INTRACELLULAR SIGNALLING IN THE HGT-1 GASTRIC CELL LINE AND IN RAT ISOLATED PARIETAL CELLS.

by

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The work presented in this thesis was undertaken to increase understanding of the intracellular mechanisms regulating acid secretion by gastric parietal cells. Investigation of the effects of protein kinase C on secretory activity induced by a variety of agents was a major objective. A further aim was to establish the sites at which epidermal growth factor (EGF) acts to stimulate prostaglandin E_2 (PGE_2) production and to inhibit acid secretion. These investigations were carried out by using the HGT-1 human gastric cancer cell line and freshly isolated rat parietal cells.

In HGT-1 cells, the cyclic AMP response to histamine and to truncated glucagon-like peptide 1 (TGLP-1) was reduced when protein kinase C was activated by 12-O-tetradecanoylphorbol 13-acetate (TPA). Receptor-binding studies and experiments in which cyclic AMP production in HGT-1 cells was stimulated by gastric inhibitory polypeptide, cholera toxin and forskolin suggested that the effect of TPA was mediated by uncoupling of the histamine H_2 receptor from the guanine nucleotide regulatory protein G_s, possibly by phosphorylation of the receptor. An involvement of protein kinase C α in this effect was suggested because an antibody to this isoform specifically prevented the inhibitory effects of TPA on histamine-stimulated adenylate cyclase activity in a membrane fraction prepared from HGT-1 cells.

Carbachol-stimulated secretory activity in parietal cells was specifically inhibited by Ro 31-8220, a bisindolylmaleimide inhibitor of protein kinase C. Thus protein kinase C may play a role in the activation of the secretory response to carbachol.

In parietal cells prelabelled with [3H]-arachidonic acid or [3H]-myristic acid, EGF did not affect [3H]-fatty acid or [3H]-diacylglycerol content. No evidence for effects of EGF on phosphatidylinositol glycan-specific phospholipase C, phospholipase A_2 or on low K_m cyclic AMP phosphodiesterase activities were found.

Key words: Parietal cell. HGT-1 cell line. Gastric acid secretion. Protein kinase C. Epidermal growth factor (EGF).

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For Penelope and Christopher

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ABBREVIATIONS.

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

ANOVAR	Analysis of variance
APR	aminopyrine accumulation ratio
BSA	bovine serum albumin
CA	carbonic anhydrase
cAMP	$3',5'$-cyclic adenosine monophosphate
DAG	diacylglycerol
DMSO	dimethylsulphoxide
ECL	enterochromaffin-like
EGF	epidermal growth factor
FFA	cis-unsaturated fatty acid
Fig.	figure
Fura-2AM	Fura-2 acetoxymethylester
GIP	gastric inhibitory polypeptide
GTP	guanosine triphosphate
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	concentration of agent producing a half-maximal inhibitory effect
IP_3	inositol 1,4,5-trisphosphate
kDa	kilodalton (dalton = $1/12$th of the mass of 1 atom of nuclide ^{12}C)
M. Wt.	molecular weight
OAG	1-oleoyl 2-acetyl glycerol
PBS	phosphate buffered saline
PDBu	phorbol 12,13-dibutyrate
PGE_2	prostaglandin E_2
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cyclic AMP-dependent protein kinase (protein kinase A)
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
RIA	radioimmunoassay
S.E.M.	standard error of the mean
SH2	*Src* homology 2
TLC	thin layer chromatography
TGFα	transforming growth factor α.
TGLP-1	truncated glucagon-like peptide 1
TPA	12-O-tetradecanoylphorbol 13-acetate
4α-TPA	4α-phorbol 12-myristate 13-acetate
VIP	vasoactive intestinal peptide

CHAPTER 1

INTRODUCTION.

The objective of this chapter is to provide a brief overview of gastric acid secretion and to identify the aims of the thesis. More detailed coverage of the background to specific topics is provided in the introduction to various subsequent chapters.

1.1   THE RAT GASTRIC MUCOSA.

1.1.1   Cell types present in the mucosa.

The mammalian, non-ruminant stomach acts as a reservoir in which fragments of food, after mixing with gastric juice, are chemically broken down to produce chyme. In the rat stomach, several distinct regions may be identified. In the non-glandular, cardiac region, which is a continuation of the orifice of the oesophagus, mucus-containing cells are predominant. Adjoining the cardia, and extending down to the pylorus and duodenum, is a glandular area which includes first the corpus, and then the antral glandular regions. These regions contain respectively, the gastric glands, in which the acid-secreting parietal cells are found, and the pyloric glands, which contain the gastrin-secreting G cells.

The surface epithelium of the gastric mucosa consists of mucus- and bicarbonate-secreting simple columnar cells which extend down to the gastric pits. The surface of the stomach is punctuated with about 100 of these pits per mm^2, each of which opens into several gastric glands. The glands are elongated and contain several types of cell (Fig. 1.1). In the neck region are found mainly mucoid cells which resemble the epithelial cells of the mucosa. It is from here that regeneration of the epithelial cells

Figure 1.1
A simplified diagram showing the location of the cell types present in a gastric gland.

gastric pit

isthmus

neck

gastric gland

base

surface mucous cells

mucous neck cells

parietal cells

endocrine cell

chief cells
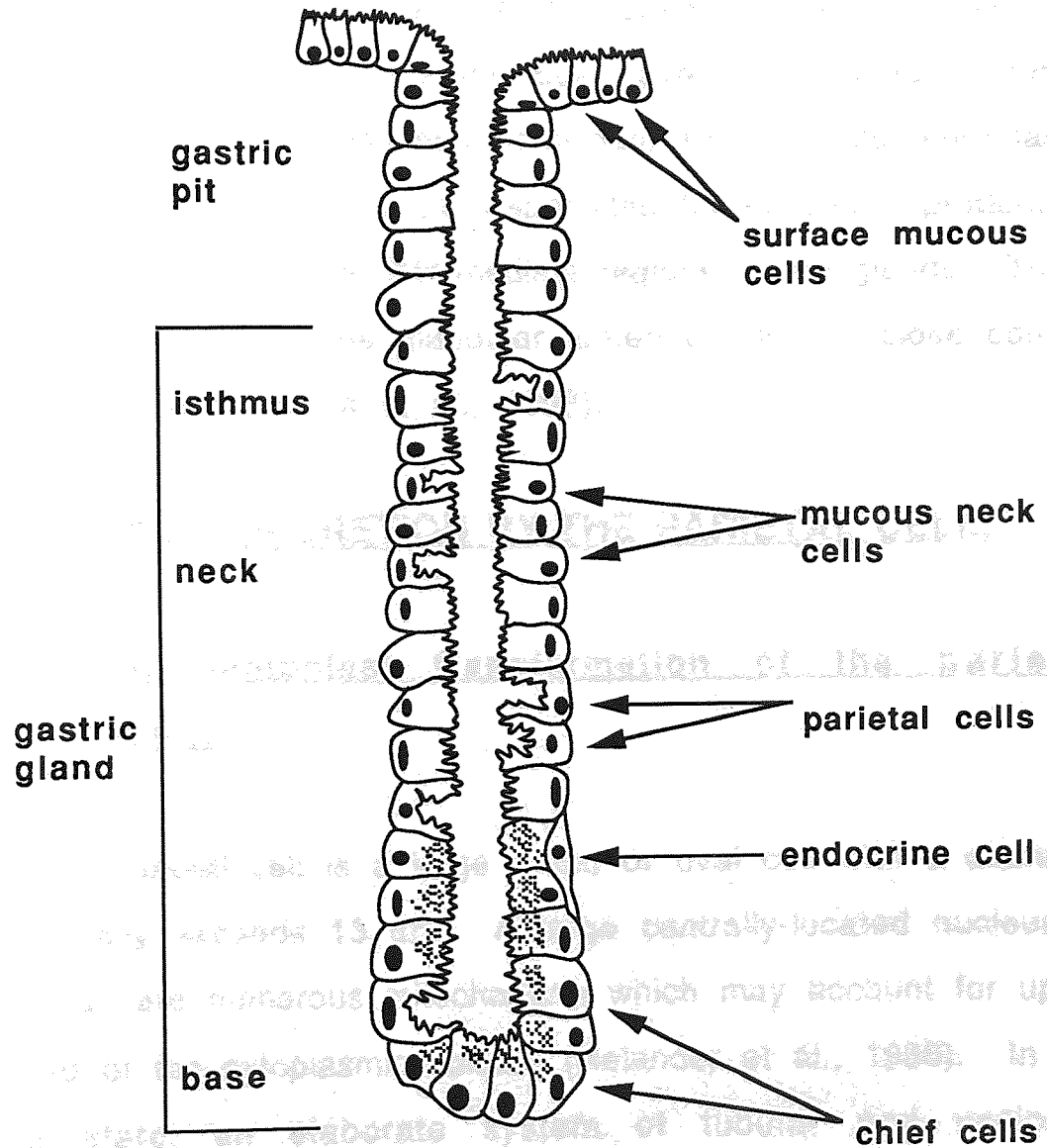
takes place. Parietal cells predominate at the neck and isthmus of the gland, and, apart from being responsible for the secretion of acid, also produce intrinsic factor which is necessary for the absorption of vitamin B_{12} (cyanocobalamin). Pepsinogen-secreting chief cells are located primarily at the base, whereas as many as nine different types of endocrine cell have been found scattered throughout the gland (Grube and Forssman, 1979). Enterochromaffin-like (ECL) cells, thought to be responsible for histamine production, are found in the deep and intermediate regions of the glands. These cells lack contact with the glandular lumen but are in close contact with parietal cells (Solcia et al., 1993).

1.2   ACID SECRETION BY THE PARIETAL CELL.

1.2.1   Morphological transformation of the parietal cell.

The parietal cell is a large round or oval cell with a diameter that normally exceeds 13 μm. A large centrally-located nucleus is evident, as are numerous mitochondria which may account for up to one third of the cytoplasmic volume (Helander et al., 1986). In the resting state, an elaborate system of tubular and vesicular structures, known as tubulovesicles, is prominent. Actively secreting parietal cells are characterized by a reduction in tubulovesicles, and the appearance of secretory canaliculi which constitute an interconnecting system of channels, lined by microvilli, that eventually open onto the apical (luminal-facing) surface of the cell (Fig. 1.2). These morphological changes, which become apparent within three minutes after stimulation (Forte et

Figure 1.2
Diagrammatic representation of the morphological changes occurring within the parietal cell following stimulation.

Resting parietal cell

Tubulovesicles

Mitochondria

Nucleus

Actively secreting parietal cell

Secretory canaliculus
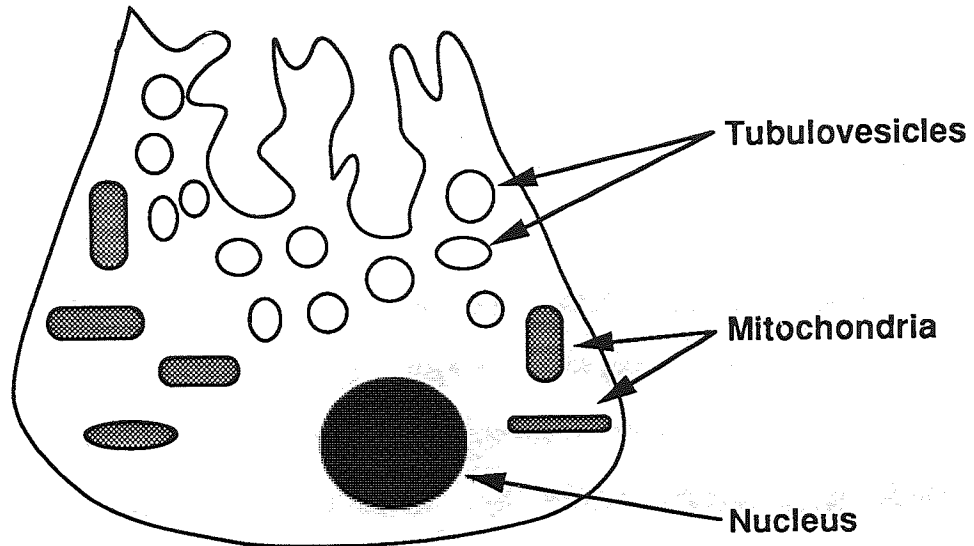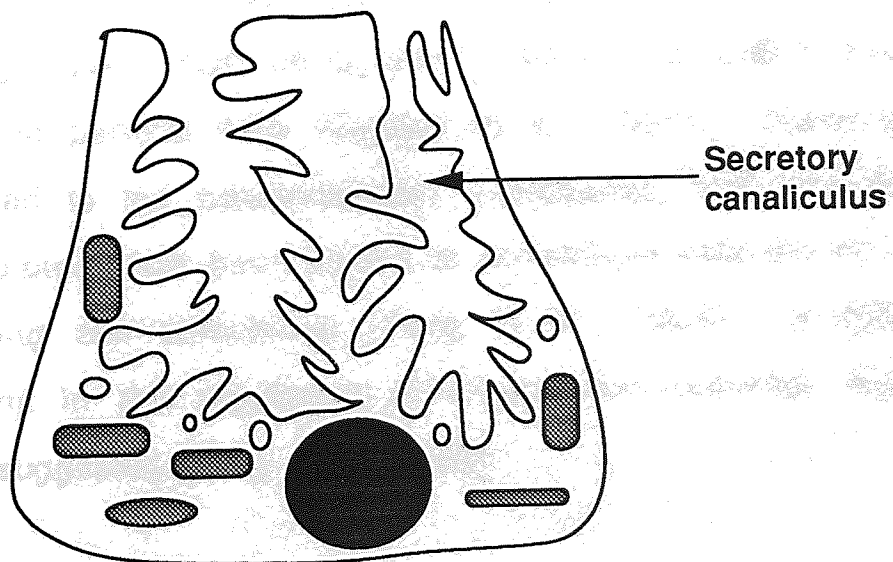
al., 1981) and result in up to a 10-fold increase in the secretory surface area (Helander et al., 1981), may be explained by an exocytotic membrane-recycling mechanism (Forte et al., 1981) which suggests that, upon stimulation, tubulovesicles containing the H^+/K^+-ATPase migrate to, and fuse with the apical membrane. In support of this, immunocytochemical studies have demonstrated the presence of the H^+/K^+-ATPase predominantly on the tubulovesicular membrane in unstimulated cells, whereas stimulated cells display immunoreactivity on the microvilli of the secretory canaliculi (Smolka et al., 1983; Mercier et al., 1989). Furthermore, studies using macromolecular tracers and freeze-fracture electron microscopy techniques have shown, following stimulation, dissemination of 8-12 nm intramembrane particles from the tubulovesicular membrane to the apical membrane (Black et al., 1980). These particles could possibly represent H^+/K^+-ATPase molecules.

A novel class of 20 - 30 kDa GTP- binding proteins, which are structurally dissimilar from the classical membrane-bound GTP-binding proteins such as G_S and G_i, have also recently been identified in rabbit parietal cells (Basson et al., 1991). These proteins are localized to the tubulovesicular membranes, and one of these, the rab2 protein, has been shown to redistribute with the H^+/K^+-ATPase following cell stimulation (Tang et al., 1992). A role for these proteins in the regulation of intracellular vesicular trafficking has been suggested (Tang et al., 1992).

1.2.2   Production of H⁺ within the parietal cell.

The parietal cell contains significant amounts of the enzyme, carbonic anhydrase which is responsible for the interconversion of H_2O and CO_2 with H^+ and HCO_3^- ions (Fig. 1.5). This enzyme is present on both the basolateral and apical membranes, but is especially prominent in the cores of the microvilli (Sugai and Ito, 1980) and thus is well situated to supply H^+ to the H^+/K^+-ATPase.

1.2.3   The H⁺/K⁺-ATPase proton pump.

1.2.3.1   Structure of the H⁺/K⁺-ATPase.

The protein directly responsible for the secretion of acid is an ATP-dependent ion pump known as the H^+/K^+-ATPase. This pump, which appears to be unique to the parietal cell, is transiently phosphorylated in the course of ATP hydrolysis (Waldenhaug et al., 1985) and is therefore classed as a member of the family of P-type (phosphorylated) ion pumps.

The H^+/K^+-ATPase is composed of a 100 kDa α-subunit which incorporates between 7 and 10 membrane-spanning regions, and a smaller glycosylated 33 kDa β-subunit which contains only 1 transmembrane domain (Fig. 1.3). The binding sites for ATP, the transported ions and all known inhibitors, are located on the α-subunit which therefore appears to be responsible for all the activities directly involved in pumping. The presence of the β-subunit is required for activity, however its precise function is unknown (Shull, 1990).

Figure 1.3
Diagrammatic representation of the gastric acid pump.

▨ α-subunit

▣ β-subunit

ATP-binding
site

Phosphorylation
site

COO^-

NH_3^+

NH_3^+

Cytoplasm

Membrane

Canaliculus

SCH 28080
SK&F 96067
SK&F 96356

Omeprazole

COO^-
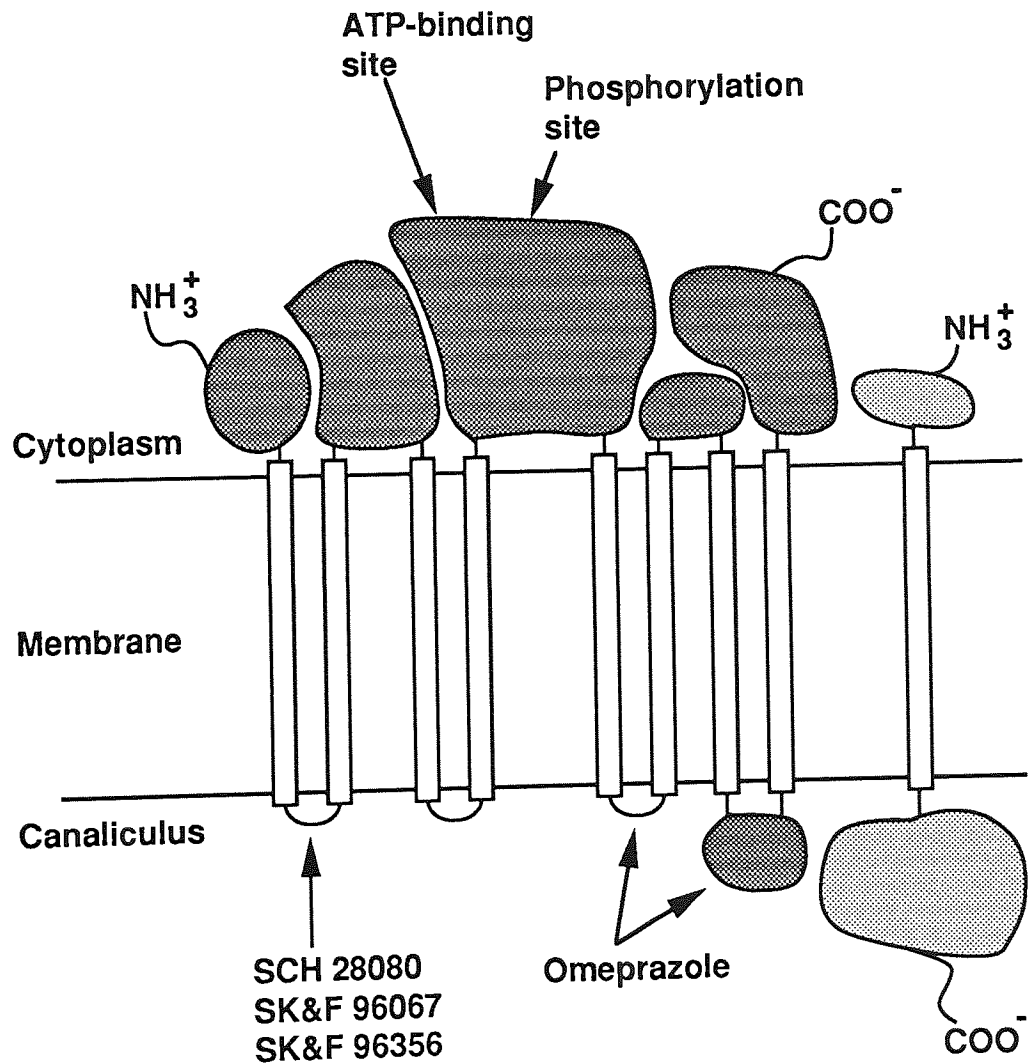
Eight transmembrane regions are shown for the α-subunit, whereas between seven and ten are thought to exist. The proposed sites of interaction of reversible and permanent inhibitors of the enzyme (see text) are shown, as are the locations of the ATP-binding and phosphorylation sites. (Helander and Keeling, 1993).

1.2.3.2   The mechanism of acid secretion.

The currently accepted model for the mechanism of acid secretion is shown in Fig. 1.4. The process of ion transport by the H^+/K^+-ATPase is sequential and begins with the binding of both H^+ and ATP to the α-subunit. Subsequent phosphorylation of this subunit, following ATP hydrolysis, induces a conformational change in the enzyme which promotes the export of H^+ into the secretory canaliculus. Upon release, the enzyme binds K^+ with high affinity at the luminal face which induces dephosphorylation of the phosphoenzyme intermediate. The return of the enzyme to its original conformation results in the transport of K^+ from the extracellular medium into the cytosol. The mechanism by which H^+ and K^+ are transported through the pump is unclear, but it is possible that the membrane-spanning domains provide a channel through the membrane that is selective for these ions.

The presence of a high concentration of HCl (0.16 M) in the canaliculus causes an osmotic efflux of water from the cytosol (Forte and Wolosin, 1987), which in turn, increases the hydrostatic pressure within the canaliculus. This results in a flow of secretions, firstly, into the lumen of the gastric gland, and then out into the stomach. In isolated parietal cells or gastric glands, some of the secreted acid remains within the secretory canaliculus, thereby enabling the accumulation of a weak base such as aminopyrine (Section 2.4.1) to act as an index of secretory activity (Berglindh et al., 1980a).

