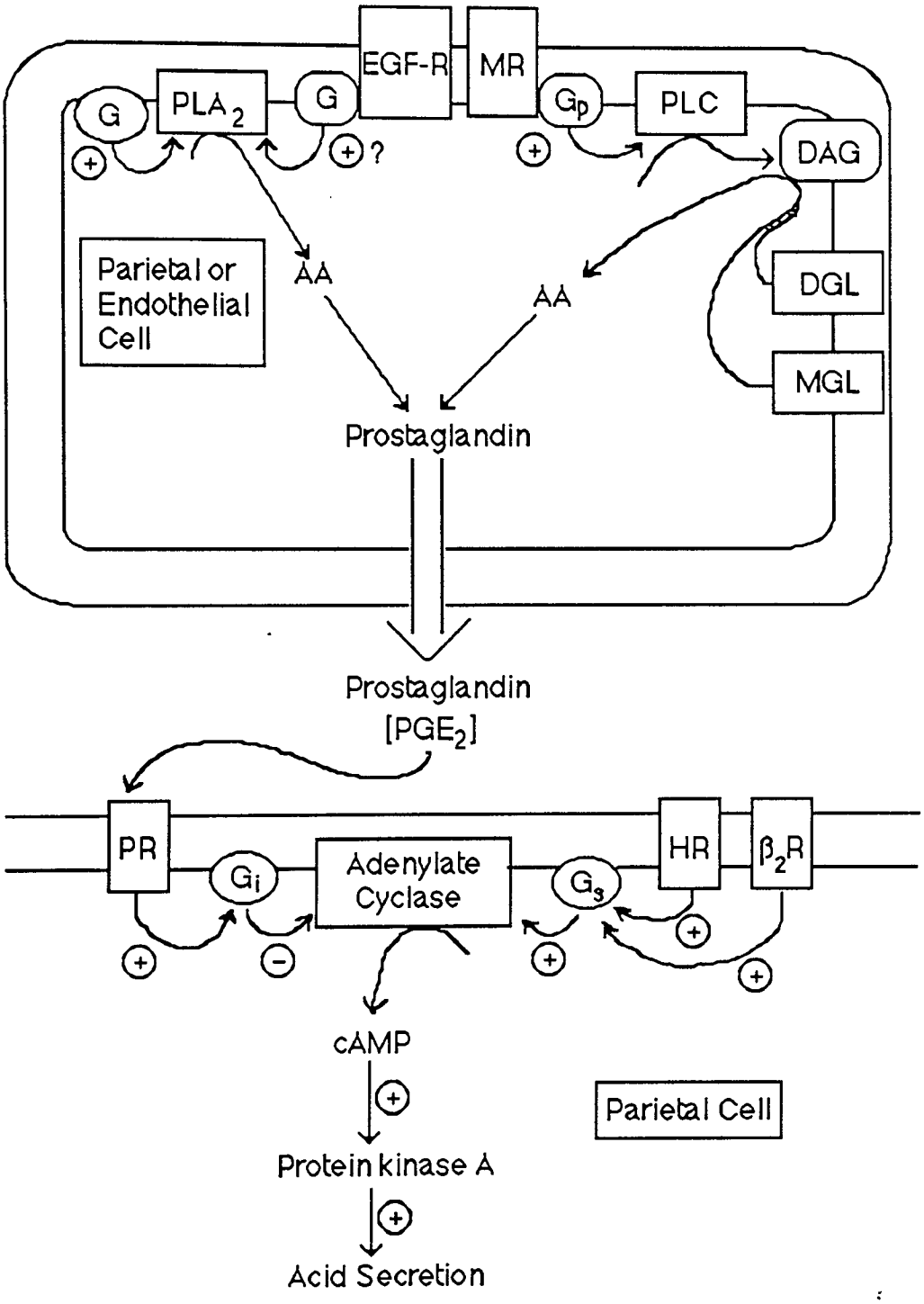
The interaction of PGE_2 with its own receptor on the parietal cell membrane leads to an inhibition of parietal cell secretory activity. This appears to be mediated by the activation of a pertussis toxin sensitive G-protein (G_i) (section 3.3.3) which then dissociates to liberate the $G_{i\alpha}$ and $\beta\gamma$ subunits with the consequent inhibition of adenylate cyclase activity. Thus PGE_2 only directly inhibits secretagogues which mediate their action by increasing adenylate cyclase activity, that is histamine (section 3.3.3) and the β_2 adrenergic receptor agonist isoprenaline (Rosenfeld *et al.*, 1986). A diagrammatic representation of the action of PGE_2 on parietal cells is shown in Fig. 7.1.

It is suggested that there are two possible physiological roles for prostaglandins as inhibitors of acid secretion. Firstly it seems likely that prostaglandins have a role as paracrine inhibitory modulators of acid secretion. This is supported by the observation that feeding increases prostaglandin content in the intact gastric mucosa of the dog (Bunnett *et al.*, 1983b). Furthermore cholinergic stimulation of isolated gastric cells induces the release of prostaglandins (Payne and Gerber, 1987). Cholinergic stimulation is obviously an essential feature of the secretory response to feeding (section 1.3.1.1). The second role is the inhibition of acid secretion during damage to the gastric mucosa. Prostaglandins are known to be produced when tissue is damaged and indeed damage caused by resuspension may be responsible for the high prostaglandin content of enriched parietal cell suspensions before incubation (Table 3.17 and Fig. 3.15). Recently specialised glands which release EGF have been found to form close to sites of damage in the stomach (Wright *et al.*, 1990) and this EGF could generate a localised release of prostaglandins (see section 7.1.3).

Although PGE_2 appears to be the most potent antisecretory prostanoid it might not be present in the highest concentration. In fact the breakdown product of prostacyclin, 6-oxo-$PGF_{1\alpha}$, was produced in greater quantities in the rat gastric mucosa (Whittle and Vane, 1987). EGF may stimulate the production of prostacyclin (Vane *et al.*, 1987) which could then inhibit parietal cell secretory activity (Soll and Whittle, 1981).

Figure 7.1

A speculative scheme of the production and action of prostaglandin E_2.



