REGULATION OF ACID SECRETION BY PARIETAL CELLS ISOLATED FROM THE RAT STOMACH.

by

Jean Frances Hatt

A thesis submitted for the

degree of

Doctor of Philosophy

ASTON UNIVERSITY

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This study was undertaken to further understanding of the mechanisms which regulate acid secretion by isolated parietal cells. The particular aims of this work were to investigate the sites at which calcium-sensitive and phospholipid-dependent protein kinase, protein kinase C, could act to modulate acid secretion, and to establish the site, and mechanism by which epidermal growth factor (EGF) inhibits acid secretion. The intracellular accumulation of the weak base aminopyrine was used as an index of parietal cell secretory activity.

Experiments using 12-O-tetradecanoylphorbol-13 acetate (TPA) and 1-oleoyl-2-acetylglycerol (OAG) to activate protein kinase C suggested three sites of action of this enzyme. TPA dose-dependently inhibited the histamine plus 3-isobutyl-1-methylxanthine (IBMX) stimulated increase in cellular cyclic AMP implicating a site of action at, or close to, adenylate cyclase. A site of action distal to adenylate cyclase was also suggested since TPA was an effective inhibitor of aminopyrine accumulation in cells stimulated by dibutyryl cyclic AMP. TPA and OAG transiently increased the aminopyrine accumulation ratio in cells incubated in a medium containing 100mM-K^+. This effect of TPA and OAG did not involve histamine, acetylcholine or changes in cellular cyclic AMP levels. It is therefore possible that a stimulatory site of action of protein kinase C exists within these cells.

Activators of protein kinase C inhibited secretory activity under conditions where EGF was ineffective. Although EGF stimulated the production of prostaglandin E_2 by a parietal cell-enriched preparation under basal conditions, there was no effect with histamine present. EGF dose-dependently inhibited the histamine-induced increase in the aminopyrine accumulation ratio and cyclic AMP content. This effect was blocked by IBMX, and it is therefore possible that the antisecretory effect of EGF was due to an increase in cyclic AMP phosphodiesterase activity.

Key Words: Gastric acid secretion. Parietal cell. 12-O-tetradecanoylphorbol-13 acetate (TPA). Epidermal growth factor (EGF).

For my Families

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ABBREVIATIONS

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

ANOVAR	Analysis of variance
cDNA	complementary DNA
dbcAMP	dibutyryl cyclic AMP
DMSO	dimethylsulphoxide
d.o.f.	degrees of freedom
EGF	epidermal growth factor
Fig.	figure
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	concentration of agent producing a half-maximal inhibitory effect
kDa	kilodalton (dalton = $\frac{1}{12}$th of the mass of 1 atom of nuclide ^{12}C)
min	minutes
Mr	relative molecular mass (a dimensionless number)
OAG	1-oleoyl-2-acetylglycerol
4αPDD	4-α-phorbol-12,13,-didecanoate
PGE_2	prostaglandin E_2
protein kinase A	cyclic AMP-dependent protein kinase
protein kinase C	calcium-sensitive, phospholipid-dependent protein kinase
S.E.M.	standard error of the mean
TPA	12-O-tetradecanoylphorbol 13-acetate

Chapter One

INTRODUCTION.

1.1 THE PARIETAL CELL

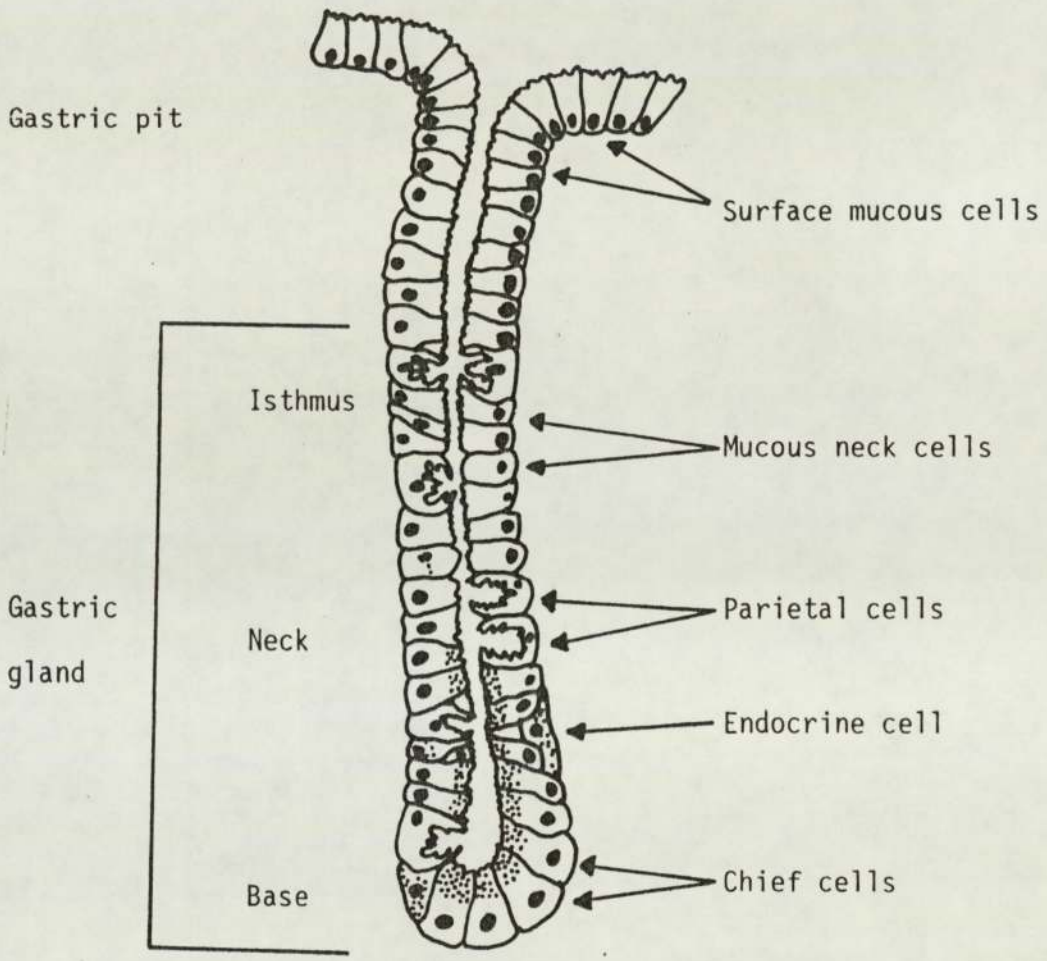
1.1.1 The location of the parietal cell in the gastric mucosa.

The mammalian stomach of most non-ruminant animals is a single chambered sac in which ingested food is temporarily retained. The stomach secretes acid which may help to sterilise ingested food, and pepsinogen, which in the acid environment is converted to the protease pepsin. The rat stomach can be broadly divided into the non-glandular or cardiac region which is immediately distal to the entrance of the oesophagus, and the glandular area which extends down to the pyloric region and the duodenum.

Gastric glands containing parietal cells are located in the fundic glandular region and pyloric glands containing the gastrin-secreting G-cells are located in the antral glandular region, closer to the pylorus. The surface of the gastric mucosa is highly convoluted and is covered by a simple columnar epithelium of mucous cells which also extends down into gastric pits. These cells secrete mucus and bicarbonate thereby creating a mucus-bicarbonate barrier which may in part serve to protect the mucosa from damage by luminal acid and pepsin (Garner et al., 1984). Gastric glands, containing a variety of cells (Fig. 1.1) empty into gastric pits which provide a channel along which secretions can pass to the lumen of the stomach. At the top of the gastric gland another type of mucus-secreting cell, the mucous neck cell is found. The parietal or oxyntic cell is usually located in the neck or isthmus of the gastric gland and is responsible for the production of hydrochloric acid. Endocrine cells may be scattered throughout the gland and as many as nine different types have been identified (Grube and Forssmann, 1979). The endocrine cells responsible for the production of histamine vary between species, thus in the rat histamine is synthesised by endocrine-like cells which are

Figure 1.1

A simplified schematic diagram of a gastric gland from a mammalian stomach.



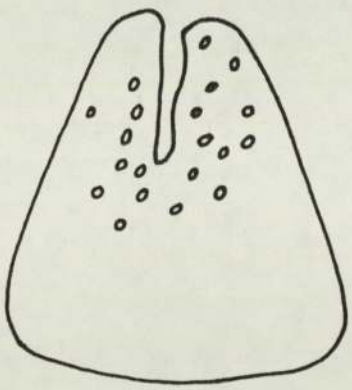
distinct from the mast cells found in the dog (Soll et al., 1981). Chief cells are usually located at the base of gastric glands, and they have been demonstrated by immunofluorescence to be the source of pepsinogen (Samloff, 1971).

1.1.2 Ultrastructural changes of the parietal cell consequent upon secretagogue-stimulation.

The ultrastructure of the parietal cell undergoes pronounced changes when it is stimulated to secrete acid (Fig. 1.2). These changes involve narrow canals called secretory canaliculi which run from the apical (luminal-facing) membrane of the cell. In the resting cell these secretory canaliculi are poorly developed and are lined by short, stubby microvilli, and a system of tubular and vesicular membranes, tubulovesicles, are prominent around the apical region of the cell. As soon as three minutes after stimulation of the cell with secretagogues the morphological changes become apparent (Forte et al., 1981). The microvilli, lining the now prominent secretory canaliculi, elongate, and the increase in the surface area of the invaginated apical membrane correlates with the decrease in surface area of tubulovesicle membranes (Helander and Hirschowitz, 1972). In order to explain this transformation, two hypotheses have been proposed. The first is a membrane-recycling hypothesis in which tubulovesicles containing the H^+/K^+ ATPase (1.1.3) fuse with the apical membrane (Forte et al., 1981). This idea is supported by immunocytochemical studies showing that in resting cells the enzyme is predominantly located in tubulovesicles, but in stimulated cells staining for the enzyme was found on the microvilli of the secretory canaliculi (Smolka et al., 1983). The second hypothesis, termed the osmotic expansion hypothesis (Berglindh et al., 1980a), suggests that the tubulovesicles are effectively

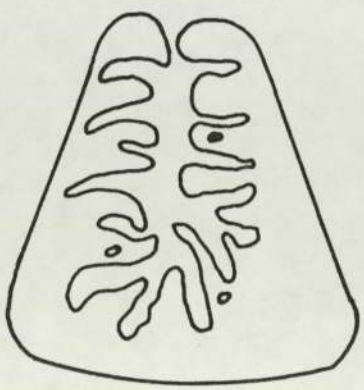
Figure 1.2

A diagrammatic representation of the gross morphological changes in the parietal cell which occur as a result of secretagogue stimulation.



Resting parietal cell

Numerous tubulovesicles are present in the apical region.



Actively secreting parietal cell

The tubulovesicles decrease in number, and the secretory canaliculus is enlarged.

For clarity the only cellular structures shown are the tubulovesicles and a secretory canaliculus.

collapsed canaliculi. The accumulation of HCl within the lumen of the acid-secreting spaces generates a hypertonic environment with the consequent movement of water out of the cell by osmosis into this space thereby inducing the expansion of the previously collapsed secretory canaliculi. This hypothesis is largely based on the finding that when cells are incubated in a 108mM-K^+ containing medium with 1mM-aminopyrine, intracellular vacuolisation is induced. However, morphometric studies (Gibert and Hersey, 1982) show that there is no decrease in the density of tubulovesicular membranes under such conditions. Thus it is rather unlikely that osmotic expansion alone can induce the morphological changes observed upon secretagogue-stimulation, and it is probable that a combination of these proposed mechanisms occurs.

Evidence has been provided that the secretory canaliculi are the site of secretagogue-induced acid secretion by incubating rabbit gastric glands with acridine orange (a fluorescent dye which distributes across membranes according to the pH gradient) and observing the glands by fluorescence and differential interference-contrast microscopy. A red fluorescence, indicative of a low pH was localised to the secretory canaliculi (Berglindh et al., 1980a).

1.1.3 The mechanism of acid secretion.

The membrane bound enzyme, the H^+/K^+ ATPase (EC 3.6.1.36) is responsible for pumping protons across the apical membrane of the parietal cell into the secretory canaliculi. Since the concentration of H^+ in blood is ~ 40nM and that in gastric fluid is ~ 150mM, a proton gradient exceeding 10^6 fold is developed. The H^+/K^+ ATPase has an absolute requirement for K^+ (4.1.1.1). Evidence that ATP is the primary source of energy for acid

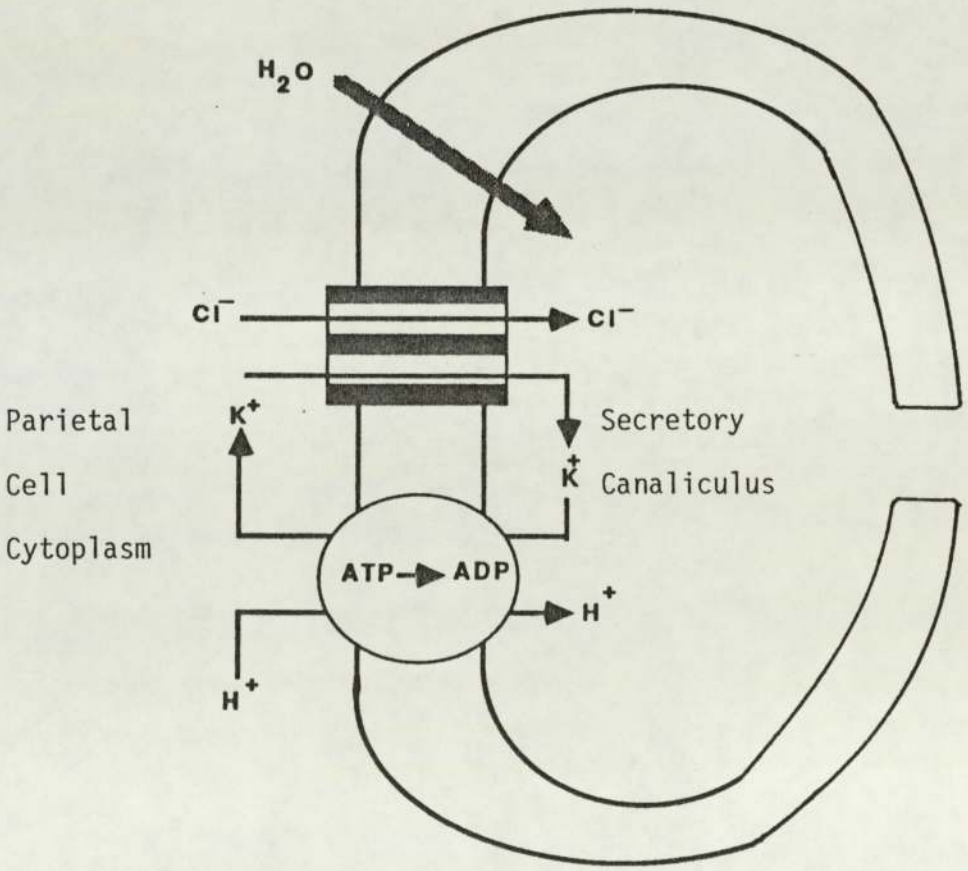
secretion is the demonstration that ATP alone can sustain aminopyrine accumulation in shock-permeabilised and metabolically inhibited gastric glands incubated in a medium containing 108mM-K^+ (Berglindh et al., 1980b).

A currently accepted model for the mechanism of acid secretion is shown in Fig. 1.3 (Wolosin, 1985). The H^+/K^+ ATPase extrudes H^+ into the secretory canaliculus in exchange for K^+ which is transferred back into the cytoplasm of the cell. In order for this enzyme to function and for H^+ to be pumped, K^+ is required in the lumen of the canaliculus. This is achieved by the presence of a conductive pathway for K^+ in the apical membrane. A Cl^- conductance is also present which is functionally independent yet both conductances may reside within a single physical unit (Wolosin and Forte, 1985). H^+/K^+ ATPase activity generates the transfer of ions from the cell into the lumen of the secretory canaliculus which is accompanied by the osmotic movement of H_2O from the cell. Normally the backflux of H^+ across the apical membrane is minimal. However compounds like SCN^- can combine with H^+ to form the lipid permeable HSCN, and thereby induce a rapid dissipation of the H^+ gradient. In isolated parietal cells or gastric glands some of the HCl remains within the secretory canaliculi thereby enabling the accumulation of a weak base to act as an index of acid secretion (1.3.2.3). However in intact tissue it is proposed (Berglindh et al., 1980a) that the basement membrane, connective tissue and muscularis mucosa together restrict cellular swelling thereby forcing the expulsion of HCl from the cell into the lumen of the gastric gland.

The mechanism by which the H^+/K^+ ATPase and K^+ and Cl^- conductances become active in the apical membrane is not fully understood to date; three of the models which have been suggested will now be summarised. (a) As a result of experiments using isolated membrane vesicles (4.1.1.1) it has been proposed that acid secretion occurs as a result of the fusion of tubulovesicles containing the

Figure 1.3

Model for pumping of HCl across the membrane of the secretory canaliculus.



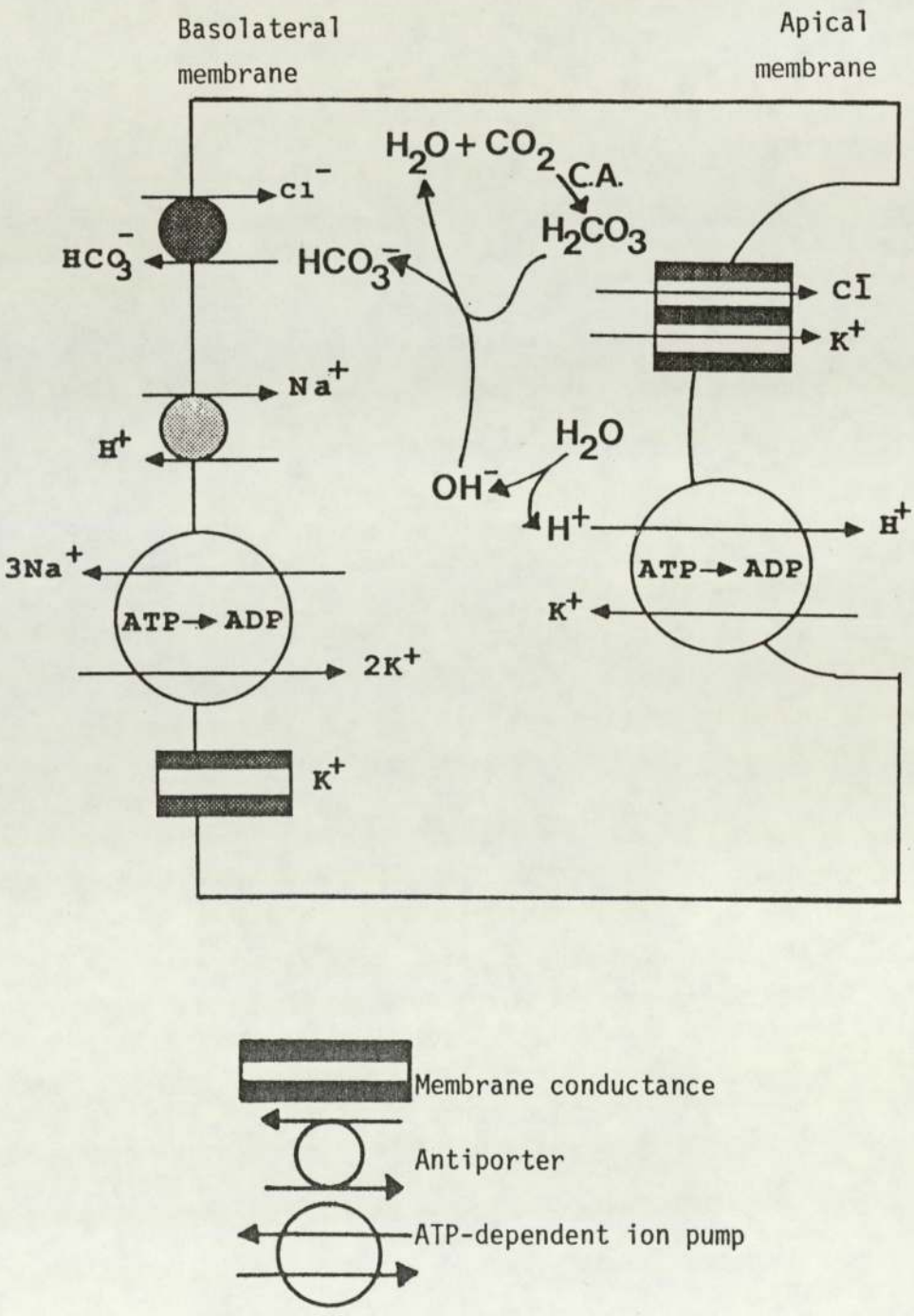
The H^+/K^+ ATPase extrudes H^+ into the secretory canaliculus in exchange for the inward movement of K^+. K^+ and Cl^- enter the lumen of the secretory canaliculus via conductance pathways in the apical membrane. A restrictive luminal duct minimises the loss of K^+ from the secretory canaliculus.

H^+/K^+ ATPase with the apical membranes containing the $K^+ + Cl^-$ conductances (Forte and Wolosin, 1987). (b) $K^+ + Cl^-$ conductances are contained in a light membrane vesicle population, distinct from the tubulovesicles, which along with tubulovesicles containing the H^+/K^+ ATPase become recruited into the apical membrane as a result of secretagogue stimulation (Im et al., 1985). (c) K^+ and Cl^- conductances are present in tubulovesicles together with the H^+/K^+ ATPase, stimulation with secretagogues activates a Cl^- conductance and simultaneously induces fusion of the tubulovesicles with the apical membrane (Takeguchi and Yamazaki, 1986). Whichever of these mechanisms operates there is no evidence for direct modulation of H^+/K^+ ATPase activity. Thus Malinowska and Cuppoletti (1988) using membrane Cl^- conductance inhibitors suggest that acid secretion may be regulated by a modulation of the ion conductances and that activation of the H^+/K^+ ATPase is secondary to the appearance of K^+ at the luminal face of the pump. The regulation of acid secretion is discussed in more detail in section 1.2.

In order for the parietal cell to function efficiently, the intracellular pH has to be closely regulated, and this is achieved by the operation of separate Cl^-/HCO_3^- and Na^+/H^+ exchangers located on the basolateral membrane of the cell (Paradiso et al., 1987; see Fig. 1.4). The Cl^-/HCO_3^- exchanger facilitates the expulsion of HCO_3^- (produced from the reaction between OH^- ions and H_2CO_3) and is believed to be the main regulator of intracellular pH when the cell is actively secreting. The Na^+/H^+ exchanger is probably important in terms of pH regulation in cells under resting conditions where H^+ produced as a result of metabolism needs to be extruded from the cell (Madshus, 1988). The Na^+/K^+ ATPase provides K^+ to the interior of the cell which is required to replace any which has been lost from the lumen of the secretory canaliculus. In order for the Na^+/K^+ ATPase to operate, intracellular Na^+ is required which is provided by the activity of the Na^+/H^+ exchanger.

Figure 1.4

Electrolyte transporters in both faces of the stimulated parietal cell.



C.A. Carbonic anhydrase

1.2 PHYSIOLOGICAL REGULATION OF GASTRIC ACID SECRETION.

The parietal cell secretes acid in response to direct stimulation by acetylcholine, gastrin or histamine (Soll, 1978). Acetylcholine is released from post-ganglionic nerve endings located on, or near to the parietal cell (neurocrine delivery), whilst gastrin is released into the bloodstream from the G-cells in the antral mucosa and the first part of the duodenum (endocrine delivery). Histamine is released from the endocrine-like cells in the rat, or from mast cells in the dog (Soll et al., 1981) into the extracellular space surrounding the parietal cell (paracrine delivery).

The stimulation of acid secretion in response to eating will be considered initially, followed by a more detailed description of the intracellular events which occur as a result of the interaction of secretagogues with the parietal cell.

1.2.1 The stimulatory phases of gastric acid secretion.

The physiological stimulation of gastric acid secretion can be considered in terms of closely related phases depending upon the site at which the stimulus is initiated. The cephalic phase, initiated by the sight, smell or taste of food, is mediated entirely by the vagus nerve and is abolished by truncal vagotomy (Debas, 1987). In dogs, vagal stimulation effects the release of gastrin from antral G-cells in addition to having a direct stimulatory action on the parietal cell (Debas et al., 1974). The gastric phase of acid secretion is initiated

by distension of the stomach by food or liquid which induces a small change in the serum gastrin concentration, and also exerts a direct effect on acid secretion possibly as a consequence of activation of vagovagal and local intramural reflexes (Debas et al., 1974). Chemical agents such as protein breakdown products also stimulate acid secretion by acting within the stomach, probably as a result of stimulation of gastrin release (Lam et al., 1980). The intestinal phase of gastric acid secretion does not involve the vagus but is stimulated by distension of the duodenum, or the presence of protein breakdown products in this region (Konturek et al., 1978). It has been proposed that a hormone mediates the intestinal phase of acid secretion, and since this has not yet been identified it has been tentatively named enterooxyntin (Grossman, 1974).

1.2.2 Stimulus-secretion coupling in the parietal cell.

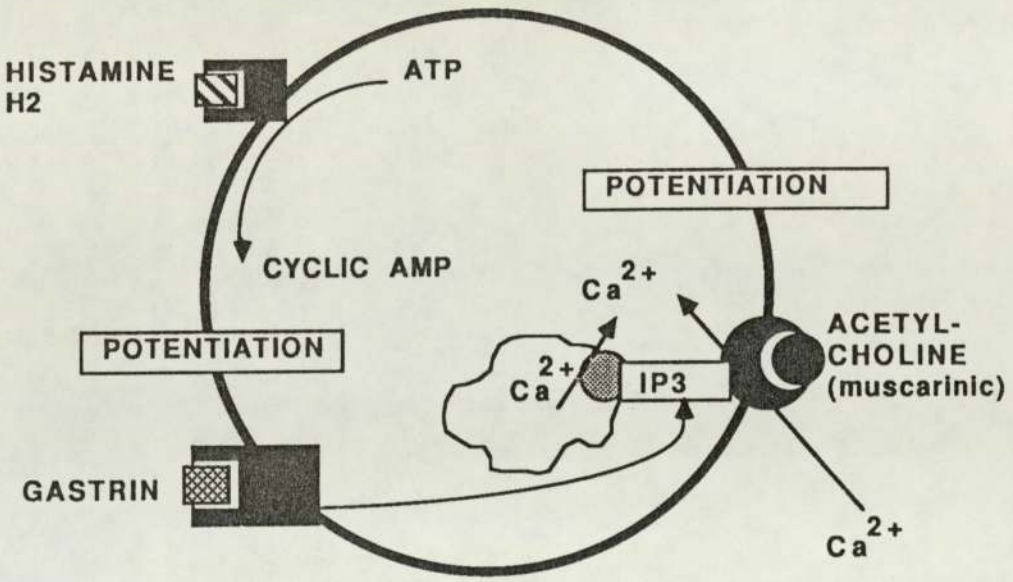
The interactions between secretagogues and their membrane receptors are shown in schematic form in Fig. 1.5.

1.2.2.1 Histamine.

Stimulation of acid secretion in response to histamine occurs as a result of the interaction of histamine with an H_2-receptor on the surface of the parietal cell (Batzri et al., 1982). The action of histamine can be competitively inhibited by incubation with an H_2-receptor antagonist such as cimetidine (Soll, 1980a). It has been demonstrated that histamine activates adenylate cyclase (EC 4.6.1.1) in isolated parietal cells

Figure 1.5

Interactions between secretagogues and membrane receptors in the parietal cell.



(Schepp and Ruoff, 1984) thereby elevating cellular cyclic AMP levels (Soll and Wollin, 1979). Activation of adenylate cyclase is probably achieved due to a coupling of the receptor and adenylate cyclase by a stimulatory GTP-binding protein (Gs) (Graziano and Gilman, 1987, review). Further evidence that stimulation of parietal cell function can be related to an elevation of cyclic AMP content is provided by the observation that forskolin, a diterpene which directly activates adenylate cyclase (Chew 1983a), and cyclic AMP analogues such as dbcAMP (Soll, 1980a) both increase aminopyrine accumulation.

The effect of histamine on the intracellular calcium concentration is rather controversial with reports that histamine induces small increases in intracellular Ca^{++} concentration in rabbit gastric glands (Chew, 1986) and within individual parietal cells in rabbit gastric glands (Negulescu and Machen, 1988), whilst in isolated canine parietal cells (Muallem and Sachs, 1984) histamine was found to have no effect on intracellular Ca^{++} concentration. Observations on single cells in rabbit gastric glands that dbcAMP together with the phosphodiesterase inhibitor IBMX can stimulate acid secretion without elevating intracellular Ca^{++} concentration suggests that the cyclic AMP-pathway can operate independently of Ca^{++} to stimulate acid secretion (Negulescu and Machen, 1988).

The intracellular events which occur in-between the elevation of cyclic AMP levels and the increased activity of the H^+/K^+ ATPase have not been fully identified but probably involve changes in protein phosphorylation. Thus histamine has been shown to activate a Type 1 cyclic AMP-dependent protein kinase isozyme in rabbit isolated parietal cells (Chew, 1985) and incubation of rabbit gastric glands with histamine plus IBMX has been shown to induce the phosphorylation of proteins present in apical membrane fractions (Urushidani et al., 1987). Furthermore, recent work using highly enriched preparations of rabbit isolated parietal cells (98% parietal cells) has

demonstrated an increased phosphorylation of 27 and 40kDa proteins in response to incubation with 0.1mM-histamine (Chew and Brown, 1987). It is interesting that the 27kDa protein was found in a 4,000g particulate fraction similar to that into which H^+/K^+ ATPase was redistributed following stimulation of rabbit gastric glands with histamine (Urushidani and Forte, 1987).

1.2.2.2 Carbachol (an acetylcholine analogue).

Carbachol interacts with muscarinic M_2-receptors on isolated parietal cells (Pfeiffer et al., 1988) and raises cytoplasmic Ca^{++} levels by effecting an initial release of Ca^{++} from intracellular stores, which is followed by a more sustained influx of Ca^{++} across the cell membrane (Negulescu and Machen, 1988). Incubation of parietal cell-enriched preparations with carbachol stimulates the formation of diacylglycerol (Pfeiffer et al., 1987) and inositol phosphates (Pfeiffer et al., 1988) implicating a M_2-receptor-mediated activation of a phosphatidylinositol phosphodiesterase (phospholipase C) and hydrolysis of phosphatidylinositol 4,5-bisphosphate (reviewed by Berridge, 1987, also see Fig. 3.3). It is possible that the carbachol-induced increase in cellular Ca^{++} levels was mediated in part by inositol 1,4,5-trisphosphate (IP_3) since addition of this inositol phosphate (5μM) to a subcellular fraction obtained from guinea-pig mucosa has been shown to cause a mobilisation of $^{45}Ca^{++}$ (Tsunoda et al., 1988). Inositol 1,3,4,5-tetrakisphosphate has recently been implicated in the activation of Ca^{++} entry into sea urchin eggs (Putney, 1987) although its involvement in the elevation of intracellular Ca^{++} induced by carbachol remains to be substantiated.

Changes in protein phosphorylation could be induced by at least two mechanisms in response to stimulation by carbachol. The increased intracellular Ca^{++} concentration may activate specific Ca^{++} - sensitive protein kinases (Shaltz et al., 1981), whilst protein kinase C has been shown to be activated in response to incubation with carbachol (Park et al., 1987). Protein kinase C phosphorylates cellular proteins (3.1.2) and has been shown to phosphorylate an 89kDa protein present in the 100,000g cytosolic fraction of the rat parietal cell (G. P. Shaw and P. J. Hanson, unpublished observations). The role of protein kinase C in the regulation of gastric acid secretion has been investigated in this work (Chapters 3 and 4).

1.2.2.3 Gastrin.

Gastrin stimulates acid secretion, in part, by interacting with a specific receptor on the surface of the parietal cell (Soll et al., 1984). Like carbachol, gastrin has no effect on cellular cyclic AMP levels (Soll and Wollin, 1979). The increased intracellular Ca^{++} concentration produced in response to gastrin is unlikely to be due to an enhanced influx of this cation across the cell membrane (Soll, 1981b). However, since gastrin increases the formation of inositol polyphosphates in the rat isolated parietal cell (Puurunen and Schwabe, 1987) it is possible that inositol 1,4,5-trisphosphate may stimulate the mobilisation of Ca^{++} from intracellular stores (Tsunoda et al., 1988) thereby elevating cellular Ca^{++} levels.

1.2.2.4 Potentiation.

Potentiation is the phenomenon when two secretagogues act simultaneously to produce a response which is greater than the sum of the individual responses (Grossman, 1967). A two-way potentiation between histamine and carbachol and between gastrin and histamine has been demonstrated in isolated canine parietal cells (Soll, 1982). Although the mechanism involved in producing potentiation is unknown, since it has been shown to occur between dbcAMP and carbachol or gastrin (Soll, 1982) it probably occurs distal to receptor interactions. The two-way potentiations previously described add further support to the view that the second messenger for histamine (cyclic AMP) is different from that for gastrin or carbachol (Ca^{++} release, possibly due to inositol 1,4,5-trisphosphate).

1.2.3 Inhibition of gastric acid secretion.

The termination of gastric acid secretion, stimulated in response to eating, can be divided into phases in a similar manner to those described for the stimulation of acid secretion. Preshaw (1973) showed that in dogs with Heidenhain pouches, sham-feeding inhibited pentagastrin-stimulated acid secretion supporting the proposal that the vagus nerve releases an inhibitor of gastrin-stimulated acid secretion (Debas, 1987). Mechanisms acting within the stomach to inhibit acid secretion are likely to include acidification of the gastric mucosa (Konturek et al., 1975) and antral distension (Yamagishi and Debas, 1977). Instillation of acid, fat or hyperosmolar solutions into the duodenum effects an inhibition of gastric acid secretion although

the mechanism(s) involved remain largely unresolved (Debas, 1987). Although the presence of acid in the duodenum stimulates secretin release, it is unclear whether a physiological stimulus for secretin release would achieve a sufficient plasma concentration for this peptide to be an effective inhibitor of gastric acid secretion (Kleibeuker et al., 1984). The hypothetical hormonal agent enterogastrone is implicated in the inhibition of gastric acid secretion which occurs in response to fatty acids or monoglycerides in the small intestine. One candidate for enterogastrone was gastric inhibitory polypeptide (GIP), the release of which is stimulated by fat in the small intestine (Brown et al., 1975). However GIP is only a weak inhibitor of acid secretion in man (Maxwell et al., 1980) and the physiological role of this polypeptide in the regulation of gastric acid secretion is therefore uncertain. Another, possibly more likely candidate for enterogastrone, is neurotensin which is released by the presence of fat in the small intestine (Rosell and Rokaeus, 1979) and which, like fat is a more potent inhibitor of the vagally innervated than of the denervated stomach (Andersson et al., 1980).

Somatostatin, oxyntomodulin, adenosine, prostaglandin E_2(PGE_2) and epidermal growth factor (EGF) are agents which may also be involved in the physiological regulation of gastric acid secretion. Somatostatin inhibits gastrin-induced histamine release in rabbit gastric glands, and also has a direct action on the parietal cell (Chew, 1983b) which is probably due to an inhibition of adenylate cyclase (Schepp et al., 1983a) by a mechanism involving a pertussis-toxin sensitive G-protein (Atwell and Hanson, 1988). Oxyntomodulin which is a potent inhibitor of pentagastrin-stimulated acid secretion in rats (Dubrasquet et al., 1982) is similar in structure to glucagon although is extended by 8 amino acid residues. The site of action of oxyntomodulin remains to be established. Adenosine interacts with A_1 adenosine

receptors on the surface of canine isolated parietal cells and inhibits histamine-stimulated aminopyrine accumulation (Gerber and Payne, 1988). However, since rat parietal cells probably do not possess A_1 adenosine receptors (Puurunen et al., 1987b) it remains to be determined whether adenosine is a universal regulator of gastric acid secretion. The antisecretory properties of EGF (section 5.1.4.1) and PGE_2 (section 6.1.2.3.1) are described elsewhere, and will not be discussed in any detail here. The mechanism by which EGF inhibits parietal cell secretory activity remains to be determined and forms part of this work (Chapters 5 and 6).

1.3 INVESTIGATION OF THE MECHANISMS CONTROLLING ACID SECRETION USING ISOLATED PARIETAL CELL PREPARATIONS.

1.3.1 Isolated cell preparations.

An isolated parietal cell preparation was used in this study to investigate the intracellular mechanisms involved in the regulation of parietal cell activity. This system has the following advantages:

1. Removal of systemic factors such as hormonal influences, blood supply and nervous activity facilitates a study of the effect of a single agent on secretory activity, and facilitates interpretation of the site of action of an agent.

2. The absence of an extracellular space which may provide a barrier to the diffusion of oxygen and nutrients facilitates the maintenance of cells under appropriate conditions for many hours.

3. The incubation medium can be modified enabling a precise manipulation of the environment surrounding the cells.

4. Technical advantages afforded by isolated cell preparations include ease of manipulation and the potential for taking identical multiple samples at a required time.

5. Isolated cell preparations have the potential for enrichment of a particular cell-type.

However, isolated cell preparations are not without faults. Disadvantages include the possibility that the removal of the intercellular connections may modify the behaviour of a cell, whilst the loss of polarity of the isolated cell means that the acid secretion *per se* cannot be measured directly.

Several methods for isolating parietal cells from a variety of species have been developed and some are summarised in Table 1.1. The procedure used to routinely prepare isolated parietal cells from the rat stomach is detailed in section 2.1.2, and is a development of the method of Lewin *et al*. 1974.

1.3.2 Measurements of acid secretion by isolated parietal cells.

Acid secretion cannot be measured directly when using isolated parietal cells since for every H^+ extruded across the apical membrane, a HCO_3^- passes across the basolateral membrane (Fig. 1.4). However various techniques have been developed which enable an indirect measurement of parietal cell-secretory activity.

Table 1.1

A Summary of some methods used to prepare isolated parietal cell preparations.

Method	Species	Authors
Everted stomach sac injected with pronase-containing medium, incubated with exposure to EDTA.	Rat	Lewin et al., 1974
Pronase digestion of the gastric mucosa interspersed with vigorous shaking in pronase-containing medium.	Mouse	Romrell et al., 1975
Blunt dissection of mucosa from sub-mucosa. Mucosa minced with scissors and digested with collagenase with transient exposure to EDTA.	Dog	Soll, 1978
Mucosa scraped from muscularis, and pressed through a stainless steel grid. Mucosal fragments digested with collagenase and EDTA.	Guinea Pig	Batzri and Gardner, 1978
Mucosa separated from sub-mucosa by high pressure perfusion, minced and digested with subsequent incubations in pronase- and collagenase- containing medium.	Rabbit	Brown and Chew, 1987

1.3.2.1 Morphological transformation.

The distinct morphological changes which occur as a result of secretagogue-stimulation (1.1.2) can be used as an indicator of acid secretion. Thus when parietal cells are observed by electron microscopy, the surface density of the tubulovesicles and of the secretory canaliculi can be determined by stereological methods and it can be demonstrated that morphological changes occur in response to stimulation by histamine, carbachol and dbcAMP (Berglindh et al., 1976). Although this technique has been used by some workers to assess regulation of gastric acid secretion (e.g. Fellenius et al., 1982), it is only semi-quantitative and is not particularly suitable for detecting any small changes in acid secretion.

1.3.2.2 Oxygen consumption.

The rationale behind using oxygen consumption as an indicator of secretory activity is that acid secretion has a high energy requirement, which is reflected by the observation than mitochondria occupy 30-40% of the parietal cell volume (Helander and Hirschowitz, 1972). Thus, changes in parietal cell activity will be reflected in changes in cell metabolism. Soll (1978) showed that O_2 consumption by a preparation of isolated canine parietal cells was increased in response to incubation with histamine, gastrin, carbamylcholine and IBMX. However, unless the preparation contains only parietal cells, interpretation of changes in oxygen consumption as reflections of changes in acid secretion can be misleading.

1.3.2.3 Accumulation of aminopyrine.

Aminopyrine is a weak base with a pKa of 5.0 and when radiolabelled, it can be used to quantify sequestered acid (Soll and Berglindh, 1987). The basis of aminopyrine accumulation lies in the pH-partition hypothesis. At a physiological pH (7.4) aminopyrine is able to cross the plasma membrane of a parietal cell and passes into the acidic regions of the secretory canaliculi where it becomes protonated and trapped (Fig. 1.6). The accumulation of aminopyrine within secretory canaliculi has been demonstrated by Berglindh *et al*. (1980a) and by Gibert and Hersey (1982). After allowing sufficient time for equilibration of aminopyrine between the acidic secretory canaliculi and the medium, the cells are separated from the medium and the accumulation of aminopyrine within the cells can be determined (2.3). The aminopyrine accumulation ratio can be expressed in the following way:

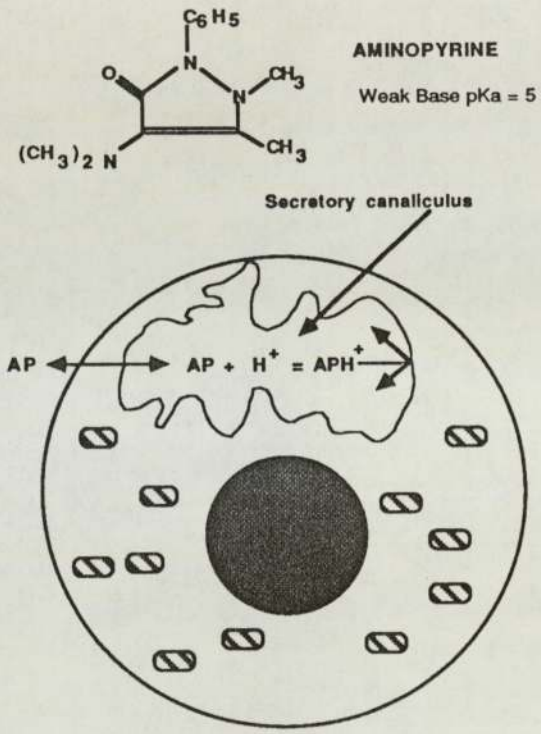
$$\text{Aminopyrine accumulation ratio} = \frac{[AP]\ \text{cell}}{[AP]\ \text{medium}} = \frac{1 + 10^{(pKa - pH\ \text{cell})}}{1 + 10^{(pKa - pH\ \text{medium})}}$$

It must be emphasised that the accumulation of aminopyrine does not measure the rate of acid secretion but instead reflects acid sequestration.

Aminopyrine accumulation is used widely by workers investigating the regulation of gastric acid secretion, (Table 2.4). It has proven to be a reliable index of acid of secretion which enables low levels of acid secretion to be detected (Soll, 1980a), and dose-response curves to be generated (e.g. Fig. 3.6). Chew *et al*. (1980) found that changes in the aminopyrine accumulation ratio correlated linearly with oxygen consumption, although Soll (1980a) found that these two indices did not entirely correlate in terms of the concentration of agent required to produce a

Figure 1.6

Intracellular accumulation of aminopyrine (AP) by parietal cells is an index of acid secretion.



Protonated form of aminopyrine is trapped inside the secretory canaliculus

Aminopyrine accumulation ratio = [intracellular aminopyrine] /[extracellular aminopyrine]

maximal effect. However, no reference can be found in the literature to a situation where an increased aminopyrine accumulation ratio, in response to physiological secretagogues, is not reflected by an increase in acid secretion as measured by other parameters. The accumulation of aminopyrine as an index of acid secretion is used extensively throughout this work, in conjunction with measurements of cellular cyclic AMP, to attempt to further understand the mechanisms which are involved in regulating parietal cell secretory activity. The two particular aims of this work are:-

1. To identify the site(s) at which protein kinase C may act to modulate parietal cell secretory activity.

2. To characterise the site, and mechanism, by which EGF inhibits secretory activity.

Chapter Two

GENERAL METHODOLOGY

2.1 PREPARATION OF ISOLATED CELL SUSPENSIONS.

2.1.1 Preparation of an everted stomach sac.

A fed rat (200-250g body weight) was anaesthetised by an intraperitoneal injection of sodium pentabarbitone (Sagatal) at 60 mg/kg body weight. A midline incision was made to expose the stomach. The oesophagus was ligated and the stomach was removed by cutting both above this ligature and across the duodenum. The animal was immediately killed by puncturing the diaphragm.

The stomach was gently rinsed in ice-cold saline (NaCl, 9 g/l) whilst holding the non-glandular region with a pair of forceps. Everted stomach sacs were then prepared (Dikstein and Sulman, 1965; see Fig. 2.1). Pronase, dissolved in medium A (Table 2.1) at a concentration of 1000PUK units/ml, was injected into the sac using a 26-gauge hypodermic needle until the sac was inflated (1.5 ml was usually sufficient).

2.1.2 Routine preparation of a crude parietal cell suspension.

Throughout this work the term "crude" will be used to refer to cell preparations containing approximately 20% parietal cells. The procedure used to isolate parietal cells is similar to that of Trotman and Greenwell (1979) which is a modification of the method of Lewin et al., (1974). It was usually necessary to utilise two everted sacs to provide sufficient cells for one experiment using a crude parietal cell preparation whilst four sacs were

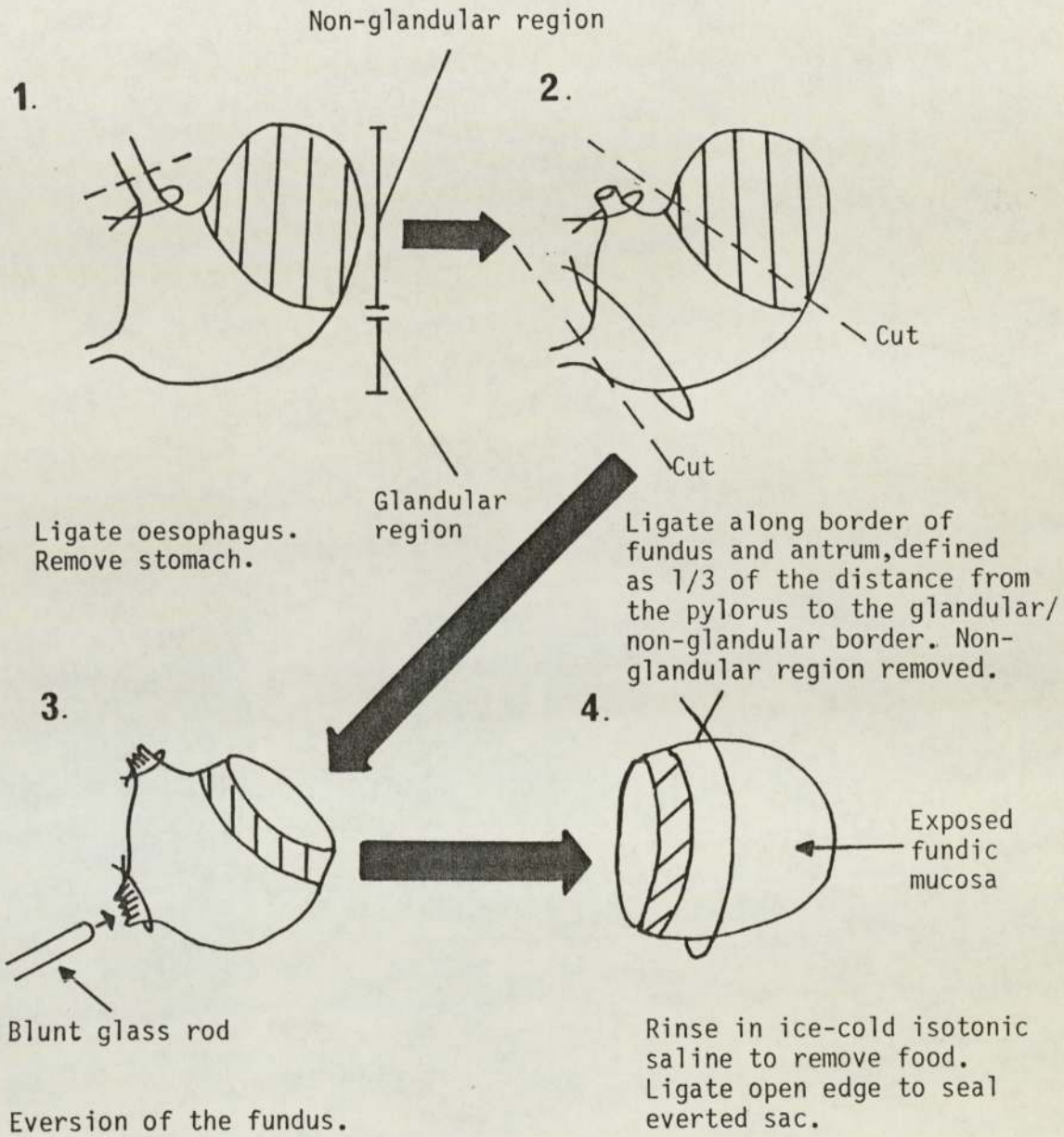


Figure 2.1 Preparation of an everted fundic sac from the rat stomach.

Table 2.1

Composition of media for isolation, incubation and purification of parietal cells.