Figure 1.4
Model showing the transport of H^+ and Cl^- into the secretory canaliculus.
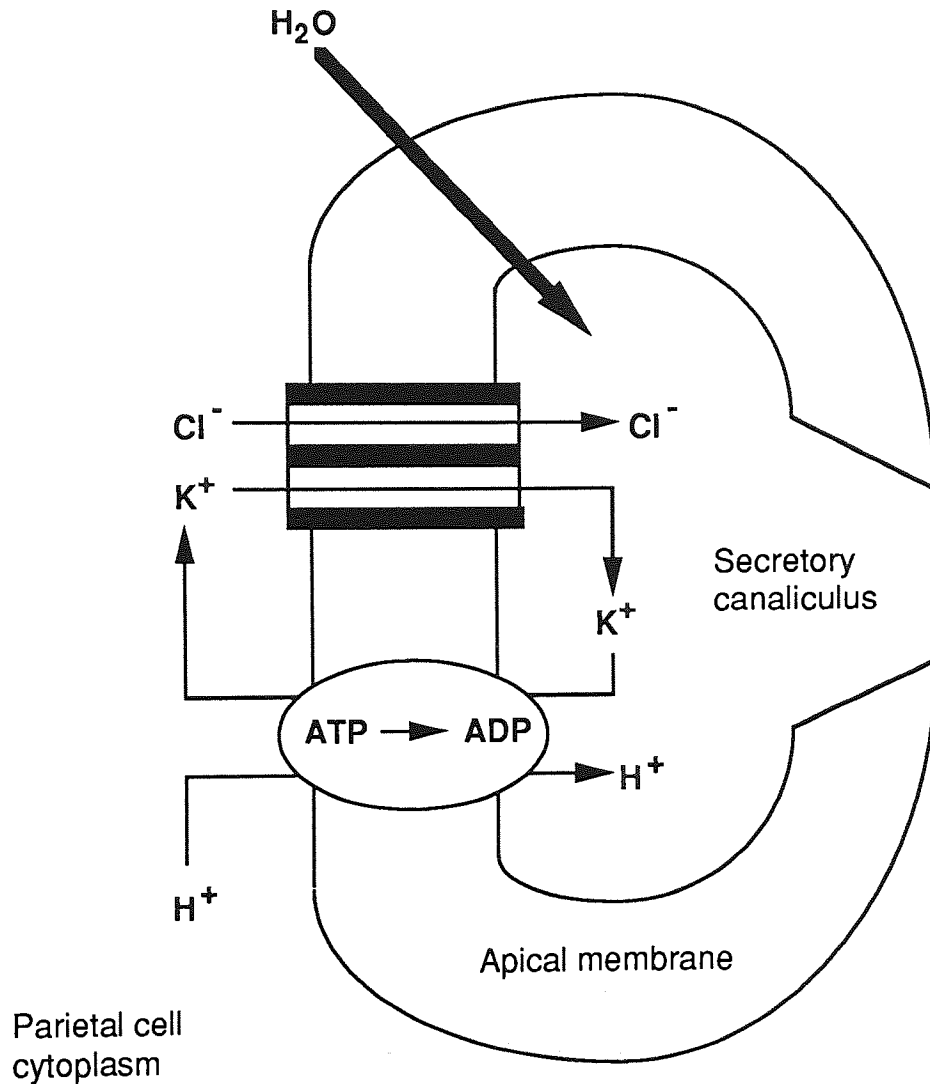
Independent conductance pathways for K^+ and Cl^- in the apical membrane are responsible for the movement of these ions from the cytosol into the secretory canaliculus. The K^+ is returned to the cytosol in exchange for the outward movement of H^+ by the activity of the H^+/K^+-ATPase. The buildup of ions in the canaliculus causes this environment to become hyperosmotic with respect to the parietal cell cytoplasm, which results in an osmotic flow of water into the canaliculus and consequently into the lumen of the gland.

The parietal cell contains large numbers of mitochondria which ensure a ready supply of ATP and thus H^+ in the cytosol. The activity of the H^+/K^+-ATPase appears to be limited solely by the supply of K^+ in the lumen of the canaliculus, and passage of K^+ from the parietal cell cytosol across the canalicular membrane is therefore required for operation of the pump. This is achieved by the presence of a conductive pathway for K^+ in the apical membrane. The presence of a Cl^- conductance in the secretory membrane, which allows electrical neutrality to be maintained, has also been demonstrated in parietal cells (Perez et al., 1989) and it has been proposed that, although functionally independent, the K^+ and the Cl^- conductances may reside within the same physical unit (Wolosin and Forte, 1985; Fig. 1.5).

Modulation of either the K^+ or Cl^- ion conductance could be responsible for regulating pump activity (Malinowska and Cuppoletti, 1988). However neither the means of activation, nor the location of these conductances in the resting parietal cell are known (Scott et al., 1993). One possibility is that they reside in the tubulovesicular membranes along with the H^+/K^+-ATPase and become activated when the vesicles fuse with the apical membrane (Takeguchi and Yamazaki, 1986). Alternatively, the conductances may be permanently located in the apical membrane and are simply inactivated in order to prevent unnecessary loss of KCl from the cell (Forte and Wolosin, 1987). A third possibility is that they are taken up into a separate class of vesicles that contain no acid pump (Im et al., 1985). The ion conductances may be regulated by phosphorylation (Im et al., 1987) and it has been suggested that the

Figure 1.5
Diagram showing ion transport across both apical and basolateral membranes of the stimulated parietal cell.
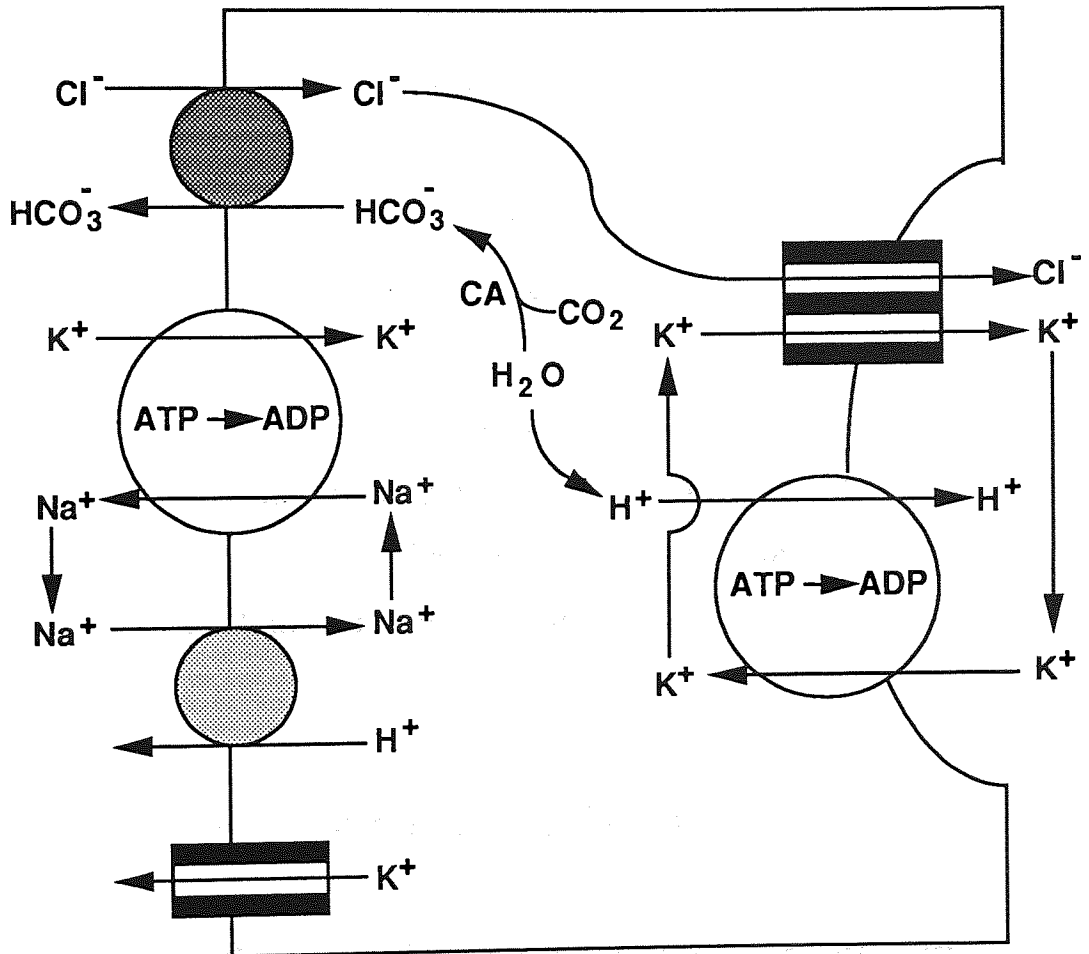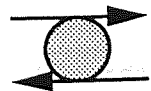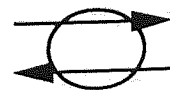
Cl⁻ conductance is regulated by a cyclic AMP-dependent protein kinase (Soumarmon et al., 1980).

In order to maintain the cytosolic pH, the secretion of H^+ must be matched by an equivalent removal of base from the parietal cell. This is largely accomplished by a Cl^-/HCO_3^- exchanger, situated in the basolateral membrane (Fig. 1.5), which becomes activated during stimulation of the cell (Muallem et al., 1988). The activity of this protein is also responsible for replenishing the Cl^- secreted across the apical membrane. In the resting state, H^+, produced as a product of metabolism, is extruded by a Na^+/H^+ exchanger (Madshus, 1988) which is also situated in the basolateral membrane (Paradiso et al., 1987; Fig. 1.5). A Na^+/K^+ ATPase provides K^+ to the interior of the cell in order to replace any which has been lost from the lumen of the secretory canaliculus (Fig. 1.5). The intracellular Na^+ generated by the activity of the Na^+/H^+ exchanger is removed by the Na^+/K^+ ATPase (Fig. 1.5).

1.2.3.3   Inhibition of the H^+/K^+-ATPase.

Two types of inhibitor are generally used to prevent proton pump activity:

(i)   The benzimidazoles such as omeprazole and pantoprazole are weak bases (pKa = 4) which, in the acid environment of the canaliculus, become activated and trapped as sulfenamides. Sulfenamides covalently react with cysteine residues on the α-subunit of the pump (Fig. 1.3), which results in a permanent inactivation.

(ii)   Imidazopyridines such as SCH 28020 (reviewed by Helander and Keeling, 1993), and the substituted quinoline derivatives SK&F 96067 (Ife et al., 1992) and SK&F 96356 (Leach et al., 1992) are potent inhibitors of acid secretion (Fig. 1.3) that, in the protonated form, competitively block the K^+-stimulation of H^+/K^+-ATPase activity.   This inactivation is therefore reversible.

1.3   STIMULATION OF ACID SECRETION.

The main secretagogues which act directly on the parietal cell are histamine, acetylcholine and gastrin (Soll, 1978).   In the rat, histamine is released from glandular ECL cells (Fig. 1.6), and also possibly from mucosal mast cells, into the interstitial fluid and local circulation and thus, exhibits a paracrine mode of action. Acetylcholine displays a neurocrine action and is released from intramural postganglionic nerve terminals located on or close to the parietal cell, whereas gastrin, released from the antral G cells into the bloodstream, acts hormonally.

1.3.1   The phases of acid secretion.

The secretion of acid is controlled immediately before, during and after eating and can be considered in terms of three closely related phases which overlap in time (Debas, 1987).

The cephalic phase of secretion which is initiated by the sight, smell, taste, or even the expectation of food, is mediated entirely by vagal activity.   Excitation of the vagus nerves results in a release of acetylcholine which, apart from its direct effect on the parietal

cell, also stimulates the release of gastrin from the antral G cells, and the release of histamine from fundic mucosal stores (Debas, 1987; Sandvik and Waldrum, 1991; Fig. 1.6). The gastric phase of secretion is initiated by moderate stretching of the antrum and by the presence in the stomach, of the products of digestion. Distension activates long vagovagal, and short intramural reflexes, which promote acid secretion via stimulation of G cells. Amino acids and dipeptides also stimulate the release of gastrin. The intestinal phase of secretion is characterized by an initial increase, followed by an inhibition of acid secretion. The initial increase is brought about, possibly, by the presence of recently consumed, non-acidified food in the duodenum which induces a release of gastrin from the duodenal G cells. Inhibition of secretion follows the entry of acidified chyme into the duodenum, and the release of enteric peptides such as gastric inhibitory polypeptide (GIP), vasoactive intestinal peptide (VIP) and cholecystokinin from the duodenal mucosa may play a role in this effect.

1.3.2   Histamine.

Histamine is stored in the gastric mucosa in both mast cells and in ECL cells, however, it is believed that only the latter source plays a significant role in the normal physiological stimulation of acid secretion in rats (reviewed by Helander and Keeling, 1993). Histamine, released from these stores interacts with an H_2 receptor (which may be defined as a histamine receptor at which the binding of histamine and related agonists is competitively inhibited by antagonists such as metiamide and cimetidine) on the surface of the parietal cell (Soll, 1978). Receptor activation results in a G-

Figure 1.6
Diagram illustrating the receptors involved in the stimulation of acid secretion and the main intracellular pathways activated.
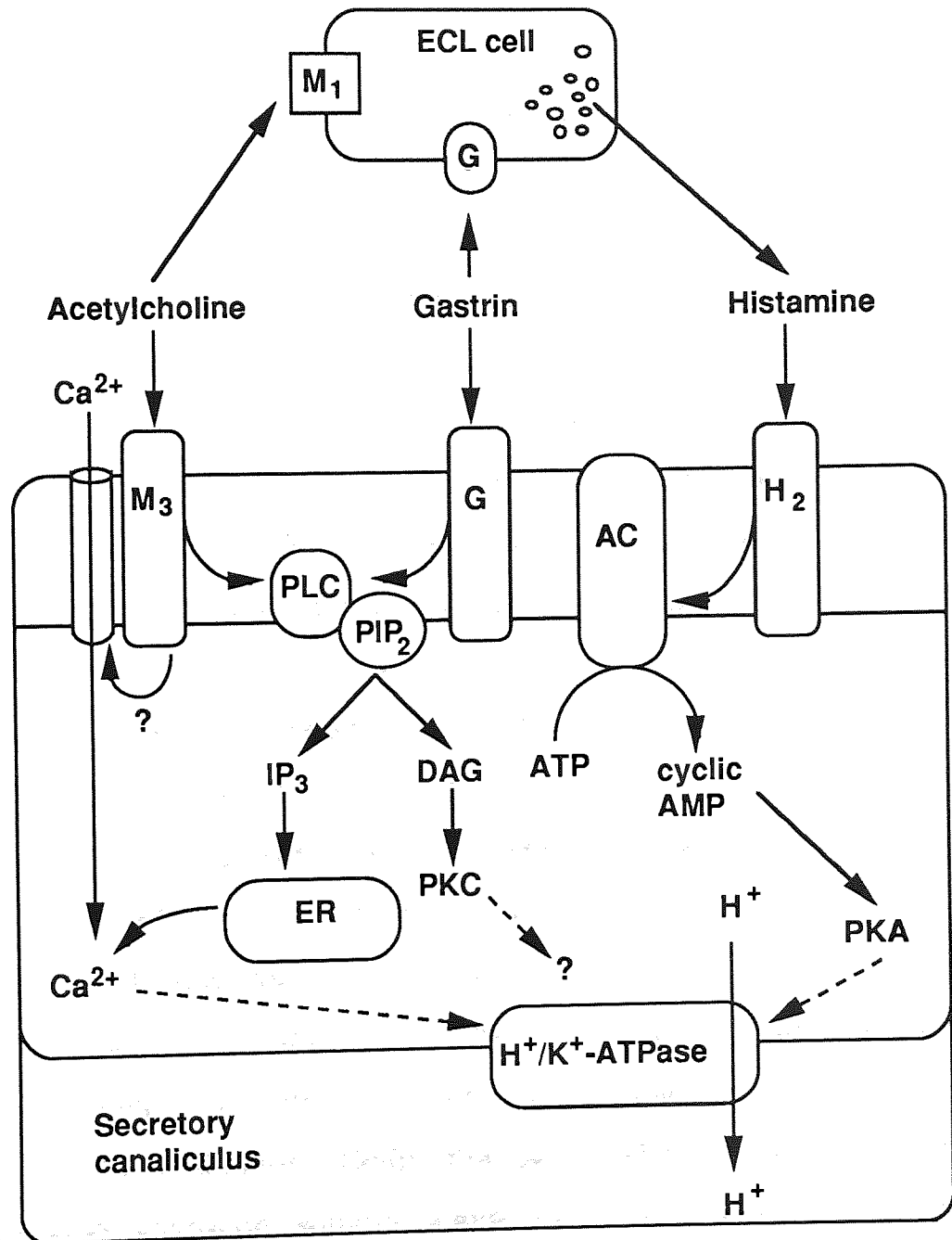
For simplicity, the involvement of GTP-binding proteins in the activation of adenylate cyclase (AC) and phospholipase C (PLC) is not shown. (ECL) enterochromaffin-like cell, (M_1 and M_3) muscarinic cholinergic M_1 and M_3 receptors respectively, (H_2) histamine H_2 receptor, (G) gastrin receptor (PIP_2) phosphatidylinositol 4,5-bisphosphate, (IP_3) inositol 1,4,5- trisphosphate, (ER) endoplasmic reticulum, (PKA) cyclic AMP-dependent protein kinase, (PKC) protein kinase C, (DAG) diacylglycerol. The muscarinic cholinergic receptors present on ECL-cells were suggested, but not proved to be the M_1 type (Helander and Keeling, 1993).

protein-mediated activation of adenylate cyclase and consequently the production of cyclic AMP (Fig. 1.6).

The activation of cyclic AMP-dependent protein kinases in parietal cells has been shown to result in the phosphorylation of four proteins with molecular weights of 27 kDa, 30 kDa, 40 kDa and 80 kDa (Chew, 1991; Hanzel et al., 1991). The largest of these has been identified as ezrin (Hanzel et al., 1991). This protein is associated with the apical membrane of the stimulated parietal cell, and may be involved in altering the cytoskeletal structure of the cell during the transition from the resting to the stimulated state. Ezrin has not been detected in epithelial cells, mucous neck cells, or chief cells isolated from the gastric mucosa (Hanzel et al., 1989).

Histamine increases the cytosolic Ca^{2+} concentration following H_2 receptor activation in individual parietal cells of rabbit gastric glands (Negulescu and Machen, 1988; Negulescu et al., 1989). Whether this increase is directly involved with the stimulation of acid secretion is not at present clear. Since the analogue of cyclic AMP, dibutyryl cyclic AMP, stimulates acid secretion without an effect on the intracellular Ca^{2+} concentration (Negulescu and Machen, 1988), the cyclic AMP pathway can clearly initiate acid secretion without elevation of intracellular Ca^{2+} being necessary.

1.3.3. Acetylcholine.

The stimulatory effect of acetylcholine on parietal cell acid secretion is mediated via activation of a muscarinic M_3 receptor

(Pfeiffer et al., 1990; Kajimura et al., 1992). Stimulation of this receptor in parietal cells has been associated with the activation of a phospholipase C, and consequently, the breakdown of phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol (Pfeiffer et al., 1989) and inositol 1,4,5-trisphosphate (Pfeiffer et al., 1990; Chiba et al., 1989). Diacylglycerol activates protein kinase C, the properties of which are reviewed in the introduction to Chapter 3. Understanding of the involvement of protein kinase C in the pathway by which carbachol stimulates acid secretion is an aim of this thesis which is examined in Chapter 5.

The rise in inositol 1,4,5-trisphosphate is coupled to an initial rapid increase in intracellular Ca^{2+} (Leonard et al., 1990) which is due to a release of Ca^{2+} from an intracellular pool (Negulescu and Machen, 1988; Fig. 1.6). This initial spike, which was shown in single, isolated rabbit parietal cells to be independent of extracellular Ca^{2+} (Negulescu and Machen, 1988; Ljungstrom and Chew, 1991), is followed by a fall to a plateau level that is maintained above the basal concentration by an influx of Ca^{2+} across the plasma membrane (Wilkes et al., 1991). The mechanism regulating this influx is not however known. A recycling of Ca^{2+} between the cytosol and the internal Ca^{2+} stores has also been demonstrated (Negulescu and Machen, 1988), which indicates the presence on the membranes of the internal stores of a Ca^{2+}-ATPase pump. A Ca^{2+}-ATPase, located in the plasma membrane is likely to be responsible for extrusion of Ca^{2+} from the cell (Muallem and Sachs, 1985).

Cholinergic stimulation of parietal cells results in the phosphorylation of three proteins with molecular weights of 28 kDa, 36 kDa and 66 kDa (Brown and Chew, 1989; Chew, 1991). The latter two proteins are also phosphorylated following direct activation of protein kinase C, whereas the 28 kDa protein is phosphorylated following activation of a Ca^{2+}-dependent protein kinase (Helander and Keeling, 1993). Thus, at least two protein kinases appear to be activated following cholinergic stimulation. An inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II almost completely inhibited carbachol-stimulated aminopyrine accumulation without affecting intracellular Ca^{2+} concentrations (Tsunoda et al., 1992), thus suggesting that the effects of Ca^{2+} are exerted partly through an activation of this kinase.

1.3.3   Gastrin.

The role of gastrin on parietal cell secretory activity has been reviewed by Hanson and Hatt (1989). The presence of a functional gastrin receptor on canine parietal cells has been demonstrated (Cabero et al., 1992), and an enhancement of secretory activity following exposure to gastrin has been shown in parietal cells isolated from dog (Soll, 1980a), rabbit (Chew and Brown, 1986) and guinea-pig (Tsunoda, 1987). The effect of gastrin on canine parietal cell secretion was unaffected by the pharmacological blockade of either histamine H_2 or muscarinic cholinergic receptors and thus, gastrin probably exerts, at least in part, a direct effect on the parietal cell. Gastrin elevated intracellular inositol trisphosphate levels (Chiba et al., 1988), and appears to mobilize the same intracellular Ca^{2+} pool as acetylcholine (Hanson and Hatt, 1989;

Chew and Brown, 1986). These effects, coupled with the fact that gastrin increased the activity of protein kinase C in a canine parietal cell membrane fraction, suggest that the action of gastrin is mediated via activation of a phospholipase C.

1.3.5   Truncated glucagon-like peptide-1.

Glucagon-like peptide-1 (GLP-1) is one of two glucagon-like peptide sequences found in the C-terminal portion of mammalian proglucagon. Only a fraction (approximately 40 %) of the total GLP-1 produced by human and rat small intestine however occurs as the entire GLP-1 sequence (proglucagon 72 - 108). The rest is accounted for by a fragment which is produced by the removal of the first six amino acid residues from the N-terminus which results in a truncated form of the peptide, known as TGLP-1 (proglucagon 78 - 108).

TGLP-1 is released postprandially and has been shown, at physiological concentrations, to inhibit pentagastrin-stimulated acid secretion _in vivo_ in humans (Schjoldager et al., 1989). It has also been found to increase somatostatin release, and to inhibit the release of gastrin in the perfused rat stomach (Eissele et al., 1990) which supports an inhibitory function of TGLP-1 in the _in vivo_ regulation of gastric acid secretion.

In contrast to the above, a stimulatory effect of TGLP-1 on aminopyrine accumulation in an enriched preparation of rat parietal cells has been demonstrated (Schmidtler et al., 1991a and 1991b). This effect, which was not mediated via activation of an H_2

receptor, correlated with an increase in the production of cyclic AMP from the same pool as that activated by histamine (Schmidtler et al., 1991a). High-affinity TGLP-1 receptors are present in isolated rat gastric glands (Uttenthal and Blasquez, 1990). Interpretation of the above data suggests that, in vivo, a direct stimulation of parietal cells by TGLP-1 may be counterbalanced by indirect inhibitory mechanisms that are excluded from the in vitro environment (Schmidtler et al., 1991a).

1.3.6   Interactions between the main secretagogues.

Acetylcholine and gastrin have been shown to cause a release of histamine from canine ECL cells (Chuang et al., 1990) and to increase plasma histamine concentrations in the dog (Gerber and Payne, 1992) and therefore, may exert indirect stimulatory effects on the parietal cell (Fig. 1.6). It is thus likely that for both acetylcholine and gastrin, direct and indirect mechanisms of action co-exist.

Potentiation between carbachol and histamine, and between gastrin and histamine has been demonstrated in isolated canine parietal cells (Soll, 1982). Since dibutyryl cyclic AMP potentiates the actions of carbachol or gastrin (Soll, 1982), the site of interaction between the secretory pathways probably occurs distal to the production of cyclic AMP.

1.4 INHIBITION OF ACID SECRETION.

Several mechanisms operate within the stomach to inhibit, or terminate gastric acid secretion. Thus, sham-feeding of dogs has been demonstrated to inhibit pentagastrin-stimulated secretion (Preshaw, 1973), which supports the notion that vagal activity results in the release of an inhibitor of gastrin-stimulated acid secretion (Debas, 1987). Antral distension (Yamagishi and Debas, 1977) and acidification of the gastric mucosa (Konturek et al., 1975) also inhibit secretion, as does the introduction of fat or acid into the duodenum. The mechanisms involved in these responses remain unresolved (Debas, 1987), although the presence of fat in the small intestine has been shown to induce the release of gastric inhibitory polypeptide (GIP) which is a weak inhibitor of acid secretion in man (Maxwell et al., 1980). The physiological role of GIP in the regulation of acid secretion is discussed below (Section 1.4.2).