Key: + = stimulation; - = inhibition; EGF-R, EGF receptor; MR cholinergic receptor; PLC, phospholipase C; DAG, diacylglycerol; DGL, diacylglycerol lipase; MGL, monoacylglycerol lipase; AA, arachidonic acid; PLA_2, phospholipase A_2; PR, PGE_2 receptor; HR, histamine H_2 receptor; β_2R, adrenergic β_2 receptor; G, G-protein; G_p, G-protein activating phospholipase C; G_i, inhibitory G-protein; G_s, stimulatory G-protein.

Continuation of Figure 7.1

PGE_2 may be produced by muscarinic cholinergic receptor stimulation which results in diacylglycerol formation via increased phospholipase C activity. Diacylglycerol may be converted to arachidonic acid by diacylglycerol lipase or monoacylglycerol lipase. Alternatively phospholipase A_2 can generate arachidonic acid. Interaction of EGF with its receptor on the cell surface leads to increased PGE_2 release which may be mediated by increased phospholipase A_2 activity. PGE_2 interacting with its membrane bound receptor appears to inhibit histamine stimulated acid secretion by the G-protein mediated inhibition of adenylate cyclase activity.

7.1.2 The physiological role of somatostatin as an antisecretory agent in the rat stomach.

Somatostatin inhibits parietal cell secretory activity by interaction with its own receptors on the cell membrane and possibly by interacting with intracellular receptors (section 3.1.2.2). Preincubation with pertussis toxin blocked the inhibitory effect of somatostatin on histamine-stimulated secretory activity and thus it appears that somatostatin receptors are coupled through G_i to inhibition of adenylate cyclase in parietal cells (section 3.3.4). Somatostatin also may inhibit secretory activity at a site downstream from cyclic AMP as it inhibits secretory activity stimulated by carbachol without altering phospholipase C or protein kinase C activity (Park *et al.*, 1987). This site may be located where the two signal transduction systems converge. Indeed somatostatin was found to be an activator of cytosolic phosphoprotein phosphatases in rat fundic mucosa (Reyl and Lewin, 1982) (section 3.1.2.3). A diagrammatic representation of the possible action of somatostatin on parietal cells is illustrated in Fig. 7.2.

Somatostatin therefore appears to inhibit acid secretion, at least in part, by a similar mechanism to that exhibited by PGE_2. As both somatostatin and PGE_2 activate the same pathway it seems possible that they may do so under different circumstances. Thus carbachol which stimulates PGE_2 production (Payne and Gerber, 1987) actually inhibits somatostatin secretion in cultured D-cells from the dog (Chiba *et al.*, 1989b). Indeed any agent which inhibits somatostatin release will be an indirect activator of acid secretion in the intact stomach.

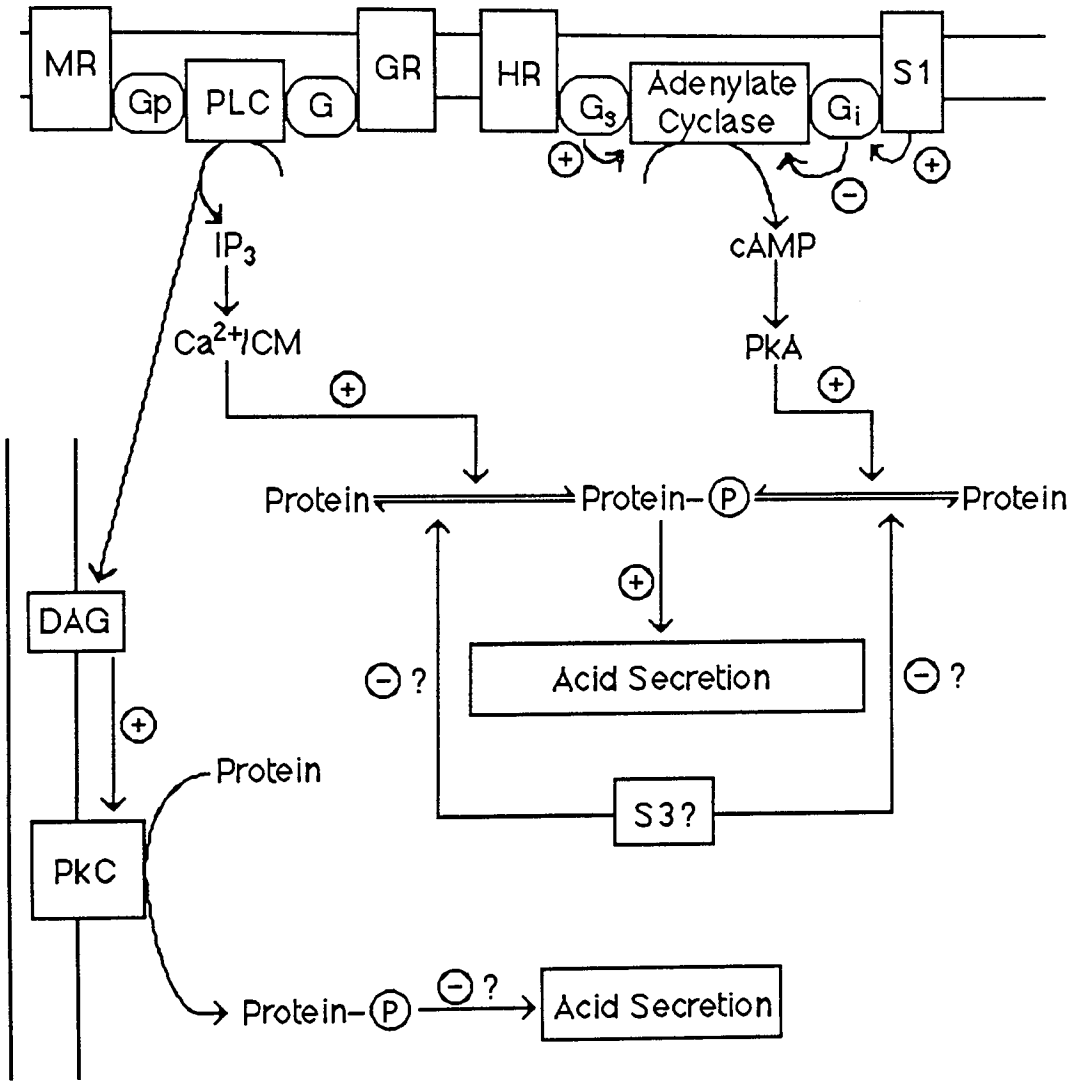
A major role of somatostatin is to mediate the inhibitory action of many indirect inhibitors of acid secretion. In the introduction (section 1.4.1) the following agents were presented as being able to induce the release of somatostatin: glucagon, truncated glucagon-like peptide 1, secretin, vasoactive intestinal polypeptide, cholecystokinin octapeptide and calcitonin gene-related peptide.