The following additions were made to Eagle's Minimum Essential Medium containing 25mM-$NaHCO_3$ and 20mM-HEPES (Appendix A2.1).

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	EGTA (3.0mM) Dithiothreitol (0.5mM) Bovine serum albumin, fraction V (1mg/ml)

required to produce parietal cell-enriched fractions (2.2.2). The sacs were placed in a plastic bottle containing 40ml medium A (Table 2.1) and incubated for 30 minutes in a shaking water bath (60 cycles/min) at 37°C with continuous gassing using 95% O_2/5% CO_2 . This mixture was used throughout to gas the cell suspension. The sacs were blotted free from medium A and transferred to 20ml of medium B in a covered plastic beaker (50ml capacity).

Incubation of the sacs in medium B was for 30 minutes at room temperature with continuous gassing of the airspace above the medium, and the sacs were gently stirred using a magnetic follower. The cells released into B were filtered 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 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/min) and continuous gassing. The sacs were incubated for a further 2 hours with changes from incubation in medium A to cell harvesting in medium B every 30 minutes. The cell fractions were pooled and centrifuged at 100g at 15°C for 5 minutes and the resulting cell pellet was resuspended in 10ml of the appropriate incubation medium prior to a second centrifugation step and final resuspension. Preparation of isolated cell suspensions using this method typically produced a cell-fraction containing 21.3 $\pm$ 0.9% parietal cells (n = 10 cell-batches) and the average number of cells isolated per stomach was 7.8 $\pm$ 0.9 x 10^7 (n = 10 cell-batches).

2.1.3 Identification of parietal cells and assessment of their viability using the trypan blue exclusion test.

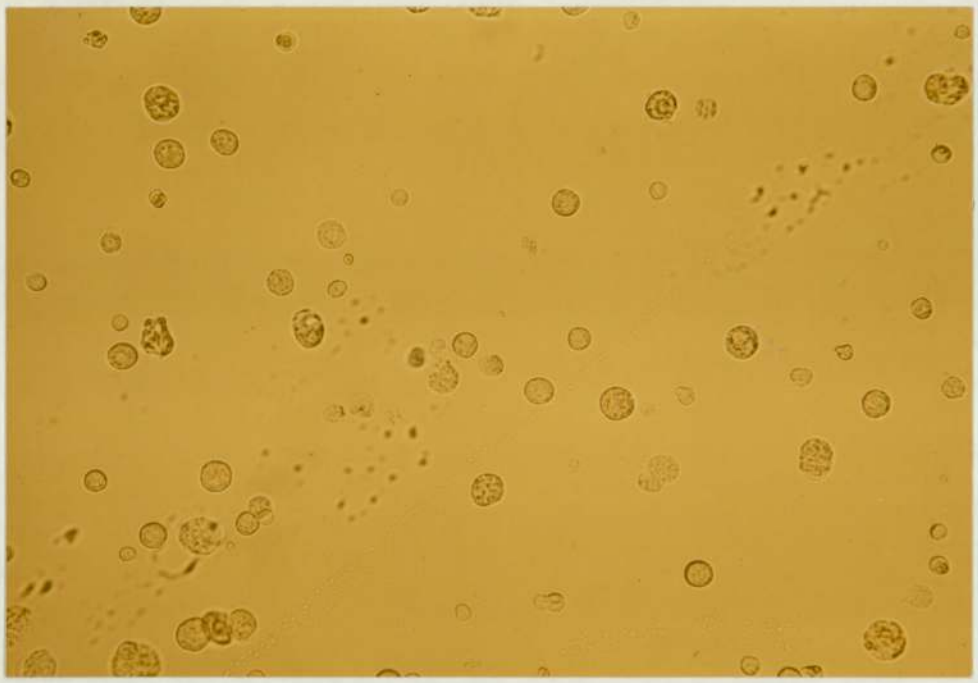
Parietal cells present in the isolated cell preparation can be identified using the light microscope (x 400) based on their size and characteristic morphology (Plate 2.1). Parietal cells are usually the largest cell-type present as they have a diameter exceeding 13µm. Furthermore these cells are distinctive since they display a large centrally-located nucleus and the large number of mitochondria in the cytoplasm generates a granulated appearance.

As an indicator of the structural integrity of the isolated parietal cells, the ability of the cells to exclude the dye trypan blue was measured. A 20µl aliquot of the cell sample in the appropriate incubation medium was mixed well with an equal volume of trypan blue (4mg/ml) in saline (NaCl, 9g/l). Cells were counted using a haemocytometer (E. Leitz, Wetzlar) by light microscopy. On each occasion at least 200 cells were counted and the percentage of cells retaining the ability to exclude trypan blue was calculated. Only cells which showed no sign of taking up the dye were considered viable. If less than 85% of cells were able to exclude trypan blue, the cell preparation was not used. Using the isolation procedure described in section 2.1.2, 97.1 $\pm$ 0.3% (n = 20 cell-batches) of the whole cell preparation and 95.9 $\pm$ 0.8% (n = 20 cell-batches) of the parietal cells were judged to be viable as determined by the ability to exclude trypan blue. It must be emphasised that this test is not a direct measurement of the viability (the ability of cells to produce acid is the ultimate test of viability) and artificially high readings may be obtained if there is insufficient contact time of cells with the dye (Elliot, 1979) or in the presence of a high

Plate 2.1

The appearance of isolated parietal cells under the light microscope. The photograph was taken on a Carl-Zeiss Photomicroscope using a 100 ASA colour transparency film.

Magnification × 375.



concentration of bovine serum albumin which can bind trypan blue (Seglen, 1976). However this test was only performed on cells suspended in medium B´, high K^+-medium or Na^+-free medium each of which only contained 1% bovine serum albumin.

2.1.4 Attempts to improve the responsiveness of crude parietal cell preparations by modifications of the isolation procedure.

2.1.4.1 Effect of short-term culture on the aminopyrine accumulation ratio in response to stimulation with histamine.

The cell pool generated using the procedure described in 2.1.2 was resuspended in medium B´ (2.2 x 10^6 cells/ml) and divided into three equal fractions. Fraction A was used immediately for the determination of the aminopyrine accumulation ratio (2.3). Fraction B was pre-incubated for 4 hours, whilst shaking at 60 cycles/min with continuous gassing of the airspace, in Medium 199 (Sigma) containing 25mM-$NaHCO_3$, 20mM-HEPES, 100µg/ml-gentamicin and 10% foetal calf serum (pH 7.4) at a concentration of 2.2 x 10^6 cells/ml. The cells were then washed free from the medium by centrifugation at 100g for 5 mins at 15°C, resuspended in medium B´ and incubated to determine the aminopyrine accumulation ratio (2.3). Fraction C was pre-incubated in Medium 199 plus components under sterile conditions for 16 hours in petri dishes (2.2 x 10^6 cells/ml) maintained at 37°C in a humidified atmosphere of 95% air/5%CO_2 . Following the 16 hour pre-incubation period, the cells which had not adhered to the petri dishes were removed by gently pouring off the medium and replacing it with fresh medium 199 plus components which was also poured off. The fractions were pooled and washed free from the medium by centrifuging at 100g for 5

min at 15°C. Cells were resuspended in medium B´ and a sample was removed for counting and for the trypan blue exclusion test (2.1.3) before determination of the aminopyrine accumulation ratio. Cells which had adhered to the petri dishes were removed by the addition of 1ml trypsin/EDTA solution (Flow Laboratories, U.K. 0.05% w/v; 0.02% w/v) and incubated for 6 - 10 minutes at 37°C to effect suspension of cells. The action of trypsin was inhibited by subsequent addition of 10ml of medium 199 containing the components as described above. The cell fraction containing any cells which had adhered was washed free from the medium by centrifuging at 100g for 5 min at 15°C. The original aim was to perform an aminopyrine accumulation test on this fraction but since it contained only 12% parietal cells it was not worthwhile.

The consequences of introducing a pre-incubation period prior to the determination of aminopyrine accumulation are summarised in Table 2.2. Neither of the methods used appeared to enhance the responsiveness of the cell preparation to stimulation by 0.5mM-histamine as compared to the control.

2.1.4.2 Effect of cell isolation using EGTA rather than EDTA.

Ethylenediamine tetraacetic acid (EDTA) is used in medium A (Table 2.1) in order to chelate Ca^{++} although it also chelates Mg^{++} and other metal cations (Garvan, 1964) and will therefore probably deplete cellular Ca^{++} and Mg^{++} during cell isolation. Ethyleneglycol-bis-(β-amino-ethylether) N, N´-tetraacetic acid (EGTA) is a more specific Ca^{++} chelator which has little affinity for

Table 2.2
Effect of modifications to the procedure described in 2.2.1 for the isolation of parietal cells on the aminopyrine accumulation ratio in response to stimulation with 0.5mM-histamine.

Experimental details (section)	Modifications	Aminopyrine accumulation ratio Basal	0.5mM-histamine	Yield of cells/ stomach	Parietal cells as a fraction of cell pool %	% of parietal cells able to exclude trypan blue
2.1.4.1	None (Fraction A)	5.8 (5.4, 6.2)	22.9 ± 1.0	6.20×10^7	22.9	99.9
2.1.4.1	4 hour pre-incubation prior to aminopyrine accumulation (Fraction B)	6.6 (6.3, 6.9)	23.3 ± 0.7	6.20×10^7	22.9	97.9
2.1.4.1	16 hour pre-incubation prior to aminopyrine accumulation (Fraction C)	3.0 (3.1, 2.9)	22.3 ± 1.2	6.20×10^7	40.82	65.3
2.1.4.2	EDTA in medium A substituted by EGTA	2.0 ± 0.5	10.2 ± 1.2	5.15×10^7	21.4	98.9
2.1.4.3	Incubation time in medium A reduced to 15 mins	4.3 ± 0.7	20.8 ± 0.9	3.78×10^7	22.5	98.5

Results are means $\pm$ S.E.M. of 4 determinations from a single cell-batch. Where only 2 determinations were made, both values are shown.

When present at 0.5mM, histamine is near-maximally effective at stimulating aminopyrine accumulation in rat parietal cells (Shaw et al., 1987).

Mg^{++} (Schmid and Reilley, 1957) and the possibility that the replacement of EDTA in medium A with EGTA may yield cells of greater responsiveness deserved investigation. Therefore cells were isolated using the procedure detailed in 2.1.2 with 2mM-EGTA used in the medium A in place of 2mM-EDTA. However this modification did not appear to enhance the responsiveness of isolated parietal cells to stimulation by 0.5mM-histamine (Table 2.2).

2.1.4.3 Effect of a reduced incubation time in medium A.

Since medium A contains 2mM-EDTA it is essentially a Ca^{++} -free medium and it was possible that a reduced contact time of cells in this medium may yield cells which were more responsive to histamine-stimulation. The incubation time of sacs in medium A was therefore reduced from 30 minutes to 15 minutes for each of the 3 cycles. This modification did not improve the aminopyrine accumulation in response to 0.5mM-histamine (Table 2.2), and it appeared to reduce the cell yield per stomach when compared with that obtained using 3 x 30 minute isolation periods in medium A.

In conclusion, the modifications described in this section did not markedly improve the aminopyrine accumulation ratio in response to stimulation with 0.5mM-histamine.

2.2 ENRICHMENT OF PARIETAL CELLS.

2.2.1 Techniques used for enrichment of parietal cells.

Preparations of isolated gastric mucosal cells obtained using the method described in 2.1.2 are heterogeneous and contain approximately 20% parietal cells. Such cell suspensions are suitable for the determination of aminopyrine accumulation since this base only accumulates within acidic spaces of parietal cells (1.3.2.3). However in order to study other aspects of parietal cell secretory activity, such as the measurement of cyclic AMP content (2.4.2.2) or prostaglandin production (2.4.3.2) it is necessary to use preparations in which the parietal cell content has been increased relative to other cells. This procedure is commonly termed parietal cell-enrichment and 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 1981a). A variety of cell separation techniques have been used to achieve enrichment of parietal cells, examples of which are shown in Table 2.3 Enrichment techniques based on velocity sedimentation include use of the elutriator rotor or unit gravity sedimentation. Velocity sedimentation is based upon Stoke's Law (equation 2.1) and since cell size is a major influence on sedimentation velocity (the equation involves the square of the radius) the larger parietal cell sediments more rapidly than other mucosal cells.

$$SV = (\frac{2}{9})r^2 \ (\rho p - \rho m) \ \frac{g}{\eta} \qquad (2.1)$$

Table 2.3

Examples of procedures used to enrich the parietal cell content of isolated mucosal cell preparations.

Procedure	Species	% parietal cells in enriched preparation	Reference
Centrifugal elutriation (using an elutriator rotor)	Dog	Up to 65%	Soll, 1978
Unit gravity sedimentation	Dog	Up to 95%	Major and Scholes, 1978
Density gradient using Percoll	Rat	83.8 $\pm$ 1.2% (n=20)	This work.
	Rat	70 - 90%	Sonnenberg et al., 1979.
	Rat	65 - 85%	Schepp et al., 1983a, b
Density gradient using Nycodenz	Dog + Rabbit	70 - 90%	Berglindh and Sachs, 1985
Combination of elutriation and Nycodenz density gradient	Rabbit	~ 95%	Brown and Chew, 1987

SV = sedimentation velocity

ρp = density of cell

ρm = density of medium

r = radius of cell

η = viscosity of medium

g = gravitational field

$\frac{2}{9}$ = shape factor constant for a sphere

Isopycnic separation is dependent upon variations in cell density and is achieved by centrifuging cells until they encounter a medium of density equivalent to their own.

For this work, parietal cell-enriched preparations were obtained by isopycnic centrifugation of a crude cell suspension in Percoll using a method developed by G. P. Shaw and P. J. Hanson which is closely related to that used by Sonnenberg et al., (1979). Percoll is a medium composed of colloidal silica particles coated with polyvinylpyrrolidone (PVP) which can form self-generated density gradients within 10-30 minutes. It is particularly suitable for use in density separation since it is non-toxic to cells (Pertoft et al., 1977). Although it is possible that cell-types of a similar density to the parietal cell may co-purify with the parietal cell (Soll and Berglindh, 1987), this is reduced by introducing a second 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 a cell suspension enriched in parietal cells.

20ml of iso-osmotic Percoll was prepared by mixing 18ml of Percoll with 2ml of 10 x tissue culture medium (prepared by dissolving the contents of 1 bottle of Eagle's Minimum Essential Medium powder in 100ml of double-distilled water). Following the addition of 25mM-$NaHCO_3$, the iso-osmotic Percoll was gassed for 15 minutes at room temperature whilst stirring and the pH adjusted to 7.4. 6 x 3ml aliquots of this mixture were placed in separate polycarbonate tubes (10ml capacity) immediately before use. The crude cell isolate in medium B (2.1.2) was centrifuged at 100g for 5 min at 15°C to separate the cells from the medium, and the resulting cell pellet was resuspended in medium C (Table 2.1) at a final concentration of 4-5 x 10^6 cells/ml (30ml of medium C was usually used). 4.5ml of this cell suspension was added to each of the tubes containing the iso-osmotic Percoll solution to generate a cell suspension in 40% Percoll (v/v). After gently inverting the tubes to ensure thorough mixing, the tubes were 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 the contents of each tube was carefully removed using a Gilson pipette, this layer corresponded to a density of approximately 1.03g/ml, and contained 84.4 $\pm$ 2.1% parietal cells (n = 20 separate occasions). Each 1.5ml sample was placed in a plastic centrifuge tube and the total volume made up to 10ml using medium B′ (Table 2.1) before centrifugation at 100g at 15°C for 5 min. The supernatant containing Percoll and cell-types of similar density to, but smaller than parietal cells was discarded and the cell pellets were resuspended in 10ml of supplemented tissue culture medium (2.2.3.2) at a concentration of 1-2 x 10^6 cells/ml. Lower down the density gradient there was a band of cells corresponding to a density of approximately

1.06g/ml which contained 12 ± 1% (n = 5) parietal cells. This cell fraction was used in one set of experiments (6.3.3) and was processed in the manner described for the parietal cell-enriched fraction.

The enrichment procedure as described above routinely produced cell fractions containing 83.8 ± 1.2% (n = 20) parietal cells with a total mean yield of 3.6 ± 0.4 x 10^6 cells/stomach (n = 20).

2.2.3 Short-term culture of parietal cell-enriched preparations.

2.2.3.1 Use of short term culture to increase responsiveness of parietal cell-enriched preparations.

Soll et al. (1986) incubated canine gastric mucosal cells which had previously been enriched in parietal cells in tissue culture medium containing various components for 2-18 hr and noted an improved responsiveness of the cells to subsequent stimulation by secretagoges. Results of a preliminary experiment using a medium similar to that used by these workers supported this observation. Thus preparations which had been enriched in parietal cells according to the method described in 2.2.2 were either used immediately, or were pre-incubated in medium B′ (Table 2.1) plus 8µg/ml-insulin, 10nM-hydrocortisone, 50µg/ml-gentamicin and 5%-foetal calf serum for 2 hours prior to stimulation with 0.5mM-histamine. Thus the aminopyrine accumulation ratio was greatly enhanced following a 2 hour pre-incubation (47.1, 39.9) compared to that obtained with no pre-incubation (4.6, 5.4). A 2 hour pre-incubation of parietal cell-enriched preparations was routinely performed. It is interesting that unlike parietal cell-enriched fractions, a short-term culture of

crude parietal cell preparations as described in 2.1.4.1 or in the supplemented tissue culture medium described above (M. M. Atwell and P. J. Hanson, unpublished observations) failed to improve cellular responsiveness. The implication is that the Percoll purification procedure produces a temporary inhibition of cellular responsiveness to histamine.

2.2.3.2 Routine pre-incubation of parietal cell-enriched preparations.

The parietal cell-enriched fraction which had been washed free from Percoll (2.2.2) was resuspended at a concentration of 1-2 x 10^6 cells/ml in a supplemented tissue culture medium which consisted of medium B´ (Table 2.1) containing 8µg/ml-insulin, 10nM-hydrocortisone, 50µg /ml-gentamicin and 5% foetal calf serum. The cell fraction was maintained for 2 hours at 37°C in a capped polycarbonate conical flask (25ml capacity) with shaking (60 cycles/min) and continuous gassing of the air-space above the cells. Every 30 minutes the cells were dispersed by gentle resuspension using a plastic transfer pipette. After 2 hours the cells were washed by centrifugation at 100g for 5 min at 15°C and resuspension in 10ml of experimental incubation medium followed by centrifugation and final resuspension in the same medium. Assessment of the ability of the cells to exclude trypan blue was performed at this stage.

It appears that the enrichment procedure followed by the 2 hour pre-incubation may have induced some membrane damage since the average ability of cells to exclude trypan blue (2.1.3) was 94.1 $\pm$ 0.9% (n = 20) which was significantly lower ($p < 0.01$, paired t-test) than the 98.7 $\pm$ 0.3% (n = 20) which were able to exclude trypan blue immediately prior to enrichment.

2.3 ROUTINE DETERMINATION OF THE AMINOPYRINE ACCUMULATION RATIO.

1.0 or 1.5ml aliquots of cell suspension at a concentration of 2-5 x 10^6 cells/ml in appropriate incubation medium were added to plastic 20ml incubation vials containing [^{14}C] aminopyrine (0.1 μCi/ml: 0.9 μM) , [^{3}H] polyethylene glycol (0.4μCi/ml) and required secretagogues plus agents. The plastic vials had been previously soaked in double-distilled water for 18 hours and dried. Secretagogues and agents were dissolved in saline (NaCl; 0.9% w/v) unless indicated otherwise and were present in small volumes (less than 20μl) to minimise any dilution of the cell suspension, the final volume in each vial was the same since equal volumes of vehicle were added to control vials. Each vial was gassed for 5 seconds with 95% O_2 /5% CO_2 , whilst gently swirling the contents to ensure dispersion of cells with agents, capped and incubated at 37°C for 30 min with continuous shaking (120 cycles /minute). A 30 min incubation period was chosen (except for time-course studies) as the accumulation of aminopyrine in response to stimulation of rat isolated parietal cells with secretagogues had been demonstrated to reach a plateau after this time (work by N. G. Anderson in Shaw et al., 1985).

After the incubation period, duplicate aliquots of cell suspension (0.4ml or 0.5ml) were placed in microfuge tubes (L.I.P. Ltd., Shipley, W. Yorks, England) and centrifuged in a Beckman microfuge at 10,000g for 30 seconds. A 50μl sample of the supernatant from each tube was transferred to glass scintillation vials and the remainder of the supernatant removed from each tube and discarded. The cell pellets were washed once in B´ without resuspension and the tip of each tube, containing the pellet, was cut off using a heated scalpel and placed in individual glass scintillation vials. 0.5ml of

Protosol was added to each vial which was incubated at 37°C overnight. The following morning 10ml of Econofluor was added to each vial and the contents were mixed by inversion. The radioactivity of all samples was determined by liquid scintillation counting as described in Appendix A.3.

The aminopyrine accumulation ratio can be calculated using the formula shown in equation 2.2.

$$\text{Aminopyrine accumulation ratio} = \frac{A - \frac{BC}{D}}{CE} \quad (2.2)$$

where:

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

Since the total [^{14}C] dpm in each pellet includes both the aminopyrine which has become trapped inside the cells and that in the extracellular space associated with the cell pellet, a correction needs to be made for the latter. This is achieved by using [^{3}H] polyethylene glycol (approx m.w. = 4,000 da) to estimate the volume of the extracellular space. Equation 2.2 requires a value for the volume of intracellular fluid which can be calculated using the relationship 2µl of intracellular fluid is equivalent to 1mg dry weight of cells (N. G. Anderson, personal communication). Dry weights were determined for each isolated cell preparation by centrifuging duplicate volumes of cell suspension at 10,000g for 1 minute in a Beckman microfuge. The tip of the microfuge tube containing the cell pellet was removed and placed in an oven at 90°C for 24 hours. The dried cell pellets were weighed on a microbalance (Mettler). The measured weight was corrected for dilution of the cell suspension upon

addition to incubation vials and also for the contribution of dissolved salts and albumin in the extracellular space associated with the pellet (e.g. medium B′ contains 17.54mg/ml). When using parietal cell-enriched preparations, to maximise the number of cells available for use in the experiment, a value for dry weight was calculated from the number of cells present in the pellet rather than measured directly. Thus 1×10^6 cells were found to have an average dry weight of 0.233 ± 0.003mg (mean of 4 separate determinations). Table 2.4 compares the aminopyrine accumulation ratio obtained in this work with that from other workers.

Table 2.4

A summary of some aminopyrine accumulation ratios obtained using isolated parietal cell preparations stimulated by histamine ± a phosphodiesterase inhibitor.

Species	Aminopyrine accumulation ratio		% parietal cells	Reference
Rat	Basal	Histamine (0.5mM) + IBMX (0.1mM)		
	2.4 ± 0.8 (3)	135 ± 32.4 (7)	19 - 22	This work †
	3.0 ± 0.3 (4)	198.3 ± 31.1 (7)	80 - 85	
Rabbit	Basal	Histamine (3µM)	95%	Brown & Chew, 1987
	44 ± 7	271 ± 49		
Dog	Basal	Histamine (10µM) + IBMX (10µM)	45 - 60	Soll, 1980a
	1.8 ± 0.3	44.7 ± 6.1		
Guinea Pig	Basal	Histamine (10µM) + RO 20-1724	70 - 80	Batzri & Dyer
	0.6	2.5 (100µM)		1981

† Values quoted are for cells incubated in medium B′ (Table 2.1).

A high basal aminopyrine accumulation ratio appears to be specific to rabbit preparations.

2.4 MEASUREMENT OF THE CYCLIC AMP CONTENT AND PROSTAGLANDIN E_2 CONTENT OF PARIETAL CELL-ENRICHED PREPARATIONS BY THE USE OF RADIO-IMMUNOASSAY (RIA).

2.4.1 Radioimmunoassay.

Radioimmunoassay (RIA) is a highly sensitive and specific technique used for the quantitative determination of hormones and other biologically active samples present in body fluids and tissue extracts. The principle of RIA is based upon the competition between a known amount of isotopically labelled antigen and an unknown quantity of non-radioactive antigen for a fixed amount of antibody binding sites. At equilibrium with antigen in excess, there will be free antigen and antigen bound to the antibody as summarised below:

Unlabelled antigen (free) + Specific antibody ⇌ Complex of unlabelled antigen plus antibody

+

Labelled antigen (free) ⇌ Complex of labelled antigen plus antibody

An increased amount of unlabelled antigen in the sample produces a proportionate decrease in the amount of labelled antigen bound to the antibody, consequently the level of radioactivity associated with the antibody - antigen complex is inversely proportional to the concentration of unlabelled antigen in the sample. Separation of the bound and free antigen is required, and this can be achieved for the radioimmunoassay of cyclic AMP by a number of methods including charcoal adsorption (e.g. Harper and Brooker, 1975), alcohol precipitation

(e.g. Frandsen and Krishna, 1976) or a pre-reacted primary antibody/secondary antibody complex (this study, Wollin et al, 1979). The concentration of antigen in a sample is determined by interpolation from a standard curve which is constructed using known amounts of unlabelled antigen with fixed amounts of labelled antigen and antibody. The standard curve should always be generated at the same time and under the same experimental conditions used for the assay of the samples.

2.4.2 Radioimmunoassay of cyclic AMP.

2.4.2.1 Introduction.

The cyclic AMP content of the isolated parietal cell preparations was determined by using the acetylated procedure of the RIANEN^{TM} radioimmunoassay kit (Du Pont) which utilises a double antibody complex to separate the bound and free antigen. Steiner et al. (1972) found that substitution of cyclic AMP at the 2´ 0 position enhanced the affinity of the cyclic nucleotide for its antibody. Prior acetylation or succinylation of cyclic AMP samples and standards has been shown to increase the sensitivity of a radioimmunoassay (Frandsen and Krishna, 1976) thereby enabling the measurement of low concentrations of cyclic AMP in a sample without the requirement for purification or concentration of that sample. Acetylation of the extracts of the isolated parietal cell suspensions was routinely performed as described in 2.4.2.2

In this work, cyclic AMP was extracted from the cell preparations using ethanol at a final concentration of 50%. This method gives similar results to those involving extraction with trichloroacetic acid (Soll et al., 1986). The contents of the radioimmunoassay kit were reconstituted as described in Appendix A4.1.

2.4.2.2 Routine determination of the cyclic AMP content of parietal cell-enriched preparations.

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

A modified assay buffer consisting of 10ml sodium acetate buffer plus 50µl of acetylation reagent was prepared. This was required in the "blank" and in the preparation of the cyclic AMP standards. The cyclic AMP stock standard reagent (5,000 pmole/ml) was diluted in sodium acetate buffer to produce a 40 pmole/ml solution. 200µl of this solution was acetylated using 10µl of the freshly prepared acetylation reagent and incubated at room temperature for 3 minutes before being diluted with 1.8ml of sodium acetate buffer to generate a standard containing 4 pmole cyclic AMP/ml. This standard was then diluted using the modified assay buffer (sodium acetate buffer plus acetylation reagent) to produce standards of the following concentrations; 2.0, 1.0, 0.5, 0.25 and 0.10 pmole/ml. The standards and samples were always prepared for radioimmunoassay 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 were made to polypropylene test-tubes as shown in Table 2.5 and the contents were mixed using a vortex mixer. All tubes were covered and incubated at 2-8°C in a refrigerator for 16-18 hours. 0.5ml of cAMP precipitator at 2 - 8°C was then added to each tube except the total count tubes (1 and 2) which were placed directly in the racks for insertion in the gamma counter. The contents of each tube was mixed thoroughly using a vortex mixer and centrifuged for 15 minutes at 1250g at 4°C. The tubes were placed (in groups of 12) into plastic holders and decanted by inversion over a radioactive disposal sink. Whilst inverted, any liquid remaining around the rim of the tubes was removed using an aspirator and the tubes were allowed to drain at an angle of 30° onto absorbent paper for approximately 1 minute. Following gentle blotting, the tubes were re-inverted and the activity of [^{125}I] remaining 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 (Appendix A4.2) and the concentration of cyclic AMP in the sample tubes calculated using a curve fitting package associated with the gamma counter. The performance characteristics of the extraction procedure and radioimmunoassay are summarised in Table 2.6. The efficiency of the extraction procedure was assessed by determining the recovery of [^{3}H] cyclic AMP. Thus an aliquot of [^{3}H] cyclic AMP (0.1m Ci/ml) was added to medium B´ which was mixed with an equal volume of 100% ethanol and evaporated to dryness in a vacuum oven at 55°C. The residue was dissolved in a known volume of acetate buffer and was solubilised by overnight incubation at 37°C in 0.5ml Protosol, and the radioactivity determined using a Packard Tri carb scintillation counter with Econofluor as the scintillant. A second aliquot of [^{3}H] cyclic AMP was similarly diluted in B´ and the radioactivity determined without prior extraction in absolute ethanol. By expressing the "extracted" counts as

Table 2.5

A summary of the protocol for the addition of reagents in the radioimmunoassay of cyclic AMP.

Tube description	Tube No.	Modified assay buffer	Standards	Sample	Working tracer	Anti-serum complex
Total counts	1,2	-	-	-	100	-
Blank	3,4	200	-	-	100	-
0.1pmol/ml standard	5,6	-	100	-	100	100
0.25pmol/ml standard	7,8	-	100	-	100	100
0.50pmol/ml standard	9,10	-	100	-	100	100
1.0pmol/ml standard	11,12	-	100	-	100	100
2.0pmol/ml standard	13,14	-	100	-	100	100
4.0pmol/ml standard	15,16	-	100	-	100	100
Samples	17,18 etc	-	-	100	100	100

All volumes are in microlitres.

Table 2.6

Performance characteristics of the extraction procedure and radioimmunoassay of cyclic AMP.

Characteristic	Value
Recovery of [^{3}H] cAMP	85 $\pm$ 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 $\pm$ 0.2%(n = 6)
Cross-reactivity:	
cyclic GMP	0.01%
GMP	0.01%
ATP	0.01%
ADP	0.01%

a percentage of the control counts, the recovery of [^{3}H] cyclic AMP could be determined. All data were routinely corrected for the recovery of cyclic AMP and were expressed as pmol cyclic AMP/10^6 cells in suspension. However, no correction was made for any contribution to the cyclic AMP measurements due to interference by medium B´ in antibody binding since this was a constant for each experiment and was found to be a small percentage (0.25% - 1.17%) of the total cyclic AMP values. Thus, the interference by medium B´ in the assay gave a "cyclic AMP" measurement of 0.01 $\pm$ 0.00 pmol cyclic AMP in the medium associated with 10^6 cells (n = 3 separate determinations).

2.4.3 Radioimmunoassay of Prostaglandin E_2 (PGE_2).

2.4.3.1 Extraction of PGE_2 prior to radioimmunoassay

It is usually necessary to extract PGE_2 from the incubation medium prior to radioimmunoassay since prostaglandins are generally produced in nanogram quantities and need to be purified from milligram quantities of other biological material present, furthermore protein, especially albumin tends to bind prostaglandins (Heinsohn et al., 1987) which would result in an under-estimation of the true prostaglandin content. Most extraction procedures are based on the principle that the carboxylic acid group on the prostaglandin is ionised at high pH but not at low pH therefore extraction into an organic solvent is most efficient when the aqueous phase is at an acid pH. Following the extraction procedure, the required prostaglandin is often purified by using thin-layer chromatography (Richelsen, 1987; Boney et al., 1978) before radioimmunoassay. However purification is not a prerequisite for the measurement of PGE_2 by radioimmunoassay

and was not performed by a number of workers using incubation medium derived from isolated cell preparations, similar to the procedure used in this study (e.g. Schepp et al., 1986). The procedure routinely used in this work for extracting PGE_2 was similar to that used by Zenser and Davis (1978).

0.5ml aliquots of cell suspension were removed from the incubation vials and immediately centrifuged at 10,000g for 1 minute in a Beckman microfuge and placed on ice. A 0.4ml sample of the cell-free supernatant was rapidly removed from each tube and added to 14µl of 1M HCl in a polypropylene test-tube reducing the pH of the sample to 3.0-3.3. 1.2ml of ethylacetate was added to each tube which was capped and vortexed for 15 seconds prior to centrifugation at 100g at 15°C for 1 minute. The non-aqueous layer (containing prostaglandin) was carefully removed using a Gilson pipette and transferred to a clean glass scintillation vial. A second 1:2ml aliquot of ethylacetate was added to the acidified sample which was vortexed and centrifuged as described previously. The non-aqueous layer was removed, pooled with the first fraction, and evaporated to dryness under a stream of N_2 at room temperature. The samples were stored at -20°C for less than one week prior to radioimmunoassay.

2.4.3.2 Routine determination of PGE_2 production using radioimmunoassay.

PGE_2 production by rat isolated parietal cell preparations was quantified by using a Prostaglandin E_2 [^{125}I] radioimmunoassay kit (New England Nuclear) in which separation of the bound and free antigen is achieved by precipitation with polyethylene glycol (PEG 6000). The contents of the radioimmunoassay kit were reconstituted as described in Appendix A4.3.

The dried extracts (2.4.3.1) were reconstituted in 0.5ml assay buffer (50mM phosphate, pH 6.8) and mixed thoroughly using a vortex mixer. A range of PGE_2 standards (0.25-25pg/0.1ml) were prepared on the day of use by serial dilution of a 100ng/ml PGE_2 standard with assay buffer. The tracer, [^{125}I] PGE_2 (2.6µCi /ml) was diluted 1 : 20 (v/v) with assay buffer immediately before each assay. Additions were made to polypropylene test-tubes as shown in Table 2.7 and the contents were mixed using a vortex mixer. All tubes were covered and incubated for 16-24 hours at 2-8°C. At the completion of the incubation period, all tubes except numbers 1 and 2 (total count tubes) were transferred to an ice bath (0 - 2°C) and 1 ml of cold precipitating agent containing 16% polyethylene glycol was mixed with the contents of each tube. After a 20-30 minute incubation, the tubes were removed from the ice-bath and centrifuged at 1500g at 4°C for 30 minutes to effect precipitation of the antibody-bound tracer. The tubes were decanted, the precipitates counted and PGE_2 estimated as described for cyclic AMP (2.4.2.2). The following precautions were observed during extraction and radioimmunoassay of PGE_2 :-

1. Prostaglandins have the tendency to adhere to surfaces and to minimise this effect polypropylene test-tubes (Luckham Ltd, Burgess Hill, Sussex, U.K.) and poly-propylene pipette tips (L.I.P. (Equipment and Services) Ltd, Shipley, W. Yorks) were used and all glassware was previously siliconised with dimethyldichlorosaline.

2. Solvent extraction was performed, where possible, in an ice-bath and centrifugation at 4°C since raised temperatures can result in degradation of prostaglandins, especially those of the E, A and C series.

Table 2.7

A summary of the protocol for the addition of reagents in the radioimmunoassay of prostaglandin E_2 .

Tube description	Tube No.	Buffer	Standard	Samples	Tracer	Anti-body
Total counts	1,2	-	-	-	100	-
Blank	3,4	200	-	-	100	-
0.25pg/0.1ml standard	5,6	-	100	-	100	100
0.5pg/0.1ml standard	7,8	-	100	-	100	100
1.0pg/0.1ml standard	9,10	-	100	-	100	100
2.5pg/0.1ml standard	11,12	-	100	-	100	100
5.0pg/0.1ml standard	13,14	-	100	-	100	100
10.0pg/0.1ml standard	15,16	-	100	-	100	100
25.0pg/0.1ml standard	17,18	-	100	-	100	100
Samples	19,20 etc	-	-	100	100	100

All volumes are in microlitres.

3. Samples were stored dry at -20°C to minimise degradation of PGE_2 .

The performance characteristics of the extraction procedure and radioimmunoassay are summarised in Table 2.8. The recovery of [^{3}H] PGE_2 was determined by taking an aliquot of [^{3}H] PGE_2 (0.1m Ci/ml) through the appropriate extraction procedure in a method which is directly comparable to that described for cyclic AMP (2.4.2.2). The figure of 89 $\pm$ 3% recovery was similar to the value of 85-95% reported by Zenser and Davis (1978) using a similar extraction procedure. The values obtained from the 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).

Table 2.8

Performance characteristics of the extraction procedure and radioimmunoassay of PGE_2.

Characteristic	Value
Recovery of [^{3}H] PGE_2	89 $\pm$ 3% (n = 4)
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 $\pm$ 0.3% (n = 5)
Cross-reactivity:	
PGE_1	3.7%
6-oxo PGF_{1_α}	0.03%
PGB_2	0.01%
PGF_{2_α}	0.01%
Arachidonic acid	0.01%

Chapter Three

INHIBITION OF PARIETAL CELL SECRETORY ACTIVITY BY AN ACTIVATOR OF PROTEIN KINASE C.

3.1 INTRODUCTION.

3.1.1 Protein kinase C.

3.1.1.1 Multiple forms of protein kinase C exist.

Calcium-sensitive phospholipid-dependent protein kinase, protein kinase C, is involved in the regulation of the activity of many types of cell (Nishizuka, 1986). The term protein kinase C represents a complex family of closely related structures, and recent analysis of complementary DNA clones has indicated that there are separate gene transcripts which upon translation could give rise to the enzyme. The transcripts designated α, β and γ are each encoded by a separate gene (Coussens et al., 1986). A further division of the β species can be made into βI and βII based on the difference in nucleotide sequence at the carboxyterminal region. Ono et al. (1986) suggest that this difference has arisen as a consequence of alternate splicing of a single gene. Protein kinase C activity has been separated into three fractions by hydroxyapatite chromatography. Fraction I has a structure encoded by the γ-cDNA sequence, Fraction II is a mixture of the enzymes encoded by βI and βII -cDNAs whilst Fraction III corresponds to protein kinase C enzyme encoded by α-cDNA (Kikkawa et al., 1987). The subspecies of the enzyme display different rates of phosphorylation of the EGF receptor of A431 epidermoid carcinoma cells, and there is also a difference in the tissue distribution of the subspecies (Ido et al., 1987).

Since it is unclear at the present time which subspecies of protein kinase C are present in rat parietal cells, the general term protein kinase C will be used throughout this work. It is however important to emphasise that it is not strictly speaking a single enzyme. The properties of protein kinase C are summarised in Table 3.1.

Table 3.1

Summary of some properties of protein kinase C.

Property	Author(s)
Widespread tissue distribution	Kuo et al., 1980
Primary structure composed of a single polypeptide chain with separate catalytic and regulatory domains	Parker et al., 1986
Three distinct subspecies have been identified Mr76000 - 78000	Coussens et al., 1986 Ono and Kikkawa, 1987
Phosphorylates serine and threonine hydroxyls	Nishizuka, 1983
Activity affected by calcium ions, certain diacylglycerols and certain phospholipids, although independent of calmodulin	Nishizuka, 1984, 1986
Certain phorbol esters can substitute for diacylglycerol and activate protein kinase C in isolated form and in intact cells	Castagna et al., 1982

3.1.1.2 Activation of isolated protein kinase C.

The activity of protein kinase C is influenced by phospholipid, calcium ions (Ca^{++}), diacylglycerols and certain phorbol esters. Kinetic analysis indicates that activators of protein kinase C, such as diacylglycerols and phorbol esters, can increase the affinity of the enzyme for Ca^{++} and phospholipid (Kishimoto et al., 1980).

The activity of protein kinase C is entirely dependent on phospholipid. Phosphatidylserine is demonstrated to be the most effective phospholipid out of phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine when activating a complex of isolated enzyme, phorbol ester and calcium (Kikkawa et al., 1983). Other phospholipids have been shown to modulate the activation of protein kinase C by phosphatidylserine (Kaibuchi et al., 1981). When phospholipid-interacting compounds such as chlorpromazine are added to platelets, the activation of protein kinase C by phorbol ester is inhibited (Uratsuji et al., 1985). It appears that a specific lipid-protein interaction is required for activation of the enzyme.

Other studies of isolated protein kinase C have demonstrated that diacylglycerols containing one or more unsaturated fatty acid either at position 1 or 2 are equally effective at activating the enzyme in the presence of phospholipid and Ca^{++} by enhancing its affinity for Ca^{++} and phospholipid (Mori et al., 1982). More recent work using lipid vesicles (Boni and Rando, 1985) demonstrated that the two stereoisomers 2,3-sn-diacylglycerol and 1,3-sn-diacylglycerol neither activate nor inhibit the enzyme where as 1,2-sn-diacylglycerol is highly effective.

Phorbol esters are potent tumour promoters; that is although not carcinogenic themselves they enhance the formation of tumours by carcinogenic substances. Castagna et al. (1982) noted a direct correlation between the ability of phorbol esters to promote tumours and to activate partially purified protein kinase C. The phorbol ester which was the most effective in activating the enzyme was 12-O-tetradecanoylphorbol 13-acetate (TPA). This molecule has a region which is similar in structure to diacylglycerol (Fig. 3.1). The major effect of phorbol esters is to reduce the Ca^{++} requirement for activation of the isolated enzyme in the presence of phospholipid (Castagna et al., 1982). It is probable that at least part of the effect of phorbol esters is due to the fatty acyl groups at positions 12 and 13 because phorbol itself is not an activator of protein kinase C. However other parts of the molecule are also important for activation of the enzyme, for example phorbol 12,13 didecanoate is quite a good activator of protein kinase C, but 4α-phorbol 12, 13-didecanoate is completely inactive (Castagna et al., 1982), and is often used as a control compound.

In addition to the reversible activation of protein kinase C described previously, the enzyme can also be activated by limited proteolysis by a Ca^{++} - dependent protease (Inoue et al., 1977). The enzyme irreversibly activated in this way is termed protein kinase M and requires neither Ca^{++} nor phospholipid for activity. The physiological role of protein kinase M is presently unclear; it could be a means of maintaining protein phosphorylation when cellular diacylglycerol levels have returned to normal, or it could be the first step in a pathway of downregulation of protein kinase C.

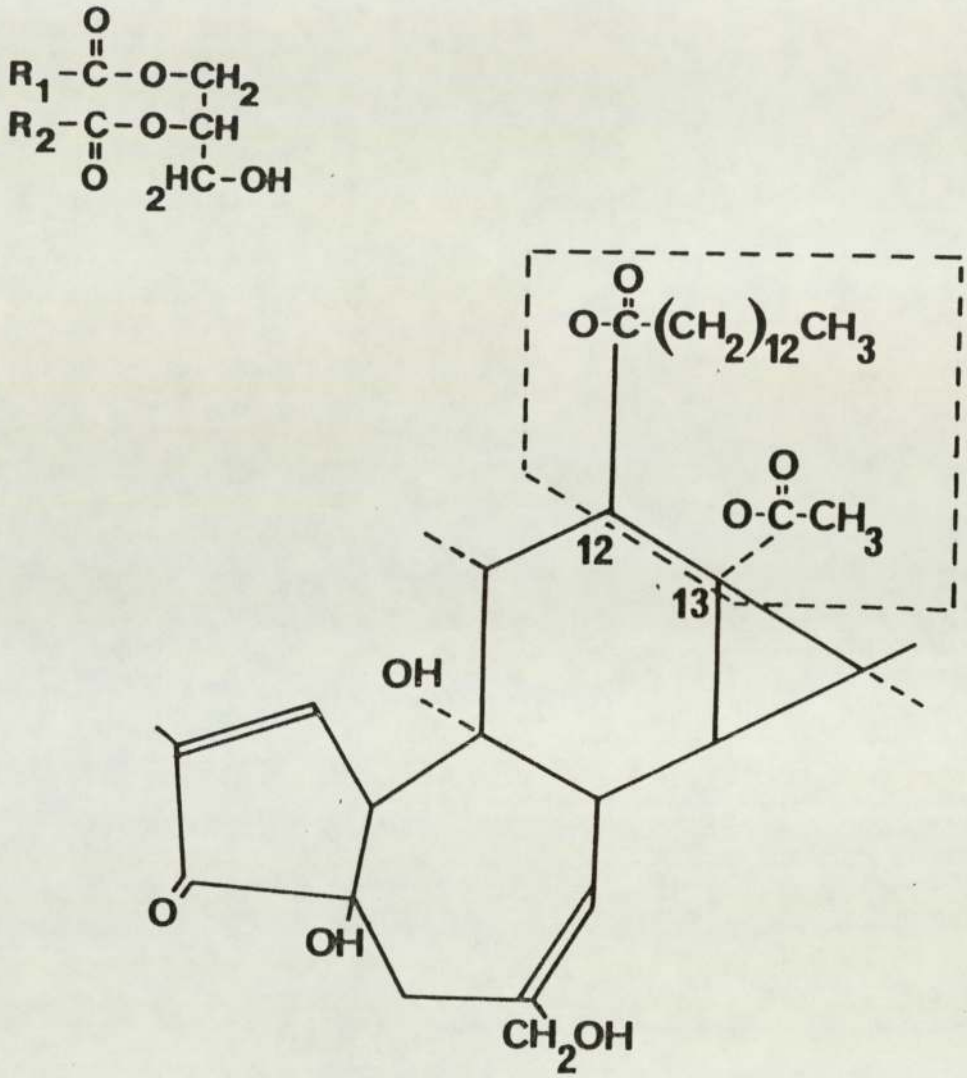


Figure 3.1

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

3.1.1.3 Activation of protein kinase C in intact tissue.

In intact cells in the resting state, protein kinase C is largely located in the cytosol. As a result of stimulation of the enzyme by hormones or by phorbol esters there is a rapid redistribution of the enzyme to the cell membrane [e.g. thyrotropin-releasing hormone stimulation of GH_3 cells (Drust and Martin, 1985); carbachol-stimulation of canine parietal cells (Park et al., 1987); treatment of parietal cell yolk sacs with TPA (Kraft and Anderson, 1983)]. Once protein kinase C is translocated to the cell membrane it is activated, probably because it comes into contact with phospholipids (Fig. 3.2).

Experiments in vitro with the isolated enzyme (see 3.1.1.2) suggest that the binding of protein kinase C to membranes can be effected by phorbol esters and also by diacylglycerols. In a physiological environment in intact tissue, the enzyme would probably be activated by diacylglycerols with long chain fatty acids esterified in position 1 or 2. 1,2-sn-diacylglycerol is normally almost absent from membranes but is transiently produced as a consequence of hormonal activation of a phosphatidylinositol phosphodiesterase (phospholipase C) resulting in the breakdown of polyphosphoinositides (reviewed by Berridge, 1984; also see Figs. 3.3 and 3.4).

Figure 3.2

Activation of protein kinase C by TPA.

The phorbol ester TPA probably induces activation of protein kinase C by causing the enzyme to be intercalated into the cell membrane where it comes into contact with phospholipids. The triangular space represents the active site of the enzyme which becomes accessible to substrate when the enzyme is bound to the cell membrane.