1.4.1 Somatostatin and Prostaglandin E_2.

In many respects, the actions of PGE_2 on parietal cell secretory activity resemble those of somatostatin. Thus, both PGE_2 and somatostatin have been shown to inhibit histamine-stimulated, aminopyrine accumulation, cyclic AMP content and adenylate cyclase activity in isolated parietal cells and gastric glands (reviewed by Hanson and Hatt, 1989). Furthermore, the inhibitory effects of both PGE_2 and somatostatin on histamine-stimulated aminopyrine accumulation were shown to be prevented by prior treatment of the cells with pertussis toxin (Hanson and Hatt, 1989). The above data strongly suggest that these agents exert direct inhibitory effects on

parietal cell adenylate cyclase activity, and that these effects are mediated through activation of a pertussis toxin-sensitive G-protein of the G_i class.

Apart from direct inhibition, somatostatin has also been proposed to exert indirect inhibitory effects on the parietal cell. Thus, an inhibition by somatostatin of gastrin-induced histamine release in rabbit gastric glands has also been shown (Chew, 1983).

The similarity of response and mechanism of action raises the possibility that PGE_2 mediates the effects of somatostatin, however the fact that indomethacin did not prevent the inhibitory effect of somatostatin on histamine-stimulated rabbit gastric glands (Nylander et al., 1985) suggests that this is unlikely.

1.4.2   Gastric inhibitory polypeptide (GIP).

GIP is a 43-amino acid peptide (Mol. Wt. 5105), chemically related to the secretin-glucagon family of hormones (Smith, 1983) which is released from the K-cells of the duodenal and jejunal mucosa. GIP has been reported to inhibit gastric acid secretion both in vivo (Pederson et al., 1972) and in vitro (Schepp et al., 1985), and in the isolated rat stomach was shown to cause a release of somatostatin from gastric D-cells (Schepp et al., 1983). At micromolar concentrations, GIP has been demonstrated to inhibit parietal cell histamine-stimulated adenylate cyclase activity, although this is probably not a physiological response (Schepp et al., 1985).

1.4.3   Protein kinase C.

The inhibitory effect of protein kinase C, which is the name given to a family of enzymes which can be subdivided into isoforms, on parietal cell secretory activity is a subject of this thesis and is reviewed in detail in Section 3.1.

1.4.4   Epidermal growth factor (EGF).

The inhibitory effect of EGF on parietal cell secretory activity is a subject of this thesis and is reviewed in detail in Section 6.1.

1.5   THE HGT-1 CELL LINE.

The human gastric cancer cell line (HGT-1) was originally isolated from a poorly differentiated gastric adenocarcinoma (Laboisse et al., 1982). The cells have a population doubling time of approximately 20 h, show an epithelial-like morphology, and ultrastructural studies have revealed the presence of microvilli and tight junctions (Laboisse et al., 1982). The plasma membrane of these cells expresses high conductance K^+ channels which are thought to represent the basolateral K^+ conductance that operates in parietal cells during HCl secretion (Fraser et al., 1990; Fig. 1.5). In addition, the presence of a cyclic AMP-activated Cl^- channel that may represent the apical Cl^- channel of the parietal cell has also been reported (Sandle et al., 1990).

HGT-1 cells do not secrete acid and therefore cannot be considered a complete model for the parietal cell. They do, however,

express receptors, linked to the production of cyclic AMP such as histamine H_2 and TGLP-1 receptors (Table 1.1) which are known to exert a stimulatory effect on acid secretion (Section 1.3). In particular, the H_2 receptors have been shown to be pharmacologically indistinguishable from those present in human gastric glands (Emami et al., 1983), and this cell line can therefore be considered a suitable system in which to study the interactions of drugs or regulatory agents on histamine H_2 receptor-stimulated adenylate cyclase activity. The expression of a number of other receptors coupled to adenylate cyclase in these cells (Table 1.1) allows a comparison to be made, of the effects of certain intracellular mediators, such as protein kinase C (Chapter 3), on adenylate cyclase activity following activation of different receptors. Such comparisons are useful when attempting to identify the sites of action of these mediators. Experiments with cultured cells do not require the time-consuming preparative procedures involved with experiments using freshly isolated rat parietal cells.

Table 1.1

A summary of the receptors identified on HGT-1 cells.

Receptor	Reference
Histamine H_2	Laboisse et al., 1982 Gespach et al., 1988
Truncated glucagon-like peptide-1 (TGLP-1)	Hansen et al., 1988
Gastric inhibitory polypeptide (GIP)	Gespach et al.,1984 Emami et al., 1986
Vasoactive intestinal polypeptide (VIP)	Gespach et al.,1984 Emami et al., 1986
Somatostatin	Reyl-Desmars et al., 1986, 1989

All of the above receptors are coupled, either positively (H_2, TGLP-1, GIP, VIP) or negatively (somatostatin) to adenylate cyclase.

1.6 AIMS OF THIS THESIS.

The particular aims of the work presented in this thesis were:

1.   To attempt to identify the site, in HGT-1 cells, at which protein kinase C modulates receptor-stimulated adenylate cyclase activity.   In this study, the histamine H_2 receptor was especially of interest.

2.   To identify the isoform of protein kinase C responsible for inhibiting the stimulatory effect of the activated histamine H_2 receptor on adenylate cyclase activity in HGT-1 cells.

3.   To investigate the involvement of protein kinase C in the response of parietal cells to muscarinic cholinergic receptor stimulation.

4.   To attempt to identify intracellular mediators involved with the actions of EGF in parietal cells.

CHAPTER 2

GENERAL METHODOLOGY.

2.1   INTRODUCTION.

This chapter contains information on general procedures utilised throughout this work. Procedures associated with particular experiments are described in the appropriate chapters.

2.2   PREPARATION OF SUSPENSIONS OF CELLS ISOLATED FROM THE RAT CORPUS MUCOSA.

2.2.1   Preparation of an everted stomach sac.

A fed rat (see appendix A.6) with a body weight of approximately 250 g was anaesthetised using an intraperitoneal injection of sodium pentabarbitone (Sagatal) administered at a dose of 60 mg/kg body weight. A midline incision of the abdomen was made to expose the stomach. The gastrosplenic and hepatogastric ligaments were cut in order to free the stomach from the surrounding viscera, the oesophagus was ligated and the stomach then removed by cutting both above this ligature and across the duodenum. The animal was immediately killed by rupturing the diaphragm.

The stomach was gently rinsed in ice-cold saline (NaCl, 9 g/l) and everted sacs of corpus prepared as shown in Figure 2.1 (Dikstein and Sulman, 1965). Approximately 1.5 ml of medium A (Table 2.1) containing pronase (isolated from *Streptomyces griseus*) at a concentration of 1000 PUK[1]/ml, was injected into the everted sac

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1 1 PUK unit is that which produces, after hydrolysis of casein at 40°C in phosphate buffer (pH 7.4) for 10 min, followed by precipitation of excess protein in trichloroacetic acid, an absorbance of 1.0 at 660 nm with Folin and Ciocalteu's reagent.

Figure 2.1
Preparation of an everted sac of rat corpus.

1. Ligate oesophagus and remove stomach

2. Ligate along the border of the fundus and the antrum (defined as one third of the distance from the pylorus to the glandular/ non-glandular border). Remove the non-glandular region.

Non-glandular region

Cut

Glandular region

Cut

Exposed corpus mucosa
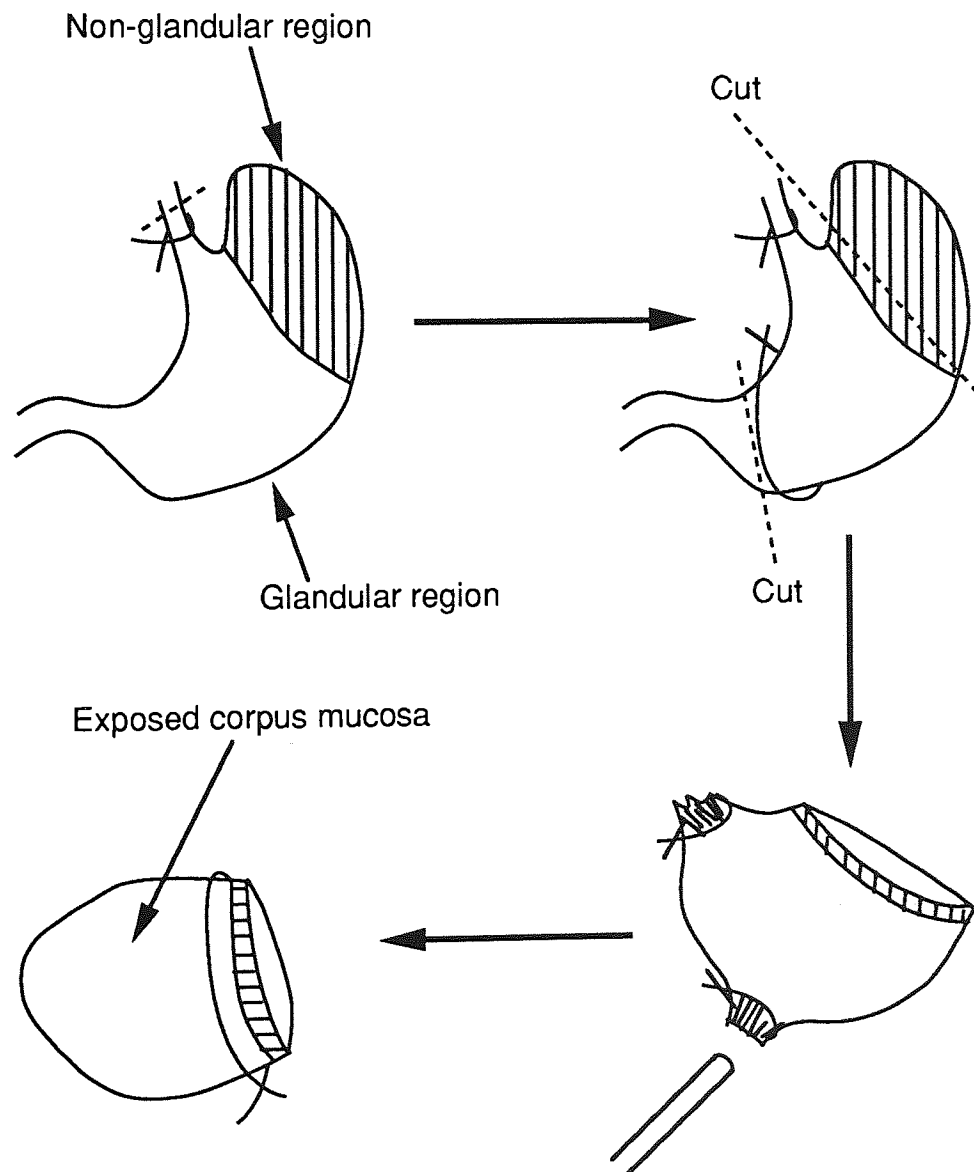
4. The corpus mucosa is rinsed in ice-cold saline to remove the food. The open end of the sac is then ligated and sealed.

3. The corpus is everted using a blunt glass rod.

Table 2.1

Composition of media used for isolation, incubation and enrichment of rat parietal cells.

The additions specified below were made to Eagle's Minimum Essential Medium (Appendix A.2.1) containing $NaHCO_3$ (25 mM) and HEPES (20 mM), and the pH then adjusted to 7.4. Media A, B and B′ were prepared not more than 24 h before use and were stored refrigerated in an atmosphere of 95 % O_2/5 % CO_2. Medium C was always made up from its components on the day of use.

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

using a 26-gauge hypodermic needle. Two sacs normally provided sufficient cells for a single experiment if an unenriched parietal cell preparation (Section 2.2.2) was being used, whereas four sacs were required for experiments with enriched parietal cells (Section 2.2.3).

2.2.2   Preparation of an unenriched suspension of parietal cells.

The procedure for preparing an unenriched suspension of parietal cells is similar to that of Trotman and Greenwell (1979) which itself is a modification of the methodology developed by Lewin et al. (1974). Everted stomach sacs were placed in a polyethylene bottle containing 40 ml of medium A (Table 2.1) which had been prewarmed to 37°C. The sacs were incubated for 30 min at the same temperature in a shaking water bath (60 cycles/min) with continuous gassing of the space above the medium with 95 % O_2/5 % CO_2. This mixture was used throughout the isolation procedure to gas the cell suspension. The sacs were removed, blotted free from medium A and transferred to 20 ml of medium B (Table 2.1) contained in a 50 ml plastic beaker which was then covered with plastic film.

The sacs in medium B were incubated for 30 min at room temperature with continuous gassing of the space above the medium, and gentle stirring with a magnetic follower running at 150 RPM. Cells released into the medium were filtered through nylon mesh (150 μm pore size, Sericol Group Ltd., London) into plastic centrifuge tubes, followed by centrifugation at 15°C for 5 min at

100 x g. The supernatant was discarded and the cell pellet carefully resuspended, using a plastic transfer pipette (L.I.P. (Equipment and Services) Ltd., Shipley, W. Yorks), in 10 ml of fresh medium B. This cell suspension was then stored at 37°C with continuous gassing and gentle shaking at 60 cycles/min. The sacs were incubated for a further two hours with alternating incubations in medium A and medium B every 30 min, the effect of which was to make exposure to EDTA intermittent. The harvested cell fractions were pooled and centrifuged at 100 x g at 15°C for 5 min and the resulting cell pellet resuspended in 10 ml of the appropriate incubation medium prior to a second centrifugation step and final resuspension in the same medium.

This procedure produced an average of $(7.8 \pm 0.4) \times 10^7$ cells/stomach (n = 12 cell batches) of which 20.2 ± 0.99 % were parietal cells (n = 12 cell batches).

2.2.3 Preparation of a suspension of cells enriched in parietal cells.

2.2.3.1 Techniques used for enrichment.

Parietal cells are the largest and also one of the least dense of the gastric cells due to the high proportion of membranes present (Soll, 1981). These characteristics are therefore often exploited, either individually, or in combination during enrichment procedures. Examples of enrichment procedures commonly used are shown in Table 2.2.

Table 2.2

Comparison of the parietal cell content of preparations of gastric mucosal cells enriched using different procedures.

Procedure	Species	% parietal cells	Reference
Density gradient using percoll	Rat	82.4 ± 1.2 %	This work.
	Rat	80 - 90 %	Schepp et al., 1989.
	Rat	79 - 88 %	Ostrowski and Bomsztyk, 1989.
Density gradient using Nycodenz	Dog and rabbit	70 - 90 %	Berglindh and Sachs, 1985.
Centrifugal elutriation (using elutriator rotor)	Rat	Up to 77 %	Schepp et al., 1990.
	Dog	Up to 70 %	Chiba et al., 1988.
	Rabbit	Up to 70 %	Leonard et al., 1990.
Combination of elutriation and Nycodenz/Percoll density gradient	Dog	About 95 %	Chiba et al., 1989.
	Rabbit	About 95 %	Brown and Chew, 1987.
Unit gravity sedimentation	Dog	Up to 95 %	Major and Scholes, 1978.

The enriched fractions of parietal cells used in this work were obtained by isopycnic centrifugation of a crude cell suspension utilising a method developed by G. P. Shaw and P. J. Hanson, which was modified from that used by Sonnenberg et al. (1979). This procedure however, results in cells of light density such as histamine-containing cells co-purifying with the parietal cells (Soll and Berglindh, 1987) and therefore a second low speed centrifugation step which partially separates the larger parietal cells from other cell types (Ecknauer et al., 1981) was introduced.

Percoll is a medium composed of colloidal silica particles which are coated with polyvinylpyrrolidone (PVP) and which can form a self-generated density gradient after 10 - 30 min of centrifugation. These particles do not penetrate the cell membrane, they are non-toxic to cells and the formation of the density gradient is not associated with a substantial gradient of osmolality (Pertoft et al., 1977).

2.2.3.2 Routine preparation of a cell suspension enriched in parietal cells.

A 20 ml stock solution of iso-osmotic Percoll was prepared by adding 2 ml of 10 x concentrated Eagle's Minimum Essential Medium to 18 ml of Percoll. Following the addition of $NaHCO_3$ to a final concentration of 25 mM, the solution was gassed for 15 min at room temperature with slow stirring and the pH then adjusted to 7.4. 6 x 3 ml aliquots of this stock were placed in separate polycarbonate tubes (10 ml capacity) just prior to use. A suspension of unenriched cells in medium B´, prepared as described in Section

2.2.2 was centrifuged at 15°C for 5 min at 100 x g and the resulting pellet then resuspended in 28 ml of medium C (Table 2.1). The cell concentration in this medium was normally 4 - 5 x 10^6 cells/ml. 4.5 ml aliquots of this suspension were then added to each of the tubes containing 3 ml of iso-osmotic Percoll to generate a cell suspension in 36 % ($^v/_v$) Percoll. The tubes were capped, gently inverted two or three times to ensure thorough mixing and centrifuged at 4°C for 13 min at 30,000 g_{av} using a 20° angle rotor in an MSE Superspeed 50 centrifuge. The top 1.5 ml of medium which contained the low-density (approximately 1.03 g/ml) fraction of cells was removed from each tube and added to 8.5 ml of medium B′ in a plastic centrifuge tube. The cells were gently dispersed in this medium before centrifugation at 100 x g for 5 min at 15°C. The supernatant, containing Percoll and cell types of a similar density to, but smaller than parietal cells was discarded and the cells then resuspended in an appropriate volume of supplemented tissue culture medium (Section 2.2.4) at a concentration of 2 x 10^6 cells/ml.

The above procedure produced cell fractions containing 82.4 ± 1.2 % parietal cells (n = 20) with a yield of (3.5 ± 0.5) x 10^6 cells/stomach (n = 20).

2.2.4   Short-term culture of an enriched preparation of parietal cells.

Short-term culture of a suspension of canine parietal cells following elutriation vastly improved the response to subsequent stimulation by secretagogues (Soll et al., 1986). Similar results were obtained with rat parietal cells after enrichment on Percoll

(Hatt, (1988). Ph.D. thesis). Consequently, enriched cells were resuspended at a concentration of 2 x 10^6 cells/ml in medium B′ which was supplemented with gentamicin (50 µg/ml) and foetal calf serum (5 % $^v/_v$). The cell suspension was then incubated for 2 h at 37°C with shaking (60 cycles/min) and continuous gassing of the space above the cells in a capped, 25 ml polycarbonate conical flask. The cells were gently dispersed at least once every 30 min by using a plastic transfer pipette. At the end of this recovery period, the cells were washed twice by centrifugation at 100 x g for 5 min at 15°C. A cell count was performed after the first spin, along with an assessment of the ability of the cells to exclude trypan blue. An appropriate volume of the incubation medium to be used for the particular experiment was then added to the final cell pellet and the cells resuspended at a predetermined concentration.

2.2.5   Identification of parietal cells and assessment of viability.

The parietal cell diameter normally exceeds 13 µm and they were therefore the largest cell type present. Under the light microscope (x 400) they may also be distinguished from other cells by their centrally-located nucleus and by a granular appearance which stems from the large numbers of mitochondria in the cytoplasm.

The ability of parietal cells to exclude the dye, trypan blue, was used as an indicator of structural integrity. A 20 µl aliquot of the cell sample in the appropriate incubation medium was mixed with a 20 µl aliquot of trypan blue dissolved to a concentration of 4

mg/ml in saline (NaCl, 9 g/l). Cells were counted using a haemocytometer (E. Leitz, Wetzlar) under the light microscope (x 400). A minimum of 200 cells were counted on each occasion and the percentage of cells excluding trypan blue was calculated. Only cells which showed no sign of taking up the dye were considered viable. The cell preparation was not used if less than 85 % of cells excluded trypan blue.

In an unenriched preparation of cells (Section 2.2.2), 94.1 ± 1.04 % of parietal cells (n = 10 batches of cells), were considered viable. After enrichment (Section 2.2.3.2) and following a 2 h recovery period (Section 2.2.4), 87.8 ± 1.04 % of parietal cells (n = 10) retained the ability to exclude trypan blue.

It appears that the enrichment procedure and subsequent 2 h preincubation may have caused some membrane damage in a small number of cells. Thus, a significantly smaller number of parietal cells (P < 0.05 by paired t-test, n = 10) were able to exclude trypan blue following enrichment compared to cells present in an unenriched preparation.

2.3   THE HGT-1 CELL LINE.

2.3.1   Culture of HGT-1 cells.

All the operations described below were carried out in a Gelaire BSB 4A laminar flow sterile cabinet which had been sprayed with a solution of 70 % alcohol/30 % double distilled water and left to run for at least 10 min prior to use.

2.3.1.1   Routine culture of cells.

HGT-1 cells were cultured in canted-neck polystyrene cell culture flasks (80 cm^2/260 ml) in 20 ml of Dulbecco's Modified Eagle's Medium (4.5 g/l glucose, without sodium pyruvate) to which L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and foetal bovine serum (5 %) were added. At 2 day intervals, the culture medium was removed from the flask and the cells washed once with 10 ml of fresh culture medium. Following removal of the wash medium, 20 ml of fresh culture medium which had been prewarmed to 37°C was then added. Incubation was in an atmosphere of 10 % CO_2 in an LEEC CO_2 incubator at a temperature of 37°C.

2.3.1.2   Passaging of cells.

When the cells had grown to confluence (approximately 2 x 10^7 cells/flask), the culture medium was removed and, in order to remove any trace of residual serum which would inhibit subsequent trypsinization, the cells were washed once with 10 ml of Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (PBS) (Appendix A.2.2). The wash medium was replaced after 20 s with 5 ml of PBS at a temperature of 37°C to which EDTA (0.001 g) and trypsin (0.005 g) had been added. Following a 2 min incubation in this medium, a sharp tap on the flask caused the cells to detach. 5 ml of culture medium was added and the cells were dispersed by resuspension. 1 ml of the resulting cell suspension was then added to 20 ml of fresh culture medium in a separate flask. The cells were washed and the medium changed as described in Section 2.3.1.1

approximately 6 h after passaging in order to remove residual trypsin and EDTA.

2.3.1.3   Freezing and subsequent storage of cells.

Confluent cells were detached as described above (Section 2.3.1.2). The resulting 10 ml of cell suspension was centrifuged at 100 x g for 3 min in a 25 ml capacity capped polystyrene container. The supernatant was discarded and the pellet washed with resuspension in 10 ml of fresh culture medium in order to remove residual trypsin and EDTA. Following a second wash and subsequent centrifugation step, the cell pellet was resuspended in 2 ml of Dulbecco's Modified Eagle's Medium containing foetal bovine serum (30 %) and DMSO (15 %). 1 ml aliquots of this suspension were placed in a 1 ml capacity cryotube and this tube then suspended over liquid nitrogen for 6 h to allow gradual cooling. The tube was then immersed in the liquid nitrogen for storage. All tubes stored in this way were clearly labelled with passage number, date of freezing and cell-type.

2.3.1.4   Thawing and seeding from frozen stock.

The cryotube containing the cells was removed from the liquid nitrogen and agitated in a water bath at 37°C to ensure a rapid thaw. After gentle resuspension, a 0.5 ml aliquot was transferred to a culture flask containing 20 ml of culture medium. The cells were then incubated as normal (Section 2.3.1.1). In order to remove traces of residual DMSO, the cells were washed after 6 h and fresh medium added as described in Section 2.3.1.1.

2.3.2   Preparations of suspensions of HGT-1 cells.

For experimental use, cells that had almost grown to confluence were washed as described in Section 2.3.1.1 and then detached by incubating for 3 min at 37°C in 5 ml of Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (PBS) (Appendix A.2.2) containing 0.02 % ($^w/_v$) EDTA. Trypsin was omitted from this medium because of the possibility that it may induce receptor damage. The detached cells in PBS/EDTA were centrifuged for 3 min at 100 x g to pellet the cells. The supernatant was discarded and the cell pellet resuspended in 10 ml of medium B' (Table 2.1). This cell suspension was again centrifuged and the pellet finally resuspended in medium B' at a concentration of 2×10^6 cells/ml.