7.1.3 The physiological role of EGF as an antisecretory agent in the rat stomach.

EGF inhibits the secretory activity of rat isolated parietal cells stimulated by histamine and forskolin via a G-protein sensitive to pertussis toxin (section 3.3.6). It is unlikely that the antisecretory action of EGF is mediated by a direct effect on the H_2 receptor (section 4.4.1.5). It is suggested that the mechanism by which EGF attenuates secretory activity involves the stimulation of a low Km cyclic AMP-phosphodiesterase (section 3.4 and 4.4.1.5). EGF may also act at a site downstream from cyclic AMP as aminopyrine uptake stimulated by dbcAMP was inhibited by EGF in rabbit gastric glands. It is thought that this action takes place at a different site to that

Figure 7.2

A speculative scheme of the action of somatostatin on the parietal cell.



Key: + = stimulation; - = inhibition; S1, somatostatin receptor; S3, cytoplasmic somatostatin receptor; MR muscarinic cholinergic M3 receptor; GR, gastrin receptor; PLC, phospholipase C; DAG, diacylglycerol; HR, histamine H_2 receptor; G, G-protein; G_i, inhibitory G-protein; G_s, stimulatory G-protein; G_p, G-protein associated with the muscarinic cholinergic receptor; PkC, protein kinase C; PkA, protein kinase A; IP_3, inositol 1,4,5-trisphosphate; Ca^{2+}/CM, Ca^{2+}/calmodulin-dependent protein kinase.

Interaction of somatostatin with its membrane bound receptors (S1) leads to a reduction in histamine-stimulated secretory activity. This seems to be mediated via inhibitory G-proteins acting on adenylate cyclase. Somatostatin also seems to act at a site downstream from cyclic AMP generation and hydrolysis which may be a site where the two signal transduction systems converge. This may involve increased protein phosphatase activity. Internal somatostatin receptors (S3) or the membrane bound receptors (not shown for clarity) may be responsible for this action.

on histamine-stimulation (Hanson and Hatt, 1989). Aminopyrine accumulation stimulated by carbachol was inhibited by EGF in enriched parietal cells from rabbit. This took place without altering the Ca^{2+} transient stimulated by carbachol (Lewis *et al.*, 1990 and section 3.1.3.3). EGF was reported to enhance the activity of soluble and particulate guanylate cyclase in the caecum, liver and kidney from mice (Scheving *et al.*, 1985). Nevertheless it remains unlikely that EGF inhibits parietal cell secretory activity by increasing guanylate cyclase activity as IBMX prevents the inhibitory effects of EGF (section 3.3.5) but not those of dbcGMP (section 6.3.2).

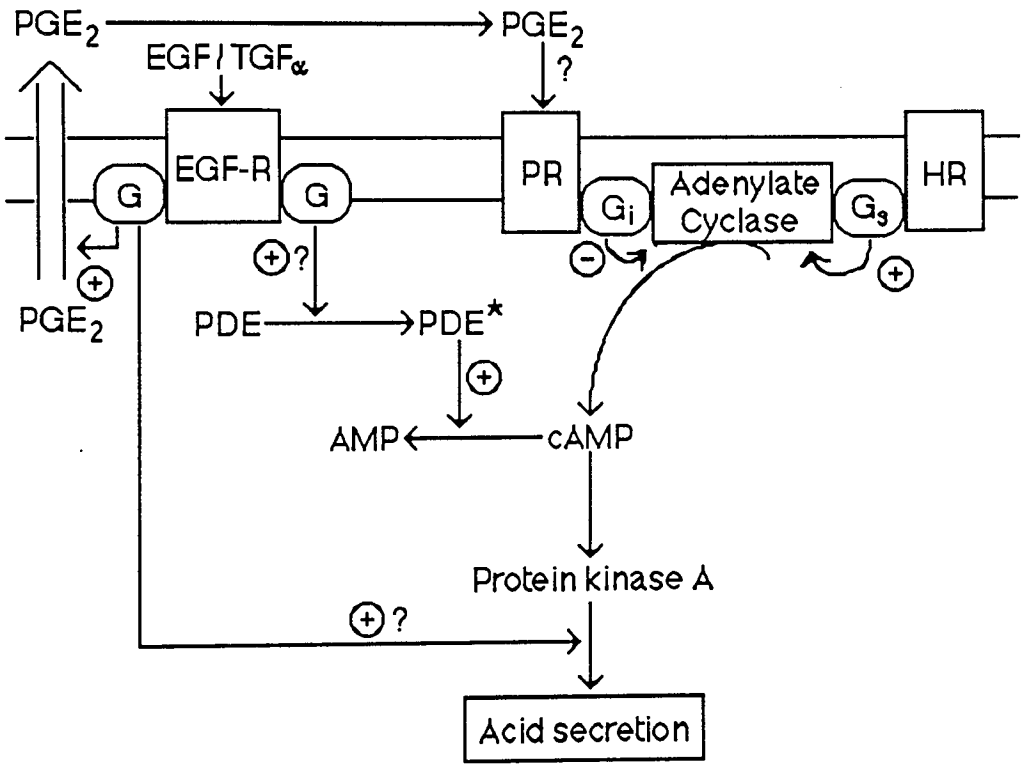
In isolated cell suspensions it seems unlikely that EGF mediates its inhibitory action via prostaglandin production. In the presence of 200nM-EGF the concentration of PGE_2 in the medium after 30 minutes incubation was 67.8 ± 13.1pM-PGE_2 whereas the half-maximally effective concentration of PGE_2 required to inhibit aminopyrine accumulation in rat parietal cells is 24nM (section 3.3.3). EGF did not mediate its inhibitory action by prostaglandin production in rat parietal cells stimulated by histamine as inhibitors of cyclo-oxygenase and lipoxygenase activity did not prevent the action of EGF (Shaw *et al.*, 1987). Histamine was found to block EGF stimulated PGE_2 release in rat parietal cells, this finding also lends further support to the suggestion that PGE_2 does not mediate the action of EGF (Hatt and Hanson, 1988). Consequently EGF must mediate its inhibitory action on isolated parietal cells stimulated with histamine by other means. Under physiological conditions prostaglandins released into the extracellular space may reach sufficiently high concentrations to inhibit acid secretion. However, as histamine will be present in acid secreting parietal cells it appears unlikely that EGF would inhibit secretory activity by stimulating prostaglandin production (Hatt and Hanson, 1988). A diagrammatic representation of the possible action of EGF on parietal cells is shown in Fig. 7.3.