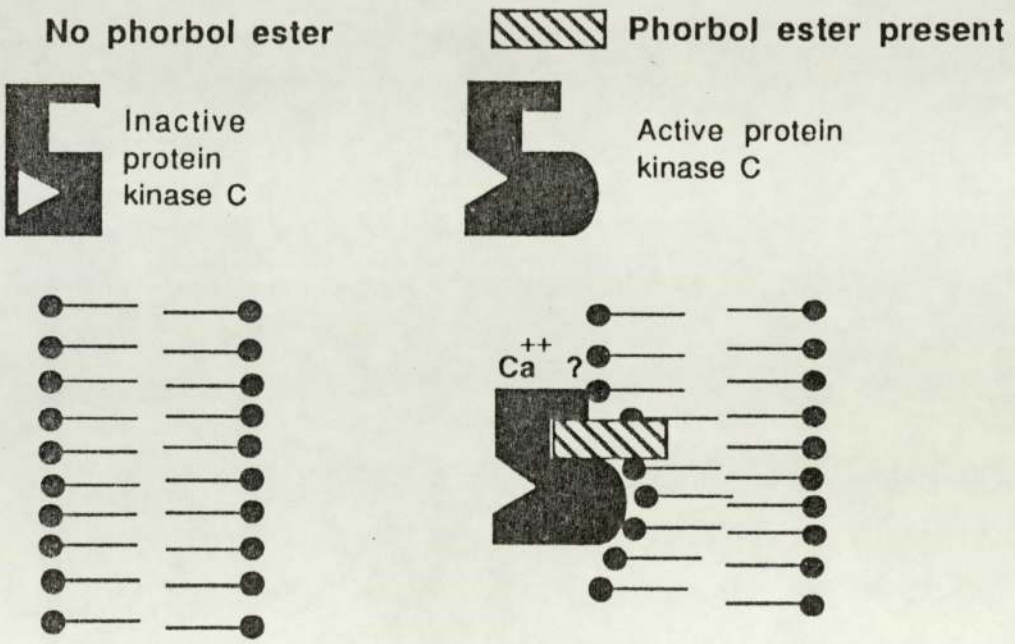
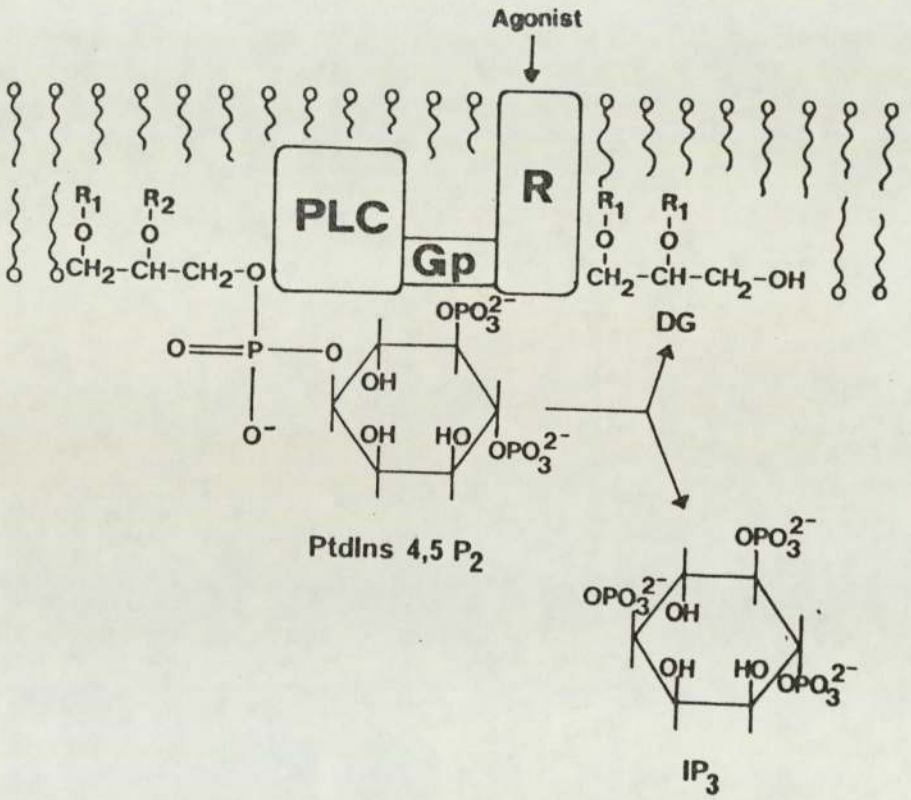


Figure 3.3

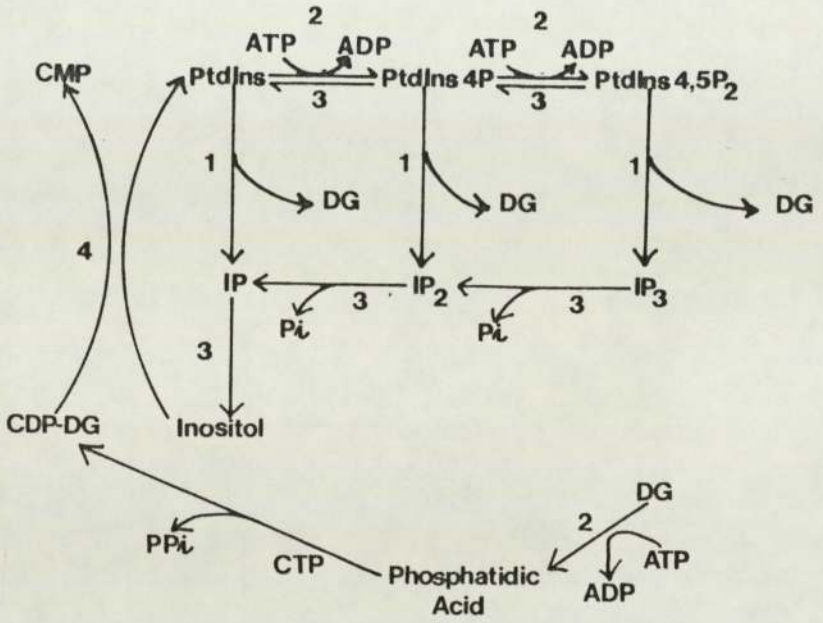
A simplified digrammatic representation of receptor-mediated breakdown of phosphatidylinositol 4,5-bisphosphate.



Agonist binding to a receptor (R) activates a phosphatidylinositol phosphodiesterase (phospholipase C,PLC) via a putative GTP-binding protein (Gp). Activation of phospholipase C causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Ptd Ins 4,5 P_2) to yield 1,2-sn-diacylglycerol (DG) and D-inositol 1,4,5-trisphosphate (IP_3). DG activates protein kinase C whilst IP_3 causes calcium ion mobilisation from a sub-fraction of the endoplasmic reticulum (Streb et al. ,1983). Ca^{2+} may then activate calcium, calmodulin-dependent protein kinases and other Ca^{2+}-dependent systems. It is necessary to replenish the phospholipid precursors and this is achieved by the cycle shown in Fig. 3.4. R_1 and R_2 represent fatty acids which are commonly stearic and arachidonic acid.

Figure 3.4

A simplified schematic representation of the cycle of inositol phospholipid turnover.



Enzymes
1 Phosphatidylinositol phosphodiesterase (phospholipase C)
2 Kinase
3 Phosphomonoesterases (phosphatases)
4 CDP-diacylglycerol inositol transferase

Compounds PtdIns (phosphatidylinositol); PtdIns4P (phosphatidyl-inositol 4-phosphate); PtdIns4,5P_2 (phosphatidylinositol 4,5-bisphosphate); DG (1,2-sn-diacylglycerol); IP_3 (inositol 1, 4,5-trisphosphate); IP_2 (inositol 1,4-bisphosphate); IP(inositol 1-phosphate); CTP (cytidine tris-phosphate); CDP.DG (cytidine diphosphate diacylglycerol); CMP (cytidine monophosphate).

The inositol phospholipids are maintained in dynamic equilibrium in the plasma membrane by the action of kinase and phosphomonoesterase enzymes. Phospholipase C hydrolyses all 3 inositol phospholipids to yield DG as a common product. DG is removed by converting it back to PtdIns. This is achieved by phosphorylation catalysed by a diacylglycerol kinase to yield phosphatidic acid which interacts with CTP to form CDP-DG and subsequently PtdIns. Alternatively, DG may be removed by diacylglycerol and monoacylglycerol lipase to form fatty acids including arachidonic acid and glycerol (not shown). IP_3 can be converted to IP_2 which in turn can be converted to IP and inositol by the action of phosphomonesterases. Lithium ions prevent the formation of free inositol by inositol-1-phosphatase. Inositol can be obtained from plasma, and is freely available to all cells except those behind the blood-brain barrier. It is evident that the pathways for metabolism of inositol polyphosphates are complicated and have yet to be fully elucidated, and therefore no attempt to include any such information here has been made.

To date, it is widely accepted that 1,2-sn-diacylglycerol produced in this way is the physiological activator of protein kinase C in intact cells. Synthetic diacylglycerols such as 1-oleoyl-2-acetylglycerol (OAG) when added to platelets cause the phosphorylation of a 40kDa protein which is also phosphorylated as a result of exposure of platelets to agonists like thrombin (Kaibuchi et al., 1983) which stimulates the breakdown of phosphatidylinositol 4,5-bisphosphate and the production of diacylglycerol. In low concentrations, TPA has been shown to activate protein kinase C in platelets (as indicated by phosphorylation of the 40kDa protein) without increasing diacylglycerol formation (Castagna et al., 1982). This indicates that TPA is able to activate protein kinase C without causing phosphatidylinositol breakdown. It has also been demonstrated that TPA can stimulate the phosphorylation of the same proteins which are phosphorylated upon exposure of cells to agonists which induce polyphosphoinositide breakdown (Garrison et al., 1984; Drust and Martin, 1985). In the liver, the translocation of protein kinase C induced by angiotensin II is mimicked by TPA (Hernandez-Sotomayor and Garcia-Sainz, 1988).

Further evidence that the effects of TPA are mediated by protein kinase C can be derived from experiments where long-term (24 hr) exposure to phorbol esters causes a loss of protein kinase activity (down-regulation). In such cells, TPA is no longer effective despite the cells remaining responsive to secretagogues (e.g. Sung et al., 1988). Evidence that protein kinase C is a major intracellular receptor for TPA has been presented by Niedel et al. (1983) and also by Leach et al. (1983) who demonstrated that the phorbol diester receptor of rat brain membranes co-purifies with protein kinase C. Kikkawa et al. (1983) examined the effect of [^{3}H] phorbol-12,13-dibutyrate on the formation of a complex of Ca^{++}, phospholipid and protein kinase C and noted that the apparent dissociation binding constant (Kd)

for the phorbol ester was equal to the apparent activation constant (Ka) for protein kinase C.

Binding of protein kinase C to the plasma membrane is Ca^{++} - dependent (Wolf et al., 1985 a, b) but there are some conflicting reports in the literature concerning the involvement of Ca^{++} since an increase in the concentration of this cation does not always seem to be required for the activation of protein kinase C. Thus following the addition of TPA to polymorphonuclear leukocytes (Christiansen et al., 1986), activation of protein kinase C occurs without an elevation of intracellular Ca^{++} concentration above resting levels (3×10^{-7} M). Schwantke et al. (1985) suggest that diacylglycerol induces the formation of phosphatidylserine clusters in the cell membrane which will bind calcium ions, and that the local concentration of these ions will be sufficient to activate protein kinase C at resting Ca^{++} levels.

3.1.2 The role of protein kinase C in cellular secretion.

To date, there are at least three different ways by which protein kinase C influences cellular secretion. Each of these will now be described with an appropriate example. In systems such as platelets, protein kinase C activation and Ca^{++} mobilisation can be induced separately by the addition of the diacylglycerol analogue OAG and a Ca^{++} ionophore such as A23187 (Kaibuchi et al., 1983). This experimental procedure has shown that there is a synergistic interaction between the two pathways, and that the activation of both is required for a full physiological response. Alternatively there is a temporal interaction between elevation of intracellular Ca^{++} concentration and activation of protein kinase C in adrenal glomerulosa cells (Kojima et al., 1985). In these

cells, the angiotensin II-stimulated secretion of aldosterone is initiated by a transient increase of intracellular Ca^{++} concentration, but secretion is sustained as a consequence of protein kinase C activation. Biological systems usually encompass a feedback control to prevent over-response to a stimulus and protein kinase C appears to have a role in feedback control. In hepatocytes, protein kinase C may exert a negative feedback effect on α1-adrenergic receptors and inhibit the calcium response and production of diacylglycerol which normally accompanies the activation of these receptors (Corvera and García-Saínz, 1984).

Protein kinase C may exert its effects on secretion by phosphorylating membrane and cytosolic proteins (Nishizuka, 1986) although relatively few substrate proteins have been identified to date. Recently it has been demonstrated that vimentin, a protein component of cytoskeletal intermediate filaments, is phosphorylated by protein kinase C (Huang et al., 1988). Furthermore, in studies using human platelets in vitro, the ability of thrombin to induce a release of serotonin is closely associated with the phosphorylation of a 40kDa protein by protein kinase C (Castagna et al., 1982).

3.1.3 The effect of TPA on the isolated parietal cell.

Although TPA stimulates secretion from a variety of cell-types, when it is incubated with parietal cells isolated from the rat stomach, this phorbol ester inhibits aminopyrine accumulation stimulated by 0.5mM-histamine plus 0.1mM-IBMX (Anderson and Hanson, 1984, 1985). It is probable that TPA exerts its action by a direct effect on the parietal cell, since the effect of TPA concentration on histamine-stimulated aminopyrine accumulation produced

a curve which was similar to a classical dose-response curve, and there was no relationship between cell concentration and the action of TPA (Anderson and Hanson, 1985).

It is likely that this inhibitory effect of TPA in rat isolated parietal cells is a consequence of protein kinase C activation. Thus, the IC_{50} for inhibition of aminopyrine accumulation by TPA is 3.3nM (Fig. 1, Anderson and Hanson, 1985) which is close to the value of 2.5nM which was required to elicit half-maximal activation of partially purified protein kinase C (Arcoleo and Weinstein, 1985). Furthermore, 4α-phorbol 12,13-didecanoate, a phorbol ester which does not activate protein kinase C has no effect on histamine-stimulated aminopyrine accumulation when it is used over the concentration range of 10-1000nM (Anderson and Hanson, 1985). The diacylglycerol analogue OAG inhibits histamine-stimulated aminopyrine accumulation in a concentration-dependent manner when added to parietal cells, and the maximal inhibition by this analogue (83%) is similar to the maximal inhibition produced by TPA (78%) (Anderson and Hanson, 1985). The presence of protein kinase C has been demonstrated in enriched preparations of parietal cells from the rat (Anderson and Hanson, 1985) and from the rabbit (Chew, 1985). Furthermore, work by G. P. Shaw (personal communication) suggests that rat parietal cells contain a substrate for protein kinase C. This proposed substrate is an 89kDa protein which is present in the 100,000g cytosolic fraction of a parietal cell homogenate, and it is phosphorylated in response to phosphatidylserine (20μg/ml) and TPA (32nM).

The aminopyrine accumulation ratio is considered normally to be a valid indicator of acid secretory activity (see 1.3.2.3; Berglindh et al., 1976). However, it is possible that a reduction in aminopyrine accumulation in cells treated with TPA could be a consequence of an increased dissipation of acid from the

secretory canaliculi or an enhanced back-diffusion of acid into the cell, and not due to a reduction in the quantity of acid secreted. This is because aminopyrine accumulation is dependent on the amount of acid sequestered within the secretory canaliculi, and it is not a direct index of the secretory rate. The perfusion of 1μM-TPA through the lumen of the rat stomach significantly inhibits acid secretion stimulated by histamine *in vivo* (Shaw and Hanson, 1986). This observation suggests that the inhibition of histamine- stimulated aminopyrine accumulation by TPA is a true reflection of a reduction in acid secretion by the parietal cell.

The aim of this part of the work was to investigate the potential site(s) at which TPA might act to inhibit secretory activity stimulated by histamine and IBMX. There are several possible means by which TPA, presumably acting via protein kinase C, might influence the activity of the parietal cell, and these are presented below.

3.1.3.1 Does TPA activate Na^+/H^+ exchange in isolated parietal cells?

TPA has been demonstrated to activate Na^+/H^+ exchange in many cell-types (Table 3.2). The Na^+/H^+ exchange system exchanges intracellular H^+ for extracellular Na^+ and helps to regulate intracellular pH which is a major determinant of the activity of the exchanger.

It was of interest to determine whether TPA activated Na^+/H^+ exchange in the parietal cell since the elevation of intracellular Na^+ concentration has been established to have an inhibitory role in the secretory activity of rabbit gastric glands (Koelz *et al.*, 1981), possibly as a consequence of Na^+ binding to the cytosolic face of the H^+/K^+ ATPase. If uncorrected, the elevation

Table 3.2

Some recent reports of stimulation of Na^+/H^+ exchange by TPA.

Cell-type	Observed effect of TPA	Approximate half-maximal stimulatory concentration of TPA	Reference
Human leukemic cell line (HL-60)	Stimulation of Na^+-dependent H^+ efflux	5nM	Besterman and Cuatrecasas, 1984
Human foreskin fibroblast cells	Elevation of intracellular pH	40nM	Moolenaar et al., 1984
Murine pre-β-lymphocyte cell line	Increased intracellular sodium concentration, elevation of intracellular pH	Used 50nM to observe effect	Rosoff et al., 1984
Cultured smooth muscle cells	Enhanced Na^+ uptake	25nM	Owen, 1985

of intracellular Na^+ might perturb some aspect of the acid secretory process. The presence of a Na^+/H^+ exchanger on the basolateral membrane of rabbit parietal cells has been demonstrated, and is apparently separate from the Cl^-/HCO_3^- exchanger (Paradiso et al., 1987).

Unfortunately, it was not possible to use amiloride, which is an inhibitor of Na^+/H^+ exchange in many cell-types, for this work. When used at concentrations which are effective in inhibiting Na^+/H^+ exchange amiloride also inhibits the activity of isolated protein kinase C (Besterman et al., 1985). An alternative procedure to investigate whether the effect of TPA was mediated by stimulation of Na^+/H^+ exchange was to incubate a preparation of cells in a medium where the Na^+ has been replaced by choline (see A.2.2 for the composition of medium used). Choline has been shown not to substitute for Na^+ in stimulation of Na^+/H^+ exchange in HL-60 cells (Besterman and Cuatrecasas, 1984). It is therefore assumed that under these conditions the Na^+/H^+ exchange will not operate in the parietal cells. Histamine was used at a concentration of 0.5mM and IBMX at 0.1mM as these are shown to be maximally effective concentrations for the stimulation of aminopyrine accumulation in rat isolated parietal cells (Shaw et al., 1987).

3.1.3.2 Does TPA inhibit aminopyrine accumulation which has been stimulated by the cyclic AMP analogue dibutyryl cyclic AMP (dbcAMP)?

It is now generally accepted that histamine stimulates secretory activity within the parietal cell as a consequence of receptor-mediated activation of adenylate cyclase and the subsequent elevation of intracellular cyclic AMP content (1.2.2.1). It is possible to mimic such an effect without involving the

receptor and activation of adenylate cyclase by the use of cyclic AMP analogues. DbcAMP contains two lipophilic fatty acid residues which have been introduced to the basic cyclic AMP molecule at position N^6 (adenine) and at position 2'-O (ribose). This molecule has been shown to activate protein kinase A in intact adipocytes and appears highly resistant to breakdown by the low km phosphodiesterase which is present in these cells (Beebe et al., 1984). The analogue dbcAMP thus appears to be an effective agent, and it has been used by other workers to stimulate aminopyrine accumulation in parietal cells (Soll 1980a). In order to determine whether TPA inhibits aminopyrine accumulation at a site distal to adenylate cyclase, the effect of TPA will be compared on cells from the same batch which have been stimulated by 0.5mM-histamine and 0.1mM-IBMX or by 1mM-dbcAMP. When present at 1mM, dbcAMP is near-maximally effective at stimulating aminopyrine accumulation in rat parietal cells (Table 2, Shaw et al., 1987).

3.1.3.3 Does TPA interfere with the elevation of cyclic AMP levels by histamine?

TPA has been reported to attenuate receptor-mediated increases in cyclic AMP content in several cells [e.g. in leydig cells (Bernier et al., 1987) and in intact hepatocytes (Heyworth et al., 1984)]. It was therefore of interest to determine whether TPA modulated cyclic AMP levels in parietal cells stimulated with 0.5mM-histamine and 0.1mM-IBMX.

The possibility that TPA attenuates secretory activity by other mechanisms such as inhibition of the proton pump present on the apical membrane of the parietal cell or by a stimulation of back-diffusion of acid into the cell is considered in Chapter 4.

3.2 METHODOLOGY.

3.2.1 Preparation of incubation media and TPA stock solutions.

The incubation media used in this section of work were either medium B´ (Table 2.1), or Krebs-Ringer bicarbonate medium. A stock solution of Krebs-Ringer bicarbonate medium was prepared by the addition of appropriate salts to double-distilled water (A.2.2) and was stored at 2-8°C. On the day of use, a 100ml sample was gassed with 95% O_2 /5%CO_2 at room temperature for 30 minutes. The addition of bovine serum albumin (1mg/ml), glucose (5mM) and isoleucine (0.1mM) was followed by adjustment of the pH to 7.4 using NaOH (KOH for the Na^+ - free media).

A stock solution of TPA was prepared in dry DMSO at 10mg/ml and aliquots were stored at -20°C. On the day of the experiment, the TPA stock was thawed at room temperature and diluted to the required concentration by serial dilutions in DMSO, and a small volume (usually 2µl) was added to incubation vials. DMSO was also added to the control vials so that the final concentration was 0.1% (v/v) DMSO in all vials. IBMX was dissolved in absolute ethanol. Ethanol was also added to control vials so that the concentration was 0.125% (v/v) ethanol in all vials.

3.2.2 The effect of TPA on aminopyrine accumulation in crude parietal cell fractions.

Parietal cell preparations containing approximately 20% parietal cells were isolated as described in section 2.1. Where it was necessary to modify the composition of the incubation medium from B´ the cell pool was split into two at this stage. The cell fractions were centrifuged for 5 minutes at 175g at 15°C. The supernatants were discarded and the cell pellets were carefully resuspended in 10ml of the appropriate incubation medium prior to a second centrifugation under the same conditions. The resulting cell pellets were resuspended in the required volume of incubation medium at a final concentration of 2-5 x 10^6 cells/ml. 1.5ml aliquots of the cell suspension were added to incubation vials and the determination of aminopyrine accumulation was performed as described previously (2.3).

3.2.3 The effect of TPA on cyclic AMP content and aminopyrine accumulation ratio in enriched fractions of parietal cells.

Fractions containing 80-85% parietal cells were prepared and pre-incubated in a supplemented tissue culture medium (2.2.3.2) for 2 hours prior to a wash with, and resuspension in either medium B´ or Krebs-Ringer bicarbonate medium before use. The cells were maintained at 37°C with a gentle flow of 95% O_2 /5%CO_2 in the gassing space above them for 10 minutes to allow a stabilisation of cyclic AMP levels. 1.5ml aliquots of cell suspension were added to incubation vials, containing appropriate test reagents, which were then gassed and capped prior to incubation at 37°C with

shaking at 120 cycles/minute. For determination of the cyclic AMP content of the cell suspension a volume of cells (usually 0.5ml), was removed, added to an equal volume of absolute ethanol at 4°C and mixed well with a vortex mixer. The cyclic AMP concentration in the supernatant of the incubation medium was determined by using samples of the cell suspension which were centrifuged at 10,000g for 5 seconds to pellet the cells. A sample of the supernatant was rapidly mixed with an equal volume of absolute ethanol. All ethanolic extracts were stored at -20°C for less than three weeks prior to centrifugation, evaporation and radioimmunoassay (2.4.2.2). When it was necessary to obtain cyclic AMP and aminopyrine accumulation measurements from the same cell sample, aminopyrine and polyethylene glycol were present throughout the incubation and the cell sample was processed as described in 2.3 to determine the extent of aminopyrine accumulation.

3.2.4 Presentation of Data.

The effect of TPA on aminopyrine accumulation was usually expressed by normalising the data to % inhibition by TPA. This was calculated by the following expression:-

$$\%\ \text{inhibition by TPA} = \frac{\text{value in absence TPA-value in presence of TPA}}{\text{value in absence of TPA}} \times 100$$

Normalisation of the data has been used by other workers using isolated cell preparations (Soll, 1980a) and enables meaningful comparisons to be made between experiments despite considerable variation in the absolute magnitude of the untransformed data. Due to the variation in aminopyrine accumulation ratios between

experiments, both control and experimental measurements were always performed on the same cell batch.

The effect of TPA on cyclic AMP content was expressed in a similar manner although the basal (unstimulated) cyclic AMP was subtracted from the histamine-stimulated value prior to calculation of % inhibition by TPA. It was necessary to correct for basal cyclic AMP since this was a much greater proportion of the stimulated value than was the basal aminopyrine accumulation ratio. For example basal cyclic AMP was typically 1 pmol/10^6 cells and the histamine-stimulated value was around 3 pmol/10^6 cells. By comparison the basal aminopyrine accumulation ratio was around 2-3 which is a small proportion of the typical stimulated value of 50-200. Thus, the subtraction of a basal aminopyrine accumulation ratio had little effect on the calculated % inhibition by TPA (Appendix A.5). However, in those experiments where both cyclic AMP content and aminopyrine accumulation were measured on the same cell batch, both results were corrected by subtraction of the appropriate basal value.

3.3 RESULTS AND DISCUSSION.

3.3.1 Effects of sodium-replacement on the inhibition by TPA of histamine-stimulated aminopyrine accumulation.

Krebs-Ringer bicarbonate medium (A.2.2) supplemented with isoleucine (0.1mM), glucose (5mM) and bovine serum albumin (1 mg/ml) was a suitable medium for incubation of parietal cells whether or not sodium was replaced by choline. Thus stimulation by histamine plus IBMX produced a control aminopyrine accumulation ratio of 50.8 ± 7.0 (n = 6 cell-batches). This was not significantly different from the aminopyrine accumulation ratio of 43.6 ± 11.2 when cells from the same batch were incubated in a medium where Na^+ had been replaced by 149mM-choline (analysis of data by paired t-test; Bishop, 1980). Replacement of extracellular Na^+ by choline had no significant effect on basal aminopyrine accumulation when 6 samples from a single cell batch were compared (Na^+-containing medium = 2.2 ± 0.0; Na^+ replaced medium = 2.4 ± 0.1, anaylsis by paired t-test). These results are in agreement with those obtained using rabbit gastric glands (Berglindh, 1978) in showing that the removal of extracellular Na^+ does not affect the secretory reponse to histamine.

Table 3.3 shows the untransformed data from six experiments. The effect of TPA is more clearly demonstrated by normalising the data to express a value for % inhibition by TPA (3.2.4); this is shown in Fig. 3.5. The inhibitory action of TPA on aminopyrine accumulation was similar whether or not the cells were incubated in a medium containing Na^+ (149mM Na^+ - containing medium; IC_{50} = 2.9 nM, maximum inhibition of aminopyrine accumulation = 77.9 $\pm$ 3%; Choline-containing medium; IC_{50} = 2.7nM, maximum inhibition of aminopyrine

Table 3.3

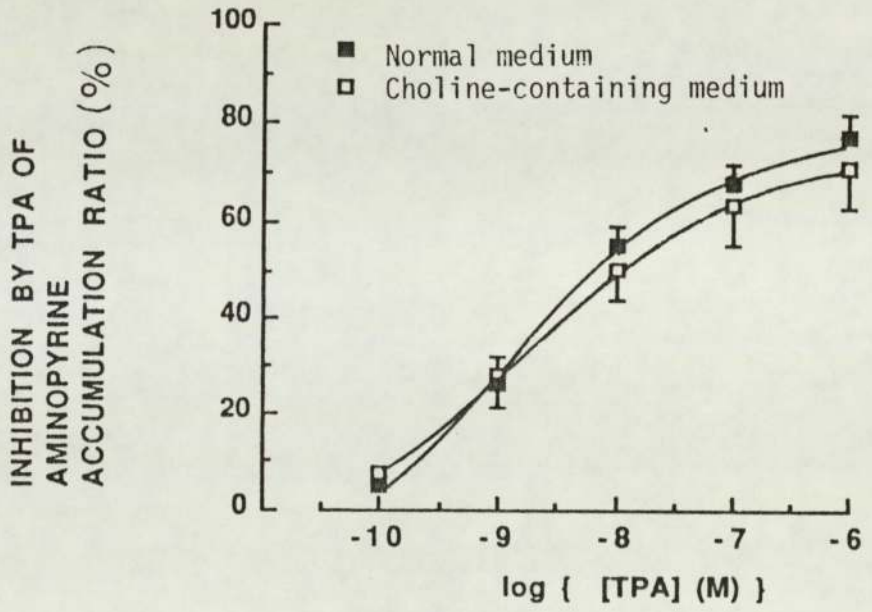
The effect of sodium-replacement by choline on the inhibition by TPA of aminopyrine accumulation stimulated by histamine (0.5mM) and IBMX (0.1mM).

Concentration of TPA (nM)	Aminopyrine accumulation ratio. Na^+ containing medium	Choline containing medium
0	50.8 ± 7.0	43.6 ± 11.2
0.1	46.4 ± 3.7	45.8 ± 13.1
1.0	36.6 ± 4.3	30.0 ± 9.6
10.0	22.4 ± 2.9	22.6 ± 9.2
100.0	15.7 ± 2.2	18.1 ± 8.8
1000.0	11.1 ± 3.2	14.9 ± 9.5

Results from 6 experiments on separate cell-batches containing 20.8 ± 0.6% parietal cells are presented as means ± S.E.M.

Figure 3.5

Effect of the concentration of TPA on the inhibition of aminopyrine accumulation in parietal cells stimulated by histamine (0.5mM) and IBMX (0.1mM) in normal medium and in a medium in which the Na^+ has been replaced by choline.



Data from Table 3.3 have been normalised to % inhibition of aminopyrine accumulation by TPA. For each concentration of TPA tested, the % inhibition was not significantly different between that measured on cells incubated in normal medium and that on cells incubated in choline-containing medium (paired t-test). The position of the lines has been calculated by using the computer programme FIT (Barlow, 1983).

accumulation ratio = 70.0 ± 4%; see A.6 for the determination of IC_{50} and maximum inhibition). There was no significant difference between the % inhibition by TPA on cells incubated in either medium at any concentration of TPA tested when the data were compared by paired t-test. The half-maximally effective concentration of TPA for the inhibition of aminopyrine accumulation stimulated by histamine plus IBMX in normal Krebs-Ringer bicarbonate medium obtained in this series of experiments was close to the value of 3.3nM previously obtained when isolated parietal cells were incubated in B´ (Anderson and Hanson, 1985).

There was no significant difference in the ability of parietal cells to exclude trypan blue after a 30 minute incubation in either medium in the presence of 1μM-TPA (% of parietal cells excluding trypan blue: Na^+ -containing medium, 93.7 ± 1.5; choline-containing medium, 94.0 ± 1.3, analysis of data from 5 cell-batches by paired t-test).

The lack of effect of the replacement of Na^+ by choline on the inhibitory action of TPA makes it unlikely that the inhibition of aminopyrine accumulation by TPA involves a stimulation of Na^+/H^+ exchange.

3.3.2 Effect of TPA on aminopyrine accumulation stimulated by 1mM-dibutyryl cyclic AMP.

Stimulation of parietal cell preparations with 1mM-dbcAMP in medium B´ produced an aminopyrine accumulation ratio of 138.8 ± 16.7 (n=4) which was not significantly different from the value of 129.3 ± 31.5 when cells from the same cell-batch were incubated with 0.5mM-histamine and 0.1mM-IBMX (paired t-test). Soll (1980a) showed that

dbcAMP dose-dependently increased the aminopyrine accumulation ratio over the concentration range of 0.1-1.0mM. The precise mechanism by which dbcAMP stimulates secretory activity in the parietal cell is unclear. When added to adipocytes, 1mM-dbcAMP has been reported to elevate cellular cyclic AMP levels (Jarett and Smith, 1974). It was suggested that this effect was a consequence of inhibition of a cyclic AMP-dependent phosphodiesterase due to competition between endogenous cyclic AMP and the dbcAMP analogue. However, Corbin et al. (1985) have shown that in rat hepatocytes, a series of cyclic AMP analogues (dbcAMP was not tested) depressed basal and glucagon-stimulated elevation of intracellular cyclic AMP. It is unlikely that dbcAMP was stimulating secretion in the parietal cells used in this study solely by elevating the cyclic AMP content as a consequence of acting as a phosphodiesterase inhibitor. This is because incubation of parietal cells with IBMX, a potent phosphodiesterase inhibitor, does not increase the basal aminopyrine accumulation ratio (Shaw et al., 1987, compare Tables 1 and 2).

There was no evidence that endogenous histamine played any part in the stimulatory effect of dbcAMP on aminopyrine accumulation. Thus the inclusion of 10μM-cimetidine did not have any significant effect (paired t-test) on the stimulation of aminopyrine accumulation by 1mM-dbcAMP or on the basal value (aminopyrine accumulation ratios with S.E.M. from three experiments; dbcAMP = 58.6 $\pm$ 10.8; dbcAMP + cimetidine = 60.8 $\pm$ 12.1; basal = 2.1 $\pm$ 0.2; basal + cimetidine = 2.3 $\pm$ 0.0). It is therefore unlikely that the stimulation of aminopyrine accumulation by dbcAMP was a consequence of interaction between dbcAMP and endogenous histamine. The most likely effect of dbcAMP is that of activation of cyclic AMP-dependent protein kinase. DbcAMP is more effective than exogenous cyclic AMP in this respect because the analogue is more lipophilic and displays a greater resistance to phosphodiesterase breakdown (see 3.1.3.2).

TPA inhibited aminopyrine accumulation stimulated by dbcAMP in a concentration-dependent manner, Table 3.4 shows the untransformed data from four experiments and Fig. 3.6 shows the % inhibition by TPA of aminopyrine accumulation stimulated by dbcAMP and by histamine plus IBMX. There was no significant difference between the inhibitory effect of any TPA concentration tested whether the stimulation was by dbcAMP or histamine plus IBMX (paired t-test). The half-maximally effective concentration of TPA against histamine plus IBMX - stimulated secretion was calculated to be 8.9nM which is close to the value of 9.6nM against dbcAMP-stimulated secretion. Although these values are similar to each other, both are apparently greater than the half-maximally effective concentration of TPA reported earlier (3.3.1) and the value of 3.3nM obtained in previous work (Anderson and Hanson, 1985). It is unlikely that the difference between these values was a consequence of larger aminopyrine accumulation ratios in this set of experiments. Thus, there was no significant relationship (linear regression analysis: Bishop, 1980) between the aminopyrine accumulation ratio stimulated by histamine plus IBMX and the concentration of TPA which in the same experiment produced half-maximal inhibition of aminopyrine accumulation (Fig. 3.7).

An inhibitory effect of TPA against dbcAMP-stimulated secretion was not observed by Brown and Chew (1987) using rabbit gastric glands although they obtained very similar results to those presented here with histamine-stimulated glands. In the gastric glands stimulated with 0.5mM-dbcAMP in the presence of 10μM-cimetidine (to prevent effects of endogenous histamine) 100nM-TPA produced a further stimulation of aminopyrine accumulation. A stimulatory effect of TPA on aminopyrine accumulation in the presence of dbcAMP was also reported on isolated parietal cells from the rabbit (Brown and Chew, 1987). Since the rat cell preparations did not

Table 3.4

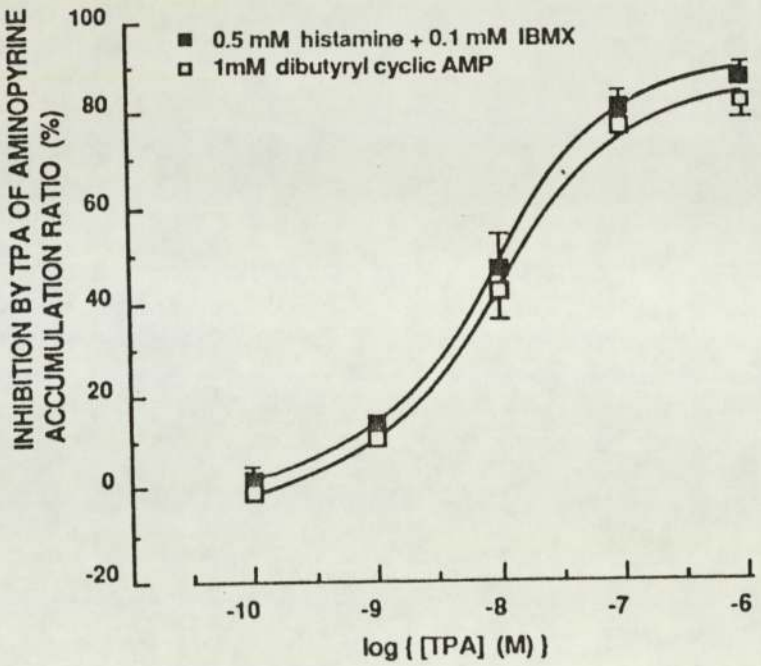
Inhibition by TPA of the aminopyrine accumulation ratio of cells stimulated by histamine and IBMX or by dbcAMP.

Concentration of TPA (nM)	Aminopyrine accumulation ratio.	
	Histamine (0.5mM) + IBMX (0.1mM)	dbcAMP (1mM)
0	129.3 ± 31.5	138.8 ± 16.7
0.1	126.1 ± 30.9	140.0 ± 18.2
1.0	112.4 ± 26.0	124.2 ± 15.7
10.0	70.7 ± 22.1	81.3 ± 13.1
100.0	26.8 ± 10.3	32.0 ± 3.7
1000.0	18.2 ± 7.2	25.4 ± 6.0

Results from 4 cell-batches containing 20.6 ± 0.7% parietal cells are expressed as means ± S.E.M. There was no significant difference between aminopyrine accumulation stimulated by histamine and IBMX or by dbcAMP (paired t-test).

Figure 3.6

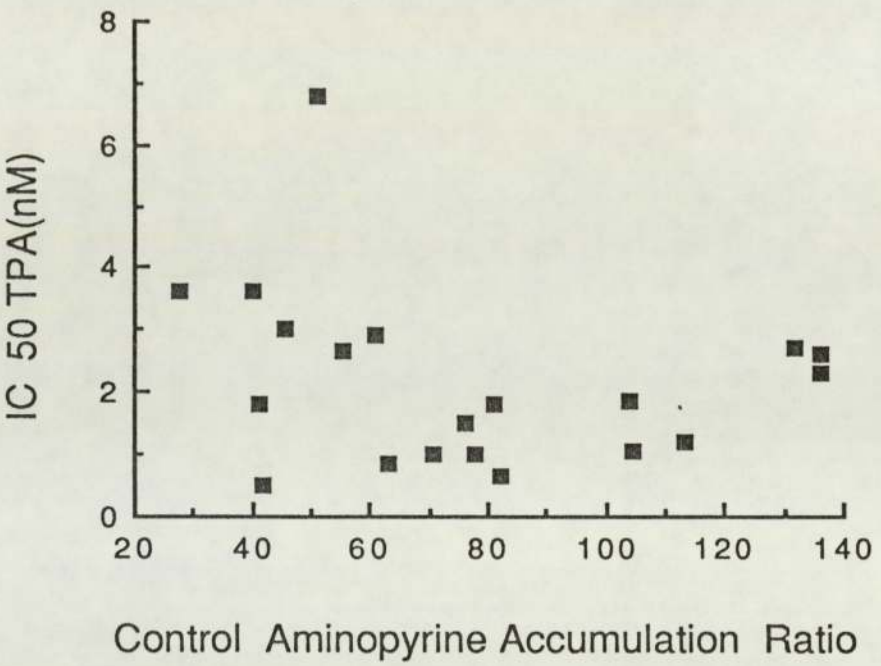
Effect of the concentration of TPA on the inhibition of aminopyrine accumulation by parietal cells stimulated with histamine (0.5mM) and IBMX (0.1mM) and with dibutyryl cyclic AMP(1.0mM).



Data from Table 3.4 have been normalised to % inhibition by TPA of the aminopyrine accumulation ratio. For each concentration of TPA tested, the % inhibition was not significantly different between cells stimulated by histamine plus IBMX and cells stimulated by dbcAMP (paired t-test). The position of the lines has been calculated by using the computer programme FIT (Barlow,1983).

Figure 3.7

Lack of relationship between the control aminopyrine accumulation ratio of cells stimulated by histamine (0.5mM) and IBMX (0.1mM) in physiological composition Krebs-Ringer bicarbonate medium and the concentration of TPA which, in the same cell-batch produced half-maximal inhibition of aminopyrine accumulation..



Results are from 20 cell-batches. The correlation co-efficient was not significantly different from zero (r = -0.228) therefore no line has been drawn.

contain any cimetidine, TPA could potentially have been inhibiting any interaction between dbcAMP and endogenous histamine. However, results of separate experiments with cimetidine (see above) suggested that this was not the case. The inhibition by TPA observed using rat parietal cells may therefore involve either a reduction in the activation of cyclic AMP-dependent protein kinase by dibutyryl cyclic AMP or a counteracting of the consequences of this activation. The difference between the results presented here and those obtained using gastric glands and isolated cells from the rabbit are so profound that they may be due to species variation. A recent abstract (Pfeiffer and Noelke, 1988) seems to confirm that in rat parietal cells TPA inhibits aminopyrine accumulation stimulated by dibutyryl cyclic AMP.

3.3.3 The effect of TPA on cyclic AMP content and aminopyrine accumulation in parietal cell-enriched preparations.

3.3.3.1 Stimulation of cyclic AMP content by 0.5mM-histamine and 0.1mM-IBMX.

Incubation of cell preparations which had been enriched in parietal cells (81.9 $\pm$ 0.7% parietal cells, n=6) with histamine plus IBMX produced an elevation of cyclic AMP content above basal levels (Table 3.5). Stimulation of the cyclic AMP content of the cell suspension above basal after a 5 min incubation was not significantly different (paired t-test) from the stimulation after a 30 minute incubation with the secretagogues. The level of stimulation of cyclic AMP by histamine was similar to the values reported by Sonnenberg et al. (1978) and Puurunen et al. (1987a) using rat cells, but lower than those obtained by Soll

Table 3.5

The effect of 0.5mM-histamine and 0.1mM-IBMX on the cyclic AMP content of enriched parietal cell preparations.

Incubation conditions	Cyclic AMP pmol/10^6 cells: Cell suspension 5 min	Cell suspension 30 min	Supernatant 30 min	Calculated cellular value 30 min
Basal	1.34 ± 0.14	1.36 ± 0.16	0.72 ± 0.32	0.76 ± 0.08
Histamine (0.5mM) + IBMX (0.1mM)	3.47 ± 0.41	3.51 ± 0.41	0.78 ± 0.12	2.73 ± 0.30

Results, from 6 batches of cells containing 81.9 ± 0.7% parietal cells are expressed as means ± S.E.M.

and Wollin (1979) using isolated canine cells (Table 3.6). The differences obtained may be a reflection of varying responsiveness to histamine depending on the species, or may be due to differential inhibition of phosphodiesterase activity by IBMX. Thus, Soll and Wollin (1979) demonstrated that the effect of IBMX on enhancing histamine-stimulated cyclic AMP accumulation in isolated canine cells was strongly dependent on the concentration of IBMX used.

The cellular cyclic AMP was calculated as the difference between the content of cyclic AMP in the cell suspension and the cyclic AMP measured in the medium accompanying those cells. The content of cyclic AMP in the medium is therefore expressed as pmole cyclic AMP/10^6 cells and refers to the cyclic AMP present in the volume of medium which was associated with 10^6 cells. Not all of the cyclic AMP present in the cell suspension after 30 minutes was retained inside the cells; 22 $\pm$ 1% of the cyclic AMP was present in the medium when cells had been stimulated by histamine. This value is similar to that reported by Batzri and Gardner (1978) although higher than the 12.5% present in the supernatant after a 5 minute incubation of canine cells with histamine and IBMX (Soll and Wollin, 1979).

3.3.3.2 The effect of TPA on histamine-stimulated cyclic AMP content and aminopyrine accumulation.

Incubation of parietal-cell enriched preparations with TPA for 30 minutes effected an inhibition of histamine-stimulated cyclic AMP content and aminopyrine accumulation. Tables 3.7 and 3.8 show the untransformed data from 6 experiments. In order to facilitate a comparison between the effect of TPA on cyclic AMP content and the aminopyrine accumulation ratio, the data

Table 3.6

The cyclic AMP content of some isolated cell preparations incubated with histamine and a phosphodiesterase inhibitor.

Secretagogue (mM)	Cyclic AMP content pmol/10^6 cells Basal	Stimulated	Species	% parietal cells	Reference
Histamine(1)+ theophylline(5)	0.2	9.1	Guinea Pig	70-80%	Batzri and Gardner, 1978
Histamine(0.1)+ theophylline(10)	0.35	1.9	Rat	43%	Sonnenberg et al., 1978
Histamine(0.01) + IBMX (0.1)	1.67	11.0	Dog	54%	Soll and Wollin, 1979
Histamine(0.01) + IBMX (1.0)	1.67	72.00			
Histamine(1) + Rolipram(0.1)	0.79	5.25	Rat	60-70%	Puurunen et al., 1987a
Histamine(0.5) + IBMX (0.1)	* 1.36 ± 0.16	*3.51 ± 0.41	Rat	80-83%	This work

*Values given are for cell suspension after 30 min incubation.

Table 3.7

Inhibitory effect of TPA on the stimulation of cyclic AMP content and of the aminopyrine accumulation ratio by histamine (0.5mM) + IBMX (0.1mM) in enriched parietal cell fractions.

TPA concentration (nM)	Cyclic AMP pmol/10^6 cells				Aminopyrine accumulation ratio
	Cell suspension 5 min	Cell suspension 30 min	Supernatant 30 min	Calculated cellular value 30 min	30 min
0	3.47 ± 0.41	3.51 ± 0.41	0.78 ± 0.12	2.73 ± 0.30	199.9 ± 43.9
0.1	3.42 ± 0.48	3.46 ± 0.38	0.71 ± 0.08	2.75 ± 0.31	186.2 ± 40.8
1.0	3.09 ± 0.44	2.95 ± 0.28	0.77 ± 0.10	2.18 ± 0.20	134.4 ± 30.5
10.0	2.91 ± 0.34	2.66 ± 0.27	0.77 ± 0.15	1.89 ± 0.16	68.7 ± 15.0
100.0	2.53 ± 0.29	2.44 ± 0.22	0.74 ± 0.17	1.70 ± 0.16	24.2 ± 5.0
Basal (unstimulated)	1.34 ± 0.14	1.36 ± 0.16	0.72 ± 0.32	0.76 ± 0.08	3.9 ± 0.2

Results, from 6 batches of cells containing 81.9 ± 0.7% parietal cells are expressed as means ± S.E.M.

Table 3.8

Inhibitory effect of TPA on the stimulation of cyclic AMP content and of the aminopyrine accumulation ratio above basal by histamine (0.5mM) and IBMX (0.1mM) in enriched parietal cell preparations.

TPA concentration (nM)	Cyclic AMP pmol/10^6 cells				Aminopyrine accumulation ratio
	Cell suspension 5 min	Cell suspension 30 min	Supernatant 30 min	Calculated cellular value 30 min	30 min
0	2.12 ± 0.41	2.16 ± 0.33	0.60 ± 0.07	1.91 ± 0.26	196.0 ± 43.8
0.1	2.07 ± 0.39	2.11 ± 0.31	0.47 ± 0.12	1.99 ± 0.32	182.2 ± 40.7
1.0	1.74* ± 0.35	1.60** ± 0.21	0.53 ± 0.08	1.42* ± 0.20	130.5* ± 30.4
10.0	1.56** ± 0.25	1.31** ± 0.19	0.53 ± 0.08	1.12** ± 0.15	64.7** ± 15.00
100.0	1.18** ± 0.20	1.09** ± 0.14	0.50 ± 0.11	0.94** ± 0.14	20.3** ± 5.0

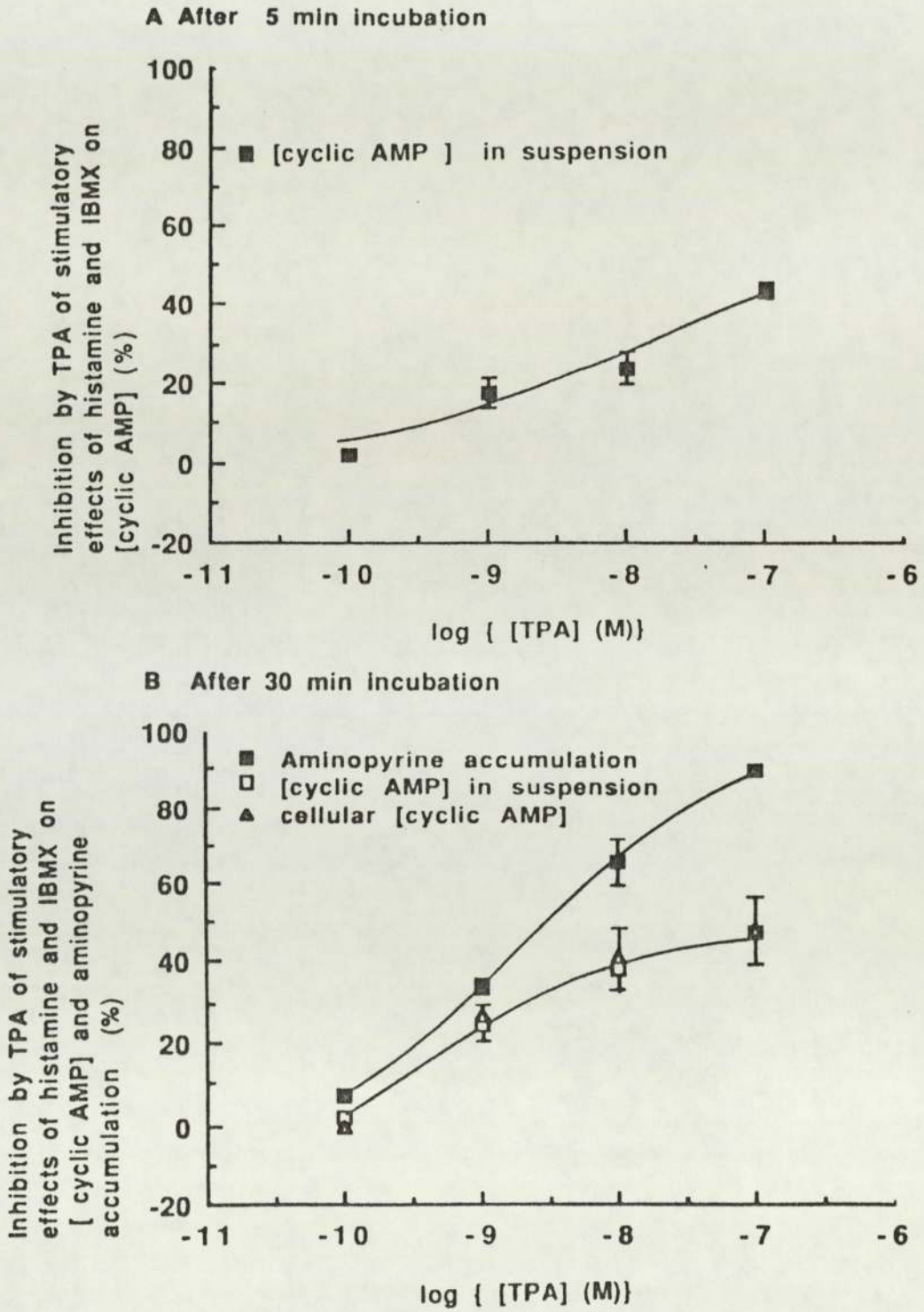
The data from Table 3.7 have been corrected for basal (unstimulated) values. The inhibitory effect of TPA on the stimulation of cyclic AMP and aminopyrine accumulation has been analysed using ANOVAR. Dunnett's Test was used to establish whether results were different from control values in the absence of TPA (**, $p < 0.01$; *, $p < 0.05$).

from Table 3.8 have been normalised. The inhibitory effect of TPA on the elevation of cyclic AMP content above basal is expressed as a % of the value in the absence of TPA (Fig. 3.8, A + B). Analysis of the data by ANOVAR and Dunnett's test (A 7.1 and A 7.2) indicated that 1, 10 and 100nM-TPA had a significant inhibitory effect on the stimulation by histamine of the cyclic AMP content of the suspension (5 and 30 minutes) and on cellular cyclic AMP and aminopyrine accumulation (30 minutes). TPA had no significant effect on the cyclic AMP content of the supernatant at any concentration tested. The consequence of TPA addition was very similar whether the cellular or cell suspension cyclic AMP content was observed (maximum inhibition by TPA; cell suspension = 45.7 $\pm$ 5.1%, cellular cyclic AMP = 44.9 $\pm$ 3.7; half-maximally effective concentration of TPA; cell suspension = 1.0nM, cellular cyclic AMP = 0.8nM). Thus, it is apparent that the effect of TPA on the cyclic AMP content of the cell suspension stemmed from changes in cellular cyclic AMP.