A cell count and trypan blue exclusion test was carried out on detached cells as described in Section 2.2.5. The typical cell yield was $(2.4 \pm 0.21) \times 10^7$ cells/culture flask (n = 20) of which 98.3 ± 0.2 % were considered viable (n = 20).

For the experiment, 1.5 ml aliquots of the above cell suspension were transferred to polyethylene scintillation vials containing the required secretagogues, agents or vehicles as appropriate. These vials were gassed with 95 % O_2/5 % CO_2 and capped prior to incubation at either 20°C or 37°C for the required time in a water bath shaking at 60 cycles/min.

2.4   TECHNIQUES USED TO ASSESS CELLULAR RESPONSE.

2.4.1   The aminopyrine accumulation ratio.

Aminopyrine is a weak heterocyclic base (Fig. 2.2) with a pKa of 5.0 which exists almost entirely in an unionised, lipid soluble form at physiological pH. As such, it is able to diffuse freely across the plasma membrane of the parietal cell and enter the membrane-bound spaces within. The lipid-solubility of the charged, protonated species is low and therefore aminopyrine becomes trapped within acidic spaces upon ionisation (Fig. 2.2). These properties allow aminopyrine to be used as a probe, the accumulation of which depends upon the average pH within the secretory canaliculi and thus, the sequestration of acid within this region. As aminopyrine accumulates only within the acidic spaces of parietal cells, it can be used as an indicator of parietal cell response in both unenriched and enriched preparations of parietal cells (Berglindh et al., 1976). It must be remembered, however, that the accumulation of aminopyrine within the parietal cell reflects sequestration of acid and not the rate of H^+ secretion and therefore can only provide an index of secretory activity (Equation 2.1).

Aminopyrine accumulation is normally expressed as the aminopyrine accumulation ratio which is determined by dividing the concentration of intracellular aminopyrine by the concentration of aminopyrine in the medium (Equation 2.1).

Figure 2.2

Sequestration of aminopyrine within the acidic spaces of the parietal cell.
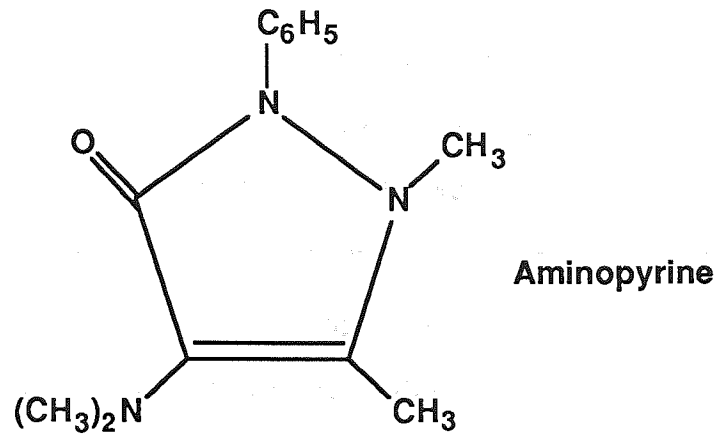
Aminopyrine

Aminopyrine (AP) is a weak base (pKa = 5) which is membrane impermeable in the protonated form. It therefore becomes trapped within the secretory canaliculi as shown in diagrammatic form below.
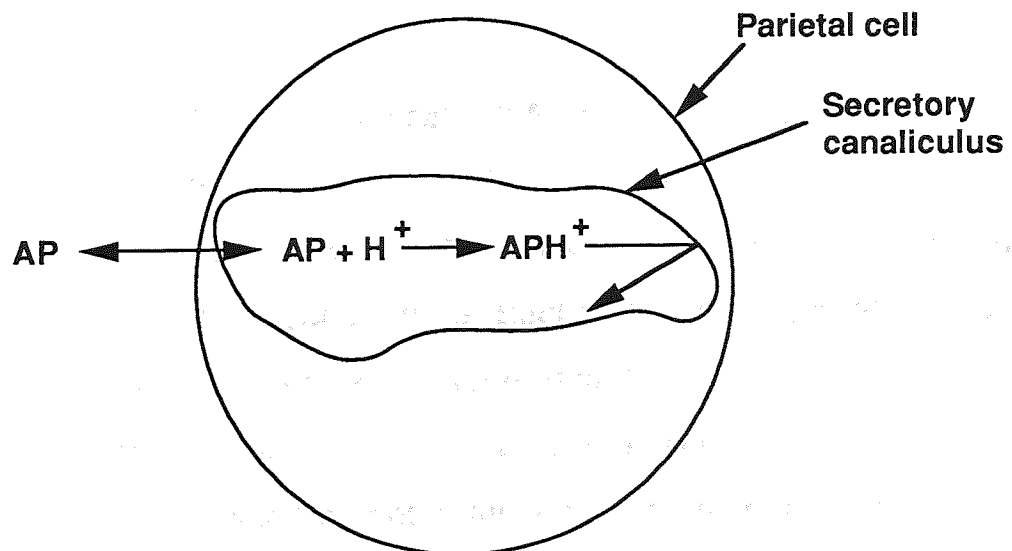
The aminopyrine accumulation ratio (APR) can be calculated from the following equation:

Equation 2.1

$$APR = \frac{\text{Intracellular [AP]}}{\text{Extracellular [AP]}} = \frac{1 + 10^{(pKa - pH_{cell})}}{1 + 10^{(pKa - pH_{medium})}}$$

where:
pKa is the dissociation constant for aminopyrine (AP).
pH$_{cell}$ is the pH inside a defined acidic compartment within the cell.
pH$_{medium}$ is the extracellular pH.

2.4.1.1   Determination of the aminopyrine accumulation ratio.

20 ml polyethylene incubation vials were prepared, containing [^{14}C] aminopyrine (0.1 μCi/ml, 0.9 μM), [^{3}H] polyethylene glycol (0.4 μCi/ml) and appropriate secretagogues and agents. Equal volumes of vehicles were added and the combined volume of secretagogues, agents and vehicles was then made up to 100 μl using saline (NaCl, 9 g/l). 1.5 ml aliquots of cell suspension at a concentration of 2 - 5 x 10^6 cells/ml were then added to each vial and the contents gassed for 5 s with 95 % O_2/5 % CO_2 whilst gently swirling to ensure thorough mixing. The vials were immediately capped and incubated at 37°C for 30 min in a water bath with continuous shaking (120 cycles/min).

After incubation, duplicate 0.5 ml aliquots of cell suspension were removed from each vial, placed in separate 1.5 ml microfuge tubes (L.I.P. Ltd., Shipley, W. Yorks, England) and immediately subjected to centrifugation in a Beckman microfuge at 10,000 x g for 30 s. A 50μl sample of supernatant from each microfuge tube was then transferred to a polyethylene scintillation vial and the remainder of the supernatant removed from each tube by aspiration. The surface of the cell pellet and the inside of the tube were washed by adding 0.5 ml of medium B′ at a temperature of 4°C to each tube and then centrifuging at 10,000 x g for 10 s. The supernatants were aspirated and the cell pellet was dissolved in 0.5 ml of NaOH (1 M) by allowing to stand at room temperature overnight. The contents of the microfuge tube were mixed by vortexing and a 450 μl aliquot transferred to a polyethylene scintillation vial containing 10 ml of

OptiPhase Hi-Safe II for determination of the radioactivity of the samples by liquid scintillation counting (Appendix A.3).

The aminopyrine accumulation ratio was calculated using the formula:

$$APR = \frac{A - \dfrac{B\,C}{D}}{C\,E}$$

Where:

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

B = pellet [^{3}H] polyethylene glycol (dpm)

C = supernatant [^{14}C] aminopyrine (dpm/μl)

D = supernatant [^{3}H] polyethylene glycol (dpm/μl)

E = volume of intracellular fluid (μl)

Since the total aminopyrine in each pellet includes both that within the cells and that in the extracellular space, a correction needs to be applied for the latter. To do this, [^{3}H] polyethylene glycol (approximate molecular weight = 4000 Da) was used to estimate the volume of extracellular fluid. In order to estimate the volume of intracellular fluid, the finding that 2 μl of intracellular fluid is associated with 1 mg dry weight of cells (Atwell, (1990). Ph.D. thesis) was used. The dry weight of the cell pellet was determined by centrifuging duplicate 0.5 ml aliquots of cell suspension at 10,000 x g for 1 min in a Beckman microfuge. The tip of the microfuge tube containing the pellet was removed and dried in an oven at 90°C for 24 h, after which the dried pellets were weighed on a microbalance (Mettler). Dry weights were corrected for

dilution of the cell suspension upon addition to incubation vials and for the dissolved salts and albumin present in the incubation medium. A comparison of the aminopyrine accumulation ratios obtained in this work with those from other workers is shown in Table 2.3.

2.4.2   Testing of different batches of pronase.

The pronase used to disperse gastric mucosal cells from the fundic mucosa was supplied as a crude mix of enzyme activities which included a variety of proteases, and trypsin-like activity. This crude mixture resulted in considerable variation in the proteolytic activity of different batches and thus, to avoid the possibility of receptor damage due to excessive proteolytic action, each new batch of pronase was tested before purchase.

Testing involved isolation of cells using the pronase under test and subsequent stimulation of an unenriched preparation of parietal cells with either 0.5 mM histamine plus 0.1 mM IBMX, or 1 mM dibutyryl cyclic AMP. The aminopyrine accumulation ratio measured in response to stimulation by histamine plus IBMX was compared with the response obtained following stimulation with dibutyryl cyclic AMP using the formula:

$$\frac{\text{Response to 0.5 mM histamine} + 0.1 \text{ mM IBMX}}{\text{Response to 1 mM dibutyryl cyclic AMP}} \times 100$$

A ratio of 70 % or less indicated that the pronase was causing some damage to the histamine receptor and the batch was rejected. A

Table 2.3
A comparison of the aminopyrine accumulation ratios obtained with isolated parietal cell preparations stimulated with either histamine or carbachol.

Species	Basal	Secretagogue	Aminopyrine accumulation ratio	% parietal cells	Reference
Rat	2.2 ± 0.3	0.5 mM histamine + 0.1 mM IBMX	125 ± 18 (n=10)	> 80	This work.
	1.7 ± 0.2	0.1 mM carbachol	4.5 ± 0.3 (n=4)	19-22	
Rat	3.1 ± 0.4	0.1 mM histamine + 0.1 mM IBMX	42 ± 4	77	Schepp et al., 1990.
		0.1 mM carbachol	9.2 ± 0.7		
Guinea-pig	2.3 ± 0.4	0.1 mM histamine	7.3 ± 0.9	Gastric glands	Kajimura et al., 1990.
		0.1 mM carbachol	3.3 ± 0.6		
Dog	6.2 ± 0.5	0.1 mM histamine	30 ± 2	70	Chiba et al., 1988.
		0.1 mM carbachol	97 ± 8		
Rabbit	16.4 ± 1.8	0.1 mM histamine	73 ± 12	Gastric glands	Berglindh et al., 1980b.
		0.1 mM carbachol	43 ± 6		

summary of the results obtained after testing of different batches is shown in Table 2.4.

2.4.3 Measurement by radioimmunoassay (RIA) of the cyclic AMP content of cells and homogenates.

2.4.3.1 Theory of radioimmunoassay.

Radioimmunoassay (RIA) combines the specificity of the immune reaction with the sensitivity of radioactive techniques. It is used for the quantitative determination of hormones and other biologically active substances 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 number of antibody binding sites. Thus, an inverse relationship exists between the level of radioactivity associated with the antibody-antigen complex and the amount of unlabelled antigen in the sample. In order for the amount of bound labelled antigen to be measured however, separation of the bound from the free antigen is first required. This was achieved in this work using a pre-reacted primary antibody/secondary antibody complex which was removed by precipitation with polyethylene glycol and centrifugation.

The cyclic AMP content of both, whole cell preparations and homogenates of HGT-1 cells were determined by using the acetylated procedure of the RIANEN^{TM} radioimmunoassay kit (Du Pont). Prior acetylation of cyclic AMP in the 2´0 position was carried out to enhance the affinity of cyclic AMP for its antibody

Table 2.4

Comparison of the response of parietal cells isolated using different batches of pronase to stimulation by either histamine plus IBMX or dibutyryl cyclic AMP.

Pronase batch number	Aminopyrine accumulation ratio		Ratio:	Batch: accepted/rejected
	0.5 mM histamine + 0.1 mM IBMX	1 mM dibutyryl cyclic AMP	Response to histamine + IBMX / Response to dibutyryl cyclic AMP	
858784OK	42	76	55.3 %	Rejected
0615180L	129	174	74.1 %	Accepted
4167570M	72	75	96.0 %	Accepted

(Steiner et al., 1972). This procedure increases the sensitivity of the assay (Frandsen and Krishna, 1976) and thus, allows the accurate determination of very low levels of cyclic AMP in a sample without the need to further purify or concentrate the sample.

2.4.3.2 Determination of the cyclic AMP content of HGT-1 cells and homogenate fractions.

Following incubation of either whole cells or homogenates, cyclic AMP was extracted by vortexing in the presence of ethanol at a final concentration of 50 % ($^V/_V$). 0.4 ml aliquots of the above mixture were then transferred to microfuge tubes and subjected to centrifugation at 10,000 x g for 1 min in a Beckman microfuge in order to pellet cellular debris. 160 μl aliquots of supernatant from extracts of whole cell suspensions (original cell concentration during incubation, 1.5 x 10^6 cells/ml), or 350 μl aliquots from extracts of homogenates (approximately 1.5 x 10^6 broken cells/ml present during the incubation) were placed into polypropylene test-tubes (Luckham Ltd, Burgess Hill, Sussex, U. K.) and the tubes capped and stored overnight at a temperature of -18°C. After thawing and evaporation to dryness in a vacuum oven at 40°C, the residue was reconstituted in 100 μl of sodium acetate buffer (pH 6.2), mixed by vortexing with 5 μl of acetylation reagent (2 volumes of triethylamine mixed with 1 volume of acetic anhydride) and then incubated at room temperature for 3 min. A further 900 μl of sodium acetate buffer was added to each sample which was again mixed by vortexing.

A 40 pmol/ml cyclic AMP standard was prepared by serial dilution with sodium acetate buffer of a 5000 pmol/ml cyclic AMP stock. 10 µl of acetylation reagent was then added to a 200 µl aliquot of the 40 pmol/ml solution and following a 3 min incubation at room temperature, addition of a further 1.8 ml of sodium acetate buffer produced a 4 pmol/ml acetylated standard. Serial dilution of this standard with modified assay buffer (prepared by the addition of 50 µl of acetylation reagent to 10 ml of sodium acetate buffer) produced a series of standards with concentrations of 2.0, 1.0, 0.5, 0.25 and 0.10 pmol/ml. These standards were always prepared for radioimmunoassay on the same day as the samples. 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 use. Additions were made to polypropylene test-tubes as shown in Table 2.5 and the contents mixed well by vortexing. The tubes were then capped and incubated for 16 - 18 hours at 2 - 8°C. Tubes 1 and 2 which were used to measure the total counts were placed in racks ready for insertion into the gamma counter and no further operations were carried out on them. Cyclic AMP precipitator (0.5 ml) at a temperature of 2 - 8°C was added to all other tubes, the contents mixed by vortexing and then centrifuged at 1250 x g for 15 min at 4°C. The tubes, 10 at a time, were placed in a plastic holder and the supernatant decanted into a radioactive waste-disposal sink. After draining at an angle of 30° onto absorbent paper for 30 s, the tubes were tapped to remove a final drop of supernatant and then capped. The radioactivity associated with the precipitate 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 A.4, Fig. A.4.1) and the concentration of cyclic AMP in the samples calculated using a curve fitting package associated with the gamma counter.

The performance characteristics of the extraction procedure and radioimmunoassay are summarized in Table 2.6. All data were routinely corrected for the recovery of [3H]-cyclic AMP in the extraction procedure and were expressed as pmol cyclic AMP/10^6 cells.

Table 2.5

Summary of reagent additions used 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	-
0.1 pmol/ml standard	5, 6	-	100	-	100	100
0.25 pmol/ml standard	7, 8	-	100	-	100	100
0.5 pmol/ml standard	9, 10	-	100	-	100	100
1.0 pmol/ml standard	11, 12	-	100	-	100	100
2.0 pmol/ml standard	13, 14	-	100	-	100	100
4.0 pmol/ml standard	15, 16	-	100	-	100	100
Samples	17 and on	-	-	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 cyclic AMP	90.6 ± 2.2 % (n = 8)
Inter-assay coefficient of variation	7.4 % (n = 6)
Intra-assay coefficient of variation	2.8 % (n = 6)
Cross-reactivity:	
cyclic GMP *	0.01 %
GMP *	0.01 %
ATP *	0.01 %
ADP *	0.01 %

* Data supplied by J. F. Hatt (personal communication).

CHAPTER 3

SITES OF INTERACTION OF PROTEIN KINASE C WITH ADENYLATE CYCLASE IN HGT-1 CELLS.

3.1   INTRODUCTION.

3.1.1   Some characteristics of protein kinase C.

The term protein kinase C refers not to a single enzyme, but to a complex family of closely related structures called isoforms, which all show protein phosphorylating activity (Asaoka et al., 1992).   Some basic information on the protein kinase C family is presented in Table 3.1.

A diagrammatic representation of the primary structure of protein kinase C shows there to be four regions of high homology between isoforms (C regions), and five more variable regions (V regions) (Fig 3.1).   The carboxy-terminal half of the protein represents the catalytic domain.   This region contains sequences, including the ATP binding site, which are homologous with sequences within the catalytic domains of other protein kinases including cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase (Nishizuka, 1988).   The amino-terminal half of the protein represents the regulatory domain, and the binding sites for lipid, and in some isoforms Ca^{2+} (Gschwendt et al., 1991) are found in this region.   The regulatory domain also contains a sequence that resembles protein kinase C phosphoacceptor substrates but in which the phosphoacceptor residue, serine or threonine, has been replaced by alanine (Table 3.2).   It is called a pseudosubstrate prototope.   Synthetic peptides containing the pseudosubstrate sequence are potent competitive inhibitors of protein kinase C. It has been suggested that the regulatory domain maintains the catalytic domain in an inactive state through

Table 3.1

General features of the protein kinase C family.

	Feature	Reference
1.	Wide tissue distribution. For some isoforms ubiquitous.	Asaoka et al., 1992.
2.	Ten isoforms so far identified.	Asaoka et al., 1992.
3.	Single polypeptide chain separated by a proteolytically sensitive hinge region into regulatory and catalytic domains.	Parker et al., 1986.
4.	Molecular mass between 77 and 84 kDa.	Asaoka et al., 1992. Liyanage et al., 1992.
5.	Preferentially Phosphorylate serine and threonine residues flanked by clusters of basic residues on both N- and C-terminal sides. See also Table 3.2	Nishizuka, 1984.
6.	Found in the cytosol, on cell membranes, and in a few circumstances for certain isoforms in the nucleus.	Nishizuka, 1986. Hocevar and Fields, 1991.

Figure 3.1
Domain structure for protein kinase C (O' Brian and Ward, 1989).
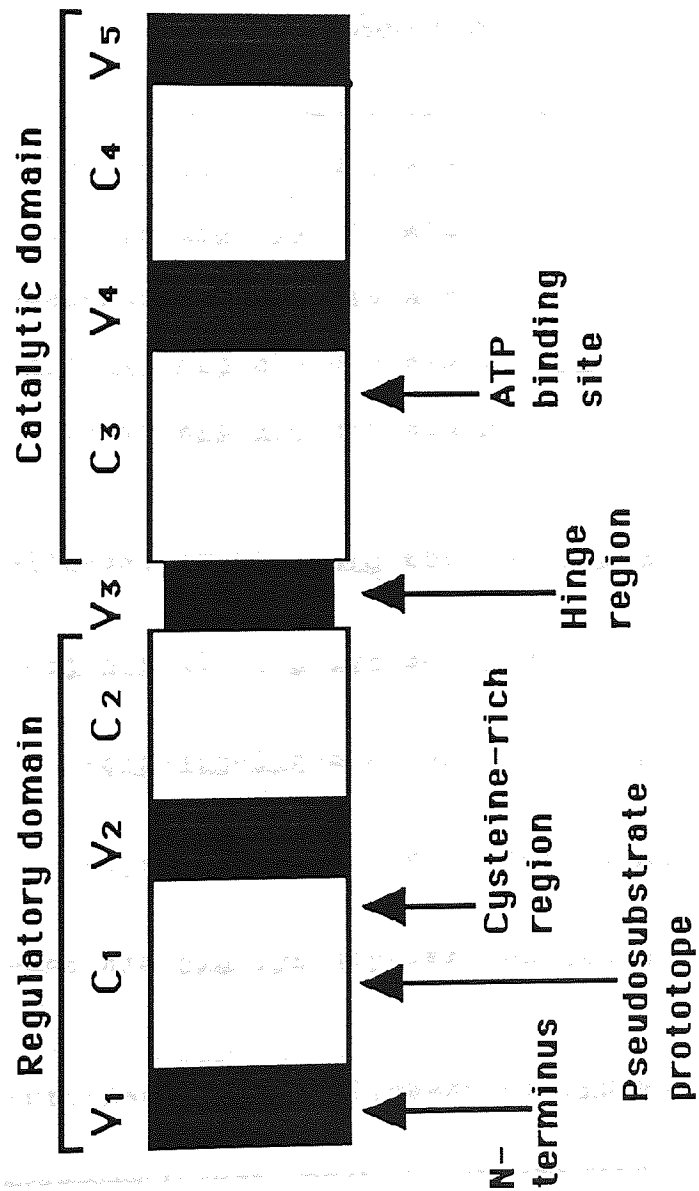
Table 3.2

Comparison of the putative pseudosubstrate prototope sites on isoforms of protein kinase C with sequences around sites phosphorylated by protein kinase C on proteins and synthetic peptides.

Isoform/Peptide	Sequence
α, βI, βII.	-phe-ala-<u>arg</u>-<u>lys</u>-gly-**ala**-leu-<u>arg</u>-glu-<u>lys</u>-asn-
γ.	-phe-cys-<u>arg</u>-<u>lys</u>-gly-**ala**-leu-<u>arg</u>-glu-<u>lys</u>-val-
δ.	-met-asn-<u>arg</u>-<u>arg</u>-gly-**ala**-ile-<u>lys</u>-glu-ala-<u>lys</u>-
ε.	-<u>arg</u>-<u>lys</u>-<u>arg</u>-glu-gly-**ala**-val-<u>arg</u>-<u>arg</u>-<u>arg</u>-val-
ζ.	-ile-tyr-<u>arg</u>-<u>arg</u>-gly-**ala**-<u>arg</u>-<u>arg</u>-trp-<u>arg</u>-<u>lys</u>-
EGF receptor	-ile-val-<u>arg</u>-<u>lys</u>-<u>arg</u>-**thr**-leu-<u>arg</u>-<u>arg</u>-leu-leu-
Interleukin-2 receptor	-<u>arg</u>-<u>arg</u>-glu-<u>arg</u>-<u>lys</u>-**ser**-<u>arg</u>-<u>arg</u>-thr-ile-
Histone H1	-<u>arg</u>-<u>arg</u>-<u>lys</u>-ala-**ser**-gly-pro-pro-val-
Transferrin receptor	-tyr-thr-<u>arg</u>-phe-**ser**-leu-ala-<u>arg</u>-
Pseudosub-strate-ser 25 peptide	-phe-ala-<u>arg</u>-<u>lys</u>-gly-**ser**-leu-<u>arg</u>-gln-<u>lys</u>-asn-
p36-1 peptide	-pro-ser-ala-tyr-gly-**ser**-val-<u>lys</u>-ala-tyr-thr-

Phosphorylation sites are in bold type; basic amino acid residues are underlined. (Hardie, 1988; Hardie, 1991; O'Brian and Ward, 1989).

interaction of the pseudosubstrate prototope with the peptide substrate binding site (House and Kemp, 1987) (Fig. 3.2).