The mechanism by which EGF inhibits acid secretion in the rat is different to that used by PGE_2 and somatostatin. Therefore it is conceivable that EGF acts in concert with another inhibitory agent. Indirect support for the hypothesis that EGF does not work alone comes from the observation that the 200nM-EGF can only reduce secretory activity by 40 to 50%. Indeed EGF may act with prostaglandins to inhibit acid secretion when the mucosa is damaged (see below).

Production of EGF occurs in Brunner's glands and submandibular glands in man. It has been suggested that EGF in the saliva protects the gastric mucosa against damaging agents. EGF, which is a stimulator of cell proliferation and differentiation, reduced ulcer formation in rats and oral doses of EGF reduced or healed ulcers in humans (Itoh *et al.*, 1988).

Figure 7.3

A speculative scheme of the action of EGF on the parietal cell.



Key: + = stimulation; - = inhibition; EGF-R, EGF receptor; PR, PGE_2 receptor; HR, histamine H_2 receptor; PDE, phosphodiesterase (basal); PDE*, phosphodiesterase (activated); G, G-protein; G_i, inhibitory G-protein; G_s, stimulatory G-protein; cAMP, cyclic AMP.

Interaction of EGF with its receptor on the cell surface inhibits histamine stimulated secretory activity by a possible reduction in cyclic AMP by the activation of a low Km phosphodiesterase. EGF also stimulates an increase in PGE_2 secretion by possibly stimulating phospholipase A_2 activation. Both these mechanisms appear to be mediated via pertussis toxin sensitive G-proteins (not shown for clarity). EGF may also mediate its inhibitory action at a site downstream from cyclic AMP generation and hydrolysis in pareital cells from species other than the rat.

Luminal EGF has an insignificant antisecretory action (section 3.1.3.3). Also the concentration of EGF in mouse plasma (0.16nM; Carpenter and Cohen, 1979) is lower than that required for half-maximal inhibition of aminopyrine accumulation stimulated by histamine (4nM; section 3.3.6). Thus although there is evidence that EGF receptors are present on the vascular-facing membranes of parietal cells (section 3.1.3.2) it appears unlikely that EGF present in plasma plays a physiological role in the inhibition of parietal cell secretory activity under normal circumstances. However, it possible that EGF inhibits acid secretion during acute injury to the gastric mucosa and blood vessels. Under such conditions EGF may be released from platelets during their aggregation and provide EGF at a local concentration sufficient to inhibit acid secretion. This action would prevent the accumulation of acid under the protective mucus and fibrin cap (Shaw *et al.*, 1987). Alternatively disruption of the the luminal surface of the stomach may allow luminal EGF to gain access to the parietal cell.

More recently it has been reported that EGF is produced by a novel cell lineage in the stomach and intestine. Ulceration apparently induces this lineage to form small glands which secrete mucin and immunoreactive EGF (Wright *et al.*, 1990). Thus EGF, which may be absorbed through damaged mucosa, may have a physiological role in stimulating ulcer healing by inhibiting parietal cell secretory activity and stimulating cell proliferation .

An alternative possibility is that TGF_{α} rather than EGF is the physiological agonist for the EGF receptor on parietal cells. TGF_{α} and EGF share structural homology and a common membrane bound receptor (section 4.5). Both peptides are acid stable and share a similar spectrum of biological activity (Beauchamp *et al.*, 1989). EGF and TGF_{α} displayed similar potencies in inhibiting acid secretion stimulated by histamine in guinea-pig mucosa (Rhodes *et al.*, 1986). High TGF_{α} mRNA expression was detected in the gastric epithelium with little detected in the gastric submucosa. Expression of TGF_{α} and TGF_{α}/EGF receptor mRNA was higher in fractions enriched with parietal cells relative to unfractionated gastric mucosal cells (5.8 and 7.8 fold increase respectively) (Beauchamp *et al.*, 1989). Only weak EGF mRNA expression was detected in in the fundus of the dog and none was detected in gastric mucosa from humans, guinea-pigs or rats. Thus if there is little or no EGF mRNA there will be little or no EGF produced by the gastric mucosa (Beauchamp *et al.*, 1989). However, EGF immunoreactivity has been detected previously in human gastric glands (Kasselberg *et al.*, 1985). Beauchamp *et al.* (1989) suggested that the EGF detected may have been produced by the salivary glands, swallowed and become bound to gastric TGF_{α}/EGF receptors, internalised and then detected by immunoreactivity. Therefore locally produced TGF_{α} may regulate acid secretion by a paracrine or autocrine mechanism.

7.1.4 The role of protein kinase C in modulation of parietal cell secretory activity in the rat.

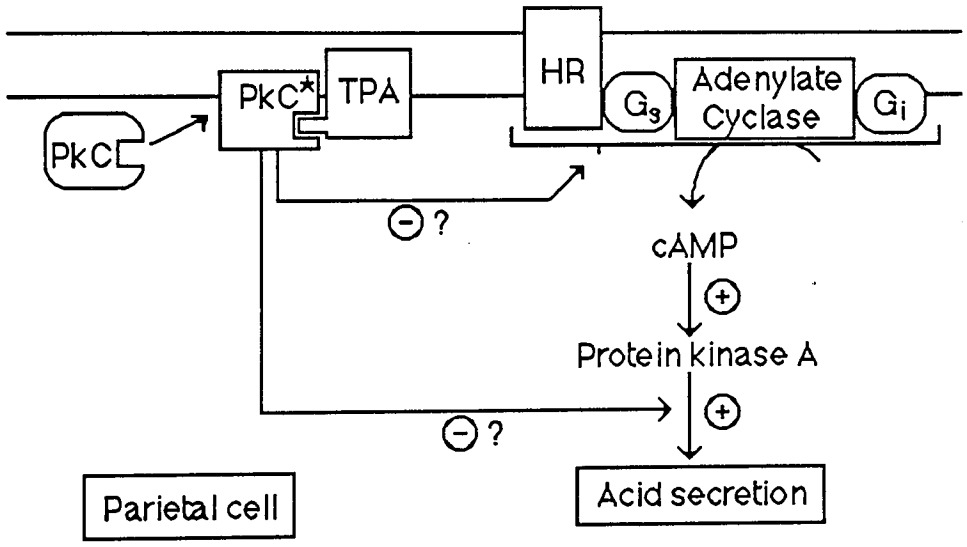
TPA was used to activate protein kinase C so that the involvement of protein kinase C in the modulation of secretory activity in the parietal cell could be determined (section 5.1). Under physiological conditions protein kinase C is activated by diacylglycerol, which may be metabolised by a number of different pathways in the cell (Pelech and Vance, 1989). In contrast TPA does not appear to be metabolised by the cell (Castagna *et al.*, 1982). Therefore prolonged and potent activation of protein kinase C by TPA may not truly mimic the effects produced by physiological agonists.