The concentration-dependent inhibitory effect of TPA on the cyclic AMP content of the cell suspension was similar after 5 or 30 minutes of incubation. When the data from Table 3.8 were analysed by a factorial analysis of variance (A 7.3) there was a significant effect of treatment with TPA ($p < 0.01$) but there was no significant effect of incubation time or of the effect of incubation time on the action of TPA. This makes it unlikely that the reduction of cyclic AMP levels by TPA was due to the accumulation of an inhibitory agent in the incubation medium, indeed any such substance would be substantially diluted. The possibility of TPA acting via an intermediate inhibitor is discussed in greater detail in the next section (3.3.4).

Figure 3.8

The inhibitory effect of TPA on the stimulation of the cyclic AMP content and of the aminopyrine accumulation ratio by histamine (0.5mM) and IBMX (0.1mM).



Data from Table 3.8 have been normalised to % inhibition by TPA, and are expressed as means $\pm$ S.E.M. The position of the lines has been calculated by using the computer programme FIT (Barlow,1983). For the sake of clarity the line for cellular cyclic AMP and the S.E. bars for 0.1nM TPA have been omitted from the figure.

TPA exhibited a dose-related inhibition of aminopyrine accumulation which was measured using samples obtained from the same incubation vial from which cyclic AMP samples were taken. After 30 minutes of incubation, histamine-stimulated aminopyrine accumulation was inhibited by TPA to a greater extent than was the cyclic AMP content (maximum inhibition of aminopyrine accumulation by TPA = 98.5%, maximum inhibition of the cyclic AMP content of suspension at 30 minutes = 45.7%) although the half-maximally effective concentrations of TPA were quite similar for both parameters (3.1nM for aminopyrine accumulation, 1.0nM for cyclic AMP content). A possible explanation for this difference is that TPA inhibited aminopyrine accumulation more effectively by acting at the proposed site distal to adenylate cyclase (see 3.3.2).

Since the half-maximally effective concentration of TPA for inhibition of the cyclic AMP content was similar to that for inhibition of aminopyrine accumulation it is arguable that the same agent, possibly protein kinase C produces both effects. Support for the specificity of the effect of TPA comes from two further sets of results. The first set were obtained using 4α-phorbol 12,13-didecanoate (4αPDD), a phorbol ester which does not activate protein kinase C (Castagna et al., 1982). Thus the stimulation of the cyclic AMP content of the cell suspension above basal after a 5 minute incubation with histamine and IBMX (1.31 ± 0.08 pmol/10^6cells) was not significantly affected by the presence of 100nM-4αPDD (1.28 $\pm$ 0.08 pmol/10^6 cells) when 4 samples from a single cell-batch were compared (unpaired t-test; Bishop, 1980). The second set of experimental data supporting the specificity of the action of TPA were that 100nM-TPA had no significant effect (paired t-test) on basal cellular cyclic AMP content after a 5 min incubation period (pmol cyclic AMP/10^6 cells; no TPA = 1.28 $\pm$ 0.06; 100nM-TPA = 1.27 $\pm$ 0.04, n = 3 batches of cells). If TPA

had been effecting a non-specific inhibition of cyclic AMP, it would be expected that TPA would also inhibit basal cyclic AMP accumulation which was not the case.

3.3.4 Does the inhibitory effect of TPA on histamine-stimulated cyclic AMP content and aminopyrine accumulation involve prostaglandins or somatostatin?

As the isolated cell preparations used in this work were not composed entirely of parietal cells, it is possible that the action of TPA was mediated via the release of an inhibitor from a second cell-type. Prostaglandins and somatostatin are inhibitors of adenylate cyclase activity in parietal cells (Schepp et al., 1983a, see 1.2.3) and may potentially mediate the inhibitory effect of TPA. This phorbol ester has been reported to stimulate the release of somatostatin from a short term culture of canine gastric somatostatin cells (Sugano et al., 1986). Furthermore Yamatani et al. (1985) have shown that TPA stimulates the release of somatostatin from the perfused rat stomach. The release of prostaglandins from non-proliferative variants of 3T3 cells (Butler-Gralla et al., 1983) and from human parietal cells (Schepp et al., 1987) has been reported to increase in the presence of TPA.

3.3.4.1 Lack of effect of flurbiprofen or of a somatostatin antagonist on the inhibition by TPA of histamine-stimulated cyclic AMP content.

Considering the experimental observations reported above, the possibility that TPA may be acting to attenuate cyclic AMP levels via the release of prostaglandins or somatostatin had to be considered. In order to simplify the experimental design, the experiments described in this section measured the cyclic AMP content of the cell suspension, as the inhibitory action of TPA was shown to be the same whether cellular or cell suspension cyclic AMP measurements were made (3.3.3.2). Cyclo (7-aminoheptanoyl-Phe-D-Trp-Lys-Thr [Bzl]) is a specific somatostatin antagonist (Fries et al., 1982), and when used at a concentration of 10nM, it completely inhibited the effect of 10nM-somatostatin on histamine-stimulated aminopyrine accumulation (P. J. Hanson, personal communication). Incubation of histamine-stimulated parietal cells with the antagonist (10nM) for 30 minutes did not significantly affect the control cyclic AMP content nor the inhibitory action of TPA (Table 3.9), data analysed by Newman-Keuls multiple comparison test (A 7.4). Flurbiprofen is a specific and potent inhibitor of cyclo-oxygenase activity in the perfused pancreas (MacAdams et al., 1984), and like the somatostatin antagonist it had no significant effect on the stimulation of cyclic AMP by histamine and IBMX. The inhibitory effect of TPA was not abolished in the presence of flurbiprofen (Table 3.9). These results made it unlikely that the inhibition of histamine-stimulated cyclic AMP content by TPA was a consequence of TPA stimulating the release of somatostatin or prostaglandins into the medium.

Table 3.9

Lack of effect of flurbiprofen or of a somatostatin antagonist on the inhibition by 100nM-TPA of the stimulation of cyclic AMP content above basal by histamine (0.5mM) + IBMX (0.1mM).

Agent	Cyclic AMP content of cell suspension (pmol/10^6 cells)			
	No Addition	100nM TPA	Agent	Agent + 100nM-TPA
Flurbiprofen (10μM)	2.03 ± 0.23	1.52** ± 0.21	1.95 ± 0.20	1.51** ± 0.21
Somatostatin antagonist (10nM)	1.80 ± 0.22	1.29** ± 0.23	2.00 ± 0.13	1.44** ± 0.21

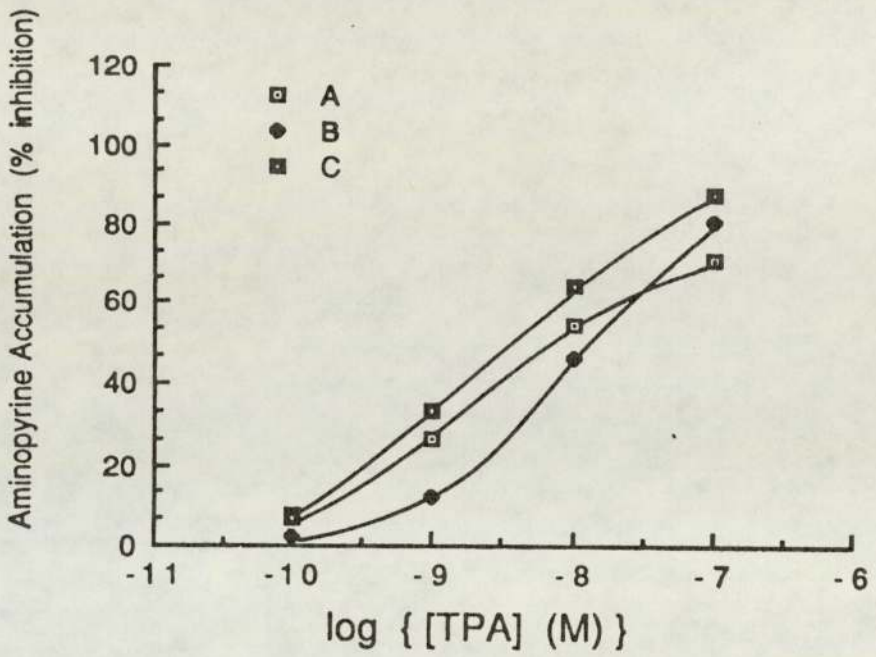
Results from 3 batches of cells are presented as means ± S.E.M. Cell preparations contained 85.3 ± 2.5%, and 83.9 ± 1.6% parietal cells for flurbiprofen and somatostatin antagonist respectively. Basal cyclic AMP content of the suspension was 0.76 ± 0.13 and 0.63 ± 0.13 pmol/10^6 cells for the flurbiprofen and somatostatin antagonist experiments respectively. **, $p < 0.01$ for significant inhibitory effects of TPA (Newman-Keuls multiple comparison test).

3.3.4.2 Lack of involvement of prostaglandins or somatostatin in the inhibition by TPA of histamine-stimulated aminopyrine accumulation.

As indicated previously (3.3.1 and 3.3.2) TPA reduced histamine-stimulated aminopyrine accumulation in preparations containing 20 - 22% parietal cells with a maximal effect of 70% or more inhibition. However it is improbable that the inhibitory effect of TPA on aminopyrine accumulation was mediated via somatostatin since near maximally effective concentrations of somatostatin only cause 15 - 25% inhibition of aminopyrine accumulation in parietal cell-enriched preparations (Schepp et al., 1983a). Previous work in this laboratory (Anderson and Hanson, 1984) indicated that the inhibitory effect of TPA was not reduced by indomethacin (a cyclo-oxygenase inhibitor) or by nordihydroguaiaretic acid (a combined cyclo- and lipoxygenase inhibitor). The lack of involvement of prostaglandins in the inhibitory action of TPA is also suggested by the fact that unlike TPA, prostaglandins do not inhibit acid secretion stimulated by dbcAMP (Soll, 1980a). If the dose-related effect of TPA on histamine-stimulated aminopyrine accumulation is compared between crude and parietal cell-enriched preparations using the data from Figs. 3.5, 3.6 and 3.8 it is apparent that there is relatively little difference between the effectiveness of TPA on these preparations (Fig. 3.9). To facilitate comparison between the cell-preparations, the data from the parietal cell-enriched fraction has not been corrected for basal aminopyrine accumulation in this figure. If TPA were effecting the inhibition of histamine-stimulated aminopyrine accumulation by inducing the release of an inhibitor from a cell of different density than the parietal cell, it would be anticipated that TPA would be less effective in parietal cell-

Figure 3.9

A comparison of the dose-response curves for the inhibition of aminopyrine accumulation by TPA in isolated cell preparations containing 20-22% and 80-83% parietal cells and which have been stimulated by 0.5mM-histamine and 0.1mM-IBMX.



This figure has been compiled using the control dose-response curves for TPA from Figures 3.5,3.6 & 3.8. The positions of the lines have been calculated by using the computer programme FIT(Barlow,1983). For the sake of clarity the error bars have been omitted.

KEY A: 20.8 $\pm$ 0.6% parietal cells incubated in Krebs-Ringer bicarbonate medium, from Fig. 3.5.

B: 20.8 $\pm$ 0.7% parietal cells incubated in medium B', from Fig. 3.6.

C: 81.9 $\pm$ 0.7% parietal cells incubated in medium B', from Fig. 3.8.

enriched fractions. Alternatively, a more pronounced inhibitory effect in parietal cell-enriched fractions would be expected if TPA was stimulating the release of an inhibitor from the parietal cell (or from a cell of similar density). Neither of these situations appear to be the case, suggesting that TPA acted directly on the parietal cell.

3.4 GENERAL DISCUSSION.

The results obtained suggest that there are at least two sites at which TPA acts within the parietal cell to effect a reduction of histamine-stimulated secretory activity. One of these sites is inhibiting the histamine-induced elevation of cyclic AMP since TPA has no effect on basal cyclic AMP nor does it increase the rate of exit of cyclic AMP from the cells. The possibility that TPA may exert part of its effect by increasing the rate of breakdown of cyclic AMP cannot be entirely discounted since the inhibition of phosphodiesterase activity by IBMX may not be complete. There are a number of mechanisms by which TPA may have acted including modulation of the number of histamine receptors capable of binding agonist, perhaps as a result of phosphorylation of the H_2 receptor. TPA has been demonstrated to reduce the affinity of the β-adrenergic receptor for isoproterenol in S49 lymphoma cells (Bell and Brunton, 1987). There is evidence from a number of experiments to suggest that protein kinase C exerts some regulatory effects distal to the hormone receptor. Data obtained by Heyworth et al. (1984) suggest that the inhibitory action of TPA on glucagon-stimulated cyclic AMP accumulation in hepatocytes was a consequence of impaired activation of adenylate cyclase by Gs. Previous work in this laboratory (Atwell and Hanson, 1988) has shown that pre-incubation of isolated parietal cells with pertussis toxin, which ADP-ribosylates and inactivates the inhibitory G protein Gi, produced a small but significant reduction of the inhibition of histamine-stimulated aminopyrine accumulation by TPA. It is therefore possible that the action of TPA on histamine-stimulated cyclic AMP content may be mediated through an activation of Gi. However, work on platelets suggests that TPA inactivates Gi and so impairs the transduction of the inhibitory signal from Gi to adenylate cyclase

(Jakobs et al., 1985) possibly by phosphorylating the α-subunit of the inhibitory G-protein (Katada et al., 1985). Further work needs to be performed using a system in which parietal cell adenylate cyclase activity is assayed to determine whether TPA affects the histamine receptor, coupling of the receptor to G-protein(s) or the interaction between G-protein(s) and adenylate cyclase.

A second site at which TPA may act to inhibit secretory activity is distal to adenylate cyclase since the inhibition by TPA is very similar whether histamine or dbcAMP was the secretagogue. It is possible that an inhibition at this site may have the indirect effect of reducing cyclic AMP content. The potential physiological significance of the inhibitory pathways described here is discussed in Chapter 7.

3.5 SUMMARY.

1. The inhibitory action of TPA against histamine-stimulated aminopyrine accumulation was not affected by the replacement of medium Na^+ with choline, and probably does not involve activation of the Na^+/H^+ exchanger.

2. Inhibition of aminopyrine accumulation in a crude preparation of parietal cells by TPA was similar whether 1mM-dbcAMP or 0.5mM-histamine plus 0.1mM-IBMX were the secretagogues. TPA apparently has an inhibitory site of action which is distal to adenylate cyclase.

3. The histamine plus IBMX-stimulated increase in cyclic AMP content of a cell suspension containing about 80% parietal cells was dose-dependently and specifically inhibited by TPA. The effect did not involve the release of either prostaglandins nor somatostatin from an intermediate cell-type, and it was a consequence of a reduction in cellular cyclic AMP.

Chapter Four

STIMULATION OF PARIETAL CELL SECRETORY ACTIVITY BY ACTIVATIONS OF PROTEIN KINASE C IN A MEDIUM CONTAINING 100mM-K^+.

4.1 INTRODUCTION.

4.1.1 Relationship between K^+ and parietal cell secretory activity.

4.1.1.1 The role of K^+ in acid secretion.

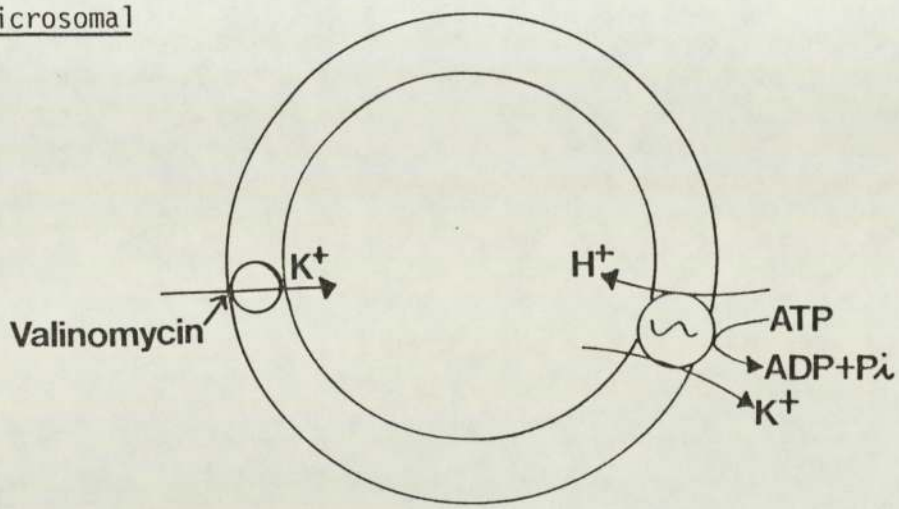
Studies using rabbit gastric glands have demonstrated that acid secretion has an absolute requirement for extracellular K^+ (Berglindh, 1978). Thus incubation of glands in a K^+ -free medium decreased the basal aminopyrine accumulation ratio from 43.6 to 2.6 and also prevented the stimulatory effect of histamine. It is generally accepted that luminal K^+ is required for the functioning of the H^+/K^+ ATPase enzyme, which is also called the proton pump (section 1.1.3).

Much of the information known about the H^+/K^+ ATPase has been obtained from studies using isolated vesicles prepared by differential and density gradient centrifugation of gastric mucosal homogenates (Wolosin, 1985). Microsomal vesicles prepared from resting mucosa are only sparingly permeable to K^+ (Fig. 4.1), and therefore only exhibit significant ATP-dependent proton accumulation in the presence of a K^+ ionophore such as valinomycin. This suggests that the proton pump can only operate if there is a supply of K^+ ions with access to the proton-releasing face of the enzyme. A different class of vesicle called a stimulation-associated vesicle can be prepared from actively secreting gastric mucosa. Accumulation of protons by these vesicles is still dependent upon K^+ and ATP, but occurs in the absence of valinomycin because these membranes possess a K^+ conductance (Wolosin and Forte, 1984). Wallmark et al. (1980) describe a "sidedness" to the proton pump due to an activating K^+ site (present on the luminal face of the

Figure 4.1

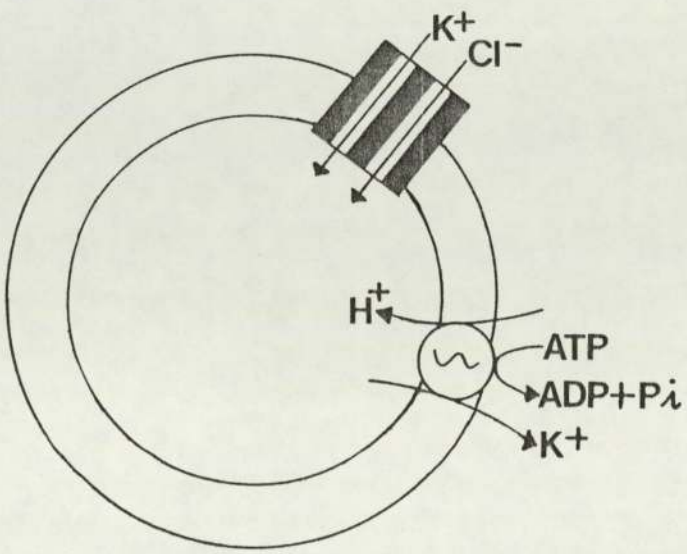
Schematic diagrams showing the ion-transport systems in microsomal and stimulation-associated vesicles.

Microsomal

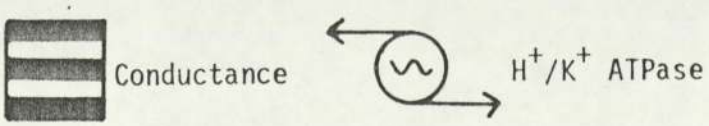


Microsomal vesicles lack a K^+ conductance therefore inclusion of a K^+ ionophore such as valinomycin is necessary to provide K^+ required for the operation of the proton pump.

Stimulation-associated



An ionic conductance provides a rapid flow of K^+ into the vesicle interior which is then available to the proton pump.



enzyme in intact cells) and an inhibitory K^+ site (present on the cytosolic face). These authors also propose that K^+ is required to stimulate the breakdown of a phosphoenzyme which is an intermediate in the reaction mechanism of the H^+/K^+ ATPase.

4.1.1.2 The effect of elevated extracellular K^+ concentration on parietal cell secretory activity.

The K^+ concentration usually used in physiological (control) media is of the range 4.5mM to 5.4mM. If this concentration is substantially elevated with a compensatory reduction in medium Na^+ concentration, there is an increase in the aminopyrine accumulation ratio. This effect is seen both in isolated parietal cells and in gastric glands and occurs in the absence of added secretagogues. This is summarised in Table 4.1 which also shows some of the characteristics of parietal cell preparations which have been incubated in a medium containing a high K^+ concentration.

In order to elucidate the site of accumulation of acid within parietal cells incubated in a medium containing 108mM-K^+, rabbit gastric glands were incubated with a high concentration of aminopyrine (1mM) which is trapped within spaces of low pH (Berglindh et al., 1980a; Gibert and Hersey, 1982). Regions in which

Table 4.1

A summary of some of the characteristics of parietal cells which have been incubated in a medium in which the K^+ concentration has been elevated above control levels.

Tissue	Concentration of medium K^+ (mM)	Effect of K^+ on (i) aminopyrine accumulation ratio (ii) oxygen consumption	Other Characteristics	Reference
Rabbit gastric glands	54	(i) increased from 45.4 to 80.3 (ii) no effect	After 45 minute incubation, a reduction of cellular Na^+ content and slight elevation of cellular K^+ content was observed	Berglindh, 1978
Rabbit gastric glands	108	(i) increased (ii) N.D.	Swelling of canaliculi. Gibert and Hersey additionally observed that there was no reduction in the density of tubulovesicles in cells incubated in a 108mM K^+-medium.	Berglindh et al., 1980a Gibert and Hersey, 1982

cont/.....

Table 4.1 cont.....

Rabbit gastric glands	100	(i) increased (ii) N.D.	Aminopyrine accumulation inhibited by SCN^- and omeprazole	Wallmark et al., 1983
Isolated parietal cells from dog and rabbit	"High" medium	(i) increased (i) N.D.	Aminopyrine accumulation could be further stimulated by secretagogues	Berglindh and Sachs, 1985
Isolated parietal cells from the rat	100	(i) increased from 2.2 ± 0.2 (n = 3) to 83.4 ± 12.1 (n = 19) as the medium K^+ was elevated from 4.5 to 100mM (ii) N.D.	Aminopyrine accumulation inhibited by SCN^-	This work

N.D. = not determined

aminopyrine accumulates become hypertonic and swell as water moves in, enabling them to be visualised by transmission electron microscopy. In the presence of 108mM-K^+ and 1mM-aminopyrine there was a pronounced swelling of the secretory canaliculi, although the density of the tubulovesicular membranes had not changed from the resting state (Gibert and Hersey, 1982). The resting state is typically characterised by a granular cytoplasm containing a large number of tubulovesicles with only a few flattened secretory canaliculi which lead to the parietal cell apical membrane. Therefore it appears that incubation of gastric glands in a medium containing an elevated K^+ concentration results in the accumulation of H^+ within pre-existing secretory canaliculi.

4.1.1.3 How does high extracellular K^+ stimulate aminopyrine accumulation?

To date, the exact mechanism by which aminopyrine accumulation is increased in response to incubation of parietal cells or gastric glands in a medium of high K^+ content is unknown. However, experimental data suggest that K^+-stimulated aminopyrine accumulation is not mediated via the activation of the histamine or of the cholinergic receptors since inclusion of atropine or of cimetidine did not significantly inhibit the elevation of aminopyrine accumulation stimulated by a high K^+ concentration in rabbit gastric glands (Fellenius et al., 1981). It is likely that the enhanced aminopyrine accumulation ratio which occurs in the presence of a high medium K^+ concentration is a consequence of activation of the proton pump since in rabbit gastric glands, K^+-stimulated aminopyrine accumulation is dose-dependently inhibited by the substituted benzimidazole omeprazole (Wallmark et al. 1983). It is conceivable that the enhanced aminopyrine accumulation ratio observed with

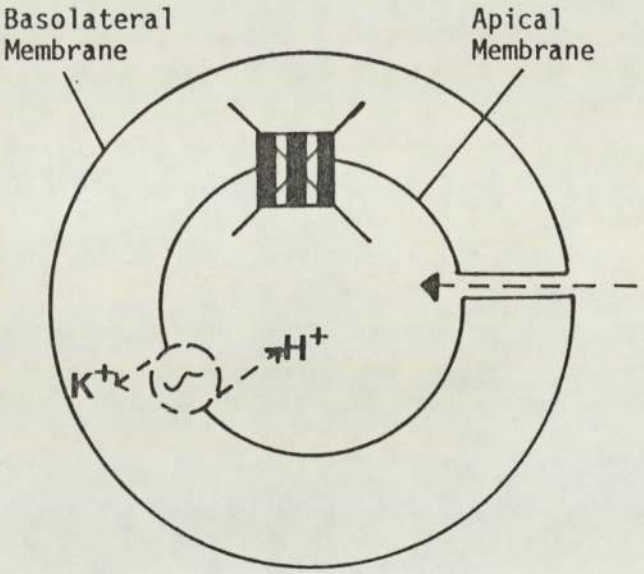
higher K^+ concentrations is a consequence of more K^+ being available to the luminal face of the H^+/K^+ ATPase. Thus Sachs et al. (1976) using vesicular membrane fractions containing the H^+/K^+ ATPase showed that the higher the ratio of internal : external K^+ concentration within the vesicle, the greater the uptake of protons into the vesicle. A possible mechanism by which high medium K^+ induces the accumulation of aminopyrine within the intact parietal cell is summarised in Fig. 4.2. The apical membrane of a parietal cell is highly membraneous with many infoldings. It can be argued that this structure may provide a barrier to the free diffusion of ions from the medium to the canalicular lumen although a restricted diffusion can, and does occur. This is supported by the observation that there is an accumulation of aminopyrine within the parietal cell indicating that protons extruded into the canalicular lumen do not freely diffuse away. However since experimental aminopyrine accumulation ratios are below the theoretical maximal values (Sack and Spenney, 1982) there is probably a loss of some protons from the cell into the surrounding medium. Therefore, it is possible that a restricted diffusion of K^+ from the medium into the preformed secretory canaliculi can, and does occur when there is a sufficient concentration gradient to drive the movement of K^+ ions.

Incubation of rabbit gastric glands with 54mM-K^+ did not stimulate O_2 consumption (Berglindh, 1978). One explanation is that the reduction in extracellular Na^+ reduced Na^+/K^+ ATPase activity and that this counter-balanced the increased activity of the H^+/K^+ ATPase so that there was no net change in ATP utilisation or oxygen consumption. Although a feasible model for K^+-induced aminopyrine accumulation is presented (Fig. 4.2), incubation of the parietal cell in a medium containing an elevated K^+ concentration will also probably depolarise the cell membrane, therefore other mechanisms may also be involved.

Figure 4.2

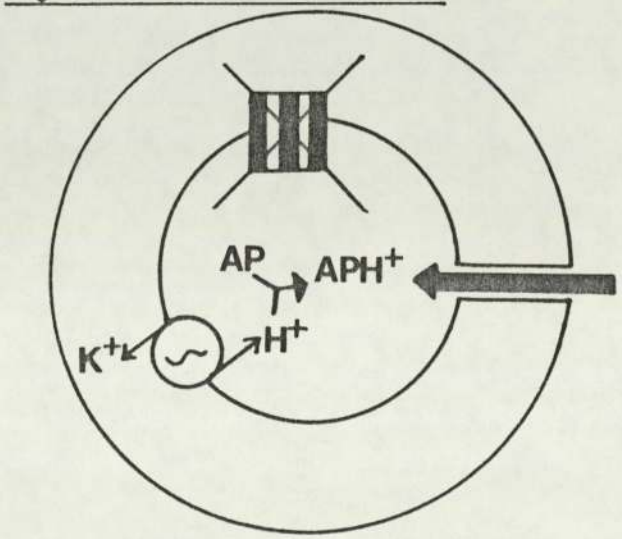
A potential mechanism by which a high extracellular K^+ concentration may stimulate aminopyrine accumulation within a pre-formed secretory canaliculus of a parietal cell.

Low medium K^+ concentration, no secretagogues present.



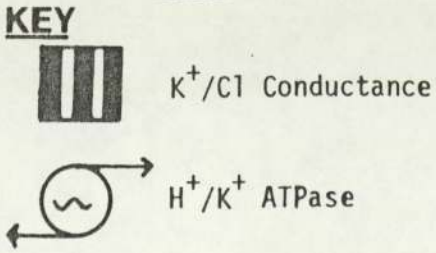
Little diffusion of K^+ into the secretory canaliculus therefore the pump will have a very low activity due to the low K^+ concentration inside the secretory canaliculus.

High medium K^+ concentration.



Restricted but finite diffusion of K^+ into the secretory canaliculus occurs as it is driven by a concentration gradient. The elevation of K^+ within the secretory canaliculus is sufficient to activate the proton pump. Aminopyrine will become protonated in the presence of H^+. There will also be a restricted exit of H^+ from the secretory canaliculus.

In both situations above it is likely that the conductance pathways will be absent or turned off.



In conclusion, a high external K^+ concentration activates the H^+/K^+ ATPase and stimulates aminopyrine accumulation by a mechanism which by-passes that involving secretagogues. It is uncertain exactly what this mechanism is and to what extent it forms part of the physiological pathway activating acid secretion.

4.1.2 Does TPA inhibit the H^+/K^+ ATPase pump?

As mentioned in the previous chapter, there was a possibility that TPA might attenuate histamine-stimulated aminopyrine accumulation by inhibiting the H^+/K^+ ATPase pump. A range of membrane proteins involved in ion transport have been proposed as substrates for protein kinase C (Nishizuka, 1986). Thus an inhibition of the proton pump would be reflected in reduced secretory activity of the parietal cell. If TPA were to act in this fashion, it should have an action similar to that of the substituted benzimidazole, omeprazole (Wallmark et al., 1983) which is a potent inhibitor of the H^+/K^+ ATPase and of K^+-stimulated aminopyrine accumulation. In order to answer the above question, the effect of TPA on the aminopyrine accumulation ratio in parietal cells incubated in a medium containing 100mM-K^+ was investigated.

4.1.3 Does TPA cause a backflux of protons into the parietal cell?

It is possible that an inhibitory effect of TPA on aminopyrine accumulation may be a consequence of the stimulation of the backflux of H^+ into the parietal cell. Normally, there is negligible backflux of protons

into the cell but if the anion thiocyanate (SCN^-) is present, it moves H^+ back across the apical membrane as HSCN (Wolosin, 1985). SCN^- therefore acts as a potent inhibitor of aminopyrine accumulation stimulated by 100mM-K^+ in rabbit gastric glands (Wallmark et al., 1983). If TPA stimulates the backflux of H^+ across the secretory canaliculus into the cytosol of rat parietal cells, then it should have a similar action to thiocyanate on K^+-stimulated aminopyrine accumulation.

The work described in this chapter was undertaken in order to try to determine whether TPA had an action on the proton pump or on proton backflux. As will become evident, not only was this objective achieved but a potential stimulatory action of TPA on secretory activity was uncovered.

4.2 METHODOLOGY.

4.2.1 Preparation of Krebs-Ringer bicarbonate media, TPA and OAG stock solutions.

The media used in this chapter for incubation of parietal cells during the experimental period were based on Krebs-Ringer bicarbonate medium. A stock solution of the required incubation medium was prepared by the addition of appropriate salts to double-distilled water and was stored at 2-8°C, the composition of the 100mM-K^+ and 4.5mM-K^+ media are given in appendix A 2.2. On the day of the experiment, a 100ml sample was prepared as described in section 3.2.1.

TPA was prepared as described previously (3.2.1). A stock solution of OAG was made in DMSO and aliquots were stored at -20°C. OAG was dispersed in the incubation medium by sonication (5 sec at an amplitude of 2 microns, MSE Soniprep) before use. DMSO was also added to control vials so that the final concentration was 0.1% (v/v) DMSO in all vials. IBMX was dissolved in absolute ethanol (3.2.1).

4.2.2 Aminopyrine accumulation experiments using crude parietal cell fractions.

In order to examine the effect of medium K^+-concentration on aminopyrine accumulation, isolated cell preparations were divided into 2 fractions and re-suspended in 4.5mM, or 100mM K^+ - containing media (Appendix A 2.2). A small sample from each cell fraction was counted under the light microscope, and the volume of the cell fractions was adjusted where necessary to ensure that the cell concentrations of both the 4.5mM-K^+ and

100mM-K^+ fractions were the same. Aliquots of cell suspension in differing proportions were added to incubation vials to produce the required intermediate K^+ concentrations, the volume of cells in all vials was 1.5ml. In order to produce a K^+ concentration of 120mM, a 20µl aliquot of a 1.52M KCl solution was added to an incubation vial containing 1.5ml of cells in 100mM-K^+ medium. The incubation conditions and the determination of the aminopyrine accumulation ratio were performed as described in section 2.3. All incubations were for 30 minutes duration except for the time course experiments where the samples were removed at the appropriate time and processed immediately.

4.2.3 The effect of TPA on the cyclic AMP content and on the aminopyrine accumulation ratio in parietal cell-enriched fractions.

Fractions containing 80-85% parietal cells were prepared and pre-incubated in a supplemented tissue culture medium (2.2.3) for 2 hours prior to a wash with, and resuspension in 100mM-K^+ containing medium. A small portion of the cells were washed and resuspended in an equivalent medium containing 4.5mM-K^+. 1.5ml aliquots of cell suspension were added to incubation vials, containing appropriate test reagents, which were then gassed and capped prior to incubation at 37°C with shaking at 120 cycles/minute. After 30 minutes, a 0.5ml aliquot of cells was removed and processed as in section 2.3 for the determination of the aminopyrine accumulation ratio. To measure the cyclic AMP content of the cells, a second 0.5ml aliquot was removed from the same incubation vial and rapidly mixed with 0.75ml of ice-cold B´ and centrifuged for 5 seconds at 10,000g in an Eppendorf microfuge. The supernatant was carefully removed and the pellet immediately mixed with 50% ethanol at 4°C. The extracts were stored at -20°C for less than three weeks

prior to centrifugation, evaporation and radio-immunoassay (2.4.2.2).

4.2.4 Presentation of data.

The effect of TPA on aminopyrine accumulation in crude and enriched parietal cell preparations was usually expressed by normalising the data to % stimulation by TPA. This was calculated using the following expression:-

$$\text{\% stimulation by TPA of the aminopyrine accumulation ratio} = \frac{\text{value in presence TPA - value in absence TPA}}{\text{value in absence TPA}} \times 100$$

A similar normalisation procedure (calculating % inhibition by TPA) was used in Chapter 3 (3.2.4).

The effect of TPA on the cyclic AMP content was expressed in a similar way although unlike the data in Chapter 3, no correction was made for unstimulated or basal cyclic AMP content. The main reason for this was that unlike histamine (Chapter 3), 100mM-K^+ did not stimulate the cyclic AMP content of the cell suspension above that obtained in control (low K^+) medium. Thus any effects of TPA or of OAG on cyclic AMP content would be seen against a cyclic AMP content in the presence of 100mM-K^+ which was effectively basal.

4.3 RESULTS AND DISCUSSION.

4.3.1 Effect of medium K^+ concentration on the aminopyrine accumulation ratio in isolated parietal cells.

Increasing the extracellular K^+ concentration from 4.5mM to 100mM produced an elevation of the aminopyrine accumulation ratio (Table 4.2). A further increase in K^+ concentration to 120mM produced an apparent reduction in the maximal aminopyrine ratio. When the same data were normalised (result at 100mM-K^+ = 100 for each cell batch) and plotted (Fig. 4.3), there was no classical dose-response relationship unlike that obtained by Soll (1980a) for the effect of histamine concentration on the aminopyrine accumulation ratio in dog parietal cells. Thus the curve shown in Fig. 4.3 was asymmetrical, and the major elevation of aminopyrine accumulation by extracellular K^+ occurred over a relatively much narrower concentration range than that obtained when histamine was the secretagogue.

The relationship obtained in this study between the elevation of extracellular K^+ concentration up to 100mM and the corresponding increment in aminopyrine accumulation ratio was similar to that previously reported for rabbit gastric glands by Berglindh *et al.* (1980a) and Dembiński *et al.* (1986).

There was considerable variation between the batches of parietal cells in their response to 100mM-K^+. An analysis of possible variables such as initial parietal cell viability, concentration of cells during incubation and % of parietal cells in the preparations showed that variation was not simply due to any of these factors.

Table 4.2

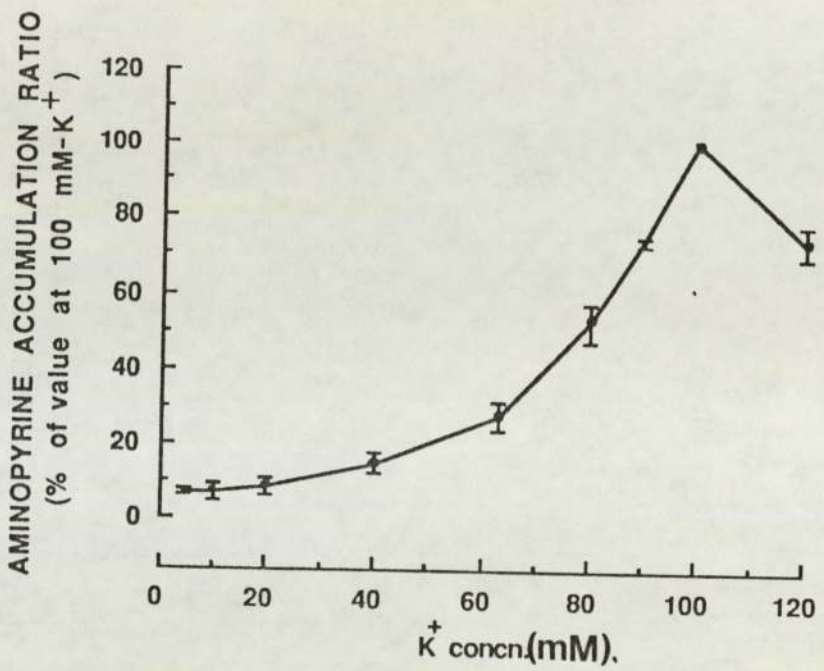
Effect of medium K^+ concentration on the accumulation of aminopyrine by isolated parietal cell preparations.

Medium K^+ concentration (mM)	Aminopyrine accumulation ratio
4.5	2.2 ± 0.3
10.0	2.6 ± 0.1
20.0	3.0 ± 0.1
40.0	4.6 ± 0.4
63.0	7.1 ± 1.3
80.0	13.7 ± 1.5
90.0	20.0 ± 3.7
100.0	36.3 ± 7.3
120.0	25.9 ± 4.2

Results are expressed as means ± S.E.M. from 5 separate cell-batches containing 21.4 ± 0.5% parietal cells. The incubation was for 30 minutes duration in all cases.

Figure 4.3

Effect of extracellular K^+ concentration on the aminopyrine accumulation ratio in isolated parietal cells.



Data from Table 4.2 have been normalised by expressing each value as a % of the aminopyrine accumulation ratio obtained with 100mM-K^+ in the same cell-batch (36.3 $\pm$ 7.3). The heights of the bars represent the means $\pm$ S.E.M.

Subsequent experiments examining the effects of TPA used a medium containing 100mM-K^+ as this concentration was shown to be maximally effective for stimulating aminopyrine accumulation. Preparations of cells incubated in this medium will be termed K^+-stimulated cells. Control cells are K^+-stimulated cells incubated in the absence of TPA or OAG.

4.3.2 Effect of incubation time on the action of 10nM-TPA on aminopyrine accumulation by parietal cells stimulated by 100mM-K^+.

The stimulation of aminopyrine accumulation by 100mM-K^+ varied with time. 10nM-TPA produced a significant stimulation of the aminopyrine accumulation ratio above that of control cells after a 30 minute incubation when the data were compared by a paired t-test ($p < 0.01$) (Table 4.3). Results which have been normalised by setting the control aminopyrine accumulation ratio at 30 minutes to 100 for each cell batch, are shown in Fig. 4.4. Incubation of parietal cells in a medium containing 100mM-K^+ is non-physiological , and this is reflected in a significant reduction in the ability of parietal cells to exclude trypan blue after a 30 minute incubation in this medium (Table 4.4). However, when cells were incubated in Krebs-Ringer bicarbonate medium of physiological composition, there was no significant reduction in the ability of parietal cells to exclude trypan blue. 100nM-TPA did not significantly affect the ability of parietal cells to exclude trypan blue after a 30 minute incubation in 100mM-K^+ media (% of parietal cells able to exclude trypan blue, control = 87.8 ± 3.0%; 100nM-TPA = 83.7 ± 2.0%, when 6 cell-batches were compared by a paired t-test). The results obtained above will be discussed separately, the effect of 100mM-K^+ will be considered

Table 4.3

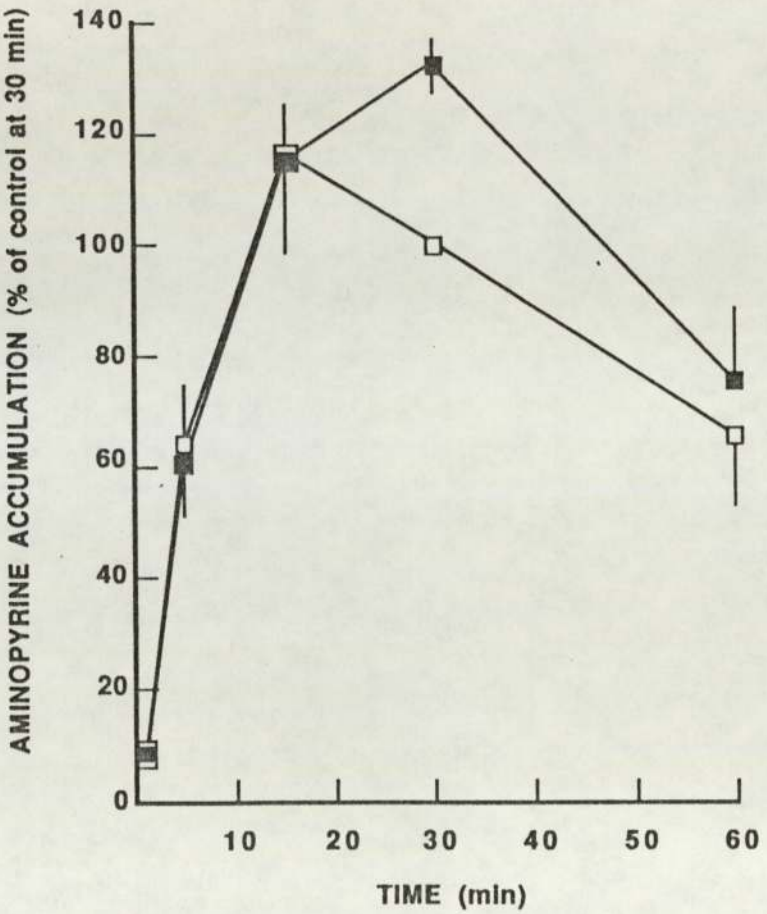
Effect of incubation time on aminopyrine accumulation stimulated by 100mM-K^+ in the absence and presence of 10nM-TPA.

Incubation Time (Min)	Aminopyrine accumulation ratio Control	10nM-TPA
1	7.7 ± 1.4	7.9 ± 1.5
5	59.0 ± 8.6	55.9 ± 6.8
15	115.6 ± 24.8	110.0 ± 18.6
30	105.8 ± 28.0	138.1 ± 34.2**
60	79.4 ± 32.1	91.0 ± 35.0

Results from 4 batches of cells containing 19.1 ± 0.5% parietal cells are expressed as means ± S.E.M. A significant effect of 10nM-TPA was observed after a 30 minute incubation (**, $p < 0.01$, paired t-test).

Figure 4.4

Effect of the incubation period on the aminopyrine accumulation ratio in isolated parietal cells incubated in a medium containing 100mM-K^+ in the presence (■) and absence (□) of 10nM-TPA.



Data from Table 4.3 have been normalised by setting the control aminopyrine accumulation ratio at 30 minutes (105.8 $\pm$ 28.0) to 100 for each cell-batch. Each point represents the mean $\pm$ S.E.M. from 4 batches of cells.

Table 4.4

Effect of a 30 minute incubation of parietal cells in normal and 100mM K^+-containing Krebs-Ringer bicarbonate medium on their ability to exclude trypan blue.

Composition of Krebs-Ringer Bicarbonate Medium	% of parietal cells which excluded trypan blue	
	(a) Immediately prior to incubation	(b) After a 30 minute incubation
Normal	98.1 ± 0.3 (15)	95.4 ± 0.8 (15)
100mM-K^+ containing	97.5 ± 0.6 (6)	87.8 ± 3.0*** (6)

Results are expressed as means $\pm$ S.E.M. with the number of cell-batches in parenthesis. Data were compared by a paired t-test (***, $p < 0.025$). There was no significant difference between the initial values for either medium (unpaired t-test).

initially followed by a discussion of the stimulation observed with TPA.

4.3.2.1 Effect of 100mM-K^+.

The results previously described show that the effect of 100mM-K^+ was transient with a maximal stimulatory effect obtained between 20 and 30 min after the onset of stimulation. Hersey et al. (1981) presented a similar time-course for the effect of 108mM-K^+ on aminopyrine accumulation in rabbit gastric glands with a maximal aminopyrine accumulation ratio of ~ 70 occurring between 20 and 30 minutes of incubation. These workers suggested that the transience of the K^+ effect was due to an initial formation of a proton gradient which was not maintained, and that the fall off in aminopyrine accumulation ratio with time was a consequence of a leakage of protons from the cell. However the ability of K^+-stimulated cells to accumulate aminopyrine when it is present at a concentration of 1mM in the medium (Gibert and Hersey, 1982) implies that there was a sustained pump activity. Thus the high concentration of aminopyrine will significantly neutralise protons and will only accumulate in the presence of their sustained production. After incubation of rabbit gastric glands in 108mM K^+-containing media, there was an increased intraglandular water content and a relatively uniform swelling of cellular organelles including mitochondria (Gibert and Hersey, 1982). Furthermore our results show an enhanced permeability to trypan blue indicating a more "leaky" cell membrane after incubation in a high-K^+ medium. It is likely that when parietal cells are incubated in a high-K^+ medium there is a retention of K^+ and Cl^- within the cell as there is no evidence to date to suggest that there is an activation of K^+ or Cl^- conductances within K^+-stimulated cells. Thus the

K^+ translocated into the parietal cell as a consequence of increased activity of the proton pump would remain trapped within the cell which would generate an increased osmolarity, with a concomitant flow of water into the cell inducing swelling. It could therefore be proposed that the decline in K^+-stimulated aminopyrine accumulation with time was a consequence of cell swelling rendering the cellular organelles less efficient. However, it has been shown that histamine can stimulate oxygen consumption above control levels when rabbit gastric glands were incubated in a medium containing 108mM-K^+ (Hersey et al., 1981) suggesting that incubation of cells in this medium did not damage the integrity of the histamine receptor nor did it substantially impair the functioning of the respiratory pathway within the parietal cell.