The regulatory and catalytic domains are joined by a proteolytically sensitive hinge region (V₃) (Fig. 3.1) which may be cleaved by calpain, a calcium-dependent protease to produce two fragments with molecular weights of approximately 30 and 50 kDa respectively (Nishizuka, 1988). Catalytic fragments generated in this way are active in the absence of activators such as Ca^{2+}, phospholipid and diacylglycerol and are subsequently degraded by proteases (Parker et al., 1989). The physiological function of this irreversible activation process is however uncertain.

The present understanding of the substrate specificity of protein kinase C is based upon studies using synthetic peptides and from sequencing known substrate proteins (Table 3.2). These studies show that basic residues may be N-terminal and C-terminal but not usually immediately adjacent to the phosphoacceptor residue itself which can be serine or threonine, but not tyrosine (Nishizuka, 1988).

3.1.2   Activation of isolated protein kinase C isoforms.

3.1.2.1   Phospholipids.

The first group of classical or conventional (cPKC) isoforms (α, βI, βII and γ) (Table 3.3) require the presence of the acidic phospholipid, phosphatidylserine for maximal activation (Parker et al., 1989). Phosphatidylinositol and lysophosphatidylserine will

Figure 3.2
Diagrammatic representation of phorbol ester-induced activation of protein kinase C.
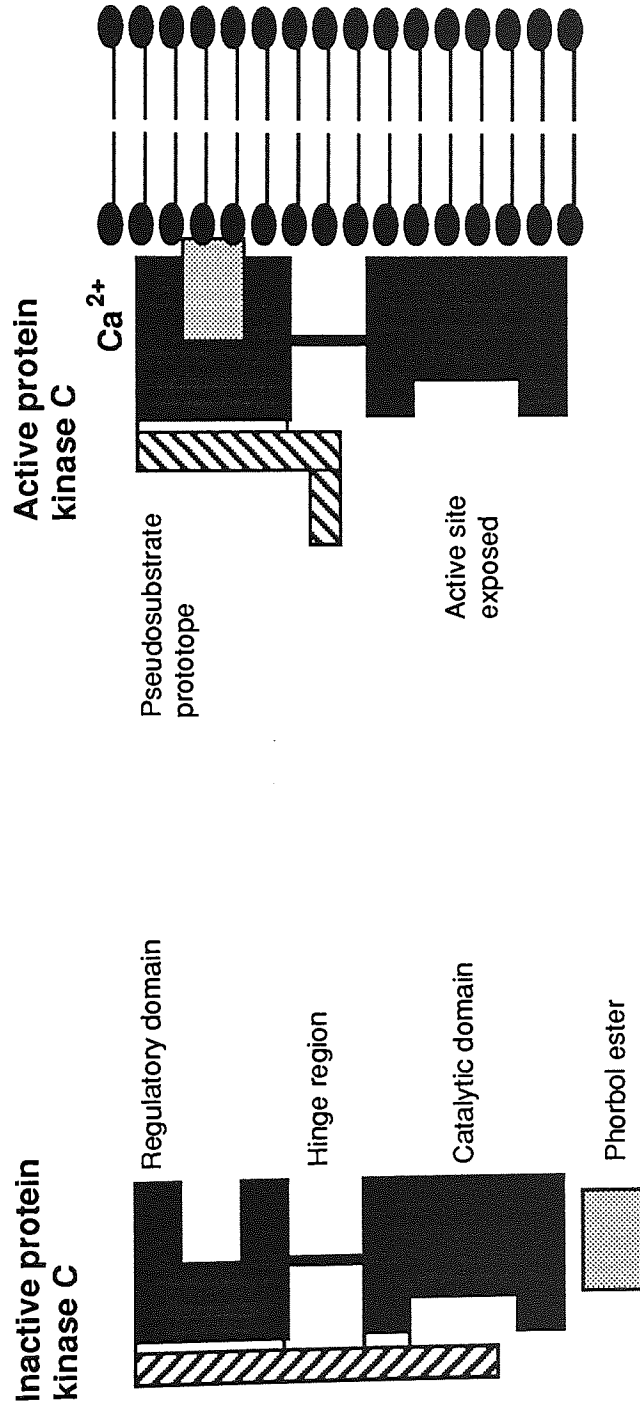
In the inactive state the pseudosubstrate sequence interacts with the catalytic domain, effectively blocking the substrate binding site of protein kinase C. Binding of effector molecules such as phorbol esters induces a conformational change that exposes the active site allowing its function to be expressed. Activation is also observed following proteolysis which again leads to loss of pseudosubstrate site interaction (Parker et al., 1989).

Table 3.3
Protein Kinase C subspecies in mammalian tissues.

Group	Subspecies	Apparent molecular mass (kDa)	Activators	Tissue expression
cPKC	α	76 799	Ca^{2+}, DAG, PS, FFA, LysoPC	Universal
	βI	76 790	Ca^{2+}, DAG, PS, FFA, LysoPC	Some tissues
	βII	76 933	Ca^{2+}, DAG, PS, FFA, LysoPC	Many tissues
	γ	78 366	Ca^{2+}, DAG, PS, FFA, LysoPC	Brain only
nPKC	δ	77 517	DAG, PS	Universal
	ε	83 474	DAG, PS, FFA	Brain and others
	η(L)	77 972	?	Lung, skin, heart
	θ	81 571	?	Skeletal muscle
aPKC	ζ	67 740	PS, FFA	Universal
	λ	67 200	?	Ovary, testis and others

The activators for each subspecies are determined with calf thymus H1 histone and bovine myelin basic protein as model phosphate acceptors. The detailed enzymological properties of the η (L)-, θ and λ subspecies have not yet been clarified. Abbreviations: DAG, diacylglycerol; PS, phosphatidylserine; FFA, cis-unsaturated fatty acid; LysoPC, lysophosphatidylcholine. (Asaoka et al., 1992).

also support activity, albeit to a lesser extent. However other membrane lipids such as phosphatidylcholine and phosphatidylethanolamine are without effect (Huang et al., 1988). The isoforms of this group are all similar in their order of preference for these phospholipids (Huang et al., 1988).

The second group consists of four new (nPKC) subspecies (δ, ϵ, η and θ). The δ and ϵ isoforms are the best characterized members of this group and are maximally activated in the presence of phosphatidylserine (Ogita et al., 1992; Koide et al., 1992), although phosphatidylinositol also potently activates protein kinase C δ (Mizuno et al., 1991). The η isoform shows little activity when stimulated with 12-O-tetradecanoylphorbol-13 acetate (TPA) in the presence of phosphatidylserine (Liyanage et al., 1992), however this result may have been influenced by the poor choice of substrate. The substrate specificities of all members of this group are known to be different to those of the more conventional protein kinase C isotypes (Liyanage et al., 1992).

The third and most recently identified group contains two atypical (aPKC) isoforms (λ and ζ). The ζ isotype is activated by cis-unsaturated fatty acids in the presence of phosphatidylserine, however the properties of this group have not yet been fully characterized (Asaoka et al., 1992).

The interaction of protein kinase C with phospholipid is not on a 1 : 1 molar basis but of the order of a lipid : protein molar ratio of between 4 : 1 and 10 : 1 (Hannun et al., 1985). This suggests that it

is the micellar structure, and presumably the surface charge distribution which govern the interaction.

3.1.2.2 Ca^{2+} and diacylglycerol.

Activation of the cPKC group of protein kinase C isoforms (Table 3.3) requires Ca^{2+} at micromolar concentrations in addition to the presence of phospholipid. Diacyglycerol activators of protein kinase C exert their effects by reducing the concentration of Ca^{2+} required for activation to as low as 0.1 μM which is close to the resting Ca^{2+} concentration in most cells. Thus, while the presence of Ca^{2+} is necessary for activity of this group of enzymes, it is the diacylglycerol that regulates this dependence of activity on Ca^{2+}. In other groups, diacylglycerols activate protein kinase C without any requirement for Ca^{2+} whatsoever (Asaoka et al., 1992).

All diacylglycerols able to bind to and activate protein kinase C _in vitro_ have the 1,2-_sn_ configuration. The fatty acid present at position 1 of the glycerol backbone is not thought to be critical, and diacylglycerols containing long or short chain, saturated or unsaturated fatty acids in this position have been shown to activate protein kinase C isoforms of the α, β and γ subtypes (Kerr et al., 1987). By contrast, the nature of the substituent at position 2 of the molecule is more critical. The probable physiological activators of protein kinase C are diacylglycerols which tend to have an unsaturated fatty acid at the 2-position such as 1-oleoyl 2-arachidonyl glycerol (Fig. 3.3). Synthetic diacylglycerols such as 1-oleoyl 2-acetyl glycerol (OAG) (Fig. 3.3) are more hydrophilic than naturally occurring ones, but still sufficiently hydrophobic to allow

Figure 3.3
Comparison of the structures of 1-oleoyl 2-arachidonyl glycerol,
OAG and TPA.

1.   1-oleoyl 2- arachidonyl glycerol.

$$H_2C—\overset{\overset{O}{\|}}{C}—(CH_2)_7\ (CH)_2\ (CH_2)_7\ CH_3$$

$$HC—\overset{\overset{O}{\|}}{C}—(CH_2)_3\ (\ (CH)_2\ CH_2)_4\ (\ (CH_2)_3\ CH_3$$

$$H_2COH$$

2.   OAG.

$$H_2C—O—\overset{\overset{O}{\|}}{C}—(CH_2)_7\ (CH)_2\ (CH_2)_7\ CH_3$$

$$HC—O—\underset{\underset{O}{\|}}{C}—CH_3$$

$$H_2COH$$
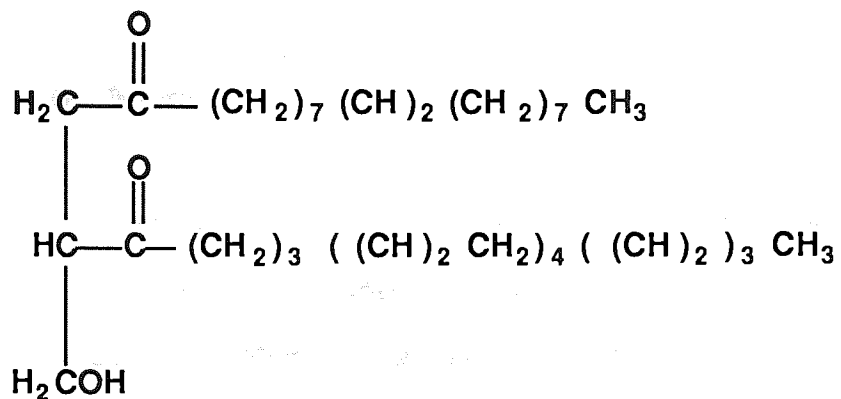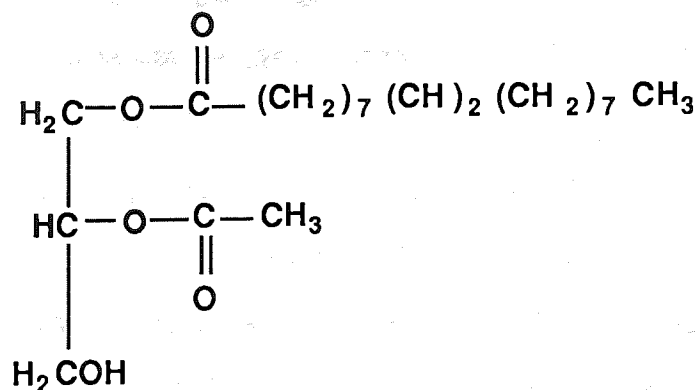
3.   TPA.
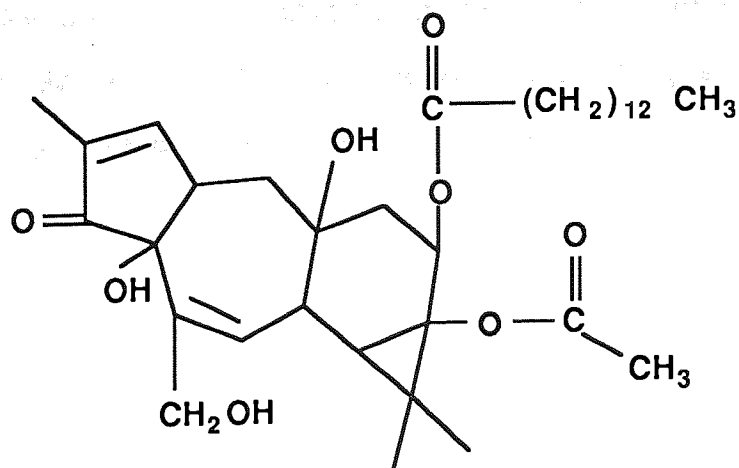
partitioning into the cell membrane (Go et al., 1987), and thereby activate protein kinase C in intact cells. The stereoisomers, 1,3- and 2,3-*sn*-diacylglycerol are ineffective both as activators and inhibitors of protein kinase C, as are the mono- and triacylglycerols (Boni and Rando, 1985).

3.1.2.3   Phorbol esters.

Although not carcinogenic themselves, phorbol esters enhance the formation of tumours by carcinogenic substances and are thus referred to as tumour promoters. Castagna et al., (1982) were the first to show that biologically active phorbol esters could mimic diacylglycerol in the activation of protein kinase C, and also that the relative potencies of these agents as tumour promoters paralleled their ability to activate protein kinase C (Castagna et al., 1982). The tumour-promoting phorbol esters have a structural moiety very similar to diacylglycerol (Fig. 3.3) and they have a similar mechanism of action in respect of their activation of protein kinase C. They directly activate protein kinase C both in vivo and in vitro, although they do not activate the ζ isoform (Asaoka et al., 1992). Their ability to specifically activate protein kinase C in intact cells make them valuable tools in investigations of the functions of this group of enzymes. 4α-phorbol derivatives are completely inactive (Castagna et al., 1982) and can be used as negative control compounds.

3.1.2.4   Lysophospholipids and cis-unsaturated fatty acids

Recent work (Asaoka et al., 1992) has demonstrated that for some isoforms, cis-unsaturated fatty acids e.g. arachidonic acid and lysophosphatidylcholine may also activate protein kinase C. This suggests a way in which products of phospholipase A_2 activity may activate this enzyme (Fig.3.4).

3.1.3   Activation of protein kinase C in intact cells.

In many cells a shift of protein kinase C from cytosol to membranes can be demonstrated in response to agonists that produce diacylglycerol. An even more pronounced redistribution is observed after treatment with phorbol ester (Anderson et al., 1984). The membrane bound enzyme becomes activated, probably because the presence of phospholipid fulfils the cofactor requirements of the enzyme and allows the quaternary complex of enzyme, Ca^{2+}, diacyglycerol and phospholipid to be formed (Fig. 3.2). Recent work however with a variety of cultured cell lines (Chakravarthy, 1991, 1992) has suggested that not all of the protein kinase C associated with membranes in resting cells may be in an activated form.

Diacylglycerol production is initiated by the hormonally induced activation of a phospholipase C which hydrolyses phosphatidylinositol-4,5-bisphosphate. The other product of this reaction, inositol-1,4,5-trisphosphate, triggers the release of Ca^{2+} from intracellular stores (Berridge and Irvine, 1984) (Fig. 3.4). Thus diacylglycerol and Ca^{2+} levels often increase simultaneously in response to the same stimulus. Elevation of intracellular Ca^{2+} is

Figure 3.4
Potential pathways for the generation of diacylglycerol from membrane phospholipids and subsequent cellular responses.
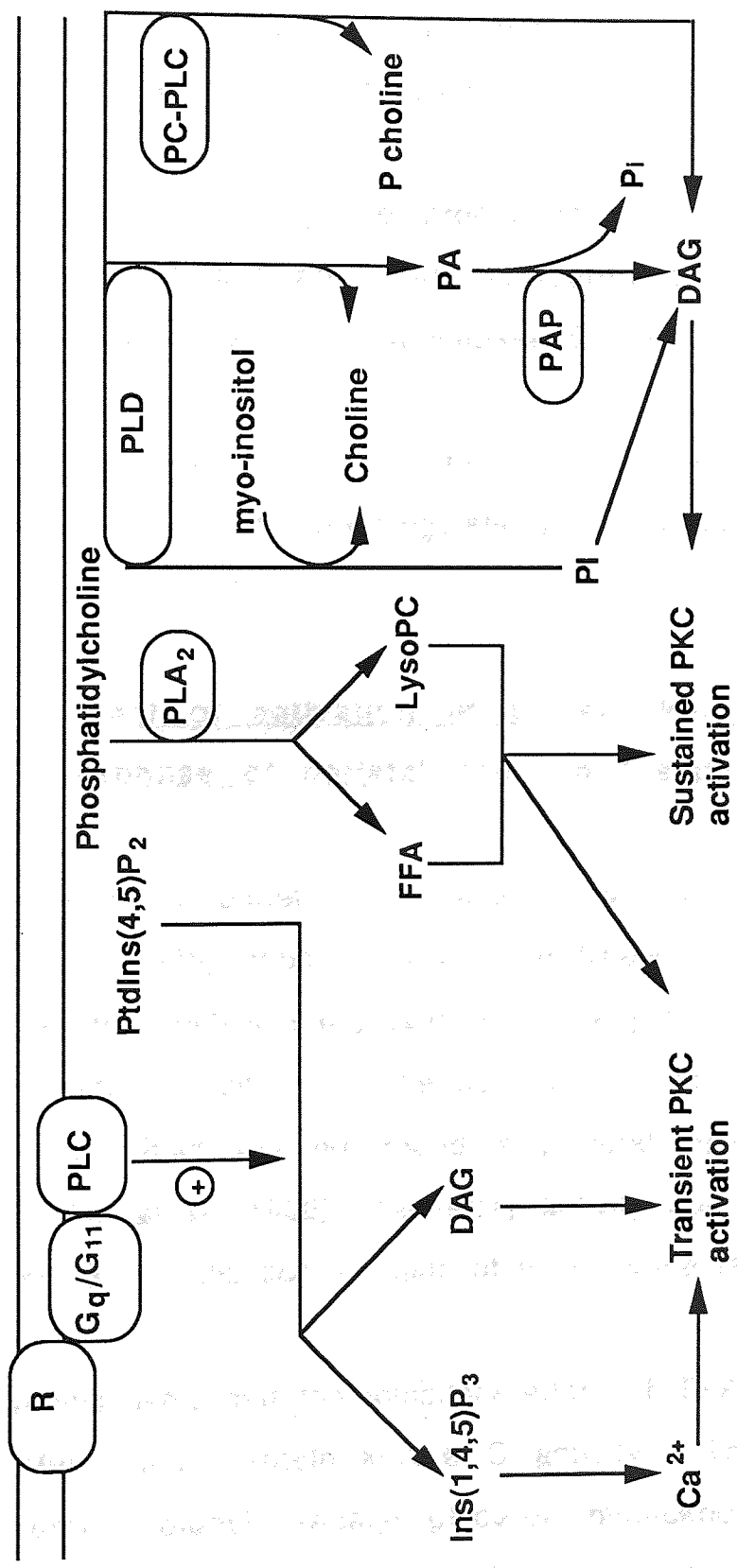
PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)P3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; FFA, cis-unsaturated fatty acid; LysoPC, lysophosphatidylcholine; PKC, protein kinase C; P-choline, phosphocholine; Pi, inorganic phosphate; PI, phosphatidylinositol; PA, phosphatidic acid; PLD, phospholipase D; PC-PLC, phosphatidylcholine-reactive phospholipase C; PLC, phosphatidylinositol-specific phospholipase C; PAP, phosphatidic acid phosphohydrolase (Asaoka et al., 1992).

often transient but sustained elevation of diacylglycerol and therefore activation of protein kinase C may be caused by secondary activation of phospholipase D (Asaoka et al., 1992) (Fig. 3.4). Diacylglycerol may also be produced by the phosphatidylcholine-reactive phospholipase C catalysed hydrolysis of phosphatidylcholine, or by the production of phosphatidylinositol from phosphatidylcholine and subsequent hydrolysis by phosphatidylinositol-specific phospholipase C (Fig. 3.4).

Prolonged exposure of cells to phorbol esters causes loss of protein kinase C activity (downregulation). This topic is discussed in more detail in Chapter 4.

3.1.4 Effect of activation of protein kinase C on the response of parietal cells to histamine.

Incubation of parietal cells isolated from rats (Anderson and Hanson, 1984, 1985), rabbits (Brown and Chew, 1987) and guinea pigs (Beil et al., 1987) in the presence of TPA produced a substantial and dose-related inhibition of the secretory response to histamine. However, this effect was not found in parietal cells isolated from the dog (Chiba et al., 1989). Secretory activity was assessed in all of these studies by the accumulation of aminopyrine (see Chapter 2).

It seems likely that the inhibitory effect of TPA was due to a specific effect upon protein kinase C activity. The analogue of diacylglycerol, 1-oleoyl 2-acetyl glycerol, mimicked the effect of TPA and produced a dose-dependent inhibition of histamine-stimulated aminopyrine accumulation, the maximal inhibition (83%)

being similar to that caused by a maximally effective concentration of TPA (78%) (Anderson and Hanson, 1985). The concentration of TPA required to produce half-maximal inhibition was 2.9 nM (Hatt and Hanson, 1989), a figure not dissimilar to the value of 2.5 nM required to promote half-maximal activation of a partially purified sample of protein kinase C from brain (Arcoleo and Weinstein, 1985). Furthermore, the inactive isomer 4α-phorbol 12-myristate 13-acetate (4α-TPA) had no effect on secretory activity, and the relative potency with which phorbol esters inhibited acid secretion stimulated by histamine paralleled their ability to activate protein kinase C from brain (Anderson and Hanson, 1985). Finally the inhibitor of TPA, 1-(5-isoquinolinyl sulfonyl)-2-methyl piperazine (H-7) partially reversed the effect of TPA on histamine-stimulated secretory activity (Ostrowski and Bomsztyk, 1989).

The effects of TPA were not thought to be mediated through activation of isoforms of protein kinase C located within cells other than parietal cells because there was no apparent relationship between cell concentration and the action of TPA. Also, the effect of TPA concentration on histamine-stimulated aminopyrine accumulation produced a curve which closely fitted a classical dose-response curve (Anderson and Hanson 1985).

3.1.4.1 Possible sites of action of protein kinase C on histamine-stimulated secretion.

In rat parietal cells, TPA inhibited aminopyrine accumulation stimulated by dibutyryl cyclic AMP with a potency similar to that for inhibition of histamine-stimulated aminopyrine accumulation

(Hatt and Hanson, 1989). This effect was not reproduced in rabbit parietal cells (Brown and Chew, 1987), but was shown in guinea pig parietal cells (Beil et al., 1987). These results suggest an inhibitory site of action of protein kinase C distal to the production and hydrolysis of cyclic AMP in rat parietal cells. A possible target is therefore the K^+-H^+ATPase pump. However, Beil et al. (1987) showed that inhibition of the proton pump in membranes isolated from guinea pig parietal cells was half-maximal at a TPA concentration of 6.5 μM. This value was almost 1000-fold higher than the concentration of TPA required for half-maximal inhibition of histamine-stimulated aminopyrine accumulation in these same cells. Furthermore, high concentrations of TPA did not inhibit aminopyrine accumulation stimulated by 100 mM K^+ (Hatt & Hanson, 1989) as would have been expected had TPA had an effect on the K^+-H^+-ATPase (see Chapter 1 for a discussion on how high K+ may stimulate aminopyrine accumulation). Thus, it seems unlikely that the effects of protein kinase C are mediated at the proton pump.