Protein kinase C may have inhibitory and stimulatory effects on acid secretion by acting on different sites within the parietal cell. The sites at which protein kinase C may inhibit parietal cell secretory activity are illustrated in Fig. 7.4. The action of TPA against histamine-stimulation can be differentiated from that of PGE_2, somatostatin and EGF in that it was only affected by preincubation with pertussis toxin to a limited extent. TPA appears to effect its inhibitory action via a different mechanism to PGE_2, somatostatin and EGF. One possible role of this inhibitory pathway is to provide a means by which muscarinic cholinergic receptor activation can negatively modulate stimulation of parietal cell activity by histamine. Thus recently it has been suggested that there are two subtypes of muscarinic cholinergic receptors present on parietal cells from the guinea-pig (Kajimura *et al.*, 1990). The high affinity receptor appeared to potentiate with histamine and the low affinity receptor-subtype appeared to inhibit the histamine response. It was suggested that the inhibitory action of carbachol may be mediated via the activation of protein kinase C though this must be established (Kajimura *et al.*, 1990). Hatt and Hanson (unpublished work) examined interactions between carbachol (0.1mM) and histamine (0.5mM) plus IBMX (0.1mM) in a crude preparation of parietal cells from the rat. The results were variable showing an additive or inhibitory effect of carbachol. Other workers have observed a synergistic interaction between carbachol and dbcAMPin enriched parietal cells (Pfeiffer *et al.*, 1987). Thus some evidence for the negative modulation of the histamine-stimulated pathway by carbachol exists but it is unclear why in some species and under some circumstances synergistic interactions between these pathways occurs.

An alternative possibility is that protein kinase C mediates the inhibitory mechanism of an inhibitor of acid secretion. Thus if a phospholipase C specific for a phospholipid other than phosphatidylinositol 4,5-bisphosphate was activated then protein kinase C may be activated without mobilisation of Ca^{2+}. It is unlikely that this ligand is EGF, somatostatin or PGE_2 as the spectrum of inhibition by these agents is different to that by TPA (see section 3.3). Unlike other physiological inhibitors of secretory activity in rat parietal cells both substance P and thyrotropin-releasing

Figure 7.4

Diagrammatic representation illustrating the potential sites at which protein kinase C, when activated by TPA, may act to inhibit acid secretion induced by histamine in the parietal cell.



Key: + = stimulation; - = inhibition; PkC, protein kinase C (inactive); PkC*, protein kinase C (active); HR, histamine H_2 receptor; G_i, inhibitory G-protein; G_s, stimulatory G-protein.

TPA becomes intercalated into the plasma membrane and activates protein kinase C. Upon activation protein kinase C may exert an inhibitory action on acid secretion at 3 sites as indicated.

hormone inhibit aminopyrine accumulation stimulated by histamine and dbcAMP (Schepp *et al.*, 1988a and b). Thus it is possible that these agents activate protein kinase C, but this remains to be investigated. Unfortunately attempts to address this problem by down-regulating protein kinase C on exposure to TPA were not successful.

PUBLICATIONS RESULTING FROM THIS WORK.

Full paper.

Atwell, M. M. and Hanson, P. J. (1988). Effect of pertussis toxin on the inhibition of secretory activity by prostaglandin E_2, somatostatin, epidermal growth factor and 12-*O*-tetradecanoylphorbol 13-acetate in parietal cells from the rat stomach. Biochim. Biophys. Acta **971**, 282-288.

Communications.

Atwell, M. M. and Hanson, P. J. (1988). Effect of pertussis toxin on the action of inhibitors of acid secretion by parietal cells of the rat. Biochem. Soc. Trans. **16**, 281.

Atwell, M. M., Hatt, J. F. and Hanson, P. J. (1989). Effect of epidermal growth factor on acid and pepsinogen secretion by rat isolated stomach cells stimulated with forskolin. Biochem. Soc. Trans. **17**, 1077.

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APPENDICES

A.1. SOURCE OF REAGENTS.

Reagent.

A. General Chemicals.	Supplier.
Acrylamide	BDH
Amphotericin B	Sigma
Bovine serum albumin (BSA), fraction V	ICN Biomedicals
Bromophenol blue	Bio-Rad
Calcium chloride	BDH
Dextran (M.Wt. 40,000Da)	Sigma
Dimethyldichlorosilane	BDH
Dimethylsulphoxide (DMSO)	Sigma
Di-potassium hydrogen orthophosphate	BDH
Di-sodium hydrogen orthophosphate	BDH
Dithiothreitol (DDT)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Ethyleneglycol-bis-(β-aminoethylether) N, N'-tetraacetic acid	Sigma
Foetal calf serum	Sigma
Folin and Ciocalteu's phenol reagent	Sigma
Gentamicin	Sigma
Glycerol	BDH
Glycine	Sigma
D-glucose	BDH
L-glutamine	Sigma
Haemoglobin	Sigma
Hydrocortisone	Sigma
N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid	Sigma
Insulin	Sigma
L-isoleucine	Sigma
Leupeptin	Sigma
Magnesium sulphate	BDH
NN'-methylenebisacrylamide	BDH
Pepstatin	Sigma
Percoll	Sigma
Potassium chloride	BDH
Potassium dihydrogen orthophosphate	BDH
Sagatal	May and Baker
Sodium chloride	BDH
Sodium dihydrogen orthophosphate	BDH