4.3.2.2 The effect of 10nM TPA.

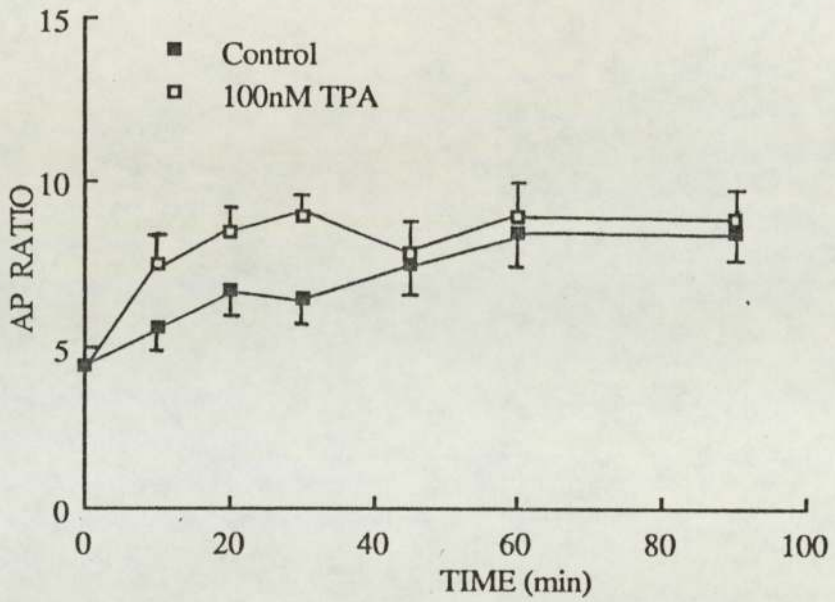
Over an incubation period of 60 minutes, incubation with 10nM-TPA did not inhibit K^+-stimulated aminopyrine accumulation at any time tested. This makes the suggestion that TPA effected an inhibition of histamine-stimulated aminopyrine accumulation by attenuating the activity of the H^+/K^+ ATPase unlikely. A rather surprising and unexpected result was that 10nM-TPA actually enhanced the K^+-stimulated aminopyrine accumulation ratio after a 30 minute incubation. This result contrasts with the inhibitory effect of 10nM-TPA on aminopyrine accumulation by cells stimulated by histamine plus IBMX (Chapter 3). The stimulatory action of 10nM-TPA was probably not related to the extent of aminopyrine accumulation as the aminopyrine accumulation ratio in cells incubated with 100mM-K^+ was 105.8 $\pm$ 28.0 whilst an inhibitory effect of 10nM-TPA was observed when the aminopyrine accumulation ratio was 129.3 $\pm$ 31.5 in the presence of 0.5mM-histamine and 0.1mM-IBMX (Table

3.4). It is unlikely that the stimulatory effect of TPA on aminopyrine accumulation by preparations of cells incubated in a medium containing 100mM-K^+ was a consequence of the reduction in medium Na^+. Work by N. G. Anderson and P.J. Hanson (unpublished) showed that an appropriate reduction of medium Na^+, by choline substitution, without an increase in medium K^+ did not affect the inhibition by TPA of histamine-stimulated aminopyrine accumulation.

A transient stimulatory effect of TPA was also observed in rabbit gastric glands which were incubated in a low-K^+ medium without added secretagogues by Brown and Chew (1987). Figure 1 from their paper has been reproduced in this thesis and is shown in Fig. 4.5. 100nM-TPA increased the basal aminopyrine accumulation in rabbit gastric glands from 12.0 ± 1.3 to 18.0 ± 1.6 after a 30 minute incubation. Thus TPA has been reported to have a transient stimulatory effect on aminopyrine accumulation ratios in rat and rabbit parietal cells, albeit under different incubation conditions. The nature of the novel stimulatory effect of TPA observed in K^+-stimulated cells isolated from the rat stomach was examined further in subsequent experiments.

Figure 4.5

The effect of 100nM-TPA on basal aminopyrine accumulation ratio in rabbit gastric glands.



This figure is a replica of Figure 1.from the paper by Brown and Chew(1987). Rabbit gastric glands containing 10μM-cimetidine were pre-incubated for 30 min prior to the addition of DMSO (0.1% v/v) (■) or 100nM-TPA (□). Aminopyrine accumulation ratios were determined on aliquots at the indicated times. Values are means ± S.E.M. for 4 separate preparations.

4.3.3 Effect of TPA concentration on aminopyrine accumulation by isolated parietal cells incubated in a medium containing 100mM-K^+.

4.3.3.1 Effect of 0.1 - 5nM TPA.

Increasing the TPA concentration from 0.1nM to 5nM dose-dependently increased the K^+-stimulated aminopyrine accumulation by rat parietal cells after a 30 minute incubation (Table 4.5). In order to separate variation due to cell-batches from that due to treatment, the data were analysed by ANOVAR (with the exception of the 2nM and 5nM TPA values which were from 3 cell-batches and so could not be compared with the remaining data which were from 7 cell-batches). Dunnett's Test indicated that 1nM-TPA and 10nM-TPA had a significant stimulatory effect ($p < 0.01$). If cells which were stimulated by 100mM-K^+ were incubated with increasing concentrations of 4α-phorbol 12,13-didecanoate, a phorbol ester which does not activate protein kinase C (Castagna et al., 1982), there was no significant effect on aminopyrine accumulation (Table 4.6). The data from Table 4.5 and from Table 4.6 have been normalised, and are presented in Fig. 4.6. The subsequent decline in the stimulatory effect of TPA as the concentration is increased above 5nM is discussed in 4.3.3.2.

The half-maximally effective concentration of TPA required to produce the stimulatory effect was 1.1nM which is similar to the concentration of 2.5nM required to activate partially purified protein kinase C from bovine brain (Arcoleo and Weinstein, 1985). Furthermore, it is interesting that the EC_{50} for the stimulatory effect of TPA observed here was also close to the value of ~ 3nM obtained in the previous chapter for the inhibition of histamine-stimulated aminopyrine accumulation. Therefore it appears that TPA can effect

Table 4.5

Effect of the concentration of TPA on the aminopyrine accumulation ratio of isolated rat parietal cells incubated in a medium containing 100mM-K^+.

Concentration of TPA when present (nM)	Aminopyrine accumulation ratio
0	92.7 ± 22.6
0.1	91.9 ± 22.3
0.3	110.8 ± 21.0
1.0	118.6 ± 31.2**
2.0	190.4 ± 54.6†
5.0	202.6 ± 47.6†
10.0	140.3 ± 33.8**
100.0	99.1 ± 22.5

† values not included in Dunnett's Test.

Results are expressed as means ± S.E.M. and are obtained from 7 different cell-batches except the 2nM and 5nM values which were obtained from 3 cell-batches. The preparations contained 19-22% parietal cells and the incubation period was for 30 minutes. The effect of TPA concentration (except at 2nM and 5nM) was analysed by ANOVAR, and Dunnett's Test was used to establish whether the results were significantly different from control cells incubated in the absence of TPA (**; $p < 0.01$).

Table 4.6

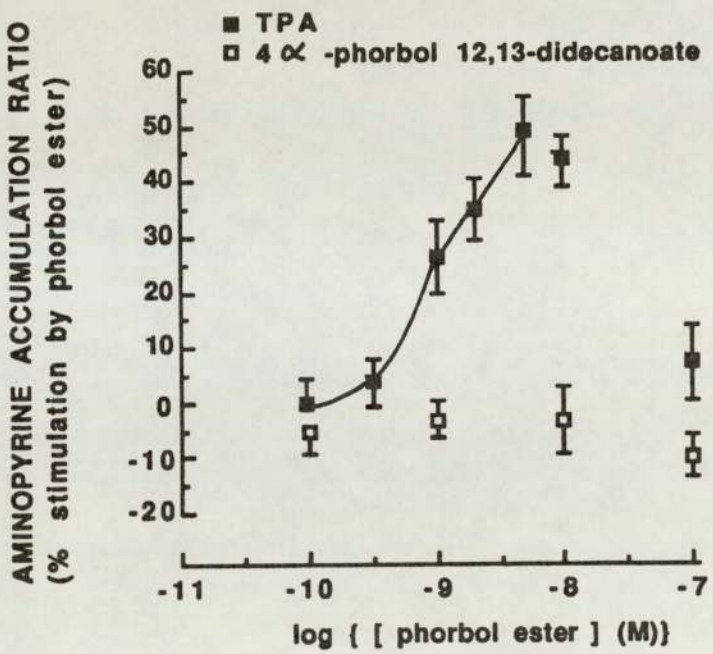
Effect of the concentration of 4α-phorbol 12,13-didecanoate (4α-PDD) on aminopyrine accumulation by rat parietal cells incubated in a medium containing 100mM-K^+.

Concentration of 4α-PDD when present (nM)	Aminopyrine accumulation ratio
0	92.4 ± 16.6
0.1	88.9 ± 18.0
1.0	90.76 ± 18.6
10.0	87.4 ± 11.6
100.0	83.6 ± 9.2

Results are expressed as means ± S.E.M. and are obtained from 4 different cell-batches containing 20.8 ± 1.2% parietal cells. The incubation period was 30 minutes. ANOVAR indicated that there was no significant effect of treatment with 4α-PDD.

Figure 4.6

Effect of the concentration of TPA and of 4α-phorbol 12,13-didecanoate on the aminopyrine accumulation ratio of cells incubated in a medium containing 100mM-K^+.



Data from Table 4.5 and from Table 4.6 have been normalised by expressing the stimulation obtained in the presence of phorbol ester as a % of the control value obtained in the same experiment in the absence of phorbol ester. The control values used for normalisation were 92.7 $\pm$ 22.6 (TPA data) and 92.4 $\pm$ 16.6 (4αPDD data). The position of the line where drawn was calculated by using the computer programme FIT (Barlow,1983), the 10nM and 100nM TPA values did not fit onto the line.

either a stimulation or an inhibition of aminopyrine accumulation depending on the mechanism operating to induce the aminopyrine accumulation. There are recent reports in the literature that treatment of certain cell-types, including parietal cells, with TPA can produce both stimulatory and inhibitory effects within the same cell (Table 4.7, see also Table 7.2). It appears that the involvement of protein kinase C with the control of cellular activity is far from simple and can be demonstrated by using rat basophilic leukaemia cells as an example. In this cell-type, Sagi-Eisenberg et al. (1985) report that not only is the stimulatory effect of TPA on histamine release dose-dependent, but that the activation of protein kinase C before antigen stimulation induces both off and on signals.

The lack of any effect of 4α-phorbol 12,13-didecanoate suggests that the effect of TPA was specific for the activation of protein kinase C. Furthermore, since the aminopyrine accumulation ratio was high in this series of experiments (the control value was 92.4 ± 16.6) there was ample room for any non-specific inhibitory effect of phorbol ester to be observed.

4.3.3.2 Effect of 5nM - 1μM TPA

As the concentration of TPA was raised above 5nM, the stimulatory effect on aminopyrine accumulation shown in Table 4.5 was reduced. This was examined in greater detail using intermediate concentrations of TPA, the untransformed data are presented in Table 4.8 and are presented in normalised form in Fig. 4.7. This effect was found to be concentration dependent with a half-maximally effective concentration of 23nM. Although 5nM

Table 4.7

Some recent reports of dual effects of protein kinase C activation, induced by TPA, on secretory activity.

Species	Tissue/Cell Type	Consequence of protein kinase C activation by TPA.	Reference
Rat	Basophilic Leukaemia Cells	Antigen-induced histamine release stimulated/inhibited depending on concentration of TPA and time at which TPA is added to cells.	Sagi-Eisenberg *et al.*, 1985
Dog	Parietal Cells	Basal AP accumulation; enhanced. Gastrin or carbachol-stimulated AP accumulation; inhibited.	Chiba *et al.*, 1986
Pig	Cultured Leydig Cells	Testosterone production; stimulated. Human choriogonadotropin-induced testosterone production; inhibited.	Bernier *et al.*, 1987
Rabbit	Gastric Glands	Basal AP accumulation; stimulated. Histamine or carbachol-stimulated-AP accumulation; inhibited.	Brown and Chew, 1987
Rat	Parietal Cells	K^+-induced AP accumulation; stimulated. Histamine or dbcAMP-induced AP accumulation; inhibited.	This work

N.B. AP = Aminopyrine.

Table 4.8

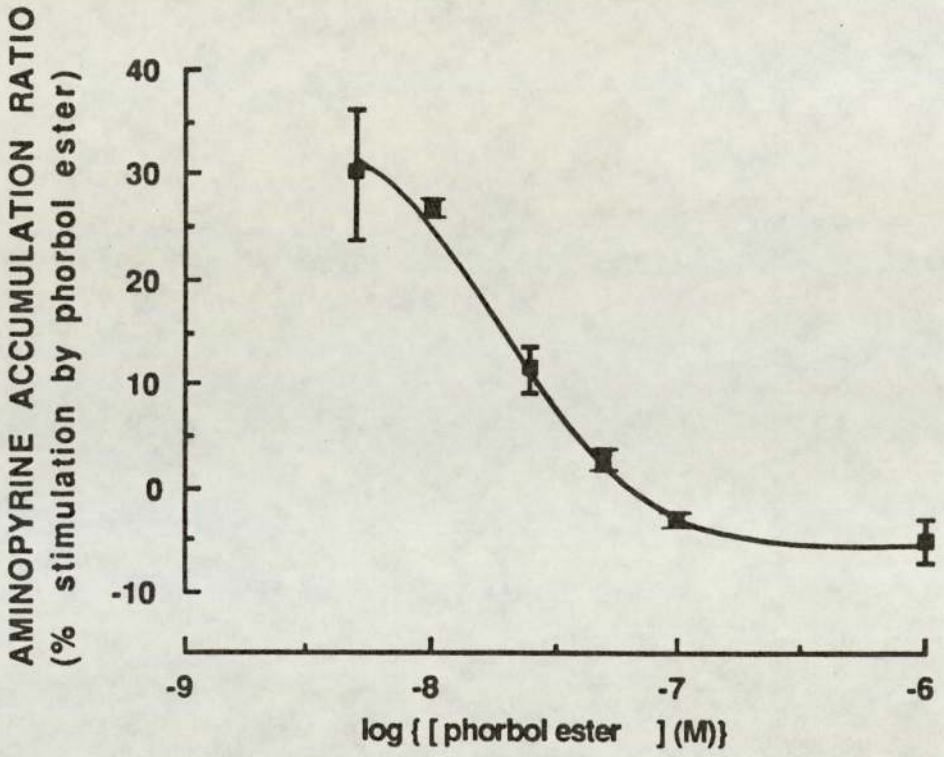
Effect of TPA concentration exceeding 5nM on aminopyrine accumulation by parietal cells incubated with 100mM-K^+.

Concentration of TPA when present (nM)	Aminopyrine accumulation ratio
0	42.7 ± 10.7
5.0	56.2 ± 16.0**
10.0	54.3 ± 13.9*
25.0	48.0 ± 12.8
50.0	44.0 ± 11.1
100.0	41.5 ± 10.4
1000.0	40.7 ± 10.3

Results from 3 cell-batches containing 20.9 ± 0.8% parietal cells are expressed as means ± S.E.M. The stimulatory effect of TPA was analysed using ANOVAR. Dunnett's Test was used to establish whether results were different from control cells incubated in the absence of TPA (**, $p < 0.01$; *, $p < 0.05$).

Figure 4.7

The effect of 5nM-1μM TPA on the aminopyrine accumulation ratio of isolated parietal cell preparations incubated in a medium containing 100mM-K^+.



Data from Table 4.8 have been normalised by expressing the stimulation of aminopyrine accumulation by TPA as a % of the value in the absence of TPA for each experiment. Each point represents the mean $\pm$ S.E.M. from 3 cell-batches. The position of the line was calculated by using the computer programme BASEFIT (Barlow,1983).

and 10nM-TPA significantly stimulated aminopyrine accumulation by comparison with control cells ($p < 0.01$ and $p < 0.05$ respectively) any further increase in TPA concentration produced a significant inhibition of the maximum aminopyrine accumulation obtained with 5nM-TPA ($p < 0.05$) when the data were analysed by Dunnett's Test. The % stimulation of aminopyrine accumulation by 10nM-TPA appears to remain consistent throughout different series of experiments despite a variation in the control aminopyrine accumulation ratio in the absence of TPA. Thus when the stimulation of aminopyrine accumulation by 10nM-TPA was compared with the aminopyrine accumulation ratio obtained with 100mM-K^+ alone in the same experiment, there was no significant relationship obtained (coefficient of correlation = - 0.175 when 13 cell-batches were compared). Normalisation of data by expressing them as % stimulation therefore largely removes the effect of variation between cell preparations.

Since an elevation of the concentration of 4α-phorbol 12,13-didecanoate did not significantly affect aminopyrine accumulation in the presence of 100mM-K^+ it appears that the reduction in the stimulatory effect of TPA with increasing concentration is also specific to an effect on protein kinase C. Beil et al. (1987) showed that 1μM-TPA inhibited the hydrolysis of ATP by H^+/K^+ ATPase isolated from guinea pig parietal cells. However, if 1μM-TPA was acting in this way in rat parietal cells stimulated by 100mM-K^+ it was not sufficiently effective to significantly reduce the aminopyrine accumulation ratio below the value obtained in control cells (100mM-K^+ alone). The present results do not support the idea that the inhibitory effect of low concentrations (nM) of TPA on histamine-stimulated cells is associated with an inhibitory action of TPA on the isolated H^+/K^+ ATPase.

A potential explanation to account for the loss of the stimulatory effect as the concentration of TPA exceeds 5nM is that of downregulation of protein kinase C activity. This has been observed in several different cell-types. For example, in pancreatic acini after a 24 hour incubation with TPA, a significant loss of cytosolic enzyme activity was first observed at a concentration of 30nM with a progressive disappearance as the TPA concentration was increased to 1μM (Sung et al. 1988). Furthermore Beil et al. (1987) demonstrated that incubation of guinea-pig parietal cells with 10nM or 30nM TPA for 30 minutes led to a decrease in cytosolic protein kinase C activity without its appearance in the particulate fraction. However, if a loss of protein kinase C activity due to downregulation was the cause of the reduced stimulatory effect with the TPA concentrations exceeding 5nM in K^+-stimulated cells, it is somewhat difficult to reconcile with the inhibitory action of TPA on histamine-stimulated cells being maintained even at 0.1μM- and 1.0μM-TPA.

Although interpretation of the data concerning the effect of TPA on K^+-stimulated aminopyrine accumulation is rather complex, the important point to consider is that over the entire concentration range tested, TPA did not significantly inhibit the aminopyrine ratio below the control level in the presence of 100mM-K^+.

4.3.4 Effect of OAG concentration on aminopyrine accumulation by isolated parietal cells stimulated by 100mM-K^+

In order to substantiate the proposal that the previous results obtained with TPA were a consequence of effects on protein kinase C activity, the action of the

diacylglycerol analogue 1-oleoyl-2-acetylglycerol (OAG) was examined. The physiological activator of protein kinase C is likely to be a diacylglycerol with an unsaturated fatty acid at position 2 on the glycerol backbone, and the analogue OAG can activate protein kinase C in intact cells (Nishizuka, 1984). The effects of OAG on aminopyrine accumulation stimulated by cells incubated in a medium containing 100mM-K^+ are shown in Table 4.9 and in a transformed mode in Fig. 4.8. Thus OAG appeared to have a similar effect to that of TPA. The concentration of OAG required for a half-maximal effect was 70μM which is quite close to the value of 57.6μM for the inhibition of histamine-stimulated aminopyrine accumulation (Anderson and Hanson, 1985). Although the experiments in this study do not prove that protein kinase C is involved, the TPA and OAG results together make a strong case that this is so. Furthermore, since stimulation of aminopyrine accumulation by 100mM-K^+ was maximal (Fig. 4.3), the stimulatory effect of TPA or OAG was not merely a consequence of a shift of the dose-response curve to the left.

4.3.5 Lack of effect of TPA and OAG on aminopyrine accumulation by isolated parietal cells incubated in a medium containing 4.5mM-K^+ and without added secretagogues.

The stimulatory effect of TPA at low concentrations which is observed in this section contrasts with the inhibitory action against histamine-stimulated aminopyrine accumulation obtained previously (Chapter 3). This inhibitory effect was obtained in medium B′ which contained a low K^+ concentration (5.3mM) and it was therefore necessary to determine the effect of TPA and of OAG on aminopyrine accumulation in the presence of a low K^+ concentration but in the absence of secretagogues.

Table 4.9

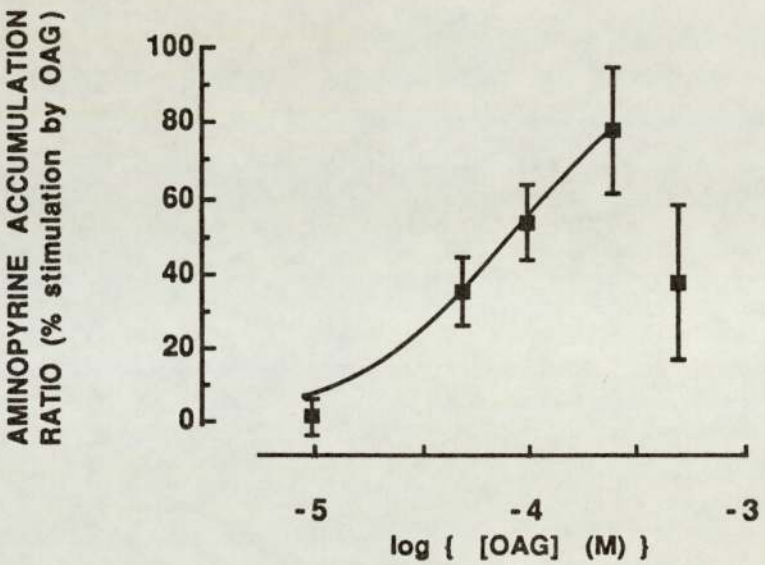
Effect of the concentration of 1-oleoyl-2-acetylglycerol (OAG) on K^+-stimulated aminopyrine accumulation.

Concentration of OAG when present (μM)	Aminopyrine accumulation ratio
0	68.2 ± 19.8
10.0	73.4 ± 29.0
50.0	95.7 ± 36.9*
100.0	105.4 ± 36.1**
250.0	121.1 ± 40.5**
500.0	103.0 ± 42.3**

Results from 5 batches of cells containing 20.7 ± 0.5% parietal cells are expressed as means ± S.E.M. The stimulatory effect of OAG has been analysed using ANOVAR. Dunnett's Test was used to establish whether results were different from control cells in the absence of OAG (**, $p < 0.01$; *$p < 0.05$).

Figure 4.8

Effect of the concentration of 1-oleoyl-2-acetyl-glycerol (OAG) on aminopyrine accumulation stimulated by 100mM-K^+.



Data from Table 4.9 have been normalised by expressing the stimulation of aminopyrine accumulation by OAG as a % of the control value (68.2 $\pm$ 19.8) in the absence of OAG for each cell-batch. Each point represents the mean $\pm$ S.E.M. from 5 cell preparations. The line has been drawn using the computer programme FIT (Barlow,1983);the 500μM OAG value did not fit onto the line.

The incubation medium used for these experiments was the physiological composition Krebs-Ringer bicarbonate medium (containing 4.5mM-K^+). The results are shown in Table 4.10.

No significant stimulatory effect of OAG or TPA was obtained when the data were analysed by ANOVAR. The stimulatory effect of OAG and TPA observed in 100mM-K^+ medium therefore seem to be a direct consequence of the elevation of the K^+ concentration of the incubation medium.

The lack of effect of TPA on basal aminopyrine accumulation in rat parietal cells (Table 4.10) contrasts markedly with the transient stimulatory effect of 100nM-TPA found in rabbit gastric glands (Brown and Chew, 1987, also Fig. 4.5 this work). The reason for this difference is uncertain at present.

4.3.6 Effect of NaSCN on aminopyrine accumulation by isolated parietal cells stimulated by 100mM-K^+.

As mentioned in 4.1.3, TPA may cause the inhibition of histamine-stimulated aminopyrine accumulation by dissipating the proton gradient due to a stimulation of backflux of protons into the cell. A 30 minute incubation of parietal cells stimulated by 100mM-K^+ with 10mM-NaSCN significantly inhibited ($p < 0.001$, unpaired t-test) the aminopyrine accumulation ratio when 4 samples from a single cell-batch were compared (control value = 89.8 $\pm$ 6.7; value with 10mM-NaSCN = 3.1 $\pm$ 0.6). When used at a concentration of 10mM, NaSCN completely inhibited histamine-stimulated aminopyrine accumulation by rabbit gastric glands (Hersey et al., 1981). The results

Table 4.10

The effect of TPA and OAG concentration on basal (unstimulated) aminopyrine accumulation by isolated parietal cell preparations incubated in physiological composition Krebs-Ringer bicarbonate medium containing 4.5mM-K^+.

TPA		OAG	
Concentration (nM)	Aminopyrine accumulation ratio	Concentration (μM)	Aminopyrine accumulation ratio
0	2.3 ± 0.2	0	2.3 ± 0.2
0.1	2.0 ± 0.3	10	2.3 ± 0.2
0.3	2.2 ± 0.2	50	2.3 ± 0.0
1.0	2.3 ± 0.2	100	2.2 ± 0.3
3.0	2.4, 2.1	250	2.0 ± 0.1
10.0	2.1 ± 0.2	500	2.0, 2.1
100.0	2.2 ± 0.1		

Untransformed data from 3 experiments containing 21.0 ± 0.8% parietal cells are presented as means ± S.E.M. except where only 2 values are given. Analysis of the data by ANOVAR indicates that there was no significant effect of TPA or OAG at any concentration tested.

obtained above make it unlikely that TPA effects an inhibition of histamine-stimulated secretion by acting in a similar way to NaSCN, since unlike TPA, NaSCN produced a significant inhibition of K^+-stimulated aminopyrine accumulation.

4.3.7 Effect of cimetidine and of atropine on the action of TPA on aminopyrine accumulation by isolated parietal cells stimulated by 100mM-K^+.

It is possible that TPA exerts its action by stimulating the release of a secretagogue or of a second agent from another cell type. Incubation of isolated cell preparations with cimetidine (0.1mM) or with atropine (10μM) did not prevent the stimulatory effect of 10nM-TPA on K^+- stimulated aminopyrine accumulation (Table 4.11). The elevation of the aminopyrine accumulation ratio by TPA (38.8 $\pm$ 2.9%) was not significantly different in the presence of cimetidine (36.5 $\pm$ 10.9%) or atropine (26.8 $\pm$ 5.6%) when the data were analysed by ANOVAR. Neither cimetidine nor atropine at the above concentrations had a significant effect (unpaired t-test) on aminopyrine accumulation ratios in the absence of TPA. Therefore it is unlikely that the stimulatory effect of TPA was mediated via the release of histamine from another cell-type as cimetidine, an H_2-receptor antagonist, did not prevent the action of TPA. Similarly, it is unlikely that the effect was due to the release of acetylcholine (possibly from any cholinergic presynaptic nerve terminals present in the cell preparation) since atropine, which is a muscarinic cholinergic receptor antagonist, had no effect on the action of TPA. The concentrations of the antagonists used in these experiments were shown to be maximally effective at inhibiting the action of the respective agonists when added to dog parietal cells (Soll, 1980a).

Table 4.11

The effect of cimetidine and of atropine on the action of 10nM-TPA on aminopyrine accumulation by isolated parietal cells stimulated by 100mM-K^+.

Antagonist	Cell-Batches	Aminopyrine accumulation ratio	
		Control	10nM-TPA
None	10	82.3 ± 10.3	118.1 ± 14.4**
Cimetidine (0.1mM)	5	83.3 ± 14.1	111.5 ± 15.5*
Atropine (10μM)	4	99.7 ± 12.4	124.6 ± 10.4**

Results are expressed as means ± S.E.M. from cell-batches containing 19-22% parietal cells. The stimulatory effect of TPA has been analysed by a paired t-test and was significant under the 3 conditions tested (**, $p < 0.01$; *, $p < 0.05$).

Although TPA has been reported to stimulate the cellular release of prostaglandins and somatostatin (3.3.4), it is unlikely that the stimulatory effect on aminopyrine accumulation observed in this work involves either of these agents since neither PGE_2 (Konturek et al., 1983) nor somatostatin (Chew, 1983b) are reported to affect K^+ -stimulated aminopyrine accumulation.

4.3.8 The effect of TPA and of OAG on aminopyrine accumulation and cyclic AMP content in parietal cell-enriched preparations incubated with 100mM-K^+.

The inhibition of histamine-stimulated aminopyrine accumulation by TPA coincides with a reduction of cyclic AMP content (3.3.3). It was therefore of interest to determine whether the effect of TPA on aminopyrine accumulation by K^+-stimulated parietal cells was also related to a change in cellular cyclic AMP. Since changes in cyclic AMP content may occur in cell-types other than parietal cells, an enriched preparation of parietal cells was used for this section of work (2.2.2).

4.3.8.1 The effect of 100mM-K^+ on aminopyrine accumulation by preparations enriched in parietal cells.

Incubation of parietal cell preparations composed of 83.7 $\pm$ 1.3% parietal cells in Krebs-Ringer bicarbonate medium containing 100mM-K^+ produced an average aminopyrine accumulation ratio of 18.6 $\pm$ 3.2 (n = 8 cell-batches) which was significantly lower than the value of

83.4 ± 12.2 (n = 19 cell-batches) obtained in preparations which had not been enriched in parietal cells ($p < 0.01$, unpaired t-test). This difference was not related to the basal aminopyrine accumulation ratios obtained with an extracellular K^+ concentration of 4.5 mM as the value of 2.3 ± 0.3 (n = 3) obtained with crude cell fractions was not significantly different from 3.1 ±0.3 (n = 8) obtained using parietal cell-enriched fractions (unpaired t-test).

As mentioned in 4.1.1.2, K^+-stimulated aminopyrine accumulation probably occurs in preformed secretory canaliculi. If the purification of parietal cells and the 2 hour culture in supplemented tissue culture medium in the absence of secretagogues reduced the number of preformed secretory canaliculi, this may explain why there was a significantly lower K^+-stimulated aminopyrine accumulation in enriched cell preparations by comparison with non-enriched preparations.

4.3.8.2 The effect of TPA and OAG on K^+-stimulated aminopyrine accumulation in parietal cell-enriched preparations.

10nM-TPA and 100µM-OAG both significantly enhanced aminopyrine accumulation by parietal cell-enriched fractions stimulated with 100mM-K^+ (Table 4.12A; $p < 0.01$, Dunnett's Test). To facilitate a comparison between the effects of these agents on crude and enriched parietal cell preparations, the data for both types of cell preparation are presented in Table 4.12. The stimulation which was observed in parietal cell-enriched preparations was significantly greater than the stimulation obtained in cell preparations containing around 20% parietal cells (10nM-TPA, $p < 0.025$; 100µM-OAG, $p < 0.01$ unpaired t-test). There was no evidence that the stimulatory effect

Table 4.12

Effect of TPA and OAG on K^+-stimulated aminopyrine accumulation in enriched and crude parietal cell preparations.

A. 10nM-TPA, 100μM-OAG

Agent (concentration)	Aminopyrine accumulation ratio Control	+ Agent	Stimulation by agent as a % of the control value	% parietal cells	No. cell batches
TPA (10nM)	23.9 ± 4.8	57.9 ± 8.4**	153.2 ± 23.7	86.4 ± 1.4	4
OAG (100μM)	23.9 ± 4.8	63.1 ± 11.4**	175.8 ± 37.5	86.4 ± 1.4	4
TPA (10nM)	92.7 ± 22.6	140.3 ± 33.8**	43.6 ± 4.8	20.3 ± 0.8	7
OAG (100μM)	68.2 ± 19.8	105.4 ± 36.1**	53.6 ± 9.7	20.7 ± 0.5	5

cont/....

Table 4.12 cont....

B. 100nM-TPA, 500μM-OAG

Agent (concentration)	Aminopyrine accumulation ratio Control	+ agent	Stimulation by agent as a % of the control value	% parietal cells	No. cell batches
TPA (100nM)	13.4 ± 2.5	12.7 ± 2.1	-0.1 ± 17.8	81.6 ± 1.5	4
OAG (500μM)	13.4 ± 2.5	16.8 ± 2.0	33.7 ± 32.5	81.6 ± 1.5	4
TPA (100nM)	92.7 ± 22.6	99.1 ± 22.5	7.4 ± 6.8	20.3 ± 0.8	7
OAG (500μM)	68.2 ± 19.8	103.0 ± 42.3**	37.7 ± 20.6	20.7 ± 0.5	5

Results are expressed as means ± S.E.M. The stimulatory effects of OAG or TPA have been analysed using ANOVAR. Dunnett's Test was used to establish whether results were different from control cells (**, $p < 0.01$).

of 10nM-TPA was related to the control aminopyrine accumulation ratio in either crude or enriched preparations of parietal cells (co-efficient of correlation with number of cell batches compared in parenthesis; crude cell preparations = -0.175 (13); enriched cell preparations = -0.145 (8)) and there is therefore no ready explanation of the greater effectiveness of 10nM-TPA or 100μM-OAG in cell preparations which had been enriched in parietal cells.

Incubation of parietal cell-enriched preparations with 100nM-TPA or 500μM-OAG did not significantly enhance K^+-stimulated aminopyrine accumulation (Table 4.12B). The effect of 100nM-TPA was therefore similar to the data obtained using preparations which had not been enriched with parietal cells. However 500μM-OAG had a significant stimulatory effect when added to crude parietal cell preparations although this effect was not significant in parietal cell-enriched preparations.

Although there were some differences in terms of the absolute effects of TPA and OAG observed in parietal cell-enriched preparations compared to crude preparations, the important point is that there was a qualitative similarity between the stimulatory effects of TPA and OAG in that they were abolished (TPA) or reduced/abolished (OAG) as the concentration of the agent was increased.

4.3.8.3 The effect of TPA and OAG on cyclic AMP content of parietal cell-enriched preparations incubated in the presence of 100mM-K^+.

The cellular cyclic AMP content of a preparation composed of 83.7 ± 1.3% parietal cells was 1.06 ± 0.11 pmol/10^6 cells (n = 8) after a 30 minute incubation in a medium containing 100mM-K^+ which was not significantly different from the value of 0.86 ± 0.09 pmol/10^6 cells obtained when cells from the same batch were incubated with a medium containing 4.5 mM-K^+ (paired t-test). Therefore elevation of the aminopyrine accumulation ratio which was observed if the medium K^+ -concentration was raised from 4.5mM to 100mM was not accompanied by a corresponding increase of cellular cyclic AMP content. This contrasts with the data showing that the increased aminopyrine accumulation in the presence of histamine + IBMX is coupled with an elevation of cyclic AMP (Chapter 3).

The cyclic AMP content was not significantly affected by any of the concentrations of TPA or OAG used (Table 4.13 A and B). These measurements were made on the same cell samples obtained from parietal cell-enriched preparations which showed significant stimulatory effects of 10nM-TPA and 100µM-OAG on aminopyrine accumulation (Table 4.12 A and B). It was possible that changes in cyclic AMP levels might not have been detected because of the degradation of cyclic AMP by cyclic AMP phosphodiesterases. Therefore further experiments were performed in the presence of the phosphodiesterase inhibitor, IBMX (0.1mM) (Table 4.14). The results obtained with the lower concentrations of TPA and OAG in the presence of 0.1mM-IBMX paralleled those obtained without the phosphodiesterase inhibitor. With IBMX present, there

Table 4.13

Effect of TPA and OAG on the cyclic AMP content of parietal cells incubated with 100mM-K^+.

A.

Agent (concentration)	Cyclic AMP pmol/10^6 cells Control	+ Agent	Stimulation of cyclic AMP content by agent as a % of the control value
TPA (10nM)	0.97 ± 0.11	1.05 ± 0.14	8.75 ± 9.81
OAG (100µM)	0.97 ± 0.11	1.20 ± 0.22	21.00 ± 10.65

B.

Agent (concentration)	Cyclic AMP pmol/10^6 cells Control	+ Agent	Stimulation of cyclic AMP content by agent as a % of the control value
TPA (100nM)	1.15 ± 0.20	1.13 ± 0.20	3.88 ± 14.30
OAG (500µM)	1.15 ± 0.20	1.26 ± 0.21	15.46 ± 17.48

Data in Tables A and B were both derived from 4 separate cell preparations which contained 86.4 ± 1.4% (A) and 81.6 ± 1.5% (B) parietal cells. All cells were incubated in medium containing 100mM-K^+ ± agent for 30 minutes duration. There was no significant effect of TPA or OAG at either concentration on the cyclic AMP content when the data were analysed by ANOVAR.

Table 4.14

Effect of TPA and OAG on the cyclic AMP content and aminopyrine accumulation by cells incubated with 100mM-K^+ plus 0.1mM-IBMX

A.

Agent (concentration)	Cyclic AMP pmol/10^6 cells IBMX	IBMX + agent	Stimulation of cyclic AMP content by agent as a % of the control value
TPA (10nM)	1.08 ± 0.20	1.14 ± 0.10	13.28 ± 16.06
OAG (100µM)	1.08 ± 0.20	1.07 ± 0.21	5.62 ± 23.10

B.

Agent (concentration)	Aminopyrine accumulation ratio IBMX	IBMX + agent	Stimulation of aminopyrine accumulation ratio by agent as a % of the control value
TPA (10nM)	13.0 ± 2.0	26.1 ± 2.1**	107.90 ± 18.49
OAG (100µM)	13.0 ± 2.0	22.6 ± 4.2*	72.28 ± 20.15

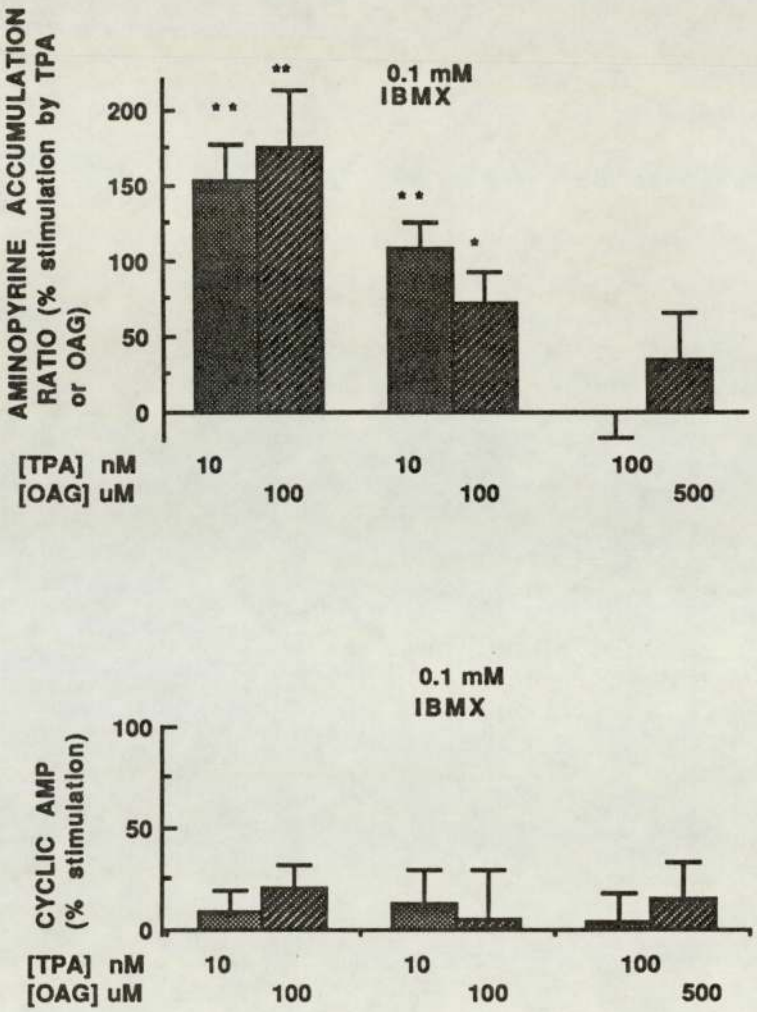
Data in Tables A and B were derived from the same cell preparations containing 81.6 ± 1.5% parietal cell (n = 4). There was a significant effect of TPA and OAG on aminopyrine accumulation when the data were analysed by ANOVAR and Dunnett's Test (**, $p < 0.01$; *, $p < 0.05$). The control values with 4.5mM-K^+ in the medium were 1.38 ± 0.14 pmol/10^6 cells (cyclic AMP content) and 2.62 ± 0.55 (aminopyrine accumulation ratio).

was still a significant increase in aminopyrine accumulation when the medium K^+ concentration was raised from 4.5mM to 100mM ($p < 0.05$) although there was no corresponding change in cyclic AMP content. The elevation of aminopyrine accumulation by TPA and OAG also occurred without a significant alteration of cellular cyclic AMP. Since the inclusion of 0.1mM-IBMX did not significantly alter the results obtained using the lower concentrations of TPA and OAG, it was decided that it would be unnecessary to perform the equivalent experiments with 100nM-TPA and 500µM-OAG.

The data obtained in this section investigating whether the stimulatory effects of TPA and of OAG on aminopyrine accumulation were accompanied by changes in the level of cyclic AMP content are summarised in Fig. 4.9.

Figure 4.9

Effects of TPA and OAG on aminopyrine accumulation and cellular cyclic AMP in parietal cell-enriched preparations incubated in a medium containing 100mM-K^+.



Results from Tables 4.12(A&B),4.13 and 4.14 are presented as means $\pm$ S.E.M. and have been normalised by expressing the stimulatory effect of agent as a % of the control value in the absence of agent. No effect of TPA or OAG on cyclic AMP content was detectable by ANOVAR. A significant effect of TPA (10nM) and OAG (100μM) on aminopyrine accumulation was observed ; **, $p<0.01$, *, $p<0.05$.

4.4 GENERAL DISCUSSION.

The question remaining to be answered is that concerning the mechanism by which the lower concentrations of TPA and OAG elevated the aminopyrine accumulation ratio in cells incubated in a medium containing 100mM-K^+. Since the aminopyrine accumulation ratio is dependent upon the space which is available for the base to accumulate in, it is possible that the stimulatory effect of TPA was merely a consequence of increasing this available space. However, in rabbit gastric glands (Brown and Chew, 1987) 100nM-TPA increased basal aminopyrine accumulation (Fig. 4.5) and also elevated basal O_2 consumption in glands and highly enriched parietal cell preparations. Thus although O_2 consumption measurements have not been made on rat parietal cells, the stimulation of aminopyrine accumulation and of O_2 consumption by TPA observed by Brown and Chew (1987) suggests that an effect of TPA on elevating aminopyrine accumulation is not a consequence of increasing the space available for aminopyrine accumulation, but instead reflects an increased rate of proton pumping as evidenced by the increased metabolic activity.

The possible physiological significance of a stimulatory role for protein kinase C in parietal cell secretory activity is discussed in Chapter 7.

4.5 SUMMARY.

1. Elevation of medium K^+ concentration from 4.5mM to 100mM significantly increased aminopyrine accumulation by isolated parietal cells, probably as a consequence of activating the H^+/K^+ ATPase.

2. TPA did not inhibit aminopyrine accumulation in a preparation of parietal cells incubated in a medium containing 100mM-K^+. It is therefore unlikely that a reduction in the aminopyrine accumulation ratio of histamine-stimulated cells by TPA involves either an inhibition of H^+/K^+ ATPase activity or a stimulation of the backflux of H^+ into the cell.

3. TPA transiently stimulated aminopyrine accumulation in a dose-related manner in cells incubated in a 100mM-K^+ medium. This effect did not involve either histamine or acetylcholine and was apparently specific to protein kinase C activation.

4. TPA did not affect the cyclic AMP content of parietal cell preparations incubated in a medium containing 100mM-K^+.

5. It is possible that a stimulatory role for protein kinase C exist within the parietal cell, and this is evident when parietal cells are incubated in a medium containing 100mM-K^+.

Chapter Five

THE EFFECT OF EPIDERMAL GROWTH FACTOR ON THE AMINOPYRINE ACCUMULATION RATIO AND CYCLIC AMP CONTENT OF ISOLATED PARIETAL CELLS.

5.1 INTRODUCTION.

5.1.1 The structure of Epidermal Growth Factor (EGF).

EGF is a polypeptide which was originally isolated from mouse submaxillary glands by Cohen (1962) and is very similar in structure to β-urogastrone, a polypeptide extracted from human urine (Gregory, 1975; see Table 5.1). Both molecules exhibit similar biological properties and it has been demonstrated that in cultured fibroblasts, β-urogastrone and mouse EGF share a common receptor (Hollenberg and Gregory, 1976). It is generally believed that urogastrone is human EGF(Gregory, 1985) and a recent study using nuclear magnetic resonance has determined the three-dimensional stucture of residues 1-48 of this molecule (Cooke et al., 1987; see Fig. 5.1). Residues 49-53 of the EGF molecule do not appear to be required for biological activity (Hollenberg and Gregory, 1980).

It is now apparent that there is a family of EGF-like molecules which possess a similar structure to mouse EGF. Transforming growth factor-α (or 1) is found in foetal tissue (Twardzik, 1985) and is also produced by cells which have been transformed with a retrovirus (Marquardt et al., 1984). The vaccinia virus protein is also similar to EGF although unlike EGF it is glycosylated; the physiological role of this protein is unknown at present (Stroobant et al., 1985).

Radioimmunoassay has detected EGF in human saliva, sweat, urine and blood (reviewed by Carpenter, 1985) and in many tissues including the stomach and duodenum (Kasselberg et al., 1985). The exact site of EGF synthesis in the human is unclear although it is unlikely to be confined to a single location.

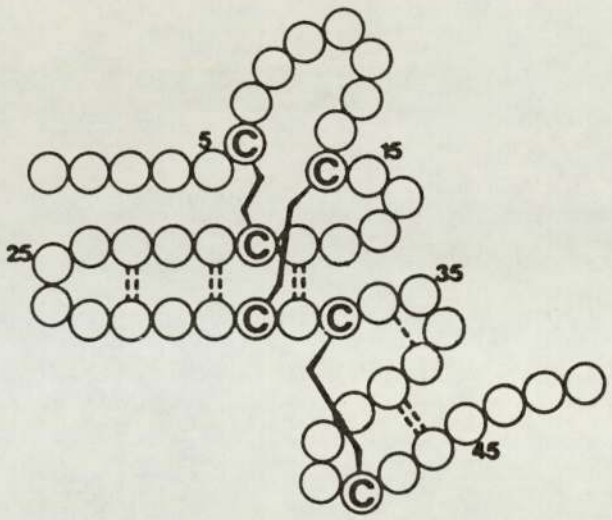
Table 5.1

A comparison of some structural features of peptides related to mouse EGF.

	Number of amino acid residues	Number of disulphide bridges	Molecular weight	% homology with mouse EGF
Mouse EGF	53	3	6045	-
Human EGF (urogastrone)	53	3	*	69.8
Transforming growth factor-α	50	3	5616	33.3
Vaccinia virus growth factor	77	3	9084	A region of 50 amino acid residues shares 36% homology with mouse EGF

* According to Sigma Chemicals there is no discreet molecular weight of urogastrone.

Figure 5.1
A schematic representation of the structure of a derivative of human EGF.



This structure was determined by Cooke et al.(1987) using nuclear magnetic resonance. The figure shows amino acid residues 1-48 of a biologically active derivative of human EGF with the disulphide bridges marked as solid lines, hydrogen bonds as dashed lines and cysteine residues are indicated as C.

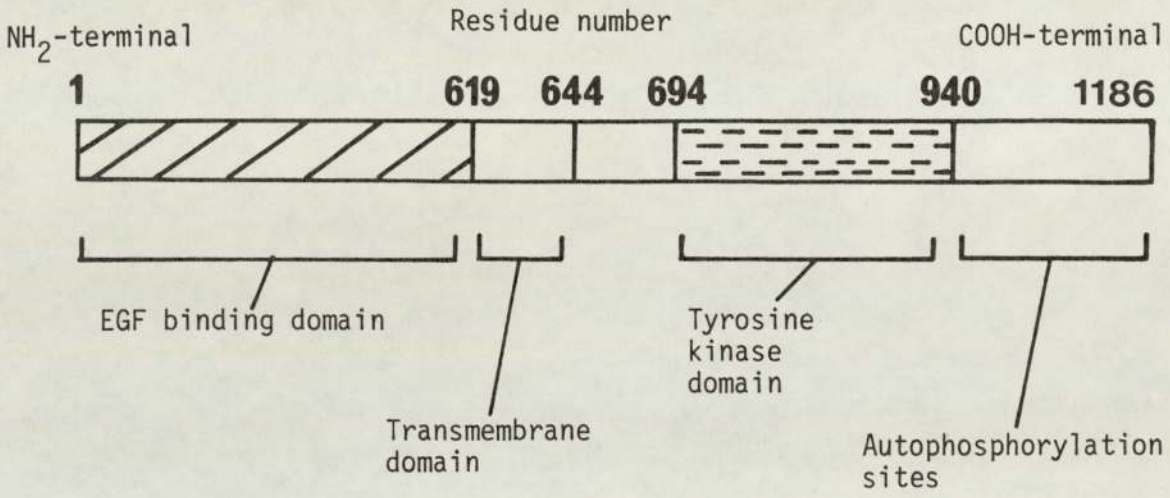
5.1.2 The EGF receptor.