A second inhibitory site of action close to adenylate cyclase has been proposed for protein kinase C in rat parietal cells (Hatt and Hanson, 1989). This is supported by data which show a 47% reduction in histamine-stimulated cyclic AMP content of parietal cells in the presence of TPA (Hatt and Hanson, 1989; however Ostrowski and Bomzstyk (1989), could not confirm this). Although 3-isobutyl-1-methylxanthine (IBMX) was present in these experiments, the concentration (0.1 mM) was probably insufficient to completely inhibit all cyclic AMP phosphodiesterase activity (see also section 3.3.1) and it is therefore unclear whether effects of TPA were on cyclic AMP production or its degradation.

3.1.5   Regulation of cyclic AMP production.

At receptors including β-adrenoceptors, histamine H_2 receptors, dopamine D_1 receptors and some 5-hydroxytryptamine receptors, the enzyme, adenylate cyclase is activated following agonist-receptor combination. This enzyme catalyses the conversion of adenosine triphosphate (ATP) into 3′,5′-cyclic adenosine monophosphate (cyclic AMP). At some α-adrenoceptors, agonist-receptor combination leads to inhibition of adenylate cyclase and a reduction in the cyclic AMP content of the cell (Convents et al., 1989). Regulation of the pathways leading to enzyme activation are described below.

3.1.5.1   Regulation of receptors linked to adenylate cyclase.

Short-term exposure of a cell to high concentrations of agonist often induces a temporary state of refractoriness during which the cell shows a reduced level of responsiveness to further treatment. This phenomenon is known as desensitization, and is termed homologous if only a single receptor is involved. Desensitization has been extensively investigated in receptors of the β_2-adrenoceptor type. The mechanism of homologous desensitization of these receptors is thought to involve phosphorylation of the receptor by a β_2-adrenoceptor kinase which is capable of achieving multiple phosphorylations of the β_2-adrenoceptor on its C-terminal tail. β_2-adrenoceptor kinase-mediated phosphorylation only occurs when the receptor contains bound hormone thus introducing specificity into the system. Phosphorylation is thought to result in an uncoupling of the agonist-

receptor complex from the regulatory protein G_s (reviewed by Houslay, 1991a).

Heterologous desensitization, which is not agonist-specific may occur via protein kinase A or protein kinase C mediated phosphorylation of the cytoplasmic loop which connects the fifth and sixth transmembrane helices of the receptor (Houslay,1991a). This process is independent of receptor occupation.

Homologous desensitization of histamine H_2 receptor activity has been observed in HGT-1 cells (Emami and Gespach, 1986a; Gespach et al., 1984) without any reduction in the affinity of the receptor for histamine. The mechanism of this desensitization is uncertain.

3.1.5.2   Regulation and mode of action of G-proteins.

A family of GTP-binding and hydrolysing proteins (G-proteins) play an essential role in the transduction of receptor-generated signals to effector proteins at the cell membrane. All members of this family have a characteristic heterotrimeric structure with subunits denoted α, β and γ (reviewed by Hepler and Gilman, 1992). The β and γ subunits coexist as a tightly associated complex which may couple to different α-subunits to form the heterotrimer. The structure of the α-subunit is more unique and is used to define an individual G-protein oligomer. To date, cDNAs that encode 21 distinct G-protein α-subunits have been cloned and these are divided into four main classes denoted: G_s, G_i, G_q and G_{12}. In addition at

least four β-, and six γ-subunits have also been described (Hepler and Gilman, 1992).

In the inactive state G-proteins exists in the holomeric form with GDP bound tightly to the α-subunit. Receptor activation causes this subunit to release its bound GDP which is then replaced by GTP, thus promoting dissociation from the $\beta\gamma$ subunits (Fig. 3.5). The free α-GTP subunit activates the adenylate cyclase catalytic subunit. The α-subunit possesses an intrinsic GTPase activity which hydrolyses bound GTP to yield bound GDP and free inorganic phosphate (P_i). Reassociation of the α, β and γ subunits and return to the inactive state then follows (Gilman, 1987). $\beta\gamma$ also inhibits the $G_{s\alpha}$-stimulated activity of type I adenylate cyclase and potentiates the stimulatory effect of of $G_{s\alpha}$ on the type II and type IV isoenzymes (reviewed by Tang and Gilman, 1992).

The α-subunits of some of the G-protein subspecies possess specific residues that can be covalently modified by bacterial toxins. Pertussis toxin catalyses the transfer of the ADP-ribose moiety of NAD to a specific cysteine residue located near the carboxyl terminus of proteins of the G_i class. ADP-ribosylation at this site prevents receptor-mediated activation of the G-protein and thus inhibition of the host cell adenylate cyclase. Similarly, cholera toxin catalyses the ADP-ribosylation of a specific arginine residue in the α-subunit of G_s resulting in an inhibition of GTPase activity and thus constitutive activation of G_s (Foster et al., 1984). Both the G_i and G_s subspecies are potential substrates for phosphorylation by protein kinase C (Houslay, 1991a; Pyne et al., 1992).

Figure 3.5
G-protein-mediated transmembrane signalling and activation of adenylate cyclase.
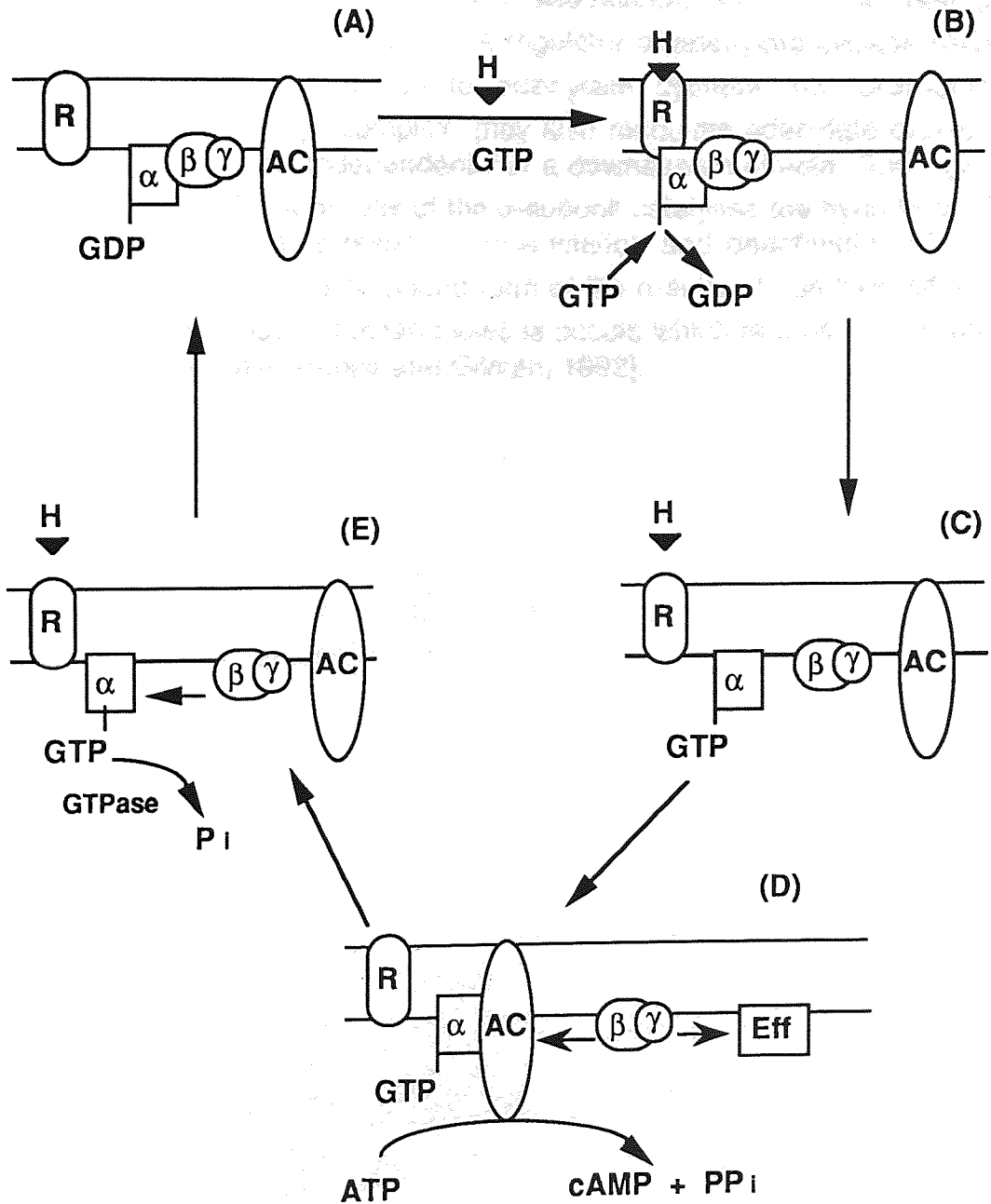
(A). In the basal state GDP is bound tightly to the α-subunit of the heterotrimeric G-protein. (B). Hormone (H) binding results in receptor (R) activation. The receptor binds to the G-protein, inducing a conformational change that promotes exchange of GTP for GDP. (C). GTP binding causes dissociation of the receptor-G-protein complex, thus

Figure 3.5 continued on next page.

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Figure 3.5 continued...

reducing the affinity of the receptor for hormone and freeing the receptor for another liason with a quiescent G-protein. GTP binding also reduces the affinity of α for $\beta\gamma$, and subunit dissociation occurs, thus freeing α-GTP to fulfil its primary role as a regulator of adenylate cyclase (AC) activity. (D). α-GTP binds to adenylate cyclase and promotes activation. The free $\beta\gamma$-complex may also modulate adenylate cyclase activity, or it may act independently at a downstream effector (Eff). (E). The intrinsic GTPase activity of the α-subunit catalyses the hydrolysis of GTP to GDP and this results in dissociation and deactivation of the active complex. The GDP-bound form of the α-subunit has high affinity for $\beta\gamma$. Reassociation of these moieties occurs which returns the system to the inactive state (Hepler and Gilman, 1992).

3.1.5.3   Regulation of adenylate cyclase.

The major enzymic component of the adenylate cyclase system is the catalytic subunit itself which utilizes ATP as a substrate for the formation of cyclic AMP and pyrophosphate. Ten distinct isoforms of the enzyme have so far been isolated. Most of these exist in a membrane-bound form, however certain bacterial enzymes are known to be cytosolic (reviewed by Tang and Gilman, 1992). All the mammalian isoenzymes are activated by G_S (Hepler and Gilman, 1992), (Table 3.4) and protein kinase C is also known to affect the functioning of the enzyme, although this is not a universal occurrence (reviewed by Houslay, 1991a). The enzyme also displays a requirement for certain divalent ions such as Mg^{2+}. Part of this dependence on Mg^{2+} possibly reflects the requirement of the substrate, ATP for this ion.

3.1.6   Breakdown of cyclic AMP.

This is achieved by cyclic AMP phosphodiesterases. This group of enzymes is reviewed in Chapter 6.

3.1.7   Use of the HGT-1 cell line to investigate effects of protein kinase C on regulation of cyclic AMP content.

The aim of the work described in this chapter was to investigate the exact site at which protein kinase C might act to inhibit stimulation of cyclic AMP content by histamine, and to determine whether such effects might take place in a model system

Table 3.4
General features of Eukaryotic Adenylate Cyclases.

Type	Amino Acid Residues	Expression	Effect of G Proteins		Ca^{2+}-Calmodulin	Forskolin
			$G_{s\alpha}$	$\beta\gamma$		
I	1134	Brain	+	-	+	+
II	1090	Brain, lung	+	+	O	+
III	1144	Olfactory	+	O	+	+
IV	1064	Brain, others	+	+	O	+
V	1184	Heart, brain, others	+	O	O	+
VI	1165	Heart, brain, others	+	O	O	+
rutabaga	2249	Mushroom body	+	O	+	+
AC-A	1407	During aggregation	?	?	?	O
AC-G	858	Fruiting body	?	?	?	O
CYR1	2026	Constitutive	O	O	O	O

Adenylate cyclase types I-VI are mammalian; rutabaga is from Drosophila; AC-A and AC-G are from Dictyostelium; CYR1 is from Saccharomyces cerevisiae. AC-A but not AC-G is activated by GTPγS however a homologue of $G_{s\alpha}$ has not been detected in Dictyostelium. CYR1 is activated by ras. +, stimulates; -, inhibits; 0, no effect. (Tang and Gilman, 1992).

expressing the histamine H_2 receptor. To this end the human gastric cancer cell line (HGT-1) was chosen. The general properties of this cell line have been reviewed in Chapter 1, but in the context of the present investigation the most important point is that the histamine H_2 receptor in these cells is pharmacologically indistinguishable from that in isolated human gastric glands (Gespach et al., 1988). The cell line was also chosen because it might enable experiments in which protein kinase C could be down-regulated (Chapter 4). This could not be achieved in rat parietal cells (PhD thesis, Atwell, 1989). Comparison of the effects of TPA on stimulation of cyclic AMP content by other agonists such as GIP and TGLP-1 with results involving histamine could help elucidate the site of action of protein kinase C on the stimulation of cyclic AMP content by histamine.

3.2 METHODOLOGY.

3.2.1 Preparation of reagents.

TPA, 4α-TPA, OAG, forskolin and staurosporine were all dissolved in dimethylsulfoxide (DMSO). DMSO was also added where appropriate to the control vials to a maximal final concentration of 0.1 % ($^v/_v$). Histamine, TGLP-1 and GIP were dissolved in saline (NaCl 0.9% $^w/_v$), and IBMX was dissolved in medium B′ (Appendix A.2) by warming the medium to 37°C. For storage, cholera toxin was dissolved to a concentration of 500 μg/ml in 50 mM tris-chloride buffer (pH 7.5), containing 0.2 M NaCl, 3 mM NaN_3 and 1 mM EDTA. This stock was diluted 1 : 10 with saline prior to use and added at a dose of 10 μl/ml cell suspension. Pertussis toxin was dissolved to a concentration of 200 μg/ml in 50% glycerol, 50% 0.05 M phosphate buffer containing 0.5 M NaCl (pH 7.5) and added to culture flasks at a rate of 2.5 μl/ml culture medium. Vehicle was added to control vials or incubation flasks where appropriate.

[^{3}H]-tiotidine was supplied dissolved in ethanol at a concentration of 1 μCi/ml (75 Ci/mmol) and was stored at -70°C under nitrogen prior to use. Unlabelled tiotidine was also dissolved in ethanol and was stored as a 3.33 mM stock at -20°C. Pyrilamine and cimetidine were dissolved in saline and were made fresh prior to use.

3.2.2   Effect of TPA on the stimulation of cyclic AMP content of suspensions of HGT-1 cells by various agonists.

3.2.2.1   Effect of TPA on the time-course of the cyclic AMP response to stimulation by agonist.

The standard protocol for experiments involving a 10 min preincubation with TPA prior to stimulation with agonist was as follows:

1.   Cells in late log phase were detached and washed as described in Chapter 2. The cells were resuspended at a concentration of 6 x 10^6 cells/ml in medium B′ and incubated in a shaking water bath (75 cycles/min) until the required temperature of either 20°C or 37°C was reached. The cell suspension was constantly gassed with a slow stream of 95% O_2/5% CO_2 (^{v}/v).

2.   t = -20 min. An equal volume of medium B′ containing IBMX at a concentration of 0.2 mM, prewarmed to the required temperature was added.

3.   t = -10 min. A 5 ml aliquot of the above cell suspension was removed and added to a flask containing an equal volume of medium B′ plus DMSO at a concentration of 0.2%. A second 5 ml aliquot was also removed and added to a second flask containing medium B′ (5 ml) plus TPA at a concentration of 200 nM.

4.   t = 0.

(a). Duplicate 0.25 ml aliquots were taken from each flask and added to 0.25 ml of ice-cold ethanol in a microfuge tube. This mixture was immediately vortexed and then stored on ice. These samples were then analysed to determine the cyclic AMP content of the cell suspension at t = 0.

(b). Duplicate 0.4 ml aliquots were also taken from each flask and added to incubation vials containing the appropriate vehicle. The airspace above the cells was gassed for 5 seconds with 95% O_2/5% CO_2 and the vials were capped. These vials were incubated for the duration of the experiment at which time an equal volume of ethanol was added. These samples were then analysed to determine the basal cyclic AMP content of the cell suspension at the end of the experiment.

(c). After the above samples had been removed, agonist (histamine, TGLP-1, GIP or forskolin) was added to each incubation flask. Duplicate 0.25 ml samples were then taken from each flask at the appropriate time. All samples were added to an equal volume of ice-cold ethanol, immediately vortexed and stored on ice. The cyclic AMP content of all the samples taken were determined as detailed in Chapter 2.

3.2.2.2   Effect of staurosporine on the cyclic AMP response to agonist in the presence and absence of TPA.

1.   The cells were resuspended at a concentration of 3 x 10^6 cells/ml in the presence of 0.2 mM IBMX as described above.

2.   t = -20 min.   A 6 ml aliquot of the above cell suspension was removed and added to a flask containing an equal volume of medium B′ plus DMSO at a concentration of 0.2%. A second 6 ml aliquot was also removed and added to a second flask containing medium B′ (5 ml) plus staurosporine at a concentration of 400 nM.

3.   t = -10 min.   Aliquots of 0.5 ml were transferred from each flask into incubation vials containing 0.5 ml medium B′ plus TPA at various concentrations. The vials were then gassed as above.

4.   t = 0.   Agonist (histamine, TGLP-1 or GIP) or vehicle were then added to each vial as appropriate. Incubations continued for 5 min and then 1 ml of ice-cold ethanol was added to the vials. The vials were vortexed and stored on ice as usual.

3.2.2.3   Effect of pertussis toxin on the cyclic AMP response to agonist in the presence and absence of TPA.

1.   Pertussis toxin or vehicle was added to cells two hours prior to detachment. The cells were detached, washed and resuspended at a concentration of 6×10^6 cells/ml in medium B′ containing 0.2 mM IBMX as usual (t = -20 min) and then transferred to flasks containing either DMSO or TPA (t = -10 min).

2.   t = 0.   0.5 ml aliquots from each flask were transferred into incubation vials containing an equal volume of medium B′ plus agonist (histamine or GIP). The vials were gassed and capped and incubations allowed to continue for 5 min. 1 ml of ice-cold ethanol

was then added to the vials. The vials were vortexed and stored on ice as usual.

3.2.3 Binding of [³H]-tiotidine to cells grown on multiwell plates.

3.2.3.1 Comparison of the effects of histamine, pyrilamine cimetidine and cold tiotidine on the binding of [³H]-tiotidine.

Cells were grown on 6-well plates until almost confluent ($1.07 \times 10^6 \pm 6.33 \times 10^4$ cells/well, n = 4). The medium was removed and the cells were washed once in medium B'. Ice-cold medium B' containing 0.5 µCi [³H]-tiotidine (6.7 nM) and either cold tiotidine (6.7 µM), histamine (1 mM), pyrilamine (100 µM), cimetidine (100µM) or vehicle was then added to each well and the plates incubated for one hour at 4°C. Incubations were carried out at this temperature in order to prevent internalization of the labelled compound. The cells were then washed three times in ice-cold medium B'. 1 ml of NaOH (1M) was added to each well and the cells allowed to solubilize overnight. The solubilizate plus washings was added to 10 ml OptiPhase Hi-Safe II for scintillation counting.

3.2.3.2 Displacement of [³H]-tiotidine by cold tiotidine.

Cells were grown on six-well plates and washed as described above. Ice-cold medium B' containing 0.5 µCi [³H]-tiotidine (6.7 nM) was then added to three of the wells and the same medium with

the addition of unlabelled tiotidine (6.7 µM) added to the other three. The cells were incubated for one hour at 4°C, then washed three times in ice-cold medium B′. Medium B′ containing only unlabelled tiotidine (6.7 µM) was then added to the cells which were incubated for a further 30 min or 60 min. The cells were washed and solubilized and the solubilizate counted as described above.

3.2.3.3 Effect of TPA on [³H]-tiotidine binding.

Confluent cells were washed gently with medium B′ and then the same medium, pre-warmed to 37°C and containing either 100 nM TPA or vehicle (DMSO) was added and the plates incubated for 10 min at 37°C. The cells were then washed twice with medium B′ at 37°C. 0.5 µCi [³H]-tiotidine, accompanied by varying amounts of unlabelled tiotidine in 1 ml of ice-cold medium B′ was then added to each well and the plates incubated for 1 h at 4°C. The medium was removed and the cells washed and solubilized prior to counting as described above. Non-saturable binding was estimated in the presence of a concentration of 6.7 µM unlabelled tiotidine. One six-well plate therefore had six different concentrations of tiotidine, with one well on each plate always containing label alone and one containing label plus 6.7 µM tiotidine for estimation of non-specific binding. Because specific binding in the absence of added cold tiotidine varied between plates, results were normalized by expressing them as the inhibition of specific-binding produced by added cold tiotidine. Use of 24-well plates was precluded by the low specific activity of the label and the small number of binding sites on the cells.

3.3   RESULTS AND DISCUSSION.

Results are presented as means ± SEM with n equal to the number of separate cell preparations.

3.3.1   Effect of incubation in the presence of histamine on the cyclic AMP content of a suspension of HGT-1 cells.

The basal cyclic AMP content of a suspension of HGT-1 cells incubated at 37°C for 20 min was 3.31 ± 0.67 pmol/10^6 cells (n = 3). This value was not significantly changed after a further 30 min incubation (2.82 ± 0.69 pmol/10^6 cells) and was unaffected by the presence of 0.1 mM IBMX (3.01 ± 0.84 pmol/10^6 cells).