Sodium dodecyl sulphate (SDS)	BDH
Sodium hydrogen carbonate	BDH
Sodium thiocyanate	Sigma
N,N,N',N'-tetramethylethylenediamine	Sigma
Trichloroacetic acid	BDH
Tris (hydroxymethyl)-methylamine	BDH
Trypan blue	BDH
Trypsin inhibitor, lyophilised from soybean	Sigma

B. Enzyme.

Adenosine deaminase (Type II)	Sigma
Pronase, 70,000 PUK/g	BDH
Pepsinogen (Porcine)	Sigma
Pepsin (Porcine)	Sigma
Pertussis toxin	Portland Down Research

C. Radiochemicals and scintillation counting.

Aminopyrine, dimethylamine-[^{14}C]	Amersham International
Cyclic AMP[^{125}I] radioimmunoassay kit (RIANEN^{TM})	Du Pont
"Econofluor"	Du Pont
[1, 2-^{3}H]-polyethylene glycol	Amersham International
Prostaglandin E_2 [^{125}I] radioimmunoassay kit	Du Pont
"Protosol"	Du Pont

D. Secretagogues and agents.

Calcitonin gene-related peptide (CGRP)	Sigma
2-chloroadenosine	Sigma
Cimetidine	Sigma
Cyclo (7-aminoheptanoyl-Phe-D-Trp- Lys- Thr-[Bzl])	
(a somatostatin antagonist)	Sigma
Dibutyryl cyclic AMP, sodium salt	Sigma
Dibutyryl cyclic GMP, sodium salt	Sigma
Epidermal growth factor	Sigma and ICI
Forskolin	Sigma
Histamine dihydrochloride	Sigma
3-isobutyl-1-methylxanthine (IBMX)	Sigma
N^6-(L-2-phenylisopropyl)adenosine	Sigma
Prostaglandin E_2	Sigma
Ro 20-1724	Hoffman-La Roche
Somatostatin	Sigma

12-0-tetradecanoylphorbol-13-acetate (TPA)	Sigma

A.2 Media preparation.

A.2.1 Preparation of Eagle's Minimum Essential Medium.

The powdered medium, obtained from Sigma, was dissolved in 100ml of double-distilled water by stirring at room temperature. The final concentration of the components was as shown below.

L-arginine (0.7mM), L-cystine (0.23mM), L-glutamine (2.0mM), L-histidine (0.27mM), L-isoleucine (0.4mM), L-leucine (0.4mM), L-lysine (0.5mM), L-methionine (0.09mM), L-phenylalanine (0.2mM), L-Threonine (0.4mM), L-tryptophan (0.05mM), L-tyrosine (0.25%), L-valine (0.4%), choline chloride (7.0μM), folic acid (3.0μM), myo-inositol (0.01mM), niacinamide (8.0μM), D-pantothenic acid (2.1μM), pyridoxal HCl (4.86μM), riboflavin (0.3μM), thiamine HCl (3.0μM), $CaCl_2$ (1.8mM), KCL (5.4mM), $MgSO_4$ (0.8mM), NaCl (116.4mM), NaH_2PO_4 (1.0mM), D-glucose (5.6mM), phenol red (0.001% ($^w/_v$)).

While the solution was stirring $NaHCO_3$ (25mM) and HEPES (20mM) were added. This medium was warmed to 37°C and gassed for 30 minutes with 95%O_2/5%CO_2. Then the pH was adjusted to 7.4.

A.2.2 Composition of stock Krebs-Ringer bicarbonate media (mM).

Salts	Physiological medium	High K^+ medium
KCl	4.5	100
$CaCl_2$	1.25	1.25
NaCl	120	24.5
$NaHCO_3$	25	25
Na_2HPO_4	1.8	1.8
NaH_2PO_4	0.2	0.2
$MgSO_4$	1	1

The media was gassed for 20 minutes with 95%O_2/5%CO_2 before the pH was adjusted to 7.4 with HCl (3M) or NaOH (3M) if necessary.

A.3 Liquid scintillation counting.

Scintillation counting was used to determine the accumulation of [^{14}C]-aminopyrine. Radioactivity causes the excitation of certain compounds or fluors which then fluoresce or scintillate. The light emitted in scintillation can be detected by photomultiplier tubes which convert the photons into electrical impulses. The magnitude of this impulse is proportional to the energy of the original radioactive event. The TRI-CARB 2600 scintillation counter (Packard) contains two counting channels which allows the simultaneous determination of [^{14}C] and [^{3}H] counts. This is possible as the energy specra for [^{14}C] and [^{3}H] are different. Thus the threshold and window of one pulse height analyser is set to detect [^{3}H] and the other [^{14}C].

The efficiency was determined using the external standards channel ratio (ESR) method. To do this a quench correction curve of the counting efficiency against the ESR is determined by counting a series of standard vials containing a known amount of [^{14}C] or [^{3}H] with varying amounts of the quenching agent chloroform. The ESR for each sample is determined and then the counts can be determined from the quench correction curve.

The activity (dpm) of the higher energy radionuclide, [^{14}C], can be calculated from the sample cpm by using the counting efficiency of [^{14}C].

$$Y = \frac{B}{E_4}$$

Y = dpm of [^{14}C].
B = total cpm in higher energy channel.
E_4 = efficiency of [^{14}C] in the high energy channel.

The activity (dpm) of the lower energy nuclide can be determined from the sample cpm as shown below.

$$X = \frac{A - E_3 Y}{E_1}$$

X = dpm of [^{3}H].
A = total cpm of the low energy channel.
E_3 = efficiency of [14C] in the low energy channel.
E_1 = efficiency of [^{3}H] in the low energy channel.

Counting [^{14}C] aminopyrine and [^{3}H] PEG in 0.5ml of Protosol and 10ml of Econofluor resulted in the following efficiencies.

[^{3}H] in the low energy channel = 23%
[^{14}C] in the high energy channel = 75%
[^{14}C] in the low energy channel = 4%

A.4 Cyclic AMP and PGE_2 radioimmunoassay.

A.4.1 Composition of reagents for the RIANEN™ cyclic AMP radioimmunoassay kit.

Reagents were reconstituted as directed in the cyclic AMP radioimmunoassay kit.

1. Sodium acetate buffer: The concentrated buffer was made up to 500ml in a volumetric flask with double-distilled water and stored at 2-8°C. The final solution (pH 6.2) contained sodium acetate (50mM), sodium azide (0.1%), EDTA (0.03%) and mannitol (0.025%).