The mature EGF receptor is a transmembrane glycoprotein composed of a single polypeptide chain with a molecular weight of around 170,000 (reviewed by Carpenter, 1985). The detailed structure of the human EGF receptor has been determined using human EGF-receptor c-DNA constructs, and on the basis of this, it has been proposed that the receptor is composed of three functional domains (Ullrich et al., 1984; see Fig. 5.2). Thus there is an extracellular ligand-binding domain, which is characterised by a large number (51) of cysteine residues, attached to a transmembrane region which links to a cytoplasmic domain containing intrinsic tyrosine kinase activity and autophosphorylation sites. Binding of [^{125}I]-EGF to its receptor in a variety of cell-lines can be interpreted in terms of high and low affinity states (see for example Livneh et al., 1986).

As a result of EGF binding to its receptor a number of events take place which have a direct effect upon the receptor. The addition of EGF to A431 human epidermoid carcinoma cells stimulates the phosphorylation of tyrosine, serine and threonine residues on the EGF receptor (Iwashita and Fox, 1984). Tyrosine phosphorylation of the receptor in cells and in intact membranes is due to an autophosphorylation (Downward et al., 1984), but the phosphorylation of serine and threonine residues is activated by EGF or TPA, and it has been proposed that protein kinase C may catalyse the phosphorylation of these residues (Cochet et al., 1984). As a consequence of EGF binding to its receptor, the receptor is internalised (Carpenter and Cohen, 1976). Results of studies examining the fate of internalised EGF and receptors suggest that both the receptor and EGF are degraded in lysosomal compartments (Stoscheck & Carpenter, 1984).

Figure 5.2

Schematic representation of the EGF receptor.



The EGF receptor is shown as a bar, and the domains are derived from the amino acid sequence determined by Ullrich et al. (1984).

The intrinsic tyrosine-specific protein kinase activity inherent in the EGF receptor is similar to that present in the gene products of the src family of oncogenes (Bishop, 1983). The protein product of the avian erythroblastis virus v-erb B oncogene is similar to the EGF receptor except that it lacks the extracellular domain responsible for binding EGF (Carpenter, 1987). It is possible that transformation of cells caused by this virus is due to the expression of a truncated EGF receptor which does not possess the extracellular control domain. The glycoprotein product of the neu oncogene shares approximately 50% homology with the EGF receptor (Bargmann et al., 1986). Although there is not yet any evidence to suggest that this molecule binds EGF, the corresponding proto-oncogene termed c-erb B-2 (to differentiate it from the EGF receptor gene, c-erb B-1) codes for a protein which possesses tyrosine kinase activity (Akiyama et al., 1986). It is an interesting possibility that the protein product of c-erb B-2 may be a receptor for an unknown ligand.

5.1.2.1 Modulation of the activity of the EGF receptor.

The EGF receptor can be regulated in at least 3 ways.

1. A loss of receptors from the cell surface due to removal of surface EGF-receptor complexes by endocytosis will effectively reduce the number of receptors available to interact with the growth factor.

2. Phosphorylation of the receptor on the threonine-654 residue reduces the tyrosine kinase activity, inhibits further autophosphorylation of the EGF receptor (King and Cooper, 1986), and causes the loss of high affinity binding sites for EGF (Friedman et al., 1984). Since EGF has been shown to stimulate phosphatidylinositol turnover in A431 cells (Sawyer and Cohen, 1981) and hepatocytes (Johnson and Garrison, 1987) and to activate protein kinase C in A431 cells (Sahai et al., 1982), it is possible that the EGF-induced activation of protein kinase C may exert a negative feedback effect onto the receptor via the phosphorylation of threonine-654.

3. The affinity of the EGF receptor for EGF is probably also influenced by aggregation of the receptors in cell membranes. Thus cross-linking of EGF receptors induced by a specific antibody converts pre-existing low affinity binding sites into binding sites which exhibit a higher affinity towards EGF (Yarden and Schlessinger, 1985).

5.1.3 Biological effects of EGF on tissues and organs other than the stomach.

As a consequence of interacting with its receptor, EGF induces a variety of biological effects which can arbitrarily be split into long- and short-term effects depending on the length of incubation with the growth factor.

5.1.3.1 Long Term Effects

EGF initiates DNA synthesis in a wide range of cultured cell-types including rat hepatocytes (Richman et al., 1976) and human vascular endothelial cells (Gospodarowicz et al., 1978). In most cases, DNA synthesis will only be initiated if EGF is present in the culture media for at least 5 hours, and an increase in synthesis can be observed after approximately 15 hours following the addition of EGF (reviewed by Carpenter and Cohen, 1979). EGF has also been shown to enhance cellular proliferation in gastric and duodenal mucosa (Dembinski et al., 1982).

5.1.3.2 Short term effects.

In addition to the events mentioned previously (5.1.3.1), incubation of cells with EGF also initiates biological reactions which are observed after a shorter duration of exposure to the growth factor than that required to initiate DNA synthesis. These effects include the phosphorylation of tyrosine residues in cellular proteins, elevation of intracellular Ca^{++} concentration and activation of metabolic enzymes (Table 5.2). It is possible that at least some of these effects may be related to the growth promoting activity of this agent.

Table 5.2

Summary of some of the rapid actions of EGF in cell types other than the gastric mucosa.

All effects were observed within 60 minutes of exposure to EGF.

Cell-type	Effect of EGF	Reference
Caecum and liver (mouse)	Increased guanylate cyclase activity	Scheving et al., 1985
Hepatocytes (rat)	(i) Activation of glycogen synthetase	Bosch et al., 1986
	(ii) Stimulation of phosphatidylinositol turnover and elevation of intracellular calcium concentration	Johnson and Garrison, 1987
	(iii) Stimulation of Na^+ - dependent alanine transport	Moule and McGivan, 1987
A431 cells	(i) Enhanced phosphorylation of tyrosine in lipocortin I	Pepinsky and Sinclair, 1986
	(ii) Stimulation of phosphatidylinositol turnover and calcium influx	Sawyer and Cohen, 1981
	(iii) Activation of protein kinase C.	Sahai et al., 1982

5.1.4 Biological effects of EGF in the stomach.

5.1.4.1 Modulation of acid secretion.

The physiological role of EGF in the regulation of acid secretion is complex and to date it is not fully understood. Parenteral administration of β-urogastrone has been shown to inhibit acid secretion in response to histamine, pentagastrin and cholinergic stimulation in dogs with Heidenhain pouches (Gregory et al., 1977). EGF exerts an inhibitory effect on histamine-stimulated acid secretion by cats when it is injected subcutaneously (Konturek et al., 1981b). It is possible that the action of EGF is exerted directly on the parietal cell as there is no change in the serum gastrin concentration despite an inhibition of acid secretion stimulated in response to sham feeding in dogs (Konturek et al., 1984). There is little evidence available at present to suggest that an antisecretory effect of EGF is observed when it is added to the lumen of the stomach (Table 5.3). These observations make it unlikely that EGF, which is present in saliva (Starkey and Orth, 1977), plays a physiological role in regulating gastric acid secretion under normal circumstances by acting from the lumen of the stomach. The precise reason for this is unclear, although it is unlikely that it is a consequence of an inactivation of the growth factor by acid or pepsin inside the stomach since luminal EGF still exhibits trophic effects (Dembiński et al., 1982).

It is likely that EGF modulates acid secretion via an action on the basolateral membrane of the parietal cell, especially in the light of recent data obtained using immuno-electron microscopy by Mori et al. (1987).

Table 5.3

A summary of experimental data indicating that EGF is not effective at inhibiting acid secretion when it is present in the lumen of the stomach.

Species	Observation	Reference
Rat	10µg/Kg EGF failed to inhibit acid secretion when given intragastrically although the same concentration had a significant effect when infused subcutaneously.	Dembiński et al., 1982
Rat	Intragastric application of EGF produced an insignificant reduction in acid secretion although the dose used was quite large (100µg/Kg).	Konturek et al., 1981a
Guinea Pig	120ng/ml EGF did not inhibit acid secretion when added to the luminal surface of isolated mucosa although the same concentration was maximally inhibitory when added to the serosal surface.	Finke et al., 1985

These workers utilised a monoclonal antibody, which reacted specifically with the human EGF receptor to demonstrate EGF-like immunoreactivity on the basolateral membrane of the parietal cell, indicating the presence of an EGF receptor on the vascular-facing membrane of these cells.

The antisecretory effect of EGF has also been demonstrated using gastric glands and isolated cell preparations. Workers using rabbit gastric glands have shown that EGF inhibits aminopyrine accumulation stimulated by histamine, sub-maximal concentrations of dbcAMP (Dembiński et al., 1986) and by carbachol (Konturek et al., 1984). However, Chen et al. (1984; abstract - no subsequent full publication) using isolated canine parietal cells reported that EGF inhibited histamine-stimulated aminopyrine accumulation, but only exerted a minimal inhibitory effect when cholinergic agents, dbcAMP or gastrin were used as secretagogues. EGF inhibits structural changes which normally occur upon stimulation of the parietal cell (Gonzalez et al., 1981). However, this result gives little clue to the site of action of EGF except that a direct inhibition of the H^+/K^+ ATPase pump is unlikely since substituted benzimidazoles do not inhibit the morphology change induced by histamine in parietal cells of the rabbit gastric gland (Fellenius et al., 1982).

5.1.4.2 Other actions of EGF within the stomach.

Non-antisecretory doses of EGF when injected subcutaneously into rats or cats significantly reduced the mean area of gastric mucosal lesions induced by aspirin + HCl (Konturek et al., 1981b). Although the

mechanism for this effect of EGF is unknown, it was claimed not to be due to the stimulation of prostaglandin release from the gastric mucosa (Konturek et al., 1981a). In addition, it has been demonstrated that 24 hours following intraperitoneal or intragastric administration to the rat, EGF induces a trophic response of the gastroduodenal mucosa (Dembiński et al., 1982). It seems that EGF may therefore have a role in maintaining the integrity of the gastric mucosa. This is supported by the experiments of Skinner et al. (1984) which showed that 30 days after the removal of the submandibular-sublingual gland complexes and ligation of the parotid gland ducts (effectively removing the major sources of luminal gastric EGF in the rat), there was a reduced rate of DNA synthesis in the rat gastric mucosa.

Since these actions of EGF are obtained in lower doses than required to produce an antisecretory effect and also occur from the luminal side of the stomach, it is probable that they are mediated by receptors and/or mechanisms which are different from those which inhibit acid secretion.

At the start of this study, neither the site nor the mechanism by which EGF inhibited acid secretion had been established. This work was therefore undertaken to investigate this problem by using aminopyrine accumulation (as an index of acid secretion) and measurements of the cyclic AMP content of parietal cells isolated from the rat stomach. In particular, the following questions were addressed:

1. Is the inhibition of aminopyrine accumulation by EGF dependent upon the nature of the secretagogue used to stimulate the parietal cell?

2. Is the pattern of inhibition of secretory activity induced by EGF comparable with that effected by TPA, an activator of protein kinase C?

3. Does EGF effect inhibition of aminopyrine accumulation by changing the cyclic AMP content of parietal cells?

Part of the data presented in this chapter were obtained in collaboration with G. P. Shaw, and it will be clearly indicated which experiments were performed jointly.

5.2 METHODOLOGY

5.2.1 Preparation of incubation media and EGF stock solutions.

The incubation media used in this section of work was medium B′ (Table 2.1). A 32μM stock solution of EGF in 0.9% NaCl was stored in aliquots at -20°C. Since the manufacturers (Sigma) recommended that repeated freezing and thawing should be avoided, a separate aliquot was used for each experiment. After thawing at room temperature, the EGF was gently mixed using a Gilson pipette and a small volume (usually 10μl) was added to incubation vials. IBMX was dissolved in absolute ethanol (3.2.1).

5.2.2 The effect of EGF on cyclic AMP content and aminopyrine accumulation.

For experiments investigating the effect of EGF concentration, the determination of aminopyrine accumulation (2.3) and cyclic AMP content (3.2.3) were performed as described previously. When it was necessary to obtain cyclic AMP and aminopyrine measurements from the same vial, aminopyrine and polyethylene glycol were present throughout.

For the time-course experiments, the cell pool was resuspended in 12ml of medium B′ (Table 2.1) at a concentration of 2 - 5 x 10^6 cells/ml and was added to 3 plastic conical flasks containing [^{14}C] aminopyrine (0.1μCi/ml: 0.9μM), [^{3}H] polyethylene glycol (0.4μCi/ml), plus saline, histamine and/or EGF as required. The air-space above the cells was gassed for 5 seconds with

95% O_2 /5% CO_2 and the flasks were capped. Incubation of the cell suspensions was at 37°C with shaking at 120 cycles/ minute. At the required time, duplicate 0.8ml samples were removed from each vial and processed to determine the cyclic AMP content (2.4.2.2) and extent of aminopyrine accumulation (2.3).

5.2.3 Analysis and presentation of data.

In order to reduce the variation due to cell-batch, the data have been normalised in the manner indicated in the legends. The effect of EGF concentration on the cyclic AMP content or aminopyrine accumulation ratio was expressed as % inhibition by EGF which was calculated by the following expression:-

$$\%\ \text{inhibition by EGF} = \frac{\text{value in absence EGF} - \text{value in presence EGF}}{\text{value in absence EGF}} \times 100$$

As indicated in section 3.2.4, it was necessary to correct the cyclic AMP data for basal (unstimulated values), and in experiments where both cyclic AMP content and aminopyrine accumulation were measured on the same cell-batch, both results were corrected by subtraction of the appropriate basal value prior to the calculation of % inhibition.

5.3 RESULTS AND DISCUSSION

5.3.1 Is the inhibition of aminopyrine accumulation by EGF in isolated rat parietal cells secretagogue-specific?

The effect of 3 concentrations of EGF on aminopyrine accumulation stimulated by near-maximally effective concentrations of various secretagogues is shown in Table 5.4. The result obtained with each secretagogue will be described and discussed in turn, all experiments were performed using cell preparations containing 19 - 22% parietal cells. EGF had no effect on basal secretion but a significant antisecretory effect was observed when aminopyrine accumulation was stimulated by 0.5mM-histamine (paired t-test). It is unlikely that the inhibitory effect of EGF in the presence of 0.5mM-histamine was merely a consequence of shifting the dose-response curve for histamine-stimulation of aminopyrine accumulation to the right. Thus the % inhibition by 200nM-EGF was similar whether aminopyrine accumulation was stimulated by 0.5mM-histamine or 5.0mM-histamine [0.5mM-histamine; aminopyrine accumulation ratio = 5.8 $\pm$ 0.8, % inhibition by 200nM-EGF = 34.8 $\pm$ 2.7% (n = 5). 5.0mM-histamine; aminopyrine accumulation ratio = 7.7 $\pm$ 1.9, % inhibition by 200nM-EGF = 36.3 $\pm$ 2.4% (n = 4). There was no significant difference between the aminopyrine accumulation ratios or the % inhibition by 200nM-EGF when the data were compared by an unpaired t-test].

Inclusion of the phosphodiesterase inhibitor IBMX (0.1mM) with histamine (0.5mM) abolished the anti-secretory action of EGF (line 3, Table 5.4). Since histamine plus IBMX produced a greater aminopyrine accumulation ratio than that obtained with histamine alone, it was a possibility that the lack of effect of

Table 5.4

The effect of EGF on the intracellular accumulation of aminopyrine by rat parietal cells incubated in the presence of various secretagogues.

Aminopyrine accumulation ratios are presented as means ± S.E.M. with the number of cell-batches in parenthesis. The effect of EGF was determined by a paired t-test (***, $p < 0.001$; *, $p < 0.05$). Experiments performed by G. P. Shaw are indicated (†).

	Aminopyrine accumulation ratio					
Secretagogues	Control	20nM-EGF	Control	200nM-EGF	Control	1μM-EGF
No addition	2.3 ± 0.5 (4)	2.1 ± 0.5 (4)	3.7 ± 0.6 (4)	3.9 ± 0.8 (4)	2.1 ± 0.4 (4)	2.0 ± 0.4 (4)
Histamine (0.5mM)	5.4 ± 0.8 (5)	4.3 ± 0.6* (5)	9.7 ± 1.0 (5)	6.3 ± 0.6* (5)	5.2 ± 1.0 (4)	3.5 ± 1.0*** (4)
Histamine (0.5mM) + IBMX (0.1mM) †	114 ± 23 (5)	115 ± 30 (5)	146 ± 21 (11)	136 ± 21 (11)	105 ± 28 (4)	99 ± 27 (4)
Carbachol (0.1mM) †	4.2 ± 0.9 (4)	4.2 ± 1.5 (4)	10.3 ± 1.1 (4)	10.5 ± 1.7 (4)	4.6 ± 0.8 (5)	4.2 ± 1.0 (5)
Dibutyryl cyclic AMP (1mM) †	147 ± 31 (5)	144 ± 30 (5)	201 ± 38 (7)	201 ± 41 (7)	126 ± 31 (4)	121 ± 33 (4)

EGF was a consequence of the increased level of stimulation obtained in the presence of IBMX. However, when a range of aminopyrine accumulation ratios was obtained by changing the concentration of histamine in the presence of 0.1mM-IBMX, there was no inhibition by EGF at any level of aminopyrine accumulation (experiments by G. P. Shaw; Fig. 2, Shaw et al., 1987). However IBMX is also an adenosine receptor antagonist although any involvement of adenosine in the above experiments is made less likely by the apparent absence of A_1 adenosine receptors from rat parietal cells (Puurunen et al., 1987b). The most straightforward explanation of these results is that if cyclic AMP-phosphodiesterase activity is inhibited, then EGF is unable to inhibit histamine-stimulated aminopyrine accumulation.

EGF was ineffective at inhibiting aminopyrine accumulation stimulated by carbachol (0.1mM) or by dbcAMP (1mM) (lines 4 and 5, Table 5.4). The absence of an effect of EGF against aminopyrine accumulation stimulated by 1mM-dbcAMP was not a consequence of the high aminopyrine accumulation ratios obtained with this agent. Thus EGF exhibited no antisecretory effect when parietal cells were stimulated by 30μM-dbcAMP generating an aminopyrine accumulation ratio of 5.5 ± 1.7 (experiments by G. P. Shaw; Table 2, Shaw et al., 1987). The lack of an effect of EGF on basal aminopyrine accumulation and on that stimulated by carbachol or by dbcAMP contrasts with the data obtained using rabbit gastric glands by Konturek et al. (1984) and by Dembiński et al. (1986). However, there is evidence that rabbit gastric gland preparations contain endogenous histamine (Chew, 1983a), and therefore the inhibition of carbachol-stimulated aminopyrine accumulation by EGF may have been directed against the potentiating interaction between endogenous histamine and carbachol. Similarly it is possible that in rabbit gastric glands, EGF inhibited a component of basal aminopyrine accumulation that was stimulated by

endogenous histamine. It is however unlikely that endogenous histamine is present in the isolated cell preparations used in this study since inclusion of the H_2-receptor antagonist cimetidine did not significantly affect the basal aminopyrine accumulation ratio (no cimetidine, 2.1 $\pm$ 0.2; 10μM- cimetidine, 2.3- $\pm$ 0.0, analysis of data from 4 cell-batches by paired t-test).

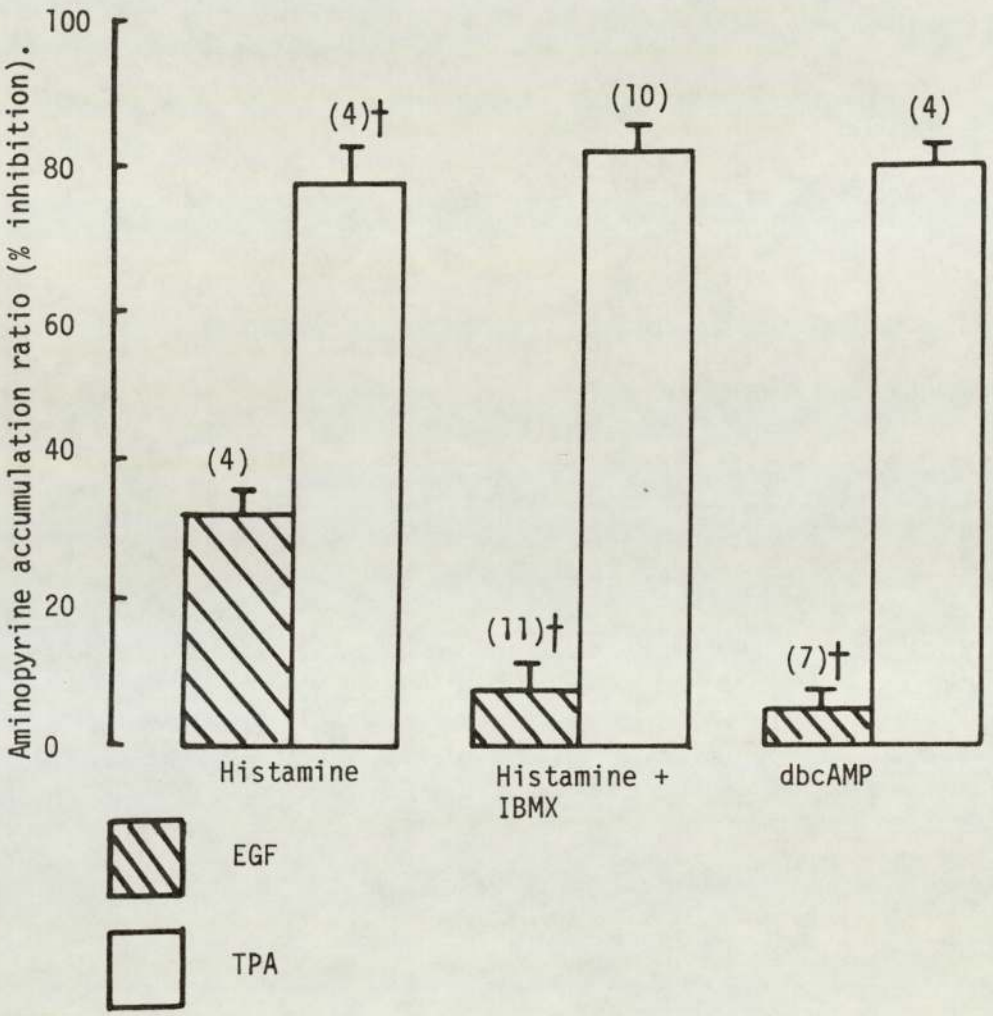
The data presented in Table 5.4 therefore demonstrate a pattern for the inhibition of secretory activity by EGF in isolated parietal cell preparations. This is similar to that reported for isolated canine parietal cells (Chen et al., 1984). In conclusion, the inhibition of aminopyrine accumulation by EGF in isolated parietal cell preparations appeared to be specific to histamine. The lack of any effect of EGF in cells stimulated by lmM-dbcAMP suggests that EGF inhibited histamine-stimulated aminopyrine accumulation by a mechanism affecting either the generation or hydrolysis of cyclic AMP within the parietal cell.

5.3.2 A comparison of the inhibitory effects of EGF and TPA on aminopyrine accumulation in isolated parietal cells stimulated with histamine, histamine + IBMX and dbcAMP.

In the light of experimental data obtained using A431 cells and hepatocytes (5.1.2.1) the possibility that the antisecretory effect of EGF may be mediated via the activation of protein kinase C deserved consideration. This was achieved indirectly by comparing the inhibitory actions of near-maximally effective concentrations of TPA and EGF (Fig. 5.3). Although both agents inhibited histamine-stimulated aminopyrine accumulation, TPA was significantly more effective than EGF ($p < 0.05$, unpaired

Figure 5.3

A comparison of the inhibitory actions of near-maximally effective concentrations of EGF and TPA on aminopyrine accumulation stimulated by histamine (0.5mM), histamine (0.5mM) plus IBMX (0.1mM), and dbcAMP (1.0mM).



The effect of TPA and of EGF is expressed by normalising the data to % inhibition, with the number of cell-batches in parenthesis. The concentration of EGF used was 200nM and TPA was present at 100nM, except for the experiments using histamine alone where it was present at 32nM.
The experiments performed by G.P.Shaw are indicated (†).

t-test). In cells stimulated by histamine plus IBMX and by dbcAMP, only TPA was an effective inhibitor. Although the control aminopyrine accumulation ratios were different for the experiments compared in Fig. 5.3, it has been previously demonstrated that the % inhibition of aminopyrine accumulation effected by EGF was not related to the extent of the histamine-stimulated aminopyrine accumulation ratio (Shaw et al., 1987), and that the concentration of TPA required for half-maximal effect was not related to the aminopyrine accumulation stimulated by histamine plus IBMX (Fig. 3.7). Since a comparison of the ability of these agents to inhibit aminopyrine accumulation under various conditions revealed a difference between the pattern of action of TPA and EGF, it is unlikely that both agents act via the same mechanism. Therefore although protein kinase C may have a role in the action of EGF in A431 cells (Sahai et al., 1982), it does not appear to mediate the antisecretory effect of EGF in isolated parietal cell preparations.

5.3.3 Time-course of the effect of 200nM-EGF on the cyclic AMP content and aminopyrine accumulation ratio in parietal cell-enriched preparations stimulated by 0.5mM-histamine.

The nature of the inhibition of aminopyrine accumulation by EGF described in 5.3.1 suggested that EGF was acting at a site close to the production or hydrolysis of cyclic AMP; this possibility therefore deserved further investigation. Since EGF was equally effective as an inhibitor of histamine-stimulated aminopyrine accumulation whether present at a concentration of 200nM or 1μM (Shaw et al., 1987) in the interest of economy, EGF was used at a concentration of 200nM for these experiments. The untransformed data are

presented in Table 5.5 (A + B) and in order to facilitate a comparison between the different incubation conditions and to reduce the variation between the samples, the data have also been normalised by expressing each value as a % of the 30 min value obtained in the presence of histamine alone (Fig. 5.4).

5.3.3.1 Cyclic AMP Data.

The basal cyclic AMP content was similar after 5 or 60 minutes of incubation, thereby resembling data obtained by other workers using preparations of cells isolated from the rat stomach (Sonnenberg et al., 1978; Puurunen et al., 1987a). Histamine elevated the cyclic AMP content of the cell suspension above basal levels, and it is generally accepted that cyclic AMP is the second messenger for histamine within the parietal cell (see 1.2.2.1). Although the preparations did not consist entirely of parietal cells, it is likely that the elevation of cyclic AMP occurred within the parietal cell since it has been previously demonstrated that there is a close correlation between histamine-stimulated cyclic AMP production and parietal cell content (Sonnenberg et al., 1978, Wollin et al., 1979). The stimulation obtained with histamine in this study with rat parietal cells was somewhat lower than that observed in cells from other species (Table 5.6). However other workers using parietal cells isolated from the rat included a phosphodiesterase inhibitor such as IBMX (Schepp and Ruoff, 1984) or rolipram (Puurunen et al., 1987a) in the medium in order to obtain a significant stimulation of cyclic AMP levels by histamine.

Table 5.5

Effect of incubation time on the action of 200nM-EGF on aminopyrine accumulation and cyclic AMP content of the cell suspension in cells stimulated with 0.5mM-histamine.

A. Cyclic AMP content of cell suspension (pmol/10^6 cells).

Incubation time (min)	Incubation conditions			Number of cell batches
	Basal (unstimulated)	Histamine	Histamine + EGF	
5	1.61 ± 0.38	2.32 ± 0.59	1.90 ± 0.45	4
30	N.D.	2.12 ± 0.45	1.73 ± 0.44	4
60	1.90 ± 0.45	2.58 ± 0.55	2.27 ± 0.57	3

B. Aminopyrine accumulation ratio.

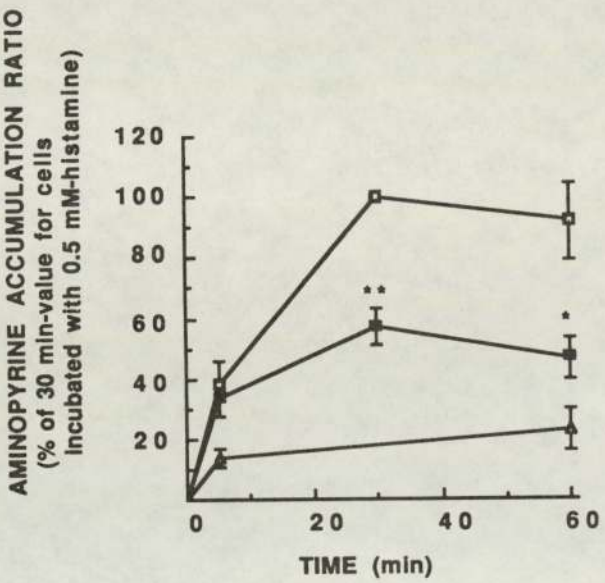
Incubation time (min)	Incubation Conditions			Number of cell batches
	Basal (unstimulated)	Histamine	Histamine + EGF	
5	1.8 ± 0.8	5.2 ± 2.4	4.5 ± 2.0	3
30	N.D.	17.3 ± 5.1	10.2 ± 3.9	4
60	2.1 ± 0.5	13.0 ± 4.9	6.7 ± 2.8	3

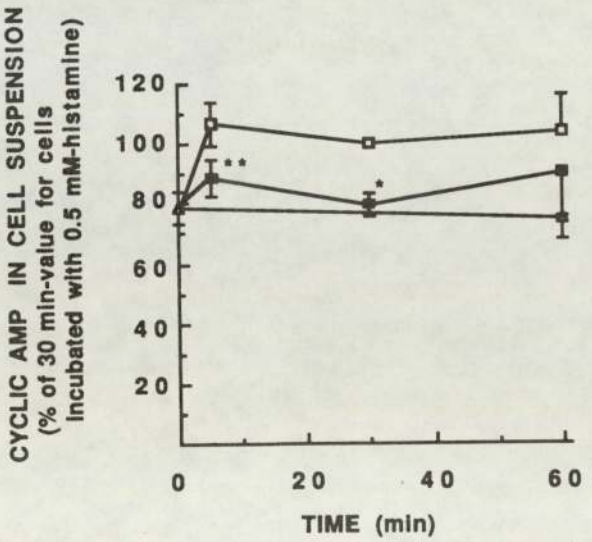
N.D. = not determined.

Values are expressed as means ± S.E.M. from cell-batches containing 77 - 81% parietal cells. Aliquots of cells analysed for aminopyrine accumulation and cyclic AMP content were obtained from the same incubation vials.

Figure 5.4

Effect of incubation period on the action of 200nM-EGF on aminopyrine accumulation and the cyclic AMP content of a cell-suspension stimulated by 0.5mM-histamine.





Data from Table 5.5 have been normalised by expressing them as a % of the value obtained at 30 min in the presence of 0.5mM-histamine (aminopyrine accumulation ratio = 17.3 $\pm$ 5.1; cyclic AMP content = 2.12 $\pm$ 0.45 p mol / 10^6 cells). Values are expressed as means $\pm$ S.E.M. □, 0.5mM-histamine; ■, 0.5mM-histamine plus 200nM-EGF; Δ, basal (unstimulated).

**, $p<0.01$; *, $p<0.05$ for a significant inhibitory effect of 200nM-EGF on histamine-stimulated values (paired t-test). The 5 min basal cyclic AMP value has been assumed to represent '0-time' value.

Table 5.6

The cyclic AMP content of some parietal cell-enriched preparations which had been stimulated by histamine alone.

Concentration of histamine (mM)	Cyclic AMP content (pmol/10^6 cells) Basal	Stimulated	Incubation period (min)	Species	Reference
0.01	0.2	1.1	20	Guinea Pig	Batzri & Gardner, 1978
0.01	1.67	2.80	5	Dog	Soll & Wollin, 1979
0.71	13.9	48.1	20	Rabbit	Takahashi *et al.*, 1983
0.1	~ 3	~ 5	5	Dog	Park *et al.*, 1987
0.5	1.61 $\pm$ 0.38	2.32 $\pm$ 0.59 (n = 4)	5	Rat	This work

The elevation of cyclic AMP above basal levels by 0.5mM-histamine was relatively constant after 5, 30 and 60 minutes of incubation which suggests that the rate of production of cyclic AMP was balanced by the rate of destruction by cyclic AMP phosphodiesterases. However, even when theophylline (10mM) a phosphodiesterase inhibitor, was present with rat isolated parietal cells stimulated by histamine (0.1mM), a similar flat time-course was obtained by Sonnenberg et al. (1978). This suggests that there may be an auto-regulatory mechanism which operates in addition to phosphodiesterases to maintain a constant cyclic AMP level over a given time period in isolated parietal cells.

Not all of the cyclic AMP was retained inside the cells, after a 30 minute incubation with 0.5mM-histamine 0.34 pmol cyclic AMP/10^6 cells was present in the supernatant which represented 28% of the total cell suspension cyclic AMP when samples from a single experiment were analysed (the supernatant value refers to the measurement of cyclic AMP in the volume associated with 10^6 cells). This value is slightly higher that the 22% recorded in previous experiments (3.3.3.1).

200nM-EGF had no effect on the cyclic AMP content of the cell suspension incubated under basal conditions in the absence of histamine for 30 minutes (cyclic AMP content, pmol/10^6 cells; no EGF, 1.16 $\pm$ 0.25; 200nM-EGF, 1.07 $\pm$ 0.21, comparison of 6 cell-batches by paired t-test). However EGF significantly reduced the histamine-stimulated cyclic AMP content of the cell suspension after 5 and 30 minutes of incubation when the normalised data shown in Fig. 5.4 were compared by a paired t-test ($p < 0.01$ and $p < 0.05$ respectively). After a 60 minute incubation period, EGF did not significantly inhibit the histamine-stimulated elevation of cyclic AMP content. The reason for this is unknown, although it may reflect the onset of downregulation of the EGF receptor, a phenomenon

where continued incubation with EGF results in a decrease in the binding-capacity of cells for EGF. This has been demonstrated by Carpenter and Cohen (1976) using human foreskin fibroblasts incubated with human EGF.

5.3.3.2 Aminopyrine accumulation data.

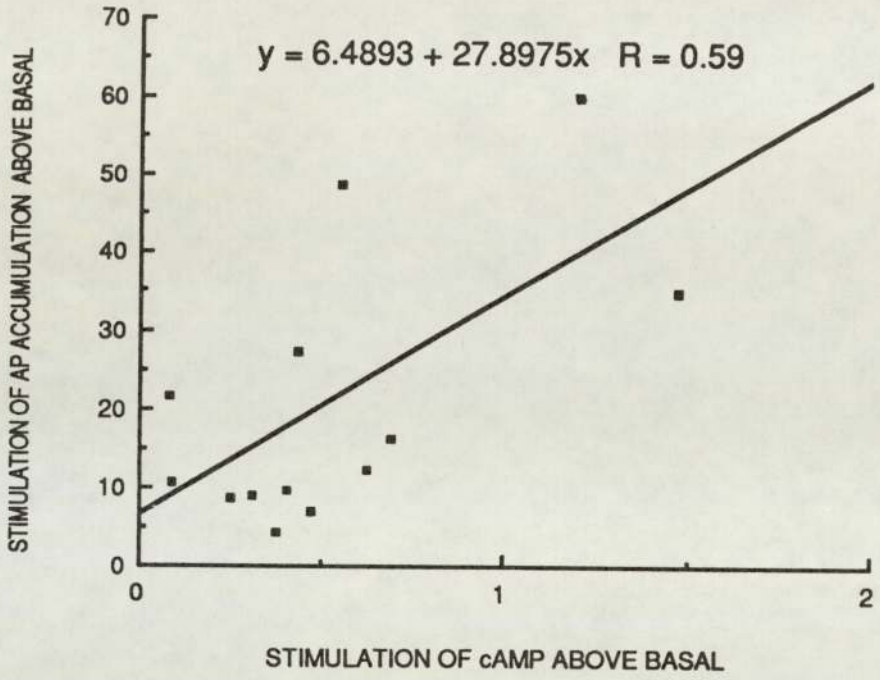
The histamine-stimulated aminopyrine accumulation ratio was similar after 30 or 60 minutes of incubation (Fig. 5.4), similar time-courses of aminopyrine accumulation were obtained using preparations of parietal cells isolated from the dog (Soll, 1980a). This "plateau" probably represents a steady-state whereby a balance has been achieved between the rate of translocation of protons from the cell into the secretory canaliculi and the rate of leakage of protons and/or aminopyrine from the secretory canaliculi to the medium. The magnitude of the elevation of the aminopyrine accumulation ratio above basal levels by 0.5mM-histamine in this study was similar to that obtained by 0.1mM-histamine in canine isolated cell preparations containing 70% parietal cells where the aminopyrine accumulation ratio was increased from 3.0 ± 0.1 to 12.3 ± 1.1 (Park et al., 1987).

After a 5 minute incubation, 200nM-EGF had no significant effect on the histamine-stimulated aminopyrine accumulation ratio although after 30 and 60 minutes of incubation, an inhibitory effect of EGF was apparent ($p < 0.01$, $p < 0.05$ respectively) when the normalised data presented in Fig. 5.4 were compared by a paired t-test. After 30 minutes of incubation, 200nM-EGF had no significant effect on the basal aminopyrine accumulation ratio (aminopyrine accumulation ratio; no EGF, 2.5 ± 0.9; 200nM-EGF, 2.3 ± 0.5, comparison of data from 6 cell-batches by paired t-test).

From the data obtained it can be suggested, although not proven, that the reduction in histamine-stimulated aminopyrine accumulation by 200nM-EGF after 30 minutes of incubation was mediated via a decrease in cellular cyclic AMP levels. To see why the absence of a significant effect of EGF on histamine-stimulated aminopyrine accumulation after 5 minutes cannot be used as an argument against this proposal, it is necessary to consider the nature of these measurements and to discuss the relationship between them. Thus the cyclic AMP content is a more immediate indicator of cellular activity representing the situation at a given time whilst the aminopyrine accumulation ratio reflects the previous history of the cell. This is demonstrated by the observation that the increase in histamine-stimulated cyclic AMP content reached a plateau before the aminopyrine accumulation ratio. A similar situation was observed in preparations of canine parietal cells stimulated with histamine plus IBMX where 85% of the maximal increase in cyclic AMP was apparent after 5 minutes [Fig. 2, Wollin et al., 1979] whilst the aminopyrine accumulation ratio plateaued after 20-30 minutes of incubation [Fig. 2, Soll 1980a]. Therefore any action of EGF on cyclic AMP content would probably not be reflected by a change in aminopyrine accumulation ratio when measurements were made after only 5 minutes since a steady-state may not have been achieved for aminopyrine accumulation. However, a significant relationship between these two parameters was clearly apparent after a 30 minute incubation of isolated parietal cell preparations with 0.5mM-histamine (Fig. 5.5). Soll and Wollin (1979) also obtained a similar correlation when using preparations of canine parietal cells which were sub-maximally stimulated with 10μM-histamine and 10μM-IBMX. Therefore it is not unreasonable to assume that a reduction in cyclic AMP content of parietal cells would produce a fall in aminopyrine accumulation after 30 minutes of incubation. A 30 minute incubation period was

Figure 5.5

Relationship between the absolute stimulation of the aminopyrine accumulation ratio and the cyclic AMP content of the cell suspension (pmol/10^6 cells) above basal levels by 0.5mM-histamine.



Each point represents the mean of 2 samples obtained from a single experiment, samples for the measurements of aminopyrine accumulation and cyclic AMP content were obtained from the same cell-batch containing 82.1 $\pm$ 1.4% parietal cells (n=13 cell-batches). The basal values were: aminopyrine accumulation ratio, 2.7 $\pm$ 0.3; cyclic AMP content, 1.3 $\pm$ 0.2 pmol/10^6 cells. The correlation co-efficient = 0.592 (11 d.o.f) and the relationship is therefore significant ($p<0.05$).

therefore chosen to investigate the dose-dependency of the effect of EGF on the cyclic AMP content and aminopyrine accumulation ratio in preparations of parietal cells stimulated by 0.5mM-histamine.

5.3.4 Effect of EGF concentration on the cyclic AMP content and aminopyrine accumulation ratio in parietal cell-enriched preparations stimulated by 0.5mM-histamine.

Results showing the effect of the concentration of EGF on the cyclic AMP content and the aminopyrine accumulation ratio in histamine-stimulated parietal cell preparations are presented in Table 5.7 and Table 5.8, and in a normalised format in Fig. 5.6. Thus the action of EGF was found to be dose-dependent, with half-maximally effective concentrations of 3.9nM and 3.0nM for the inhibition of histamine-stimulated cyclic AMP content and aminopyrine accumulation ratio respectively. The similarity between these values adds further support to the proposal discussed earlier (5.3.3) that the reduction in histamine-stimulated aminopyrine accumulation by EGF was probably mediated by a reduction of the cellular cyclic AMP content. An interesting question which remains to be answered is why 200nM-EGF inhibited the histamine-stimulated increase of cyclic AMP content by 65.6 $\pm$ 9.4% whilst the inhibition of histamine-stimulated aminopyrine accumulation was 42.3 $\pm$ 2.9%. A possible explanation is that the changes in measured total cyclic AMP content in response to EGF were over-estimates of the changes in cyclic AMP in the immediate locality of the cyclic AMP-dependent protein kinase.

Table 5.7

Effect of the concentration of EGF on the stimulation of aminopyrine accumulation and cyclic AMP content of the cell suspension by histamine (0.5mM) in parietal cell-enriched fractions.

Concentration of EGF (nM)	Aminopyrine accumulation ratio	Cell suspension cyclic AMP content (pmol/10^6 cells)
0	11.7 ± 0.8	1.15 ± 0.27
0.2	11.4 ± 1.1	1.13 ± 0.26
2.0	10.6 ± 0.6	1.09 ± 0.24
20.0	8.4 ± 0.6	0.94 ± 0.20
200.0	7.9 ± 0.6	0.96 ± 0.23

Results from 4 batches of cells containing 81.8 ± 0.8% parietal cells, are expressed as means ± S.E.M. All incubations were for 30 min. The basal (unstimulated) values were 2.5 ± 0.9 for the aminopyrine accumulation ratio and 0.83 ± 0.19 pmol cyclic AMP/10^6 cells.

Table 5.8

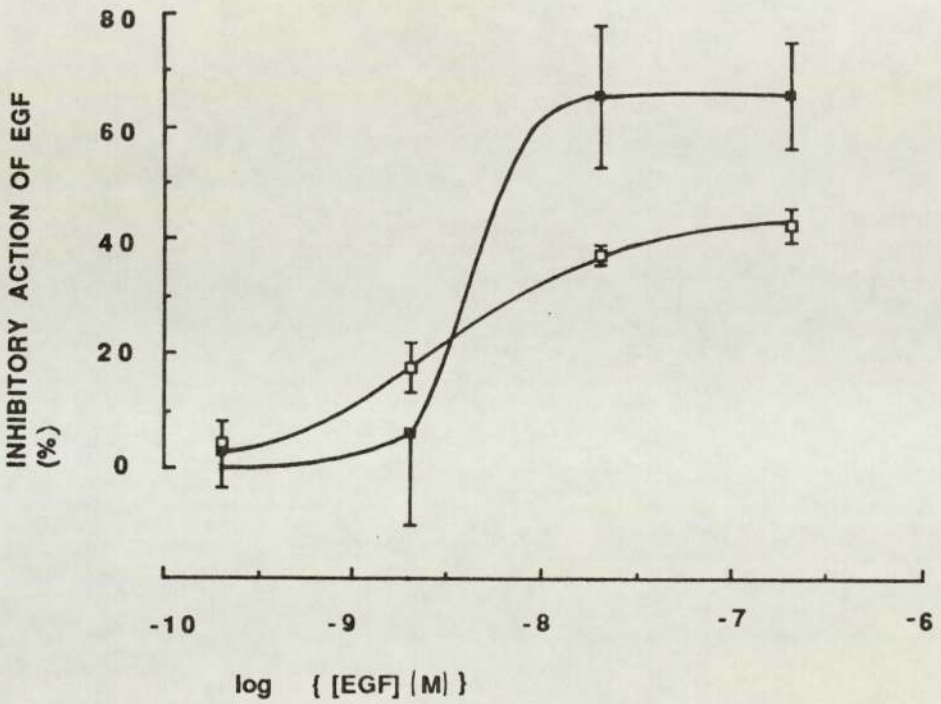
Effect of the concentration of EGF on the stimulation of aminopyrine accumulation and of the cyclic AMP content of the cell suspension above basal levels by histamine (0.5mM) in parietal cell-enriched fractions.

Concentration of EGF (nM)	Aminopyrine accumulation ratio	Cell suspension cyclic AMP content (pmol/10^6 cells)
0	9.0 $\pm$ 0.8	0.32 $\pm$ 0.08
0.2	8.7 $\pm$ 1.0	0.30 $\pm$ 0.08
2.0	7.4 $\pm$ 0.5*	0.27 $\pm$ 0.06
20.0	5.7 $\pm$ 0.5*	0.11 $\pm$ 0.06**
200.0	5.2 $\pm$ 0.6*	0.13 $\pm$ 0.05*

The data from Table 5.7 have been corrected for basal values. The inhibitory effect of EGF on the stimulation of aminopyrine accumulation and cyclic AMP content has been analysed by ANOVAR. Dunnett's Test was used to establish whether results were different from control values in the absence of EGF (**, $p < 0.01$; *, $p < 0.05$).

Figure 5.6

Effect of the concentration of EGF on the inhibition of aminopyrine accumulation (□) and of the cyclic AMP content (■) of the cell suspension in parietal cell-enriched preparations stimulated with 0.5mM-histamine.



Data from Table 5.8 have been normalised to % inhibition by EGF. The position of the lines has been calculated by using the computer programme FIT (Barlow,1983).

5.3.5 The effect of EGF on the cyclic AMP content of parietal cell-enriched preparations stimulated by histamine in the presence of IBMX.

EGF was previously shown to be ineffective at inhibiting aminopyrine accumulation stimulated by 0.5mM-histamine plus 0.1mM-IBMX (Table 5.4). The effect of IBMX on the inhibition of histamine-stimulated cyclic AMP content by EGF was therefore examined, the results are presented in Table 5.9 and in Fig. 5.7. At any concentration of histamine tested (0.5 - 500μM), EGF did not significantly reduce the cyclic AMP content of the cell suspension in the presence of a constant concentration of 0.1mM-IBMX (data compared by a paired t-test). The lack of an effect of EGF was not due to a substandard batch since EGF from the same original stock was an effective inhibitor in other experiments (e.g. data in Table 5.8).

The inclusion of IBMX with 0.5mM-histamine produced significantly higher cyclic AMP levels (3.03 ± 0.40 pmol cyclic AMP/10^6 cells, n = 5 cell-batches) than those obtained with 0.5mM-histamine alone (1.57 ± 0.21 pmol cyclic AMP/10^6 cells, n = 9 cell-batches) when the data were compared by an unpaired t-test ($p < 0.01$). A similar effect is reported by other workers (e.g. Soll and Wollin, 1979) and is probably because being a phosphodiesterase inhibitor, IBMX enhanced the histamine-induced elevation of cyclic AMP by reducing the rate at which cyclic AMP was degraded. However the elevation of cyclic AMP levels was not responsible for the lack of an effect of EGF in the presence of IBMX. This is because EGF was effective in the presence of histamine alone at cyclic AMP levels of 2.12 ± 0.45 (Table 5.5, 30 min value) and 1.15 ± 0.27 (Table 5.7) which are higher than some of the values obtained using low concentrations of histamine in the presence of IBMX (Table 5.9). These results therefore suggest that IBMX is blocking the inhibitory effect of EGF on the cyclic AMP content of parietal cell preparations incubated with 0.5mM-histamine.

Table 5.9

Effect of 200nM-EGF on the cyclic AMP content of the cell suspension in preparations of parietal cells stimulated by histamine (0.5mM) + IBMX (0.1mM).

Concentration of histamine (μM)	Number of cell batches	Cyclic AMP content of cell cell suspension pmol/10^6 cells	
		Control	+ EGF
0.5	4	1.06 ± 0.18	1.03 ± 0.19
1.0	4	1.38 ± 0.23	1.35 ± 0.23
3.0	5	1.62 ± 0.04	1.51 ± 0.13
20.0	5	1.82 ± 0.09	1.79 ± 0.13
500.0	5	3.03 ± 0.40	2.94 ± 0.32

All incubations were for 30 min. Results are presented as means ± S.E.M. from cell-batches containing 79 - 82% parietal cells. 200nM-EGF had no significant effect on basal cyclic AMP content when values were compared by paired t-test. (Values for basal cyclic AMP content, pmol/10^6 cells: control, 0.77 ± 0.09 (6); 200nM-EGF, 0.91 ± 0.19 (6)).