Stimulation with a near-maximally effective concentration of histamine (0.5 mM) (Menez et al., 1983) at 37°C resulted in a rapid but transient 4.7-fold increase in the cyclic AMP content of the suspension above basal. This response was attenuated between 1 and 10 min after addition of agonist, after which a plateau in cyclic AMP content was reached which remained significantly above basal (P < 0.05 by paired t-test) for the remainder of the experiment (Fig. 3.6). The fall in the level of cyclic AMP in the suspension must mean that complete inhibition of cyclic AMP phosphodiesterase activity had not been achieved. Indeed an isoform of cyclic AMP phosphodiesterase which is not inhibited by IBMX is present in certain cell types (Houslay, 1991a; Lavan et al., 1989). Also, since the cyclic AMP content of the cell suspension was measured, no change in the distribution of cellular and extracellular cyclic AMP

Figure 3.6
Effect of preincubation in the presence of 100 nM TPA (filled symbols) for 10 min on the time-course of the cyclic AMP response in HGT-1 cells incubated at 37°C in the presence (□■) and absence (△▲) of 0.5 mM histamine.
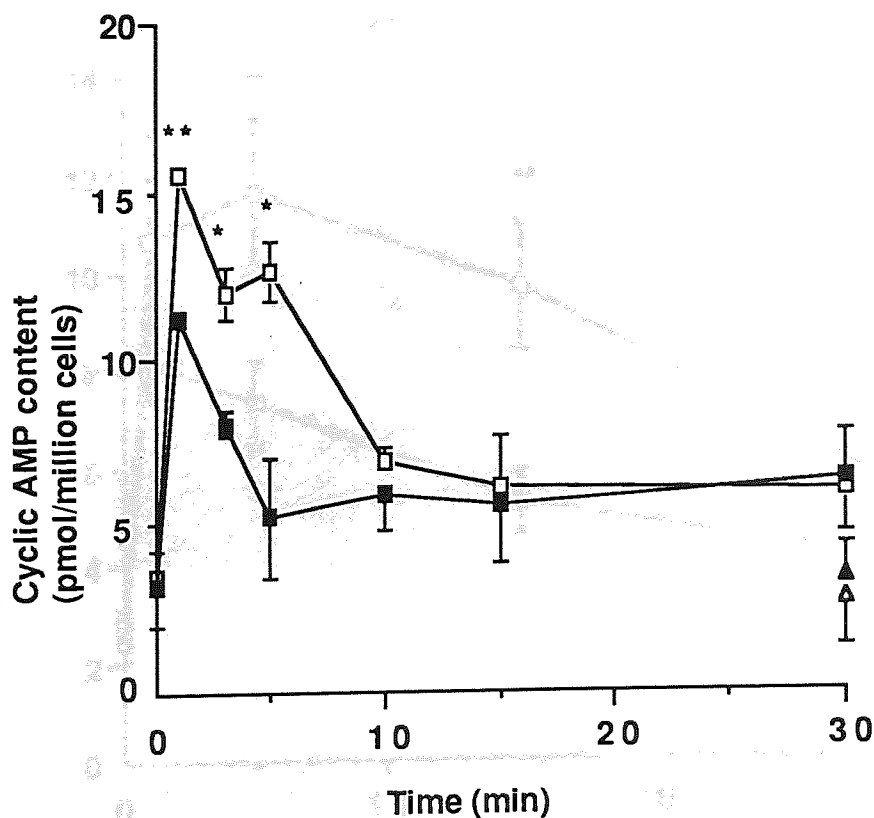
Results are means ± SEM from four experiments. Where no error bars are evident they are masked by the symbols. Significant effects of TPA were determined by ANOVAR (Appendix A.5.1) and are denoted: * $P < 0.02$; ** $P < 0.01$.

Figure 3.7
Effect of preincubation in the presence of 100 nM TPA (filled symbols) for 10 min on the time-course of the cyclic AMP response of HGT-1 cells incubated at 20°C in the presence (□ ■) and absence (△ ▲) of 0.5 mM histamine.
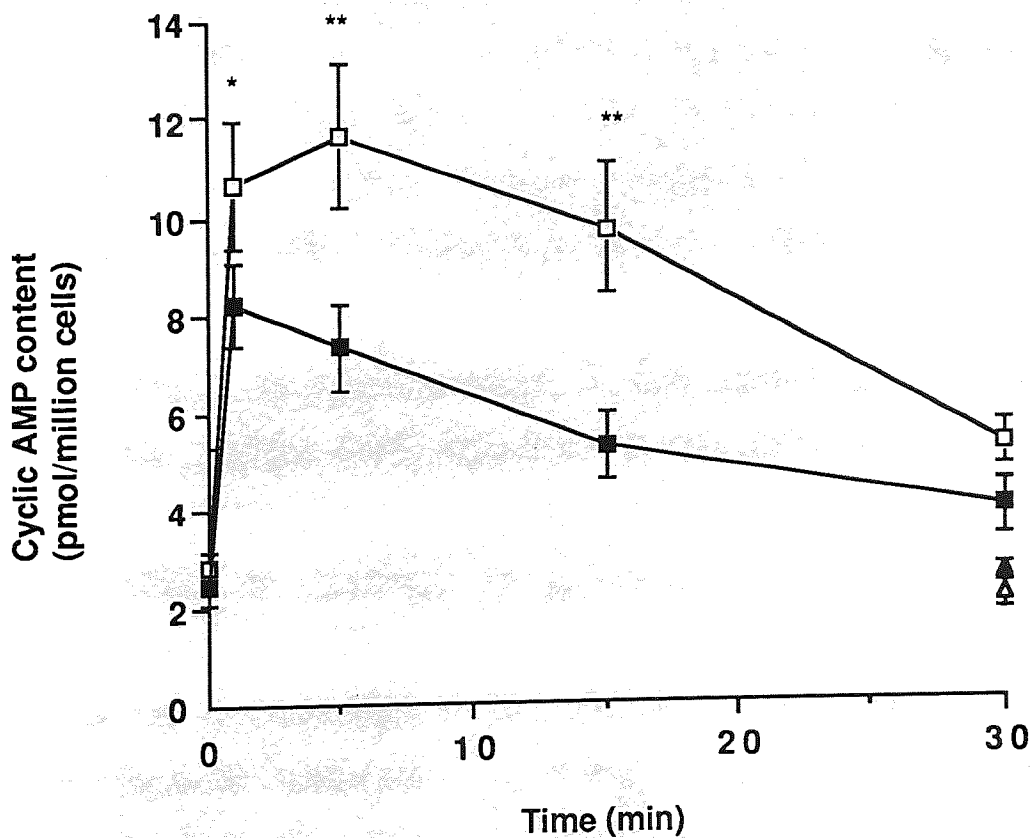
Results are means ± SEM from three experiments. Where no error bars are evident they are masked by the symbols. Significant effects of TPA were determined by ANOVAR (Appendix A.5.1) and are denoted: * $P < 0.02$; ** $P < 0.01$.

could have influenced the result. At 20°C, the level of stimulation by 0.5 mM histamine was almost unchanged with a 4.2-fold increase over basal (Fig. 3.7). The onset of the decline in cyclic AMP content was later than at 37°C and proceeded at a much slower rate.

These results are in agreement with data obtained previously with these cells (Emami and Gespach, 1986a, 1986b). The decline in cyclic AMP content at 37°C is probably explained by a decrease in adenylate cyclase activity associated with desensitization of the H_2 receptor (Prost et al., 1986). Such desensitization is much less at 20°C (Emami et al., 1983; Emami and Gespach, 1986a).

3.3.2   Effect of preincubation in the presence of TPA on the cyclic AMP response to histamine.

3.3.2.1·   Effect of incubation temperature.

A 10 min preincubation of the cells in the presence of 100 nM TPA significantly reduced the initial response to histamine measured at 37°C (P < 0.01), the effect being most apparent 5 min after addition of agonist (Fig. 3.6). This time point was therefore chosen to investigate the relationship between inhibition of stimulation by histamine and concentration of TPA (Fig. 3.8). Near-maximal inhibition occurred in the presence of 10^{-8} M TPA and resulted in 68.5 ± 10.1% inhibition. The IC_{50} was estimated by using the program FIT (Barlow, 1983) to be 0.15 nM. At 20°C, the inhibition of the response to histamine due to the presence of 100 nM TPA was prolonged, and was still significant (P < 0.01) 15 min after the addition of histamine (Fig. 3.7). The half-maximally

Figure 3.8
Effect of preincubation for 10 min in the presence of various concentrations of TPA on the cyclic AMP content of a suspension of HGT-1 cells subsequently incubated for 5 min at 37°C in the presence of 0.5 mM histamine.
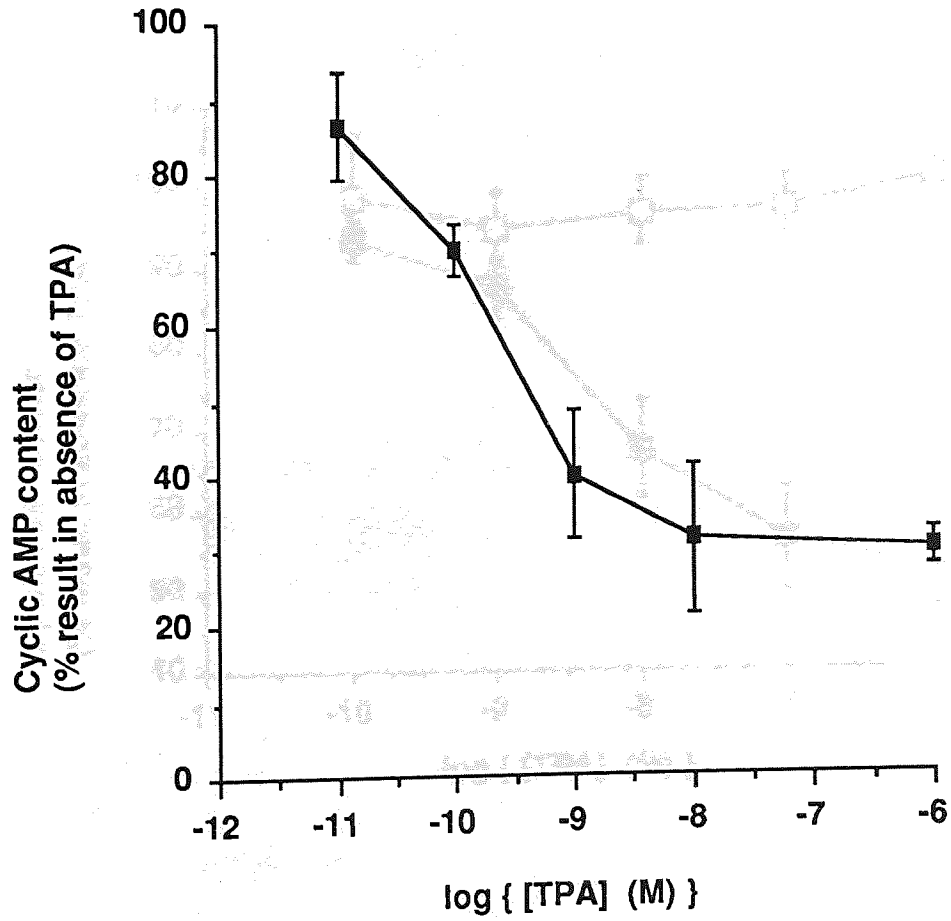
Results are means ± SEM from four experiments and are expressed as a percentage of the response to 0.5 mM histamine alone which was 13.6 ± 4.1 pmol/10^6cells.

Figure 3.9
Effect of preincubation for 10 min in the presence of various concentrations of TPA on the cyclic AMP response in cells incubated for a further 5 min in the presence of 0.5 mM histamine at 20°C. (□) 200 nM staurosporine added 20 min before histamine, (■) staurosporine absent.
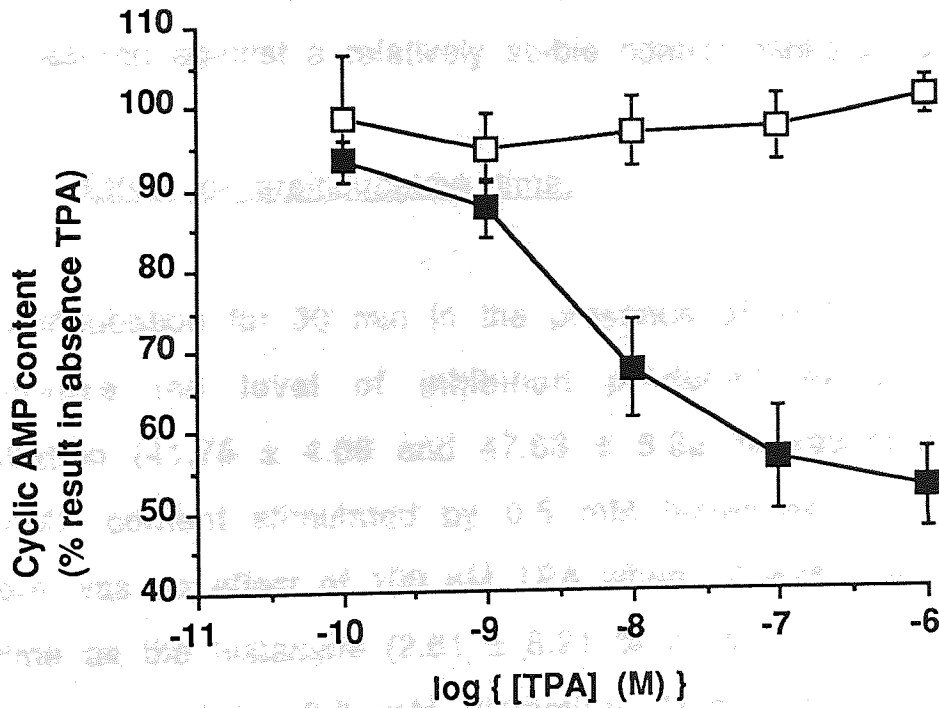
Results are means ± SEM from four separate experiments and are expressed as a percentage of the cyclic AMP response to 0.5 mM histamine alone which was respectively, 5.5 ± 0.76 and 8.3 ± 0.58 pmol/10^6cells in the absence and presence of staurosporine.

effective concentration of TPA at this temperature was 4.1 nM (Fig. 3.9). Preincubation in the presence of 100 nM TPA for 10 min produced no significant effect at either 0 or 30 min after the end of the preincubation on the basal cyclic AMP content of the cell suspension measured at 37°C (Fig. 3.6) or at 20°C (Fig. 3.7). A temperature of 20°C was preferred for virtually all subsequent experiments as it enabled effects of activation of protein kinase C to be measured against a relatively stable control background.

3.3.2.2   Effect of preincubation time.

Preincubation for 30 min in the presence of TPA at 20°C did not increase the level of inhibition produced by a 10 min preincubation (41.75 ± 4.06 and 47.63 ± 8.82 % inhibition of the cyclic AMP content stimulated by 0.5 mM histamine respectively), and there was no effect of 100 nM TPA when it was added at the same time as the histamine (2.61 ± 8.21 % inhibition of cyclic AMP content stimulated by 0.5 mM histamine) (Fig. 3.10). A 10 min preincubation period in the presence of 100 nM TPA was adopted as standard for all further experiments involving TPA.

3.3.3   Effects of preincubation with OAG, 4α-TPA and staurosporine on the cyclic AMP response to histamine.

3.3.3.1   OAG.

Preincubation with OAG, at a concentration (100 µM) at which it inhibited histamine-stimulated aminopyrine accumulation in rat

Figure 3.10

Effect of preincubation time with TPA, and of preincubation for 10 min with 1-oleoyl 2-acetyl glycerol (OAG) on the stimulation of cyclic AMP content by histamine in cells incubated at 20°C.

Present during preincubation.	Control	TPA 100 nM	TPA 100 nM	TPA 100 nM	OAG 100 μM
Time (min) agent added before histamine.		0	10	30	10
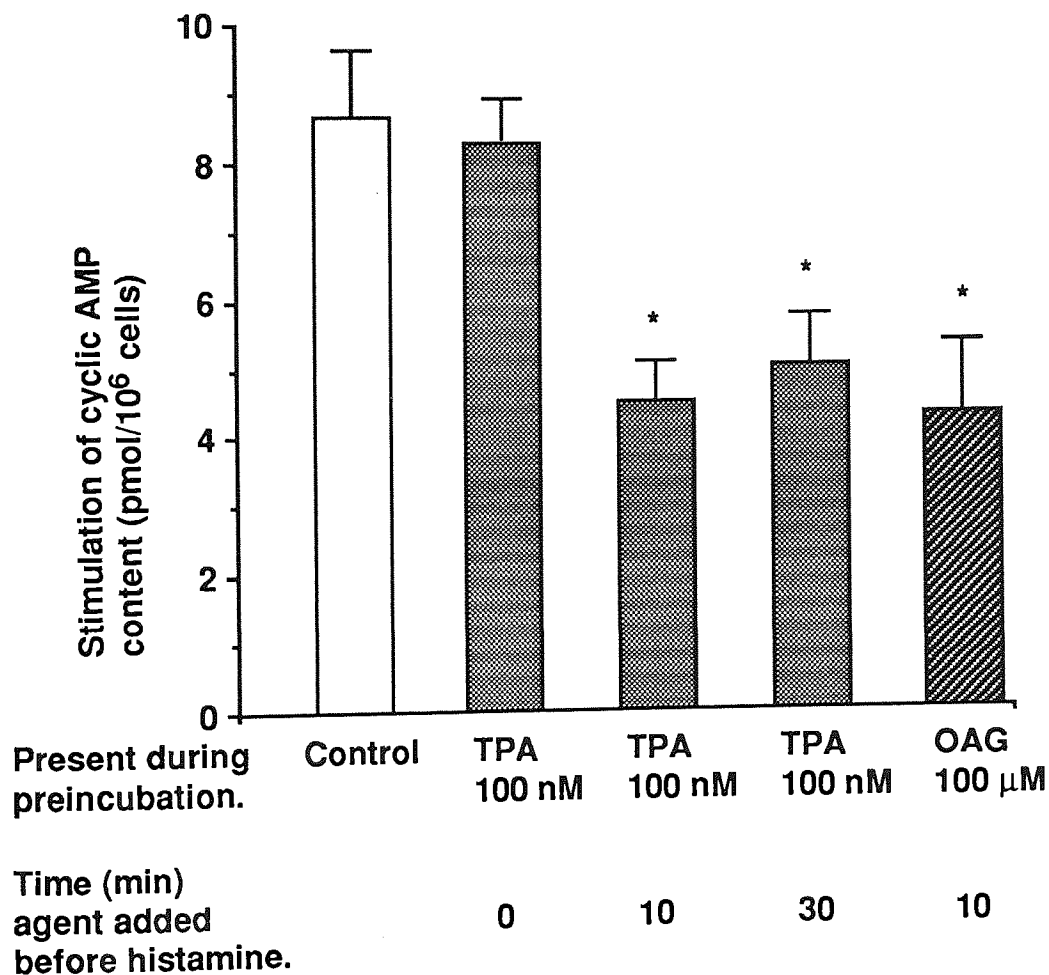
Results are means ± SEM from three experiments and are expressed as stimulation above basal by 0.5 mM histamine and 0.1 mM IBMX. ANOVAR (Appendix A.5.1) and Dunnett's test (Appendix A.5.2) were used to establish whether results were different from control cells incubated in the presence of histamine and IBMX alone, (* P < 0.01).

parietal cells (Anderson and Hanson, 1985) produced a similar inhibitory effect to 100 nM TPA on the response of HGT-1 cells to stimulation by 0.5 mM histamine (Fig. 3.10). A much higher concentration of OAG than TPA is probably required to ensure adequate entry of OAG to the cells and to maintain an intracellular concentration in the face of metabolism (Blackshear, 1988).

3.3.3.2   4α-TPA.

Phorbol esters possess lipophilic ester substituents attached to a relatively polar head group (the phorbol moiety) and as such resemble non-ionic detergents. To distinguish between specific and non-specific effects, possibly due to their detergent-like structures, 4α-TPA, an analogue of TPA that does not activate protein kinase C (Castagna et al., 1982) can be used. Preincubation with 4α-TPA (100 nM) for 10 min at 20°C resulted in a cyclic AMP response to incubation for 5 min in the presence of 0.5 mM histamine which was $102 \pm 12\%$ (n = 3) of the response measured in cells preincubated without 4α-TPA (DMSO vehicle).

3.3.3.3   Staurosporine.

Staurosporine is one of the most potent inhibitors of protein kinase C known (IC_{50} = 2.7 nM) (Tamaoki and Nakano, 1990). 200 nM Staurosporine added 10 min prior to TPA completely abolished the effects of TPA on the cyclic AMP response following exposure to 0.5 mM histamine for 5 min at 20°C (Fig. 3.9). Preincubation with 200 nM staurosporine for 20 min also enhanced the cyclic AMP response in cells incubated with 0.5 mM histamine alone (Fig. 3.9). A pilot

Figure 3.11
Effect of preincubation for 20 min in the absence (open symbols) or presence (filled symbols) of 200 nM staurosporine on the time-course of desensitization seen following stimulation of cells with 0.5 mM histamine at 37°C.
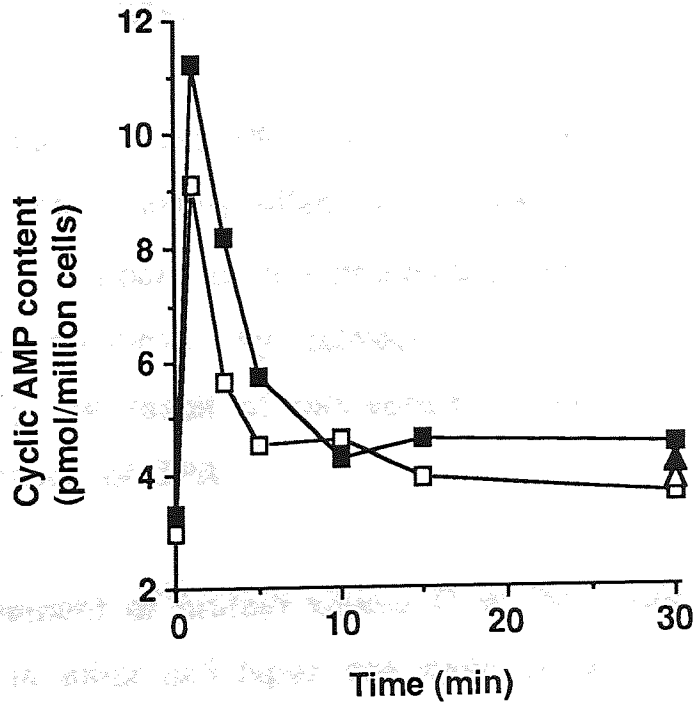
Results are means of duplicate determinations from a single experiment; (□ ■) histamine present, (Δ ▲) histamine absent.

experiment showed that incubation of cells in the presence of staurosporine (200 nM) for 20 min at 37°C had no obvious effect on the time-course of desensitization in the presence of 0.5 mM histamine (Fig. 3.11).

3.3.3.4 Interpretation of results with OAG, 4α-TPA and staurosporine.

The mimicking of the effect of TPA by OAG, the lack of effect of 4α-TPA and the inhibitory effect of staurosporine on the action of TPA provide firm support for the proposition that the effects of TPA were mediated specifically by activation of protein kinase C, and were not simply the result of non-specific effects due for example to detergent actions of TPA.

An involvement of protein kinase C in the process of receptor desensitization in other cell types has been shown (Heyworth et al., 1984; Bocckino et al., 1985), however this seems unlikely in the present studies on the histamine receptor in view of the lack of any obvious effect of staurosporine on the time-course of the cyclic AMP response to histamine stimulation at 37°C.

3.3.4 Effect of preincubation with TPA and staurosporine on the cyclic AMP response of HGT-1 cells to stimulation by GIP and TGLP-1.

In response to incubation at 20°C in the presence of near-maximally effective concentrations of peptides (100 nM) (Gespach et al., 1984; Emami et al., 1986) a significant increase in cyclic AMP

content above basal was observed (P < 0.001 for effects of agonist analysed by paired t-test, n = 3). The peak response was achieved 1 min after addition of TGLP-1 and after 5 min in the presence of GIP, (Fig. 3.12, A and B). Preincubation for 10 min in the presence of 100 nM TPA significantly reduced the cyclic AMP response measured 15 min and 30 min after addition of TGLP-1 (P < 0.01, P < 0.05 respectively for effects of TPA analysed by ANOVAR) but caused an enhancement of the response to GIP (P < 0.001) at 5 min and 15 min after the addition of agonist.

A 20 min preincubation at 20°C in the presence of 200 nM staurosporine had no significant effect on the level of stimulation of cyclic AMP produced by 100 nM TGLP-1. This treatment however, increased the stimulatory effect of GIP by 43.7 ± 13.6 % (n = 3) (Table 3.5), a result not dissimilar to the increase produced by staurosporine on the level of histamine stimulation (35.9 ± 7.1 %; n = 4) (Table 3.5 and Fig. 3.9). Since protein kinase C appears to have opposite effects on the cyclic AMP responses to GIP and histamine the similar enhancement of the response to both of these agonists by staurosporine in the absence of TPA probably does not involve protein kinase C.