2. Cyclic AMP standard: The lyophilised standard was reconstituted in 2.0ml of double-distilled water and stored at 2-8°C. The final cyclic AMP concentration was 5,000pmoles/ml in sodium acetate buffer, sodium azide (0.1%) and mannitol (0.025%).

3. Cyclic AMP [^{125}I] Tracer (Succinyl cyclic AMP tyrosine methyl ester [^{125}I]): The concentrated tracer in 1ml of propanol:water solution was mixed with 5ml of double-distilled water and stored at 2-8°C. The solution contained 1.5μCi (on the calibration date).

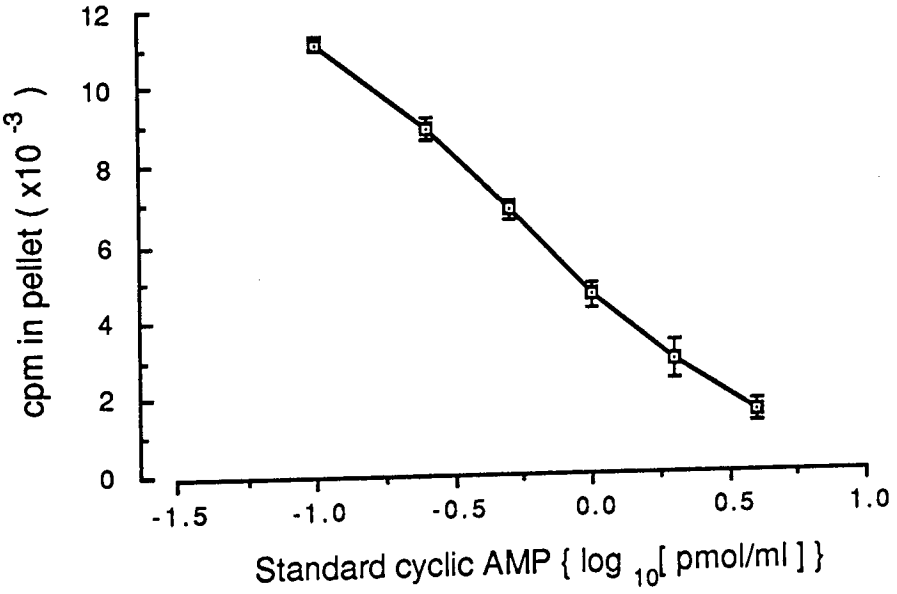
4. Cyclic AMP carrier serum: The lyophilised cyclic AMP carrier serum was reconstituted with 6.0ml of double-distilled water producing a solution (pH 6.2) containing carrier serum, sodium azide (0.1%) and a stabiliser in sodium acetate buffer.

5. Cyclic AMP antiserum complex: The lyophilised pre-reacted antibody consisted of a rabbit first and sheep second antibody and was reconstituted in 21ml of double-distilled water. This generated a solution containing the pre-reacted antibody complex, thimerosal (0.005%) and mannitol (0.025%) in sodium phosphate buffer (pH 6.0).

6. Acetic anhydride and triethylamine: Acetic anhydride and triethylamine were mixed together in a 2:1 ratio. Both agents were allowed to equilibrate at room temperature before use.

7. Cyclic AMP precipitator: The preformed complex solution in sodium acetate (50mM) contained EDTA (8mM) and NaN_3 (0.1%).

A mean standard curve for the radioimmunoassay of [^{125}I]-cyclic AMP.



Values are means ± S.E.M. of 8 separate curves. Total counts = 22100 ± 514(8) cpm and the blank = 843 ± 40(8) cpm (values expressed as means + S.E.M. with the number of determinations in parentheses). The cyclic AMP content of the samples was calculated using a single standard curve generated on the same day that the samples were assayed.

A.4.3 Composition of the reagents used in the [^{125}I] prostaglandin E_2 radioimmunoassay kit.

Reagents were made up as directed by the manufacturers of the PGE_2 radioimmunoassay kit.

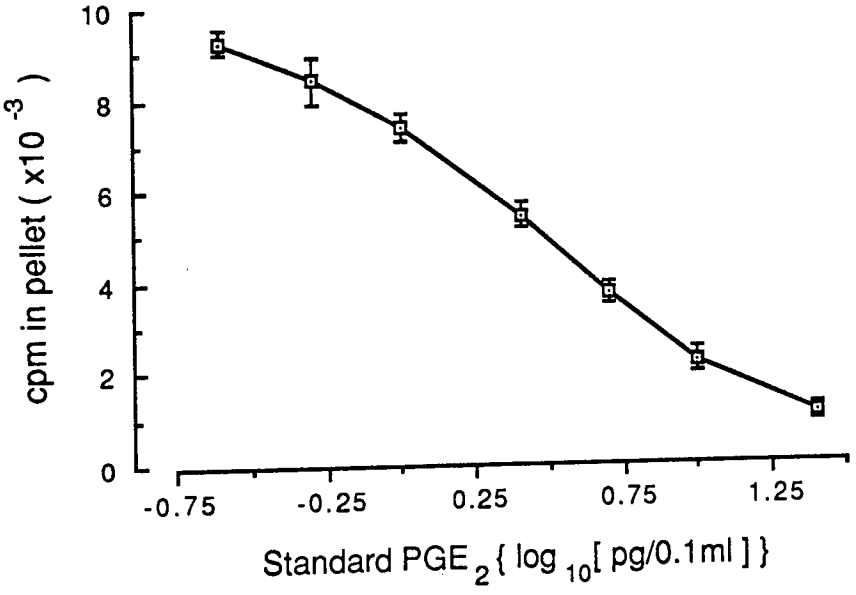
1. Assay buffer: Assay buffer consisted of NaCl (0.9%), EDTA (0.01%), bovine γ-globulin (0.3%), Triton X-100 (0.005%) and sodium azide (0.05%) in phosphate buffer (50mM).

2. PGE_2 standard: A PGE_2 solution (100ng/ml) was supplied in an organic solvent. An aliquot of this solution was diluted in assay buffer immediately prior to use. The standard concentrate was stored -20°C.

3. PGE_2 antibody: Lyophilised rabbit anti-PGE_2 antibody was reconstituted in 13ml of assay buffer and stored at 4°C.

4. Precipitating reagent: A solution consisting of polyethylene glycol (16%) (PEG 6000) and sodium azide (0.05%) in phosphate buffer (50mM) (pH 6.8) was provided. This solution was stored at -20°C.