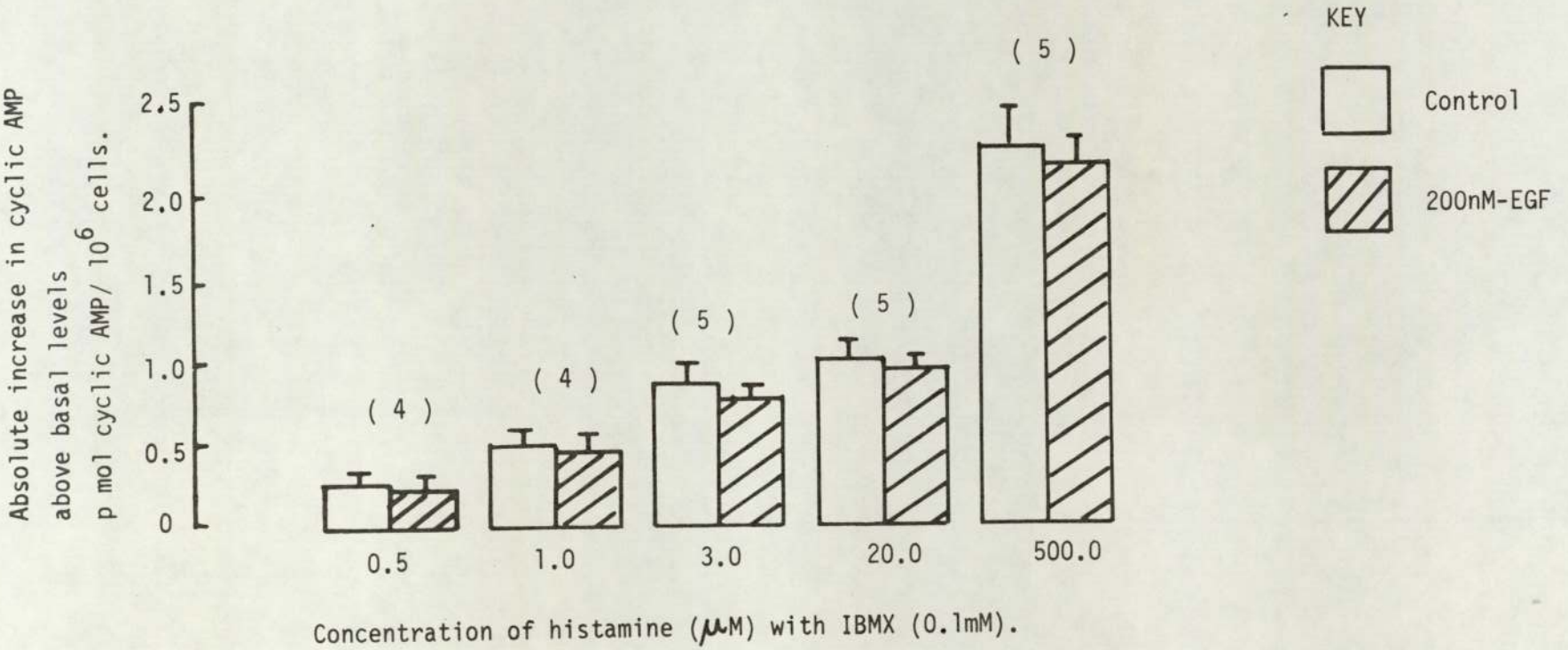
There was no significant effect of EGF at any concentration of histamine tested in the presence of 0.1mM IBMX (data compared by paired t-test).

Figure 5.7

Effect of 200nM-EGF on the stimulation of the cyclic AMP content of the cell-suspension above basal by histamine (0.5mM) plus IBMX (0.1mM).

Results from Table 5.9 have been recalculated as the absolute increase in cyclic AMP content above basal (basal values, cyclic AMP p mol/10^6 cells; no EGF, 0.77 $\pm$ 0.09; 200nM-EGF, 0.91 $\pm$ 0.19) and are expressed as means $\pm$ S.E.M.

There was no significant effect of EGF under any condition tested (paired t-test).



5.4 GENERAL DISCUSSION.

The results obtained suggest that EGF inhibits histamine-stimulated aminopyrine accumulation by effecting a reduction in the cyclic AMP content of parietal cells. The observation that the inclusion of IBMX abolishes the inhibitory effect of EGF suggests that the mechanism by which EGF acts may comprise the activation of a cyclic AMP-phosphodiesterase. In hepatocytes, insulin activates a "dense vesicle" phosphodiesterase which is blocked by IBMX (Heyworth et al., 1983). This finding is of particular interest considering that there are a number of similarities between insulin and EGF (Table 5.10). Further experiments including measuring the effect of EGF on cyclic AMP-phoshodiesterase activity are required in order to investigate this potential mechanism of action.

Although this study has implicated that the anti-secretory action of EGF in rat isolated parietal cell preparations is mediated by a reduction in cellular cyclic AMP levels, this is not a typical action of the growth factor. Thus a literature search revealed only hepatocytes (Table 5.10) and a fibroblastic cell-line (Anderson et al., 1979) in which a short term incubation with EGF reduced cellular cyclic AMP levels.

Table 5.10

A summary of some properties which are common to both EGF and insulin.

Property	Reference
Stimulation of DNA synthesis in fibroblasts	Hollenberg and Cuatrecasas, 1973
Receptors possess intrinsic tyrosine kinase activity	Ramachandran and Ullrich, 1987 review
Stimulation of glycolysis in 3T3 cells	Diamond *et al.*, 1978
Activation of glycogen synthetase and attenuation of glucagon-stimulated cyclic AMP in hepatocytes	Bosch *et al.*, 1986

5.5 SUMMARY.

1. EGF inhibited aminopyrine accumulation stimulated by histamine with an IC_{50} of 3.0nM. EGF was ineffective at inhibiting basal aminopyrine accumulation or that induced by dibutyryl cyclic AMP or carbachol.

2. EGF attenuated the histamine-induced elevation of the cyclic AMP content of a suspension containing 80-83% parietal cells with an IC_{50} of 3.9nM.

3. Inclusion of the phosphodiesterase inhibitor IBMX abolished the inhibitory action of EGF on histamine-stimulated aminopyrine accumulation and cyclic AMP content. This effect was apparently not related to the enhancement in the response to histamine induced by IBMX.

Chapter Six

THE POTENTIAL ROLE OF PROSTAGLANDINS IN THE ACTION OF EPIDERMAL GROWTH FACTOR IN ISOLATED PARIETAL CELLS.

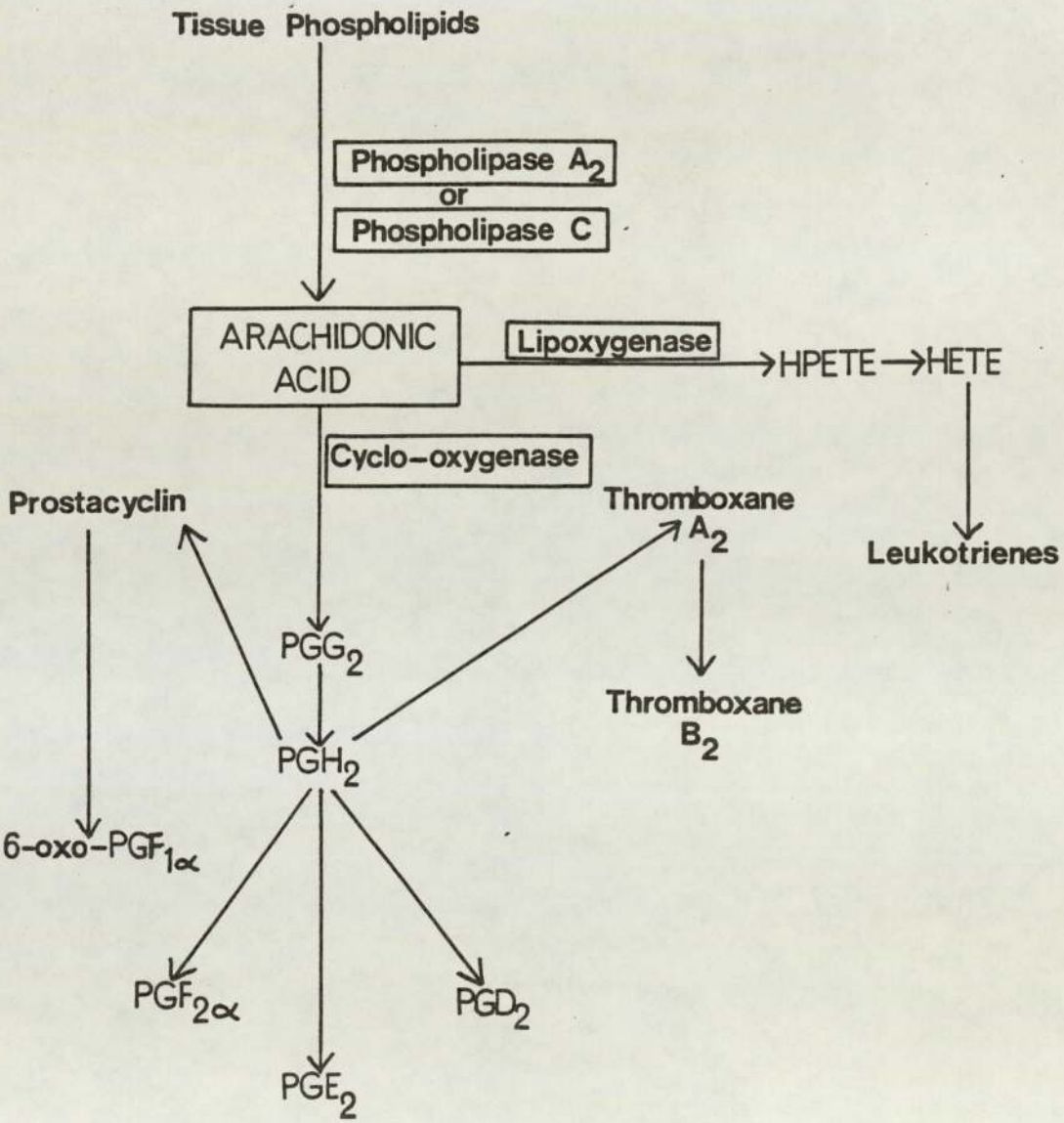
6.1 INTRODUCTION.

6.1.1 Eicosanoids.

The term eicosanoid was coined by Corey et al. (1980) and refers to the products of cyclo-oxygenase and lipoxygenase activity (Fig. 6.1). A key product in the synthesis of eicosanoids is arachidonic acid, which in mammalian cells is often found in membrane phospholipids, generally esterified in the 2-acyl position (Irvine, 1982). In order to serve as a substrate for eicosanoid synthesis arachidonic acid has to be released from the membrane phospholipids, which can be achieved by the action of phospholipase A_2 or phospholipase C. Hydrolysis of phospholipids yielding free arachidonic acid and a lysophospholipid can occur as a result of phospholipase A_2 activity. There is evidence from some cell-types that the activity of this enzyme is regulated via G-proteins which couple it to membrane receptors e.g. FRTL5, a rat thyroid cell-line (Burch et al., 1986). Although phospholipase A_2 activity has been observed in rat gastric mucosa (Tojo et al., 1988) it has not yet been demonstrated whether this enzyme contributes to the release of eicosanoids by this tissue. Alternatively, arachidonic acid can be generated from phosphatidylinositol phosphates by the action of phospholipase C (Majerus et al., 1986). Phospholipase C activity is also regulated via G-proteins coupled to membrane receptors (see Fig. 3.3). An example of how this pathway leads to arachidonic acid production is demonstrated by the stimulation of platelets with thrombin which results in the transient accumulation of diacylglycerol and inositol 1,4,5-trisphosphate (Prescott and Majerus, 1983). Diacylglycerol can then serve as a substrate for diacylglycerol lipase and monoacylglycerol lipase yielding free arachidonic acid.

Figure 6.1

A schematic summary of the biosynthesis of eicosanoids from arachidonic acid.



Key

PG = Prostaglandin

HPETE = Hydroperoxy eicosatetraenoic acid

HETE = Hydroxy eicosatetraenoic acid

To date, the exact mechanisms controlling prostaglandin production in the stomach are unknown, although it has been shown that stimulation of the muscarinic cholinergic receptor by carbachol enhances the release of PGE_2 from isolated canine parietal cell preparations by a calcium-dependent mechanism (Payne and Gerber, 1987).

6.1.2 Biological actions of eicosanoids.

The individual eicosanoids have widely varying biological effects which will now be briefly considered with particular reference to the gastric mucosa where relevant.

6.1.2.1 Leukotrienes.

Leukotrienes are synthesised via the 5'- lipoxygenase pathway and LTC_4 , LTD_4 and LTE_4 have been identified as the constituents of the slow-reacting substance of anaphylaxis (reviewed by Needleman et al., 1986). Leukotriene C_4 is generated by the gastric mucosa, where it is a powerful vasoconstrictor of the gastric microcirculation (Whittle et al., 1985) and it is also an inhibitor of histamine-induced acid secretion in vivo and in vitro (Konturek et al., 1987).

6.1.2.2 Prostanoids, excluding PGE_2 .

The term prostanoid refers to the products of cyclo-oxygenase activity, and their respective roles as regulators of gastrointestinal function has been recently reviewed by Whittle and Vane (1987). The endoperoxides PGG_2 and PGH_2 are unstable intermediates although they do exhibit biological activity and have been demonstrated to contract smooth muscle, including that from the gastrointestinal tract *in vitro* (Moncada and Vane, 1979). Thromboxane A_2 is produced by platelets and stimulates their aggregation (Hamberg *et al.*, 1975). Thromboxane A_2 has been shown to be a potent gastric vasoconstrictor in the dog and is implicated in the formation of gastric ulcers since it potentiates the formation of lesions in the gastric mucosa in the presence of acid and taurocholate (Whittle *et al.*, 1981). Prostacyclin exerts a potent vasodilator effect and is also an inhibitor of platelet aggregation (Moncada *et al.*, 1976). Prostacyclin has been shown to be effective at inhibiting histamine-stimulated acid secretion *in vitro* and *in vivo*, and when given subcutaneously to rats, it inhibited the formation of gastric erosions induced by the cyclo-oxygenase inhibitor indomethacin (Whittle *et al.*, 1978). Thromboxane A_2 and prostacyclin are both unstable compounds (approximate half-life in aqueous solution at 37°C: thromboxane A_2 , 30 sec; prostacyclin, 3 min) and decompose to form thromboxane B_2 and 6-oxo-$PGF_{1\alpha}$ respectively which have little biological activity (Hamberg *et al.*, 1975, Whittle *et al.*, 1978). In preparations of isolated canine parietal cells, PGD_2 is reported to have no effect on histamine-stimulated aminopyrine accumulation whilst the antisecretory effect of $PGF_{2\alpha}$ is apparently variable (Skoglund *et al.*, 1982).

6.1.2.3 Prostaglandin E_2 (PGE_2).

6.1.2.3.1 Antisecretory actions of PGE_2 .

PGE_2 has been demonstrated to inhibit histamine - and pentagastrin-stimulated acid secretion when infused intravenously to the anaesthetised rat (Main and Whittle, 1973). Exogenous PGE_2 inhibits aminopyrine accumulation in preparations of isolated parietal cells which have been stimulated with histamine plus IBMX (Soll, 1980b; Atwell and Hanson, 1988) but is without effect against aminopyrine accumulation in cells stimulated with carbachol, gastrin or dbcAMP (Soll, 1980b). PGE_2 (0.1nM-1μM) inhibits the histamine plus IBMX-stimulated increase in cyclic AMP content (Soll, 1980b) by a mechanism which probably involves inhibition of adenylate cyclase present on the parietal cell (Major and Scholes, 1978). Since pertussis toxin blocks the inhibitory effect of PGE_2 on histamine-stimulated aminopyrine accumulation (Atwell and Hanson, 1988) G-proteins may be involved in the anti-secretory action of PGE_2 . When present at concentrations exceeding 1μM, PGE_2 increases the cyclic AMP content of isolated gastric cell preparations probably by activation of adenylate cyclase present on non-parietal cells (Wollin et al., 1979).

6.1.2.3.2 Other actions of PGE_2 in the gastric mucosa.

PGE_2 given orally or subcutaneously to rats inhibited the formation of macroscopic gastric lesions induced by necrotising agents (Robert et al., 1979). This effect was termed "cytoprotection" and occurred at doses of prostaglandin which were ineffective at inhibiting acid secretion. However the problem of only using macroscopic analysis to demonstrate "cytoprotection" is highlighted by the detailed study of Whittle and Steel (1985). Thus when rats were pretreated with doses of the PGE_2 analogue 16,16-dimethyl prostaglandin E_2 which prevented the formation of ethanol-induced macroscopic lesions, histologic studies indicated that there was no protection of the surface epithelial cells and measurements of enzyme release indicated that cellular damage was still evident. Tarnawski et al. (1985) found that although 16,16-dimethyl prostaglandin E_2 failed to prevent any initial damage to the surface epithelial cells induced by ethanol, restoration of this layer was apparent 3 hours after ethanol instillation which was due to a protecting effect of the analogue on the mucosal proliferative zone, enabling subsequent migration of cells to the damaged region. Thus it has been suggested that in this instance, the term cytoprotection would be more correctly applied to describing the protective effect of prostaglandins on the mucosal proliferative zone cells (Tarnawski et al., 1985).

Topical application of 16,16-dimethyl prostaglandin PGE_2 to the Heidenhain pouch dog has been shown to stimulate bicarbonate and mucus secretion by the gastric mucosa (Kauffman et al., 1980). It has been suggested (Whittle and Vane 1987) that the stimulation of mucus, bicarbonate and fluid secretion by prostanoids may assist

the process of epithelial restitution which occurs after damage to the gastric mucosa (Morris and Harding, 1984).

Prostaglandin E_2 has been shown to increase mucosal blood flow (as measured by the clearance of [^{14}C]-aniline) in the rat (Main and Whittle, 1973) thereby indicating a potential physiological role for endogenous prostaglandins in the local regulation of mucosal blood flow (Main and Whittle, 1975). Experiments performed by Wallace et al. (1982) showed that pre-treatment with PGE_2 reduced the formation of haemorrhagic lesions induced by infusion of ethanol to the rat stomach, and a consequence of PGE_2 pre-treatment was a reduction in mucosal vasocongestion. Therefore the vasodilating effects of prostaglandins may be of importance in maintaining mucosal circulation under damaging conditions and assisting the removal/dilution of a noxious agent.

6.1.2 The relationship between EGF action and prostaglandin production.

6.1.2.1 Cell-types excluding those in the gastric mucosa.

EGF stimulates the production of prostaglandins from a variety of cell-types (Table 6.1). A potential scheme by which EGF could induce prostaglandin production is shown in Fig. 6.2. Thus in A431 cells, the 35kDa protein which is phosphorylated in response to EGF has been shown to be lipocortin I (Pepinsky and Sinclair, 1986). It has been demonstrated that once phosphorylated, lipocortin is no longer an effective inhibitor of phospholipase A_2 activity in vitro (Hirata, 1981). Therefore, by analogy it is feasible that EGF may induce an increase in the amount of arachidonic acid available

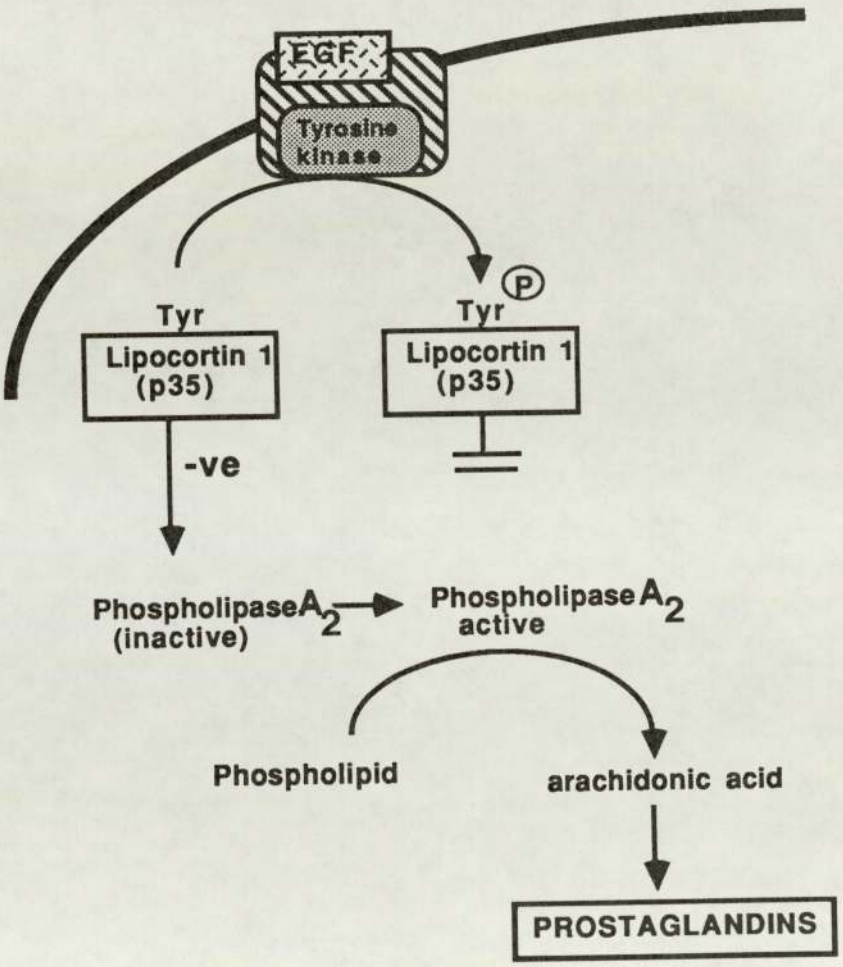
Table 6.1

A summary of some of the cell-types in which EGF stimulates PGE_2 production.

Cell-line	Half-maximally effective concentration	Reference
Kidney MDCK cells	0.1 to 8.0nM EGF was effective	Levine and Hassid, 1977
Cloned osteoblastic cells from mouse calvaria	~ 0.3nM	Yokota *et al.*, 1986
Cultured porcine thyroid cells	0.5nM	Kasai *et al.*, 1987
Cultured renal glomerulosa mesangial cells	0.52 ± 0.13nM; 100nM A23187 was required	Margolis *et al.*, 1988

Figure 6.2

A SCHEME BY WHICH EGF COULD ACTIVATE PROSTAGLANDIN PRODUCTION



This scheme is a speculative one and to date there is no evidence that it exists within the parietal cell.

for synthesis into prostaglandins by a mechanism involving the phosphorylation of lipocortin. However it should be emphasised that this model has not been entirely proven experimentally, and a recent report by Davidson et al. (1987) questions the physiological significance of lipocortin inhibiting phospholipase A_2 activity.

6.1.2.2 EGF and prostaglandin production by the gastric mucosa.

Chiba et al. (1982) demonstrated that perfusion of 100nM-EGF through the vasculature of the isolated rat stomach significantly stimulated the prostaglandin content of the venous effluent. The antiserum used by these workers displayed a 68% cross-reaction with PGE_1 therefore it was not possible to state whether the prostaglandin released was PGE_1 or PGE_2. However Konturek et al. (1981a,b) provided data suggesting that the ability of EGF to reduce the mean area of gastric mucosal lesions induced by aspirin + HCl was not due to the stimulation of prostaglandin production in the gastric mucosa by EGF. The experiments described in this chapter were undertaken to attempt to answer the following questions.

1. Do prostaglandins mediate the inhibitory action of EGF on histamine-stimulated aminopyrine accumulation?

2. Is there any evidence which can be obtained from data in vitro to support or refute the possibility that PGE_2 contributes to the antisecretory action of EGF in vivo?

In order to answer this second question, the effect of EGF on the production of PGE_2 was examined. Measurements were made of this particular prostanoid because the antisecretory action of PGE_2 on aminopyrine accumulation by isolated rat parietal cells had been characterised (Atwell and Hanson, 1988). However PGE_2 may not be the prostanoid present in the greatest concentration in the isolated cell preparations. Thus Whittle and Salmon (1983) found that 6-oxo-$PGF_{1\alpha}$ (the breakdown product of prostacyclin) was produced in greater quantity than PGE_2 by the rat gastric mucosa, whilst $PGF_{2\alpha}$ was produced in a larger quantity than PGE_2 , PGD_2 or 6-keto $PGF_{1\alpha}$ by isolated canine parietal cells (Skoglund et al., 1980).

As will become evident, there was an effect of EGF on PGE_2 production and experiments, using two slightly different approaches, were therefore undertaken to attempt to identify the cell-type reponsible. Firstly, the medium PGE_2 content was compared in cell fractions obtained from the top 1.5ml of the Percoll gradient, which were enriched in parietal cells, with parietal cell-depleted fractions obtained from the base of the Percoll gradient. Since these two fractions under comparison had originated from the same cell-pool, any variation in PGE_2 production due to cell-batch would be minimised by this procedure. Secondly, the action of EGF on PGE_2 production by these two cell fractions was compared with other agents which stimulate PGE production by particular cell-types other than parietal cells. Zymosan and bradykinin were used for this purpose and their activity in stimulating prostaglandin production will now be briefly described.

Zymosan, a yeast cell-wall carbohydrate, causes the mobilisation of arachidonic acid and the release of PGE_2 , 6-oxo-$PGF_{1\alpha}$ and acid hydrolase when incubated with cultured mouse peritoneal macrophages (Bonney et al., 1978). The stimulation of arachidonic acid release and consequently prostaglandin production may be due to the activation of a lysosomal phospholipase A_2 activity which is probably present in macrophages (Wightman et al., 1981). It is likely that the zymosan particles would be phagocytosed and internalised into lysosomal-phagocytic vesicles within the cells (Stossel, 1974b) where they may come into contact with this lysosomal enzyme. Bradykinin is a member of the kinin family (Regoli and Barabe, 1980) and exerts a variety of biological effects including stimulating PGE_2 production by human embryo lung fibroblasts and calf pulmonary artery endothelial cells (Heinsohn et al., 1987). Two mechanisms have been suggested to account for the stimulation of PGE_2 synthesis by bradykinin. Juan (1977) suggested that bradykinin induced the activation of phospholipase A_2 activity. However since both phosphatidylinositol polyphosphate hydrolysis (Derian and Moskowitz, 1986) and the release of prostacyclin from endothelial cells (Crutchley et al., 1983) are stimulated by bradykinin, it is possible that bradykinin may induce prostaglandin synthesis by stimulating phospholipase C activity.

6.2 METHODOLOGY.

6.2.1 The effect of EGF on the aminopyrine accumulation ratio and the cyclic AMP content of the cell suspension.

The aminopyrine accumulation ratio (2.3) and cell suspension cyclic AMP content (2.4.2.2) were determined as described previously. When required, IBMX, flurbiprofen and nordihydroguaiaretic acid were dissolved in absolute ethanol which was added in a small volume (usually 2µl) to the vials. The final concentration of absolute ethanol was the same in all vials (0.1% v/v) since ethanol was also added to control vials. EGF solutions were prepared and stored as detailed in 5.2.1.

6.2.2 Experiments investigating the effect of various agents on PGE_2 production.

6.2.2.1 Preparation of agents.

Powdered zymosan A was added to 50mM phosphate, 150mM NaCl, pH7.0 at a concentration of 9mg/ml and boiled at 100°C for 30 minutes. After cooling, the mixture was centrifuged at 100g for 5 minutes to pellet the zymosan and the buffer removed. This washing procedure was repeated twice using fresh buffered saline each time. Zymosan was stored in aliquots at -20°C at a concentration of 9mg/ml in buffered saline. On the day of an experiment an aliquot was thawed, mixed well using a vortex mixer, and diluted with medium B´ (Table 2.1) to produce a concentration of 0.9mg/ml. This diluted stock was sonicated on ice for 4 x 5 secs (amplitude 5 microns) immediately before the addition of a 100µl aliquot to the

appropriate incubation vials giving a final concentration of 0.1mg/ml. A 4.5 mM stock of bradykinin in 0.9% NaCl was prepared, and a small aliquot (normally 20µl) was added to the appropriate vials immediately before use.

6.2.2.2 Isolation and incubation of cells.

Cell preparations enriched in parietal cells were prepared as described in section 2.2.2. The cells remaining in the Percoll gradient following the removal of the parietal cell-enriched fraction were classed as the parietal cell-depleted fraction and contained ~ 12% parietal cells. Both fractions were washed free from Percoll by centrifugation, pre-incubated for 2 hours in a supplemented tissue culture medium (2.2.3.2) and resuspended in medium B´. 0.8ml of cells in (2-5 x 10^6 cells/ml) was added to incubation vials containing EGF, bradykinin and zymosan where appropriate. The airspace above the cells was gassed for 5 seconds with 95% O_2 /5% CO_2 prior to capping of the vials. After a 30 minute incubation at 37°C with shaking at 120 cycles per minute, 0.5ml aliquots were removed from each vial and rapidly centrifuged at 10,000g for 30 seconds in a Beckman microfuge. PGE_2 was extracted from the medium (2.4.3.1) and was measured by radioimmunoassay (2.4.3.2).

PGE_2 was measured only in the incubation medium because Schepp et al. (1986) demonstrated that PGE_2 was released into the medium rather than being retained inside human gastric mucosal cells. In this study, an increased medium prostaglandin content was presumed to reflect an increased biosynthesis of prostaglandins. A similar procedure comprising measurement of the medium prostaglandin content to reflect a change in the rate of biosynthesis of prostaglandins by isolated cell preparations has been used by other workers [e.g. mouse

peritoneal macrophages (Humes et al., 1982); rat and human adipocytes (Richelsen, 1987); renal glomerular mesangial cells (Margolis et al., 1988)].

Although it has been demonstrated that bovine serum albumin is able to trap arachidonic acid which is released from cells and thereby inhibit PGE_2 synthesis by a fibroblast cell-line (Heinsohn et al., 1987) it was decided to continue using medium B´ which contains 1.0mg/ml bovine serum albumin for the incubation as this had been used for previous aminopyrine accumulation experiments. Also, when it was present at a concentration of 1.0mg/ml, bovine serum albumin did not completely block the production of PGE_2 by the fibroblast cell-line mentioned above. Finally to avoid any assumptions about the time-course of PGE_2 release, the results have been expressed as pmole PGE_2 released per 30 minute incubation period.

6.2.3 Presentation of Data.

The effect of EGF on aminopyrine accumulation was on one occasion expressed by normalising the data to % inhibition by EGF. This was calculated using the following expression:-

$$\text{\% inhibition by EGF of the aminopyrine accumulation ratio} = \frac{\text{value in absence of EGF} - \text{value in presence of EGF}}{\text{value in absence of EGF}} \times 100$$

A similar normalisation procedure was used in Chapter 3 when calculating the % inhibition by TPA. The data were analysed using ANOVAR (A.7.1) or Friedman's Test (A.7.5) to separate out variation due to cell-batch. Friedman's Test was used to analyse the data for the effect of EGF, bradykinin and zymosan on PGE_2 production (Table 6.7). Since these results were probably not normally distributed it was decided that this test would be the most appropriate.

6.3 RESULTS AND DISCUSSION.

6.3.1 Investigation of the potential involvement of prostaglandins in the inhibition of aminopyrine accumulation or cyclic AMP content of cells stimulated with histamine.

6.3.1.1 Aminopyrine accumulation experiments.

The possibility that prostaglandins mediate the inhibition of histamine-stimulated aminopyrine accumulation by 200nM-EGF was investigated by using agents which inhibit prostaglandin synthesis. Thus nordihydroguaiaretic acid (NDGA), which inhibits both cyclo-oxygenase and lipoxygenase activity (reviewed by Higgs and Vane, 1983) and flurbiprofen, a potent and selective inhibitor of cyclo-oxygenase (MacAdams et al., 1984) were used for these experiments. The results are presented in Table 6.2. Neither flurbiprofen nor NDGA at any concentration tested abolished the inhibitory effect of EGF. Although 100μM-NDGA and 100μM-flurbiprofen both reduced histamine-stimulated aminopyrine accumulation, an inhibitory effect of EGF was still apparent. Two other inhibitors of cyclo-oxygenase, mefenamic acid (10μM) and ibuprofen (100 μM) also failed to block the inhibitory action of 200nM-EGF (G. P. Shaw, personal communication). These results therefore suggest that EGF does not effect an inhibition of histamine-stimulated aminopyrine accumulation in isolated parietal cell preparations by activating cyclo-oxygenase or lipoxygenase enzymes.

The lack of involvement of PGE_2 in the action of EGF can also be assessed by comparing the antisecretory actions of these agents. Thus although PGE_2 inhibited aminopyrine accumulation stimulated by histamine plus IBMX, a range of concentrations of EGF were ineffective (Fig. 6.3, data obtained by Atwell and Hanson, 1988). In

Table 6.2

Effect of flurbiprofen and nordihydroguairetic acid (NDGA) on the inhibitory action of 200nM EGF against aminopyrine accumulation stimulated by 0.5mM histamine.

Concentration of inhibitor (μM)	No. of cell batches	Aminopyrine Accumulation Ratio				Inhibition of amino-pyrine accumulation (%)	
		Histamine	Histamine + EGF	Inhibitor + Histamine	Inhibitor + Histamine + EGF	No Agent	Agent Present
Flurbiprofen (1.0)	4	11.1±0.8	6.1±0.4 ***	9.9±1.0	6.3±1.0 ***	43.4±6.2	37.6±4.8
Flurbiprofen (10.0)	4	12.0±1.8	6.7±0.8 ***	13.5±2.4	8.2±1.3 ***	42.9±3.7	37.3±4.4
Flurbiprofen (100.0)	4	10.3±1.2	6.4±0.7 ***	6.6±1.1	4.0±0.6 *	37.9±2.3	37.5±4.7
NDGA (10.0)	3	11.6±1.0	6.4±0.1 ***	12.0±1.5	6.9±0.6 ***	44.0±3.7	42.4±2.4
NDGA (100.0)	4	11.3±0.1	6.3±0.1 ***	6.8±0.9	3.5±0.5 ***	43.4±2.6	44.3±2.4

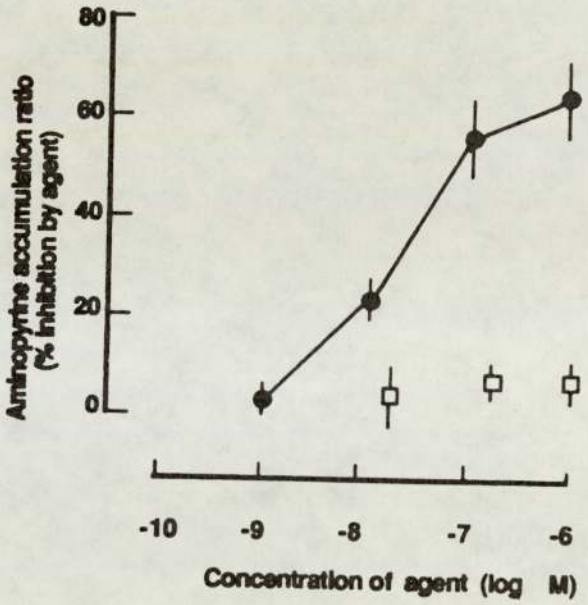
Table 6.2 cont/.....

Table 6.2 cont.....

Aminopyrine accumulation ratios are presented as means ± S.E.M. from cell batches containing 19 - 22% parietal cells. A significant effect of EGF in the presence and absence of the inhibitors was determined using ANOVAR followed by a Newman-Keuls multiple comparison test which also showed that both 100μM-flurbiprofen and 100μM-NDGA reduced histamine - stimulated aminopyrine accumulation (*, $p < 0.05$; ***, $p < 0.001$). There was no significant effect of the agents on the % inhibition by EGF (paired t-test).

Figure 6.3

The effect of PGE_2 (●) and EGF (□) on aminopyrine accumulation in crude preparations of parietal cells stimulated with histamine (0.5mM) plus IBMX (0.1mM).



These data were obtained by M.M.Atwell and P.J.Hanson and are presented in Atwell and Hanson (1988) in a similar form. Results have been normalised by expressing the inhibition by each agent as a % of the control value in the absence of agent (control values; PGE_2 data = 154 $\pm$ 34, 4 cell-batches; EGF data = 123 $\pm$ 18, 4-5 cell-batches).

view of the different abilities of EGF and PGE_2 to inhibit aminopyrine accumulation in the presence of 0.1mM-IBMX, it is unlikely that these agents inhibit aminopyrine accumulation by acting at a similar site.

6.3.1.2 Effect of EGF and PGE_2 on the cyclic AMP content of parietal cell-enriched preparations stimulated with 0.5mM-histamine plus 0.1mM-IBMX.

PGE_2 significantly inhibited the cyclic AMP content of cells stimulated by histamine plus IBMX ($p < 0.01$, paired t-test) whilst 200nM-EGF was without effect against cells isolated from the same batch (Table 6.3). A reduction in the cyclic AMP content by 10nM-PGE_2 was also observed by Soll (1980b) using preparations of canine parietal cells stimulated with 10μM -histamine plus 10μM-IBMX. The lack of effect of EGF on histamine-stimulated cyclic AMP content in the presence of 0.1mM-IBMX was obtained previously (Table 5.9) and is discussed in section 5.3.5. Thus this comparison of the actions of these agents on the cyclic AMP content of cells stimulated with histamine and IBMX provides further support that the mechanisms by which EGF and PGE_2 act on isolated parietal cell preparations are different. From the variety of data obtained, it can be concluded that it is highly improbable that the inhibition by EGF of histamine-stimulated secretory activity in preparations of isolated parietal cells was mediated by prostaglandins.

Table 6.3

Effect of PGE_2 (10nM) and EGF (200nM) on the cyclic AMP content of parietal cell-enriched preparations stimulated with histamine (0.5mM) plus IBMX (0.1mM).

Agent	Cyclic AMP content of cell suspension (pmol/10^6 cells)	
	Control	+ Agent
PGE_2 (10nM)	3.27 ± 0.41	1.70** ± 0.24
EGF (200nM)	3.27 ± 0.41	3.16 ± 0.30

Results from 4 cell-batches containing 80.4 ± 2.61% parietal cells are expressed as means ± S.E.M. The basal cyclic AMP content was 0.77 ± 0.09 pmol/10^6 cells.
**, $p < 0.01$ for effect of agent by paired t-test.

6.3.2 Could EGF inhibit acid secretion in the intact mucosa by stimulating the production of PGE_2 ?

The results described previously (6.3.1) make it unlikely that prostaglandins were involved in the antisecretory effect of EGF in isolated cell preparations containing 19-22% parietal cells. However, it is possible that prostaglandins could contribute to the antisecretory action of EGF in the intact gastric mucosa where their concentration in the interstitial fluid might be much higher than that in the medium associated with the isolated cell suspension. Initial experiments utilised parietal cell-enriched preparations containing approximately 80% parietal cells since the results obtained by Postius et al. (1985) indicated that parietal cell-enriched preparations produced significantly more PGE_2 than parietal cell-depleted preparations.

6.3.2.1 PGE_2 content of parietal cell preparations incubated under basal conditions.

PGE_2 production by parietal cell-enriched preparations varied over the range of 2.41 to 24.61 $pg/10^6$ cells/30 min with a mean value of 12.82 $\pm$ 2.50 (11 cell-batches containing 81.1 $\pm$ 1.3% parietal cells). The reason for this variation was unclear but it could not be ascribed to inter-assay variation (2.4.3.2) nor did it correlate with the ability of cells to exclude trypan blue immediately prior to incubation (correlation coefficient = 0.225,9d.o.f). The basal PGE_2 production obtained with cells in this study is compared with that reported by other workers in Table 6.4.

Table 6.4

A summary of recent experiments measuring the amount of PGE_2 released into the incubation medium under basal conditions by preparations of isolated parietal cells.

Species	% parietal cells	PGE_2 content of medium (all are pg/10^6 cells/30 min unless indicated)	Reference
Rat	20 - 25	~ 40 pg/10^6 cells/60 min	Postius *et al.*, 1985
Rat	7 71	0.79 ± 0.40 7.20 ± 1.05	Fukuda *et al.*, 1987
Guinea Pig	76	31.8 ± 8.5 pg/10^6 cells/ 60 min	Hell and Sewing 1987
Dog	60 - 75	88.6 ± 21.6	Payne and Gerber, 1987
Rat	81.1 ± 1.3	12.82 ± 2.50 (n = 11 cell-batches)	This work

Values represent the amount of PGE_2 released into the volume of incubation medium associated with 10^6 cells.

There are a number of factors which could have affected the production of PGE_2 by the isolated cell preparations.

1. The wide range of basal PGE_2 values may have in part been due to a non-parietal cell-type which co-purified with the parietal cells on the Percoll gradient and which possessed a substantial basal rate of PGE_2 production. Since the composition of the non-parietal cell-fraction was not examined, the content of such a cell-type may have varied undetected between the cell-preparations.

2. A study of the effect of homogenisation techniques on prostaglandin generation by whole-tissue mucosal preparations revealed a positive relationship between the proportion of cells which had ruptured and the content of PGE_2 released (Ahlquist et al., 1983). Therefore the extent of damage incurred to the plasma membrane of the isolated cells may influence the prostaglandin content of the medium containing the cells although there was no correlation in this study between basal PGE_2 production and the percentage of cells able to exclude trypan blue.

3. Heinsohn et al. (1987) have presented experimental data showing that bovine serum albumin present in the incubation medium is capable of trapping the released arachidonic acid thereby reducing the amount available for synthesis to prostaglandins.

4. Hydrocortisone inhibits prostaglandin production by MC5-5 fibroblasts stimulated with bradykinin or thrombin (Hong and Levine, 1976). Although the isolated cells were washed free from the supplemented tissue culture medium containing hydrocortisone (2.3.2.2) prior to the experiments, it is possible that the hydro-cortisone may have had a lasting effect on the cells, possibly reducing prostaglandin production. However, it is also possible

that the hydrocortisone may facilitate the observation of an effect of EGF. Thus if EGF acts by inducing a phosphorylation of lipocortin (Fig. 6.2), since glucocorticoids (and so probably hydrocortisone) induce the formation of lipocortin (Hirata et al., 1987), hydrocortisone may provide a background against which the effect of EGF can be observed.

However, since the concentration of bovine serum albumin and hydrocortisone were constant throughout the experiments, it is unlikely that factors 3 and 4 accounted for the variability in the basal PGE_2 production by the isolated parietal cell preparations.

6.3.2.2 Effect of EGF on PGE_2 production.

EGF significantly stimulated PGE_2 production ($p < 0.01$, one-way ANOVAR) during a 30 minute incubation with parietal cell-enriched preparations (Table 6.5). To reduce variation attributed to basal PGE_2 content the stimulation of PGE_2 production above basal levels was calculated, and it is apparent that this effect of EGF was dose-dependent with a half-maximally effective concentration of 7.5nM (Fig. 6.4). The absolute increase in PGE_2 production above basal levels by 200nM-EGF was not significantly related to the basal PGE_2 level in the absence of EGF when 11 cell-batches were analysed (coefficient of correlation = 0.308). The stimulatory effect of EGF on PGE_2 production observed in this study is in agreement with the results obtained by Chiba et al. (1982) using the isolated rat stomach preparation.

Table 6.5

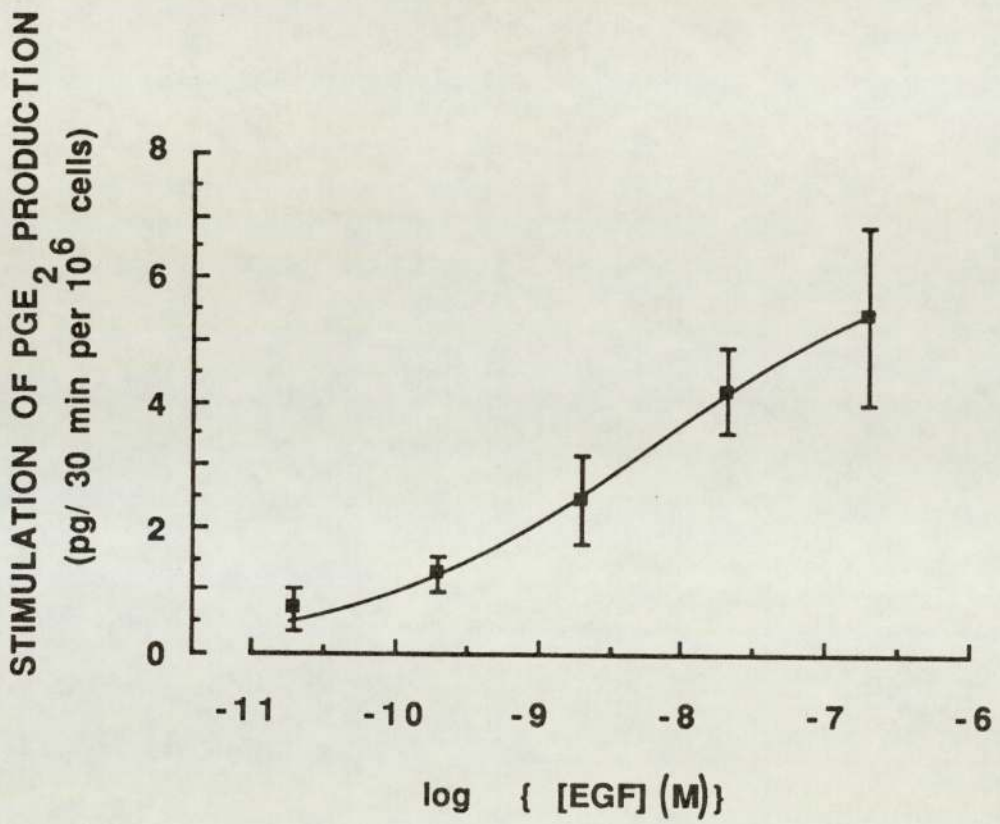
The effect of EGF concentration on the PGE_2 content of an enriched preparation of parietal cells.

Concentration of EGF (nM)	PGE_2 (pg/10^6 cells/30 min)
0	6.70 ± 2.00
0.02	7.07 ± 2.44
0.2	7.97 ± 2.54
2.0	8.88 ± 2.65
20.0	10.90 ± 2.51
200.0	12.08 ± 3.20

Results from 6 cell-batches containing 83.1 ± 1.4% parietal cells are presented as means ± S.E.M. Analysis of the data by ANOVAR (one-way) indicated a significant effect of EGF on PGE_2 production ($p < 0.01$).

Figure 6.4

Effect of EGF concentration on the production of PGE_2 by parietal cell-enriched preparations under basal conditions.



Data from Table 6.5 have been recalculated as the absolute stimulation of PGE_2 production above basal (basal value = 6.7 $\pm$ 2.00 pg/10^6 cells/ 30 min) and are expressed as means $\pm$ S.E.M. The position of the line was calculated by using the computer programme FIT (Barlow,1983).

As indicated previously PGE_2 is probably not the only prostanoid present in isolated cell preparations (Whittle and Salmon, 1983) and it is entirely possible that EGF stimulates the release of other prostaglandins which may modulate secretory activity. Thus in rat aorta smooth muscle cells, EGF stimulates the production of prostacyclin (Bailey et al., 1985).

The concentration of PGE_2 in the incubation medium obtained in the presence of 200nM-EGF was calculated to be 83.6 $\pm$ 13.2 pM (n = 11 cell-batches). This value is much lower than the concentration of 24nM-PGE_2 required for half-maximal inhibition of histamine-stimulated aminopyrine accumulation (M. M. Atwell and P. J. Hanson, unpublished observations). This finding therefore reinforces the earlier conclusion that the inhibition of aminopyrine accumulation by EGF in isolated cell preparations was not mediated by PGE_2 .

6.3.2.3 The effect of histamine (0.5mM) on the stimulation of PGE_2 production by 200nM-EGF.

EGF was not effective at stimulating the production of PGE_2 by parietal cell-enriched preparations in the presence of 0.5mM-histamine (Table 6.6). This result therefore makes it very unlikely that EGF could inhibit histamine-induced acid secretion by stimulating PGE_2 production. It also adds further support to the previous arguments that in dispersed isolated rat parietal cells, inhibition of histamine-stimulated aminopyrine accumulation by EGF is unlikely to be mediated via the release of PGE_2 .

Table 6.6

Effect of histamine (0.5mM) on the stimulation of PGE_2 production by 200nM-EGF.

All values are pg PGE_2 /30min per 10^6 cells.

Secretagogues	No EGF	EGF (200nM)	Absolute increase in PGE_2 production
None	20.16 ± 1.88	27.05*** ± 1.10	6.89 ± 1.57
Histamine (0.5mM)	20.27 ± 1.25	21.16 ± 1.51	0.89 ± 2.44

Results from 5 experiments containing 82.0 ± 1.7% parietal cells are expressed as means ± S.E.M. The effect of 200nM-EGF was analysed by a paired t-test (***, $p < 0.025$). Histamine had no significant effect on PGE_2 production in the absence of EGF (paired t-test).

An interesting point which needs to be considered is the reason for the lack of effect of EGF on PGE_2 production in the presence of histamine. One possibility is that histamine may in some way antagonise the interaction of EGF with its receptor and therefore with the production of signals mediating prostaglandin production. However, since EGF has a potent inhibitory effect on cyclic AMP content and aminopyrine accumulation in histamine-stimulated cells (5.3.4) this is rather unlikely. A more feasible explanation is that the elevation of cellular cyclic AMP levels by histamine was responsible for the lack of PGE_2 production. Thus the biosynthesis of PGE_2 by isolated human gastric cells was inhibited by forskolin or dbcAMP (Schepp et al., 1986) and that produced by renal tubular cells was inhibited by IBMX and cyclic AMP analogues (Hassid, 1982). Work by Minkes et al. (1977) suggests that in platelets, agents which elevate cyclic AMP levels inhibit the release of arachidonic acid from membrane phospholipids and thereby reduce the availability of arachidonic acid for prostaglandin synthesis.