A mean standard curve for the radioimmunoassay of [^{125}I]-PGE_2.



Values are means ± S.E.M. of 5 separate curves. Total counts = 17143 ± 743(5) cpm and the blank = 784 ± 39(5) cpm (values expressed as means + S.E.M. with the number of determinations in parentheses). The PGE_2 content of the samples was calculated using a single standard curve generated on the same day that the samples were assayed.

A.5 STATISTICAL ANALYSIS.

Some examples of the statistical tests used to analyse the results in this thesis are set out below.

A.5.1 Analysis of variance (ANOVAR).

Data from Table 3.17 were tested using a two-way analysis of variance with a randomised block experimental design. This procedure examines the effects of four different concentrations of EGF on PGE_2 release by a gastric cell suspension from the rat. In the present example EGF concentration is the fixed factor and the experiment is the random factor.

DATA

Experiments	Basal	0.2nM-EGF	2nM-EGF	20nM-EGF	200nM-EGF
1	9.270	11.229	13.704	13.311	15.127
2	12.782	13.500	16.052	18.058	16.324
3	22.699	22.883	27.372	28.140	26.531
4	26.862	25.850	31.015	32.263	30.342
5	8.122	8.486	11.695	8.657	9.979

The mean squares are determined by dividing the sum of squares by the degrees of freedom for each factor. The F-ratio is calculated by dividing the mean squares for each factor by the remainder mean squares. The F-ratio is compared with the tabulated F-ratio with the degrees of freedom associated with the remainder and the factor.

Analysis of variance summary table.

Variation	Sum Sq	DF	Mean Sq	F-test
Treatments	82.2738	4	20.5685	15.5467*
Experiments	1465.7951	4	366.4488	294.7969*
Remainder	19.8889	16	1.2431	
Total	1567.9578	24		

The tabulated F-ratio with 4 and 16 degrees of freedom is 7.94 at a significance level of 0.001%. Thus the effect of EGF was significant. *P<0.001.

A.5.2 Dunnett's test.

If the effect of treatments was found to be signifficant by ANOVAR, the results can be further analysed by a Dunnett's test. Dunnett's test (Zar, 1984) is a procedure which determines whether the control mean differs significantly from each of the treatment means. A Dunnett's test was used on data from Table 6.2 after a two-way ANOVAR on the raw data.

DATA

Experiments	Control	DbcGMP (10^{-6}M)	DbcGMP (10^{-5}M)	DbcGMP (10^{-4}M)	DbcGMP (10^{-3}M)
1	59.54	56.09	56.92	48.58	45.68
2	34.93	32.21	30.66	32.92	28.70
3	43.89	41.26	41.02	40.45	30.38
4	56.47	53.93	51.92	52.03	42.05

The data were transformed (log transformation) and subjected to a two way ANOVAR with a randomised block design.

Analysis of variance summary table.

Variation	Sum Sq	DF	Mean Sq	F-test
Treatments	0.033	4	0.008	17.285
Experiments	0.164	3	0.055	112.605
Remainder	0.006	12	0.0004844	
Total	0.203	19		

GROUP MEANS (RANKED)

1	2	3	4	5
1.678	1.651	1.643	1.632	1.556
Control	DbcGMP (10^{-6}M)	DbcGMP (10^{-5}M)	DbcGMP (10^{-4}M)	DbcGMP (10^{-3}M)

$H_O : \mu_C = \mu_A$ vs $H_A : \mu_C = \mu_A$

H_O = the null hypothesis; H_A = the alternative hypothesis; μ_C = control sample mean; μ_A = test sample mean.

The standard error is calculated as below.

$$S.E. = \sqrt{\frac{2 \times \text{remainder mean sq}}{n}}$$

The test statistic is as shown below.

$$q = \frac{\bar{X}_{control} - \bar{X}_A}{S.E.}$$

See Table A.5.2.1 for the test.

If the resulting q value exceeds the appropriate tabulated value then there is presumed to be a significant difference between those two means. In this example there was a significant effect of dbcGMP on histamine plus IBMX stimulation at 10^{-3} and 10^{-4}M-dbcGMP.

A.5.3 A paired *t*-test.

A paired *t*-test was used to analyse much of the data in this work. This test is used when the two samples to be tested are associated, in that they come from the same batch of cells. Set out in Table A.5.3.1 is an example of a paired *t*-test that was used to examine normalised data from Table 3.10.

Table A.5.2.1

Dunnett's test summary table.

Comparison	Difference	S.E.	q	p	$q'_{0.05,v,p}$	$q'_{0.01,v,p}$	Conclusion
1 vs 5	0.122	1.5563×10^{-2}	7.839	5	2.81	3.71	P<0.01
1 vs 4	0.046	1.5563×10^{-2}	2.956	4	2.68	3.58	P<0.05
1 vs 3	0.035	1.5563×10^{-2}	2.249	3	2.50	3.39	Not significant
1 vs 2	0.027	1.5563×10^{-2}	1.735	2	2.18	3.05	Not significant

p = number of means in the range being tested.
v = remainder degrees of freedom for the ANOVAR.

Table A.5.3.1

Analysis of data from Table 3.10

Normalised APR PGE_2 (10^{-6}M)	Normalised APR PGE_2 (10^{-6}M)+pertussis toxin (100ng/ml)	Difference
85.2	14.2	71.0
51.7	9.5	42.2
82.3	24.3	58.0
71.3	1.3	70.0
Mean ± S.E.M.	Mean ± S.E.M.	Mean ± S.E.M.
72.63 ± 7.59	12.33 ± 4.80	60.3 ± 6.72

H_O: $\mu_1-\mu_2=0$ vs H_A: $\mu_1-\mu_2=0$

H_O = the null hypothesis; H_A = the alternative hypothesis; μ = sample mean.

n=4 and df=n-1. So there are 3 degrees of freedom.

The test statistic is calculated as shown below

$$t = \frac{\text{mean of } \mu_1 - \mu_2}{\text{S.E. of } \mu_1 - \mu_2}$$

$$= \frac{60.3}{6.72}$$

Thus t=8.9767 and $t_{0.01}$=5.84 so there is a significant difference in this example ($P<0.01$).

A.6 Animals.

Male wistar rats were obtained from Bantin and Kingman, Hull, U. K. and were fed on Heygates breeding diet supplied by Pilsbury, Edgbaston, Birmingham, U. K.