The blockade of the effect of EGF on PGE_2 release by 0.5mM-histamine may be part of a negative-feedback effect. Nylander et al. (1986), using rabbit gastric glands, have demonstrated that PGE_2 stimulates the release of endogenous histamine, if this proves to be a physiological effect it is possible that the presence of histamine may then exert an inhibitory effect preventing any further release of PGE_2 .

6.3.3 Identification of the cell-type responsible for PGE_2 release.

The results showing the effect of EGF, bradykinin and zymosan on PGE_2 production in parietal cell-enriched and parietal cell-depleted fractions are presented in Table 6.7, the effect of each agent will be considered in turn.

PGE_2 production in the absence of any agent was significantly greater in the parietal cell-enriched fraction (A) than in the fraction which was depleted of parietal cells (B) ($p < 0.01$, paired t-test). This result is consistent with the parietal cell, or another low-density cell which co-purifies with it, being a source of prostaglandin production, and is in agreement with the data obtained by Postius et al. (1985).

The addition of 200nM-EGF significantly enhanced PGE_2 production by the parietal cell-enriched fraction ($p < 0.01$, Dunnett's Test) to an extent similar to that obtained previously despite the basal PGE_2 content being much higher than that obtained previously. Thus 200nM-EGF increased PGE_2 production (pg/10^6 cells/30 min) by 5.38 ± 1.43 when the basal value was 6.70 ± 2.00 (Table 6.5) and by a similar amount of 6.89 ± 1.57 when the basal value was 20.16 ± 1.88 (Table 6.7). However, the absolute increase in PGE_2 production by 200nM-EGF in parietal cell-depleted fractions (Fraction B) was 0.30 ± 0.37 and was not significant. These results therefore suggest that EGF stimulated the production of PGE_2 by the parietal cell, or by a cell-type of similar density which had co-purified with the parietal cell on the Percoll gradient. Furthermore since histamine-H_2 receptors are probably only found on the parietal cell in the isolated cell preparation, the blockade by histamine of the EGF-induced PGE_2 release (6.3.2.3) lends further support to the suggestion that the parietal cell is the site at which EGF acts to stimulate PGE_2 production.

Table 6.7

Effect of EGF, Bradykinin and Zymosan on PGE_2 production by parietal cell-enriched (A) and parietal cell-depleted (B) fractions.

All values are pg PGE_2 /30 min per 10^6 cells

Agent	Absolute PGE_2 production		Absolute increase in PGE_2 above basal	
	Fraction A	Fraction B	Fraction A	Fraction B
None	20.16 ± 1.88	8.34 ± 1.33	-	-
EGF (200nM)	27.05 ± 1.10*	8.64 ± 1.29	6.89 ± 1.57	0.30 ± 0.37
Zymosan (100µg/ml)	22.68 ± 1.92	8.22 ± 1.54	2.52 ± 2.44	-0.14 ± 0.38
Bradykinin (1µM)	35.74 ± 3.79**	12.92 ± 2.25*	15.58 ± 5.37	4.58 ± 1.45

Values are expressed as means $\pm$ S.E.M. from 5 cell-batches containing $82.0 \pm 1.7\%$ parietal cells (fraction A) or $11.5 \pm 0.7\%$ parietal cells (fraction B). Data for both fractions were analysed separately using Friedman's Test (A.7.5) which indicated a significant effect of treatment with agents for fraction A ($p < 0.005$) and for fraction B ($p < 0.05$). Dunnett's Test was subsequently used to compare values in the presence of agent with the control value (**, $p < 0.01$; *, $p < 0.05$).

Incubation of either type of cell fraction with zymosan failed to significantly affect the production of PGE_2 as compared to control values. This lack of effect of zymosan may have potentially been because the zymosan was not opsonised. Thus Waite et al. (1979) demonstrated that in order for zymosan to be effective at inducing the release of pre-labelled arachidonic acid from human neutrophils it had to be opsonised by prior incubation with serum. Opsonisation is the process where particles are coated with serum proteins which enhances the rate of endocytosis by phagocytes (Stossel, 1974a). However opsonisation was not used in experiments where zymosan stimulated PGE synthesis above basal levels by mouse peritoneal macrophages after a 30 minute incubation. (Humes et al., 1982). Therefore more realistic explanations of the lack of effect of zymosan are that either there were no macrophages present in these preparations, or if present, they did not produce PGE_2 in response to incubation with 100µg/ml-zymosan. This result contrasts with the information in the abstract published by Chen et al. (1986) who reported that prostaglandin production by a preparation of isolated canine gastric mucosal cells correlated with the presence of markers for macrophages and endothelial cells. However it should be noted that canine cells are produced from minced mucosa and not from everted sacs and this difference in preparation procedure may be responsible for the apparent absence of macrophages in the above experiments.

1µM-bradykinin significantly enhanced the production of PGE_2 above control levels in parietal cell-enriched ($p < 0.05$) and parietal cell-depleted fractions ($p < 0.01$, Dunnett's Test). Since this agent stimulates PGE_2 production in both fibroblasts and endothelial cells it is not clear exactly which cell type was responsible. Furthermore, the possibility that parietal cells may possess bradykinin receptors should be considered although no reference to such an effect could be found in the

literature. The results published in the abstract produced by Chen et al. (1987) indicate that a co-culture of canine submucosal fibroblasts with the "small cells" present in the elutriator fraction of canine mucosal cells exhibited a potentiated reponse to bradykinin-induced PGE_2 production. The small-cell fraction contained macrophages, mast cells and endothelial cells, and it was suggested that an interaction between these cell-types and fibroblasts was responsible for the observed effect with bradykinin.

In conclusion, the data obtained suggest that the PGE_2 produced in response to 200nM-EGF originated from parietal cells or from a cell-type which co-purified with parietal cells on the Percoll gradient. Endothelial cells and/or fibroblasts which were sensitive to bradykinin may also have been present but it is unlikely that these cells were responsible for producing PGE_2 in response to EGF as a stimulatory response to bradykinin was found in both cell fractions whilst that to EGF was restricted to the parietal cell-enriched fraction. Furthermore, experiments performed by Levine and Hassid (1977) demonstrated that neither mouse nor human fibroblast cell-lines nor a rat endothelial cell-line produced PGE_2 in response to incubation with EGF.

6.4 SUMMARY

1. Under basal conditions, EGF stimulated the production of PGE_2 by the parietal cell, or a cell-type which co-purified with the parietal cell. The isolated cell preparations also contained a cell-type which produced PGE_2 in response to incubation with bradykinin.

2. However the inhibition of histamine-stimulated aminopyrine accumulation by 200nM-EGF in isolated parietal cell preparations was not mediated by PGE_2 production. This is suported by the following observations:-

   (a) Inclusion of flurbiprofen or NDGA did not abolish the inhibitory action of 200nM-EGF against histamine-stimulated aminopyrine accumulation.

   (b) IBMX abolished the antisecretory action of EGF but not that of PGE_2 .

   (c) Although EGF stimulated the production of PGE_2 under basal conditions, the medium concentration was 83.6 ± 13.2pM and much lower than the IC_{50} of 24nM required to inhibit histamine-stimulated aminopyrine accumulation.

3. EGF did not stimulate the production of PGE_2 in the presence of 0.5mM-histamine. This finding also tends to mitigate against a role of prostaglandins in the anti-secretory action of EGF both in intact tissue and in isolated cell preparations in which acid secretion has been induced by histamine.

Chapter Seven

GENERAL DISCUSSION

The aim of this chapter is to consider the physiological significance of the data obtained concerning the effects of TPA and EGF on parietal cell secretory activity.

7.1 The role of protein kinase C in modulation of secretory activity in rat isolated parietal cells.

In order to investigate the involvement of protein kinase C in the regulation of parietal cell secretory activity, the tumour promoter TPA has been used as a tool to activate this enzyme (3.1.1.2). Under physiological conditions, protein kinase C is probably activated by diacylglycerol which is produced as a consequence of hormonal activation of a phospholipase C enzyme. TPA is probably not metabolised by the cell (Castagna et al., 1982) in contrast to diacylglycerol which can be either phosphorylated to phosphatidic acid by diacylglycerol kinase, or can be hydrolysed by diacylglycerol lipase to form monoacylglycerol and subsequently arachidonic acid (reviewed by Berridge, 1987). Thus it has been suggested (Williamson and Hansen, 1987) that the sustained activation of protein kinase C by TPA may not fully mimic the effects produced by physiological agonists. However when rat isolated parietal cells are stimulated by 0.1mM-carbachol, the cellular levels of 1,2 diacylglycerol are elevated above basal for at least 30 minutes (Pfeiffer et al., 1987) implying that the physiological activation of this enzyme is not necessarily transient. Furthermore, the diacylglycerol analogue 1-oleoyl-2-acetylglycerol (OAG), which is phosphorylated to phosphatidic acid (Kaibuchi et al., 1983), was also used in this work and produced similar results to those obtained using TPA (Table 7.1).

Table 7.1

A summary of the effects of TPA, OAG, EGF, PGE_2, substance P (SP) and thyrotropin-releasing hormone (TRH) on aminopyrine accumulation stimulated by various secretagogues in rat isolated parietal cells.

↓ = inhibition, ↑ = stimulation, - = no effect. A blank space indicates that data was not available for rat isolated parietal cells. Numbers in brackets refer to the references.

Secretagogue	Agent					
	TPA	OAG	EGF	PGE_2	SP	TRH
Histamine	↓		↓	↓		
alone	(2)		(1)	(6)		
Histamine +	↓	↓	-	↓	↓	↓
IBMX	(1,3)	(3)	(1)	(5,6)	(7)	(8)
Carbachol	↓	↓	-		-	
	(3)	(4)	(2)		(7)	
dbcAMP	↓	↓	-		↓	↓
	(1,3)	(4)	(2)		(7)	(8)
High K^+	↑	↑				
	(1)	(1)				

References:

1 = This work

2 = Work by G. P. Shaw included in this thesis

3 = Anderson and Hanson, 1984

4 = Anderson and Hanson, 1985

5 = Schepp et al., 1983a,b

6 = Atwell and Hanson, 1988

7 = Schepp et al., 1988a

8 = Schepp et al., 1988b

The results obtained from this work suggest that protein kinase C exerts both inhibitory and stimulatory effects on parietal cell secretory activity by acting at different sites within the parietal cell. The following sites at which protein kinase C acts to attenuate secretory activity (Fig. 7.1) are suggested, but not proved.

A. Results from this work.

1. Since TPA inhibits cyclic AMP stimulated by 0.5mM-histamine plus 0.1mM-IBMX (IC_{50} = 1.0nM TPA), one site of action of protein kinase C is close to the histamine-induced elevation of cyclic AMP (3.3.3).

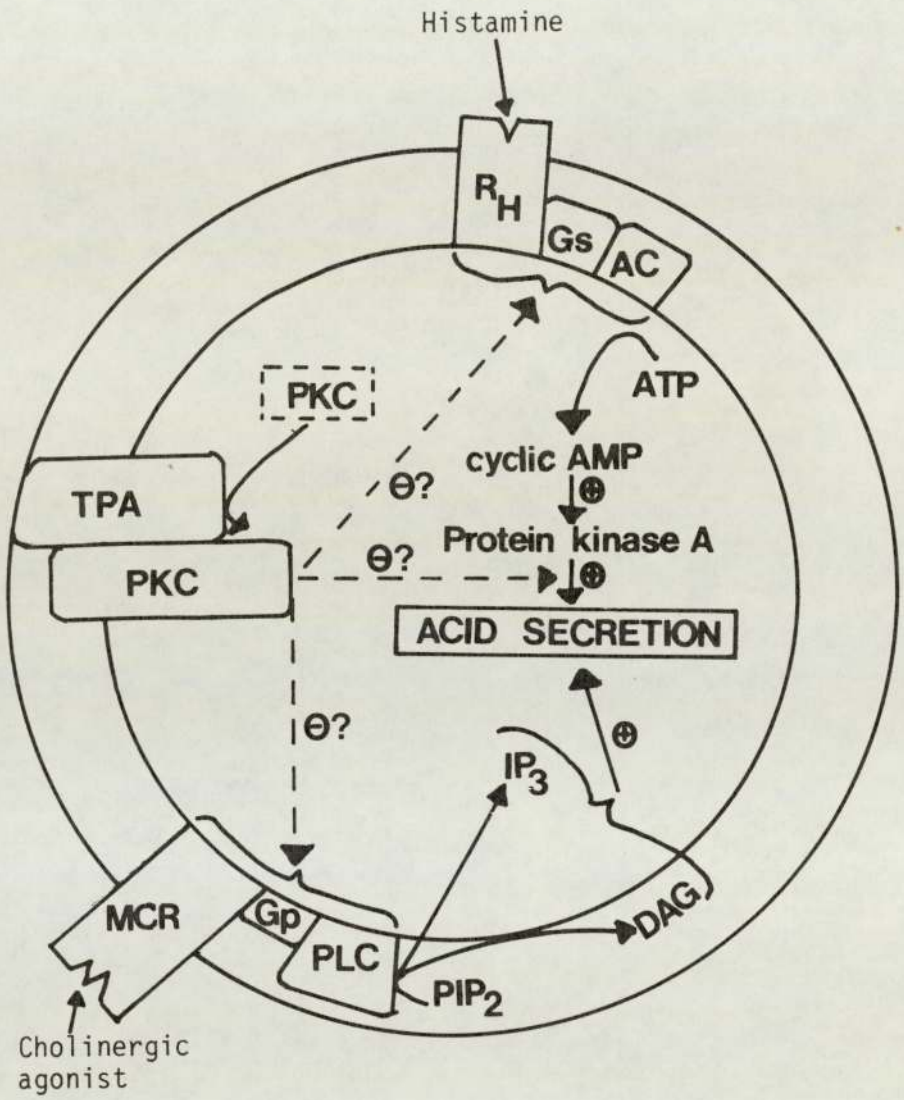
2. TPA inhibits aminopyrine accumulation induced by 1mM dbcAMP (IC_{50} = 9.6nM) implicating a second site of action which is at, or distal to cyclic AMP-dependent protein kinase (but proximal to the H^+/K^+ ATPase) (3.3.2).

B. Results from work of others.

A third site of action which was not investigated in this work may be at, or close to the muscarinic cholinergic receptor since TPA inhibits carbachol-stimulated aminopyrine accumulation by rat isolated parietal cells (Anderson and Hanson, 1984; Pfeiffer and Noelke, 1988, abstract). Furthermore, 100nM-TPA significantly inhibits carbachol-stimulated accumulation of [^{3}H] inositol phosphates in rat isolated parietal cells (Puurunen et al., 1987a). An inhibitory effect of TPA on the muscarinic cholinergic receptor has been identified in other cells, e.g. in PC12 cells, a neurosecretory cell-line (Vicentini et al., 1985). It is apparently paradoxical that protein kinase C can be activated in response to stimulation of the parietal cell by carbachol

Figure 7.1

A speculative scheme showing the sites at which protein kinase C, when activated by TPA , may act to inhibit acid secretion by the parietal cell.



Acid secretion can be stimulated by histamine interacting with an H_2-receptor (R_H) on the surface of the parietal cell coupled via a stimulatory G-protein (G_s) to adenylate cyclase (AC) which effects an activation of protein kinase A. Alternatively a cholinergic agonist binding to a muscarinic cholinergic receptor (MCR), which is coupled via a G-protein (G_p) to phospholipase C (PLC), effects the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield D-inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The DAG-induced activation of protein kinase C is not shown.

TPA becomes intercalated into the plasma membrane and activates protein kinase C which then exerts an inhibitory effect on acid secretion at the 3 possible sites indicated.

[PKC] represents the inactive form of protein kinase C.

(Park et al., 1987) where it could play a role in stimulating acid secretion, yet when activated by TPA it inhibits carbachol-induced aminopyrine accumulation. This may signify a form of negative-feedback modulation whereby protein kinase C regulates its activation by controlling the production of diacylglycerol.

It is possible that the inhibitory effects of TPA described in (1) and (2) above may represent a physiological role for protein kinase C in the negative modulation of the synergistic interaction between histamine and cholinergic agents (1.2.2.4). Under these conditions of a large stimulus for secretion, it is anticipated that the cell would require such a pathway to prevent over-production of acid or alternatively to prevent depletion of cellular ATP if the H^+/K^+ ATPase activity were to exceed the rate of ATP synthesis. It is pertinent that the results obtained by Puurunen et al. (1987a) using isolated rat parietal cells imply that there may also be another closely related feedback mechanism, since an elevation of intracellular cyclic AMP levels by histamine attenuated the carbachol-induced formation of inositol phospholipids.

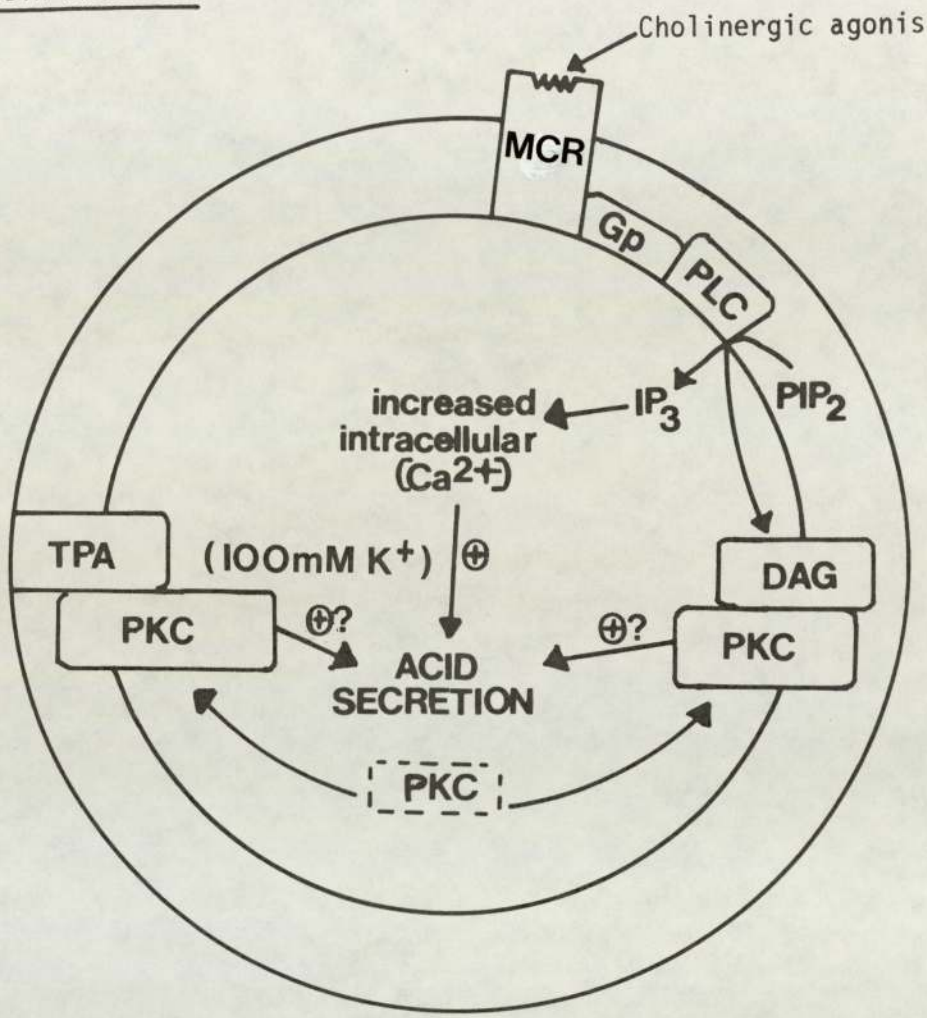
In addition to a putative role of protein kinase C in modulating receptor activity, it is possible that protein kinase C is the second messenger for an extracellular inhibitory ligand which remains to be identified. In the rat isolated parietal cell, at least two observations make it unlikely that EGF is this ligand. Thus the spectra of inhibition against various secretagogues are different for EGF and TPA (Table 7.1), and the phosphodiesterase inhibitor IBMX blocks the anti-secretory action of EGF (5.3.5) but not that of TPA (3.3.3). Recent work by Schepp et al., (1988a,b; abstracts) has examined the antisecretory action of substance P and thyrotropin-releasing hormone on rat isolated parietal cells. The similarity of the anti-

secretory pattern of these agents compared with that of TPA (Table 7.1) suggests that one, or both may activate protein kinase C, however this possibility remains to be examined.

Whilst investigating the possibility that TPA may attenuate parietal cell secretory activity by inhibiting the H^+/K^+ ATPase, a transient stimulatory effect of TPA on aminopyrine accumulation was observed when parietal cells were incubated in a medium containing 100mM-K^+ (4.3.3). The enhancement of aminopyrine accumulation by TPA under these circumstances was not due to a release of endogenous histamine or acetylcholine (4.3.7) nor did it involve an elevation of the cyclic AMP content of the cell suspension (4.3.8). The stimulatory action of TPA was reproduced with OAG (4.3.4) and was therefore apparently specific to the activation of protein kinase C. These results do not prove that TPA stimulated acid secretion when the cells were incubated in a medium containing 100mM-K^+ although it has been demonstrated that TPA is capable of stimulating the metabolic activity of the parietal cell (Brown and Chew, 1987), which is an indication of an increased rate of activity of the H^+/K^+ ATPase. It is tempting to speculate that the pathway by which protein kinase C enhances K^+ -stimulated aminopyrine accumulation may be similar to that which stimulates acid secretion in parietal cells incubated with carbachol (Fig. 7.2), however this remains to be substantiated. During the course of this work other groups have also demonstrated examples, of dual effects of protein kinase C activation on parietal cell secretory activity using cells isolated from the rat stomach; these results are summarised in Table 7.2. In conclusion the available data implicate a role for protein kinase C in both the inhibition and stimulation of parietal cell secretory activity which can be observed under different experimental conditions. Since protein kinase C is not a single enzyme but exists as at least 3 separate sub-species (3.1.1.1), it is an interesting possibility that these effects may be mediated by different sub-species.

Figure 7.2

A speculative scheme showing the putative activation of acid secretion by protein kinase C.



TPA-induced activation of protein kinase C in cells incubated in a medium containing 100mM-K^+ may induce a stimulation of acid secretion. Alternatively interaction of a cholinergic agonist with a muscarinic cholinergic receptor yields diacylglycerol (DAG) and D-inositol 1,4,5-trisphosphate (IP_3), see legend to Fig. 7.1. IP_3 elevates intracellular Ca^{++} levels, whilst DAG activates protein kinase C (3.1.1.3).

[PKC] represents the inactive form of protein kinase C.

Table 7.2

A summary of the dual effects of TPA on aminopyrine accumulation in isolated cell preparations.

Reference	Preparation	Secretagogue: None	Histamine	dbcAMP	Carbachol	Gastrin	Forskolin	High K^+
This work	Rat isolated parietal cells	-	↓*	↓				↑*
Brown and Chew, 1987	Rabbit gastric glands and isolated parietal cells	↑	↓*	↑*	↓* †		↓*	
Chiba et al., 1986	Canine isolated parietal cells	↑*			↓*	↓*		
Pfeiffer and Noelke, 1988	Rat isolated parietal cells			↓*	↑ (1-5nM) ↓ (20-100nM)			

↓ = inhibition, ↑ = stimulation, - = no effect, gaps = not determined.
† = effect only seen in glands, not cells.
* = effect also seen with OAG.

7.2 The role of EGF as an antisecretory agent and the possible involvement of PGE_2.

EGF is produced by submandibular glands and Brunner's glands in man (Heitz et al., 1978) and it has been proposed that EGF in saliva has a role in protecting the gastric mucosa from damaging agents (reviewed by Konturek, 1988). This is supported by experiments using sialoadenectomized rats (Olsen et al., 1984) in which the intragastric administration of EGF prevented the formation of gastric ulcers by cysteamine. There is gathering evidence that EGF is involved in the maintenance and repair of the gastroduodenal mucosa (Konturek, 1988) however since no experiments have been performed in this area, this discussion is confined to the antisecretory role of EGF.

EGF inhibits the secretory activity of rat isolated parietal cells stimulated by 0.5mM-histamine, apparently by effecting a reduction in cyclic AMP content (5.3.3). Since the phosphodiesterase inhibitor IBMX (0.1mM) abolishes the antisecretory action of EGF (5.3.5) it is possible that the mechanism by which EGF attenuates cellular cyclic AMP involves the stimulation of cyclic AMP-phosphodiesterase activity.

The antisecretory action of luminal EGF is insignificant (Table 5.3) and the concentration of EGF in mouse plasma (0.16nM; Carpenter and Cohen, 1979) is considerably lower than that required to effect half-maximal inhibition of histamine-stimulated aminopyrine accumulation (3.0nM; section 5.3.4). This leads to the question "Is there a physiological role for the antisecretory action of EGF?" In view of the above observations it appears unlikely that EGF has a major antisecretory role under normal circumstances. However, it is suggested that EGF may play a role in attenuating

acid secretion under conditions where the gastric mucosa and blood vessels are acutely damaged. EGF is stored in platelets (Oka and Orth, 1983) and may be released from them during their aggregation, and thereby provide a localised source of EGF which might be of a sufficient concentration to exert an antisecretory effect on the parietal cell. It is anticipated that this action of EGF would be beneficial by minimising damage caused by acid to the basal lamina which may be exposed under such circumstances. This may also be of importance in the early stages of mucosal repair during the formation of a mucoid cap which has been suggested (Ito and Lacy, 1985) to act as a localised protective barrier serving to facilitate epithelial restitution. Alternatively, in the presence of gastric ulcers, luminal EGF may be able to penetrate through the broken epithelium to parietal cells surrounding the ulcer where it could cause a localised inhibition of acid secretion.

Although prostaglandins do not appear to mediate the antisecretory effect of EGF in rat isolated parietal cell preparations (6.3.1), EGF does stimulate the production of PGE_2, possibly from the parietal cell, but only under basal conditions in the absence of histamine (6.3.2). What is uncertain at present is whether such effects occur in vivo, and if so, whether they have any physiological significance.

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APPENDICES.

A1. Source of reagents.

Reagent.	Supplier.
A. General Chemicals	
Bovine serum albumin (BSA), fraction V	Miles Labs
Calcium chloride	BDH
Choline bicarbonate	BDH
Choline chloride	BDH
Dextran (mw 40,000da)	Sigma
Dimethylsulphoxide (DMSO)	Sigma
di-potassium hydrogen orthophosphate	BDH
di-sodium hydrogen orthophosphate	BDH
Dithiothreitol	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Hopkins & Williams
Ethyleneglycol-bis-(β-aminoethylether) N,N´-tetraacetic acid (EDTA)	Sigma
Foetal calf serum	Sigma
Gentamicin	Sigma
D-glucose	BDH
L-glutamine	Sigma
Hydrocortisone	Sigma
N-2-hydroxyethylpiperazine-N´-2-ethane sulphonic acid (HEPES)	Sigma
Insulin	Sigma
L-isoleucine	Sigma
Magnesium sulphate	BDH
Percoll	Sigma
Potassium chloride	BDH
Potassium dihydrogen orthophosphate	BDH
Sagatal	May & Baker
Sodium chloride	BDH
Sodium dihydrogen orthophosphate	BDH
Sodium hydrogen carbonate	BDH
Sodium thiocyanate	Sigma
Trypan blue	BDH

Trypsin inhibitor, lyophilised from soybean	Sigma

B. Enzyme.

Pronase, 70,000 PUK/g	BDH

C. Radiochemicals and scintillation counting.

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

D. Secretagogues and agents

Atropine	Sigma
Bradykinin, triacetate salt	Sigma
Cimetidine	Sigma
Cyclo (7-aminoheptanoyl-Phe-D-Trp-Lys-Thr-[Bzl]) (a somatostatin antagonist)	Sigma
Dibutyryl cyclic AMP, sodium salt	Sigma
Epidermal growth factor	Sigma & ICI
Flurbiprofen	Boots Co PLC
Histamine dihydrochloride	Sigma
3-isobutyl-1-methylxanthine (IBMX)	Sigma
Nordihydroguaiaretic acid (NDGA)	Sigma
1-oleoyl-2-acetylglycerol (OAG)	Sigma
12-0-tetradecanoylphorbol-13-acetate (TPA)	Sigma
Zymosan A	Sigma

A2 Preparation of media.

A2.1 Preparation of Eagle's Minimum Essential Medium.

The medium was purchased in powdered form from Sigma and contained the following components (final concentration). 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 (0.25mM), L-Valine (0.4mM), Choline chloride (7.0µM), Folic acid (3.0µM), Myo-inositol (0.01mM), Niacinamide (8.0µM), D-Pantothenic Acid Ca (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 Na (0.001% w/v).

The powdered medium was fully dissolved in 1000ml of double-distilled water whilst stirring at room temperature. $NaHCO_3$ (25mM) and HEPES (20mM) were added whilst stirring. This medium was warmed to 37°C, gassed with 95% O_2/5% CO_2 for 30 minutes and the pH adjusted to 7.4.

A.2.2 Composition of Stock Solutions of Krebs-Ringer Bicarbonate Medium (mM).

The following salts were added to double-distilled water and mixed well prior to storage at 2-8°C.

Salts	Physiological Composition	Na^+-free	100mM-K^+
KCl	4.5	0.7	100
$CaCl_2$	1.25	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	1
Choline HCO_3^-	-	25	-
K_2HPO_4	-	1.8	-
KH_2PO_4	-	0.2	-
Choline Cl	-	123.8	-

Na total = 148.8
K^+ = 4.5
153.3.

148.8 µg/ml

Na^+ = 53.3.
K^+ = 100.

A.3 Liquid Scintillation Counting.

A.3.1 The principle of scintillation counting.

The radioactivity of each sample obtained for the determination of aminopyrine accumulation was measured by liquid scintillation counting. The basis of this technique is that radioactivity causes the excitation of fluors which consequently emit light (fluoresce) which can be detected using a photomultiplier tube which subsequently converts the photons into an electrical pulse. The magnitude of the electrical pulse is directly related to the energy of the radioactive emission. The TRI-CARB 2600 scintillation counter (Packard) contains two counting channels which are necessary in order to differentiate between the scintillations produced by [^{14}C] and by [^{3}H]. The limits of the low and high energy channels are set by the machine depending upon the scintillation system and the degree of quenching by the samples. The limits are set so that the counting efficiency of the lower energy nuclide, [^{3}H], in the higher energy channel is reduced to zero, and the counting efficiency of the higher energy nuclide, [^{14}C], in the lower energy channel is minimised. The activity (dpm) of the higher energy radionuclide can be calculated from the sample cpm by using the counting efficiency of this radionuclide.

Equation A.3.1

$$Y = \frac{B}{E_1}$$

Y = dpm of higher energy radionuclide
B = total cpm in higher energy channel
E_1 = efficiency of the higher energy radionuclide in the higher energy channel

The counting efficiency was calculated using the method of external standards channel ratio (ESR) by producing a curve relating efficiency and ESR. This quench correction curve was produced by counting a series of standard vials containing a known amount of radioactivity of [^{14}C] or [^{3}H] with varying amounts of chloroform as a quenching agent.

The activity (dpm) of the lower energy radionuclide can be obtained from the sample cpm using equation A3.2.

Equation A3.2

$$X = \frac{A - E_2 Y}{E_3}$$

where

X = dpm of lower energy radionuclide

A = total cpm of lower energy channel

E_2 = efficiency of higher energy radionuclide in the lower energy channel

Y = dpm of the higher energy radionuclide

E_3 = efficiency of lower energy radionuclide in lower energy channel

Counting [^{14}C] aminopyrine and [^{3}H] PEG using 0.5ml protosol and 10ml econofluor, the following counting efficiencies were achieved:

[^{3}H] in lower energy channel = 23%

[^{14}C] in higher energy channel = 75%

[^{14}C] in lower energy channel = 4%

A.4 Measurement of cyclic AMP and PGE_2 content by using radioimmunoassay.

A4.1 Reconstitution and composition of reagents used in the RIANEN™ kit for radioimmunoassay of cyclic AMP.

All reagents were reconstituted as directed in the instruction manual supplied by the manufacturers.

(a) Sodium acetate buffer.
Concentrated buffer was diluted to 500ml 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%).

(b) cAMP Standard.
Lyophilised standard was reconstituted with exactly 2.0ml of double-distilled water and stored at 2-8°C. The reconstituted solution contained 5000 pmol/ml cyclic AMP, sodium acetate buffer, 0.1% sodium azide and mannitol (0.025%).

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

(d) cAMP Carrier serum.
Lyophilised carrier serum was reconstituted with 6.0ml of double-distilled water generating a solution containing carrier serum, sodium azide (0.1%) and a stabiliser in sodium acetate buffer, pH 6.2.

(e) cAMP Antiserum Complex.

Lyophilised pre-reacted first (rabbit) and second (sheep) antibody was reconstituted in 21ml of double-distilled water. The resulting solution contained the pre-reacted antibody complex, thimerosal (0.005%) and mannitol (0.025%) in sodium phosphate buffer, pH 6.0.

(f) Acetic anhydride and triethylamine.

These agents constitute the acetylation reagent when mixed together in a ratio of 2:1 (triethylamine: acetic anhydride, v/v). Both agents are supplied in vials, and are allowed to equilibrate at room temperature before use.

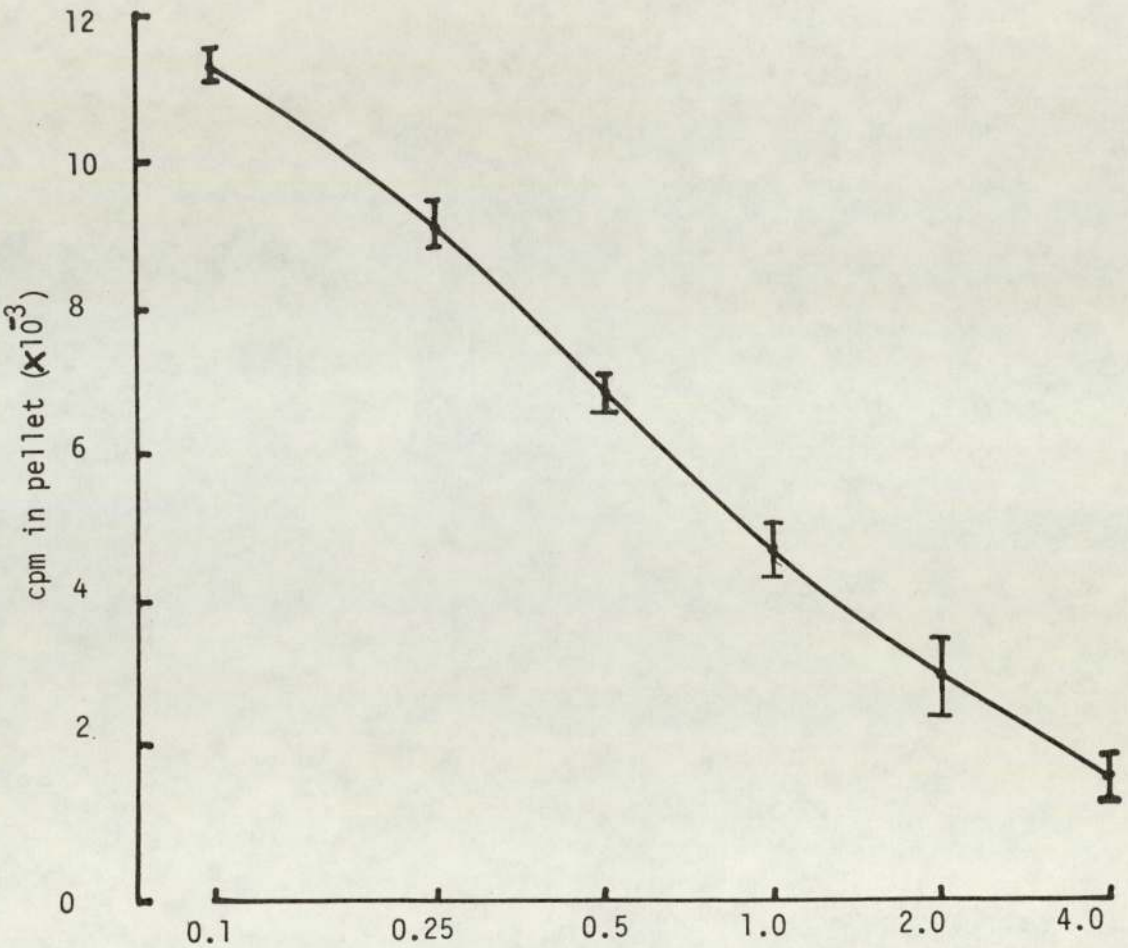
(g) cAMP Precipitator.

This consists of a precipitation enhancer (complex of non-rabbit serum and sheep anti-rabbit) in sodium acetate (50mM), EDTA (8mM) and NaN_3 (0.1%).

A 4.2

A mean standard curve (Spline function) for the radioimmunoassay of $[^{125}I]$ - cyclic AMP.

Values are means $\pm$ S.E.M. of 8 standard curves, each generated on a separate occasion. The cyclic AMP content of the samples was determined by curve-fitting with a single standard curve generated the same day that the samples were assayed.



Total counts = 22100 $\pm$ 514 (n=8) cpm
Blank = 843 $\pm$ 40 (n=8) cpm

A4.3 Reconstitution and composition of reagents used in the Prostaglandin E_2[^{125}I] radioimmunoassay.

All reagents were reconstituted as directed in the instruction manual supplied by the manufacturers.

(a) Assay buffer.
The assay buffer contained 0.9% NaCl, 0.01M EDTA, 0.3% bovine γ-globulin, 0.005% Triton X-100 and 0.05% sodium azide in 50mM-phosphate buffer.

(b) PGE_2 Standard concentrate.
A solution containing 100ng/ml of prostaglandin E_2 in organic solvent was supplied and an aliquot was diluted in assay buffer immediately before use. The standard concentrate was stored at -20°C.

(c) PGE_2 [^{125}I]-, Tracer.
The tracer concentrate contained 2μCi of [^{125}I]-PGE_2 in 0.75ml of organic solvent and was stored at -20°C.

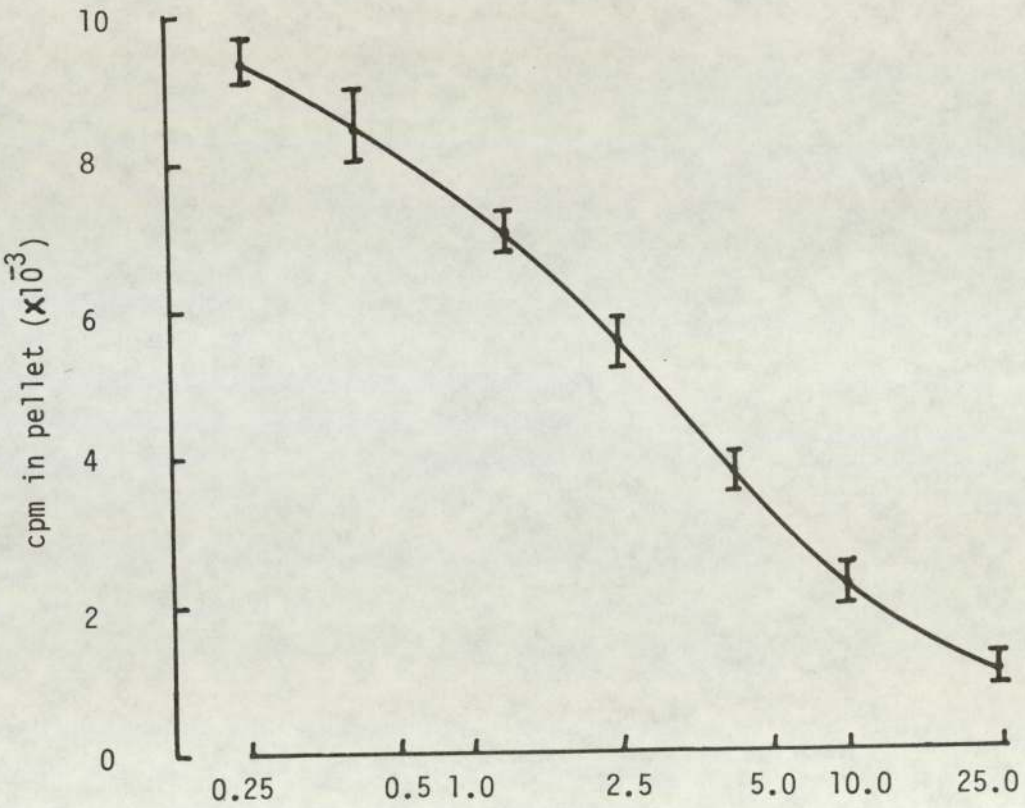
(d) PGE_2 Antibody.
Lyophilised rabbit anti-PGE_2 was reconstituted with 13ml of assay buffer and stored at 4°C.

(e) Precipitating Reagent.
A solution containing 16% polyethylene glycol (PEG 6000) and 0.05% sodium azide in 50mM-phosphate buffer (pH6.8) was provided, and was stored at 4°C.

A 4.4

A mean standard curve (Spline function) for the radioimmunoassay of $[^{125}I]$ - PGE_2.

Values are means $\pm$ S.E.M. of 5 standard curves, each generated on a separate occasion. The PGE_2 content of samples was determined by curve-fitting with a single standard curve generated on the same day that the samples were assayed.



Total counts = 17143 $\pm$ 743 (n=5) cpm
Blank = 784 $\pm$ 39 (n=5) cpm

A5 The effect of subtraction of a basal aminopyrine accumulation ratio from the stimulated value on the % inhibition of histamine-stimulated aminopyrine accumulation by TPA.

An example is given using the data from Table 3.7 (n = 6). In A there is no correction for basal, in B the basal aminopyrine accumulation ratio of 3.9 ± 0.2 has been subtracted from the histamine-stimulated value prior to calculation of the effect of TPA.

Inhibition of aminopyrine accumulation by TPA (%)		
Concentration of TPA (M)	A	B
10^{-10}	7.1 ± 1.0	7.2 ± 1.0
10^{-9}	33.6 ± 2.0	34.0 ± 2.0
10^{-8}	63.9 ± 6.0	65.7 ± 6.0
10^{-7}	87.2 ± 1.2	89.6 ± 1.2

The aminopyrine accumulation ratios in the absence of TPA were 199.9 ± 43.9 (not corrected for basal) and 196.0 ± 43.8 (corrected for basal).

It is apparent that the % inhibition by TPA is similar whether or not a basal value has been subtracted.

A6 Analysis of dose-response curve data.

In order to maximise the accuracy of determining the half-maximally effective concentration of an agent (IC_{50}) and the maximum response effected by that agent, the experimental data from dose-response curves were analysed by using the computer programme FIT (Barlow, 1983). This programme fits experimental results to the logistic expression

$$Y = M \times \left(\frac{X^P}{X^P + K^P}\right)$$

by the method of least squares

where

X = concentration of agent] experimentally
Y = response] determined values
M = maximum effect
K = IC_{50}
P = slope of the linear part of the curve relating X and Y.

This expression is similar to the hyperbola which is the logistic expression with p = 1. A graph of Y against log X is an S-shaped curve with the slope determined by the value of P and is therefore a good model for a dose-response curve.

On one occasion (Fig. 4.7) the computer programme BASEFIT (Barlow, 1983) was used to analyse dose-response data. This programme is used when there is a substantial Y value given against X = 0.

A7 Statistical Analysis.

Examples are given showing some of the statistical tests used to analyse the data in this thesis.

A7.1 Analysis of Variance (ANOVAR).

This is applied to the data from Table 3.8 on the inhibitory effect of TPA (treatments) on the stimulation of cyclic AMP content of cell suspension above basal by histamine (0.5mM) and IBMX (0.1mM) after 5 minutes incubation, and uses a two-way, random blocks design of the raw data. The mean squares are calculated by dividing the sums of squares by the degreees of freedom for each factor. The mean squares for each factor is then divided by the residual mean square to give an F-ratio. The F-ratio is then compared with tabulated F-ratios with the degrees of freedom associated with the residuals and the factor.

Variation	d.o.f	Sum Sq.	Mean Sq.	F
Experiments	4	3.57579	0.89395	12.249**
Treatments	5	14.92401	2.98480	40.899**
Residuals	20	1.45953	0.07298	
Total	29	19.95933		

**, $p < 0.01$

Since the F-ratio for the effect of treatments was significant, the data were analysed using Dunnett's Test (A7.2) to determine which concentration(s) of TPA had a significant effect.

A7.2 Dunnett's Test.

Dunnett's Test (Zar, 1974) compares a control mean to each other treatment mean. This is applied to the data in Table 3.8.

Group Means

(1)	(2)	(3)	(4)	(5)
1.18	1.56	1.74	2.07	2.12
100nM-TPA	10nM-TPA	1nM-TPA	0.1nM-TPA	control (Histamine plus IBMX)

Comparison	Difference	S.E.	q´	p	q0.01, v,p	q0.05, v,p	conclusion
5vs1	0.94	0.15	6.27	5	3.40	2.65	**
5vs2	0.56	0.15	3.73	4	3.29	2.54	**
5vs3	0.38	0.15	2.53	3	3.13	2.38	*
5vs4	0.05	0.15	0.33	2	2.85	2.09	Not significant

**, $p < 0.01$; *, $p < 0.05$.

Where:

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

p = no. means in the range being tested.

v = residual d.o.f. for the ANOVAR.

Thus the difference between the group means is calculated and divided by the S.E. If the resulting q´ value exceeds the appropriate tabulated value then there is presumed to be a significant difference between those 2 means. In this example, there was a significant effect of 1, 10 and 100nM-TPA on the stimulation of cyclic AMP content above basal by histamine + IBMX.

A7.3 Factorial Analysis of Variance.

This example is applied to data from Table 3.8 examining the inhibitory effect of TPA on the stimulation of cyclic AMP above basal after 5 and 30 minutes of incubation.

Variation	d.o.f.	Sum Sq.	Mean Sq.	F
Total	59	34.651		
Experiments (cell batch)	5	21.244	4.249	44.260***
Treatments:-	9	9.104	1.011	10.531**
TPA concentration	4	8.813	2.203	22.948**
Time	1	0.086	0.086	0.896 N/S
Time vs TPA concentration	4	0.205	0.051	0.531 N/S
Error	45	4.303	0.096	

***, $p < 0.025$; **, $p < 0.01$.

Factorial analysis of variance was used to separate out variation due to cell-batch, TPA concentration, time and effect of TPA concentration at 5 and 30 minutes. Although there was a significant effect of treatment with TPA ($p < 0.01$), there was no significant effect of time or of the effect of time on the action of TPA.

A7.4 Newman-Keuls Multiple Comparison Test.

Like Dunnett's Test (A7.2) this is applied to data which has a significant F-ratio for treatments from ANOVAR, and in this case the test is applied to the flurbiprofen data from Table 3.9 and compares each mean to every other group mean.

Group Means.

(1)	(2)	(3)	(4)
1.51	1.52	1.95	2.03
His + FB + TPA	His + TPA	His + FB	His

(His = histamine, FB = flurbiprofen)

Comparison	Difference	S.E.	q´	p	q0.01, v,p	q0.05, v,p	conclusion
4vs1	0.52	0.04	13.00	4	7.03	4.90	**
4vs2	0.51	0.04	12.75	3	6.33	4.40	**
4vs3	0.08	0.04	2.00	2	5.24	3.46	N/S
3vs1	0.44	0.04	11.00	3	6.33	4.40	**
3vs2	0.43	0.04	10.75	2	5.24	3.46	**
2vs1	0.01	0.04	0.25	2	5.24	3.46	N/S

Where:

$$S.E. = \sqrt{\frac{\text{residual mean square}}{n}}$$

**, $p < 0.01$

p = no. means in the range being tested

v = residual d.o.f. for the ANOVAR

conclusion: 4vs3 is not significant .˙. 10μM-flurbiprofen has no effect on control cyclic AMP levels. 4vs2, and 3vs1 are both significant .˙. TPA has a significant effect in the presence and absence of 10μM- flurbiprofen.

A7.5 **Friedman's Test** (Zar, 1980).

This test was applied to the data in Table 6.7 and is a non-parametric randomized block analysis of variance.

Values obtained within each experiment (block) are assigned ranks which are then summed for each experimental condition (group). An example is shown using the parietal cell-enriched fractions.

		Treatment		
Block	Control	EGF	Zymosan	Bradykinin
1	18.79	23.22	26.69	29.55
	(1)	(2)	(3)	(4)
2	24.03	27.91	17.72	27.28
	(2)	(4)	(1)	(3)
3	14.49	26.83	20.43	46.64
	(1)	(3)	(2)	(4)
4	18.89	27.31	20.93	42.69
	(1)	(3)	(2)	(4)
5	24.60	29.97	27.64	32.54
	(1)	(3)	(2)	(4)
Rank Sum (Ri)	6	15	10	19

$$Xr^2 = \frac{12}{ba(a+1)} \sum_{i=1}^{a} Ri^2 - 3b(a+1)$$

$$\therefore \quad Xr^2 = 11.64$$

Where: Ri = rank sum
a = groups
b = blocks
$p < 0.005$ for effect of treatments.

The effect of treatment as compared to control can then be examined using Dunnett's Test (A7.2). The standard error for Dunnett's Test is obtained by the following expression:

$$S.E. = \sqrt{\frac{ba\ (a + 1)}{6}}$$

A8. Animals.

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