Staurosporine completely abolished the stimulatory effect of 100 nM TPA on the cyclic AMP response to GIP, and the inhibitory effect of TPA on the response to TGLP-1 (Table 3.5). These results provide good evidence that the effects of TPA on cyclic AMP content following stimulation of HGT-1 cells by TGLP-1 and GIP were actually mediated through activation of protein kinase C.

Figure 3.12
Effect of preincubation in the absence (open symbols) and presence (filled symbols) of 100 nM TPA for 10 min on the cyclic AMP content of cells incubated at 20°C in the presence of, (A) 100 nM TGLP-1, and (B) 100 nM GIP.
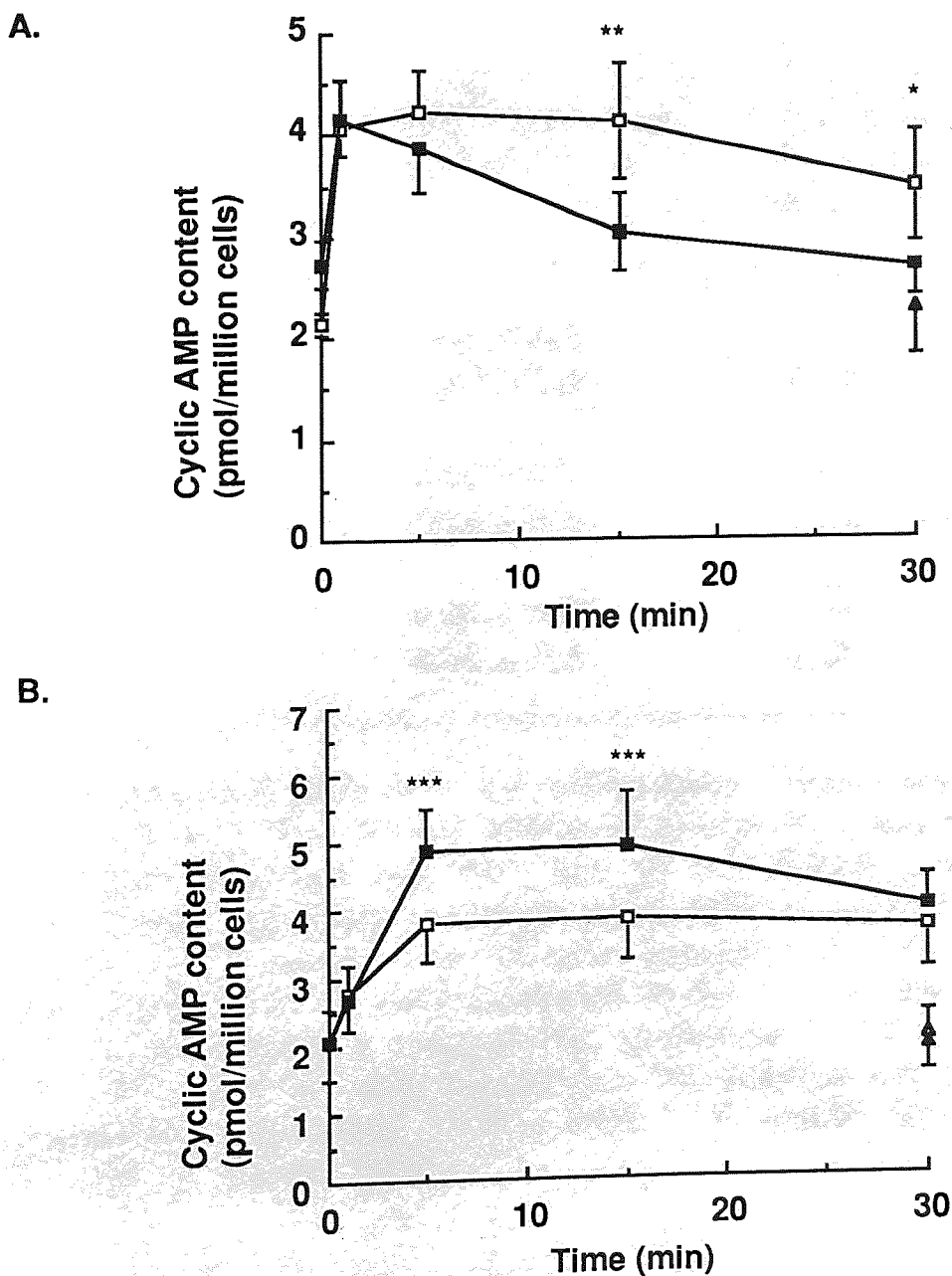
Results are means ± SEM from three experiments in each case. Where no error bars are evident they have been masked by the symbols. (□ ■) agonist present, (△ ▲) agonist absent. Effects of TPA were analysed by ANOVAR (Appendix A.5.1) and are denoted: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3.5

Effect of preincubation in the presence of 200 nM staurosporine on the stimulation of cyclic AMP content by histamine, TGLP-1 and GIP in the absence and presence of TPA.

Agonist	Stauro-sporine (200 nM)	Increase in cyclic AMP above basal (pmol/10^6 cells)	Change in cyclic AMP induced by 100 nM TPA (pmol/10^6 cells)
Histamine	-	5.09 ± 0.5	-2.17 ± 0.4
(0.5 mM)	+	6.94 ± 0.9 *	-0.16 ± 0.3 **
TGLP-1	-	2.15 ± 0.3	-1.12 ± 0.1
(100 nM)	+	2.48 ± 0.4	-0.15 ± 0.08 *
GIP	-	2.06 ± 0.6	-1.24 ± 0.2
(100 nM)	+	2.96 ± 0.8*	-0.28 ± 0.05 *

Results are means ± SEM from 3-4 cell-batches. Staurosporine was added 20 min, and TPA 10 min, before Challenge with agonist which was for 5 min (histamine and GIP) and 15 min (TGLP-1) at $20°C$. Staurosporine did not affect the basal cyclic AMP content of the cell suspensions which were, pmol/10^6 cells: (absence of TPA), 1.62 ± 0.18 and 1.59 ± 0.10, for cells incubated in the in the absence and presence of staurosporine respectively; (presence of TPA), 1.68 ± 0.21 and 1.67 ± 0.17 for cells incubated in the absence and presence of staurosporine respectively. * P < 0.05, ** P < 0.01 for effect of staurosporine by paired t-test.

The effect of TPA on the response to TGLP-1 (51.9 ± 5.3 % inhibition of the response to TGLP-1 alone, n = 3) (Table 3.5) was similar to that observed in the presence of histamine (47.63 ± 8.8 % inhibition of the response to histamine alone, n = 4) (Fig. 3.10) and it is possible that inhibition in both cases was via similar mechanisms. However, this action of protein kinase C is unlikely to be by activation of a cyclic AMP phosphodiesterase, as if this were the case, then a similar inhibition of response by TPA would be expected regardless of the agonist used to stimulate the cells. Clearly, the enhancement of the response to GIP caused by TPA argues against this (see also section 3.3.5). The effects of protein kinase C are therefore more likely to be directed towards inhibition of production of cyclic AMP rather than stimulation of its degradation, and this is supported by a direct effect of TPA on histamine-stimulated adenylate cyclase activity in an isolated membrane fraction of these cells (Chapter 4).

3.3.5 Effect of preincubation with pertussis toxin on the subsequent effects of TPA on the cyclic AMP response to histamine and GIP.

Preincubation of attached cells in culture flasks at 37°C in the presence of 500 ng/ml pertussis toxin for two hours prior to detachment prevented the stimulation of the response to GIP by 100 nM TPA. No such effect of pertussis toxin on the inhibitory action of TPA was observed when histamine was used to stimulate the cells (Table 3.6). There was also no evidence for an effect of pertussis toxin on basal cyclic AMP levels which were 3.9 ± 0.4 and 3.8 ± 0.7

Table 3.6

Effect of preincubation with pertussis toxin on the cyclic AMP content of a suspension of HGT-1 cells stimulated with histamine or GIP in the presence and absence of TPA.

Pertussis toxin	Increase in cyclic AMP (pmol/10^6 cells) above basal.				Effect of TPA (% control)	
	Histamine (0.5 mM)		GIP (100 nM)		Histamine	GIP
(ng/ml)	Control	100 nM TPA	Control	100 nM TPA		
-	6.21 ± 0.29	3.76 ± 0.39*	1.70 ± 0.21	3.42 ± 0.31**	61 ± 5	204 ± 17
500	6.84 ± 0.26	4.62 ± 0.56*	2.46 ± 0.40	2.88 ± 0.53	67 ± 7	115 ± 6

Attached cells in culture flasks were treated with pertussis toxin for 2 h at 37°C or with vehicle. Cells were then detached and measurements of the response to agonists in the presence and absence of TPA were made. Results are means ± SEM of three separate experiments using histamine and of four using GIP. Significant effects of TPA on the response to agonist (by paired t-test) are denoted as follows: * P < 0.05; ** P < 0.01.

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$pmol/10^6$ cells (n = 3) after pretreatment with vehicle or pertussis toxin respectively.

Pertussis toxin inactivates proteins of the "G_i-like" family (section 3.1.5.2). The abolition by pertussis toxin of the stimulatory effect of TPA on the action of GIP, would therefore seem to suggest an involvement of a G_i-like protein in this action (Fig. 3.13). Protein kinase C phosphorylates G_i and prevents its inhibitory action on the catalytic subunit of adenylate cyclase (Katada et al., 1985). Inactivation of G_i with pertussis toxin prior to activation of protein kinase C should therefore render any effects of protein kinase C negligible. A corollary of this argument however is that inactivation of G_i with pertussis toxin should also result in a stimulation of the response to GIP in the absence of TPA. The cyclic AMP responses to stimulation with 100 nM GIP in the absence and presence of pertussis toxin (500 ng/ml) expressed as stimulation above basal were respectively: 1.7 ± 0.21 and 2.46 ± 0.40 $pmol/10^6$ cells (n = 4). The effect of pertussis toxin was not however significant. Despite this it is possible that in these cells, an activated form of G_i regulates adenylate cyclase activity stimulated by GIP, and that this mechanism is not involved in regulation of adenylate cyclase following stimulation by histamine.

One possible explanation for the difference would be the existence of different pools or isoenzymes of adenylate cyclase, or guanine nucleotide regulatory proteins coupling to different receptors (Tang and Gilman, 1992). The existence of these pools or isoenzymes in HGT-1 cells is suggested by the additivity in the cyclic AMP responses to maximally effective concentrations of

Figure 3.13
Potential mechanism for the regulation of GIP-stimulated
adenylate cyclase activity by an activated form of Gi.
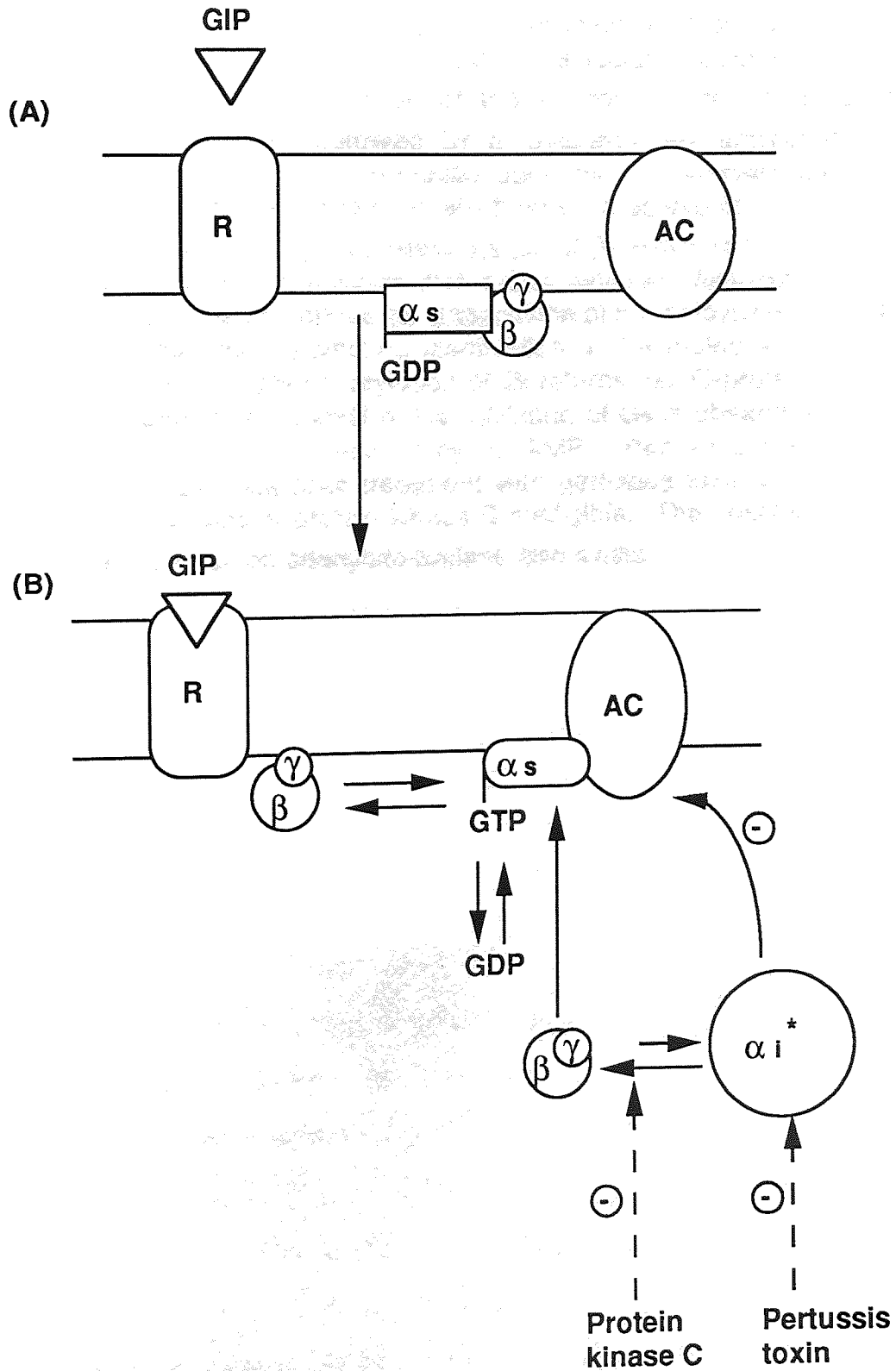
(A). In the basal state Gs exists as the heterotrimer with GDP bound tightly to the α-subunit. The GIP receptor (R) is unoccupied and the adenylate cyclase enzyme (AC) is inactive. (B). Binding of GIP to the receptor causes a receptor-Gs interaction (not shown) which results in GDP-GTP exchange and an associated conformational change in Gs. Dissociation of the G-protein into its α- and βγ-subunits ensues, followed by a Gsα-induced activation of adenylate cyclase. Superimposed upon this mechanism are the effects of an activated form of Gi which dissociates into its constituent subunits thus ensuring a constant supply of βγ-subunits to αs. This shifts the equilibrium position that exists between dissociated and undissociated Gs and drives the dissociation of the holoenzyme in the reverse order, thus promoting inactivation of the molecule. Protein kinase C-induced phosphorylation of Gi returns this G-protein to the inactive state. The removal of the inhibition of Gs is observed as an increase in the production of cyclic AMP. Pertussis toxin also inactivates Gi, thus prior treatment with pertussis toxin is likely to render any effects of protein kinase C negligible. The possibility of a direct effect of αi on adenylate cyclase also exists.

vasoactive intestinal peptide and glucagons, vasoactive intestinal peptide and GIP, and glucagons and GIP (Gespach et al., 1984). The additive response to these agents cannot be explained by the existence of distinct subpopulations of cells as this would require each individual cell type to express the receptor for only one of the agonists concerned and not the receptors for the others. This is perhaps improbable.

3.3.6 Effect of preincubation with TPA on the cyclic AMP response to forskolin and cholera toxin.

Preincubation in the presence of 100 nM TPA for 10 min had no effect on the subsequent stimulation of cyclic AMP content at 20°C by 5μM forskolin (Fig. 3.14). Incubation in the presence of cholera toxin (500 ng/ml) at the same temperature produced a negligible response and experiments were therefore carried out at 37°C in order to obtain significant stimulation. No effect of 100 nM TPA on the cyclic AMP response to 500 ng/ml cholera toxin was evident at this temperature (Fig. 3.15).

Forskolin acts directly upon the catalytic subunit of adenylate cyclase whilst cholera toxin produces a GTP-dependent irreversible activation of the enzyme by permanently activating Gs (section 3.1.5.2). The lack of effect of preincubation with 100 nM TPA on the responses to forskolin and cholera toxin suggests that the actions of protein kinase C on the cyclic AMP responses to histamine, TGLP-1 and GIP are not mediated at the catalytic subunit or at the level of the interaction between Gs and the catalytic subunit. The absence of any inhibitory effect of TPA against the stimulatory effect of GIP in

Figure 3.14

Effect of preincubation in the absence (open symbols) and presence (filled symbols) of TPA (100 nM) on the stimulation of the cyclic AMP content of cells by forskolin (5 μM) at 20°C.
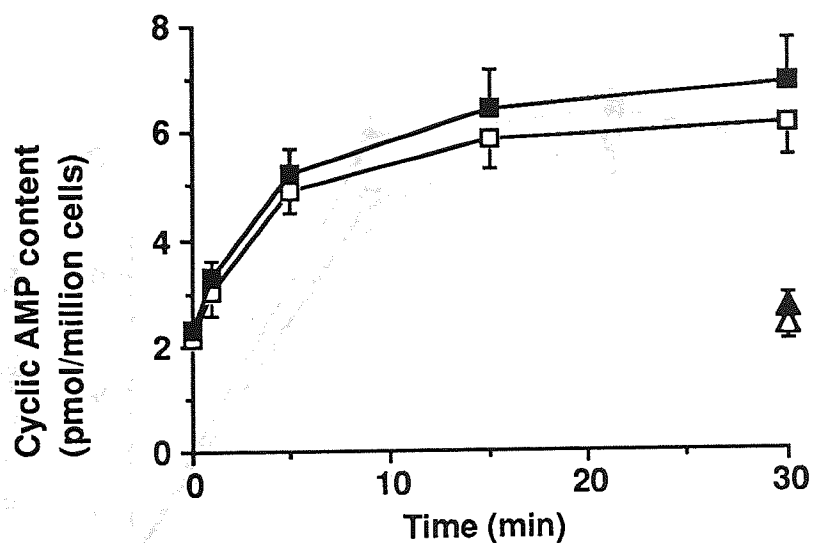
Results are means ± SEM from three experiments. (□■), Forskolin present, (△▲), forskolin absent.

Figure 3.15
Effect of preincubation in the absence (open symbols) and presence (filled symbols) of TPA (100 nM) on the stimulation of the cyclic AMP content of cells by cholera toxin (500 ng/ml) at 37°C.
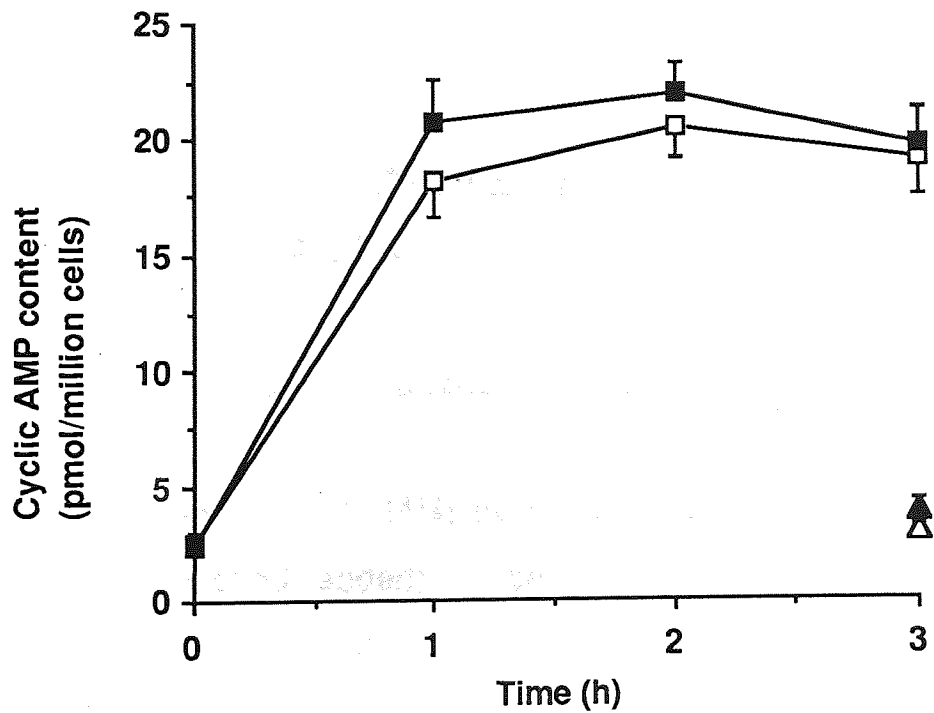
Results are means ± SEM from three experiments. (□■) Cholera toxin present, (△ ▲) cholera toxin absent.

cells preincubated with pertussis toxin (Table 3.6) also argues against an effect on the catalytic subunit or its activation by Gs, and therefore the site of action of protein kinase C is most likely to be at the level of, or close to the histamine receptor. To examine this further experiments were performed to establish whether TPA could affect the binding of an antagonist tiotidine to the histamine H_2 receptor.

3.3.7 Binding of [^{3}H]-tiotidine to HGT-1 cells incubated at 4°C.

3.3.7.1 Time-course and extent of specific binding

Specific binding of [^{3}H]-tiotidine (specific activity, 75 Ci/mmol; 6.7 pmol/ml added) to cells in suspension (6.6 x 10^6 cells/ml) was 7.3 ± 3.8% of total binding which itself accounted for 0.96 ± 0.16% of the total label added (n = 4). Using attached cells, the level of specific binding was increased to 52.5 ± 3.1% of total binding which represented 0.22 ± 0.007% of the total activity added (n = 6). The very much higher non-specific binding with cells in suspension mitigated against their use and all further work was performed using attached cells.

The level of specific binding to attached cells reached a plateau after a one hour incubation at 4°C. Specific binding was 7.32 ± 0.4 and 7.65 ± 0.1 fmol/10^6 cells after one and two hour incubations respectively (results are mean ± SEM of triplicate determinations using a single cell preparation). In all subsequent

experiments an incubation period in the presence of [3H]-tiotidine of one hour was used.

Part of the specifically bound label associated with the cells after a 1 h incubation was displaced in the presence of a 1000-fold excess of unlabelled tiotidine (Fig. 3.16). The binding of tiotidine to HGT-1 cells was therefore at least partially reversible.

3.3.7.2 Specificity of [3H]-tiotidine binding.

The reduction in the amount of [3H]-labelled tiotidine bound to attached cells in the presence of 0.1 mM cimetidine was similar to the reduction observed in the presence of 6.7 μM unlabelled tiotidine (Fig. 3.17). Cimetidine is a potent H_2 receptor antagonist and this result therefore implies that [3H]-tiotidine is binding specifically to the H_2 receptor. The reduction in binding in the presence of 1 mM histamine (Fig. 3.17) was however somewhat less than that produced by either excess cold tiotidine or 0.1 mM cimetidine. It is possible that the concentration of histamine used was insufficient to displace all of the bound tiotidine from the H_2 receptor as tiotidine has a much higher affinity for this receptor than histamine. Incubation in the presence of 0.1 mM pyrilamine had no effect on the amount of total [3H]-tiotidine bound (Fig. 3.17). Pyrilamine is a potent H_1 receptor antagonist and this lack of effect indicates that [3H]-tiotidine was not binding to this receptor should it be present on HGT-1 cells.

Figure 3.16
Displacement of bound [^{3}H]-labelled tiotidine by unlabelled tiotidine with time.
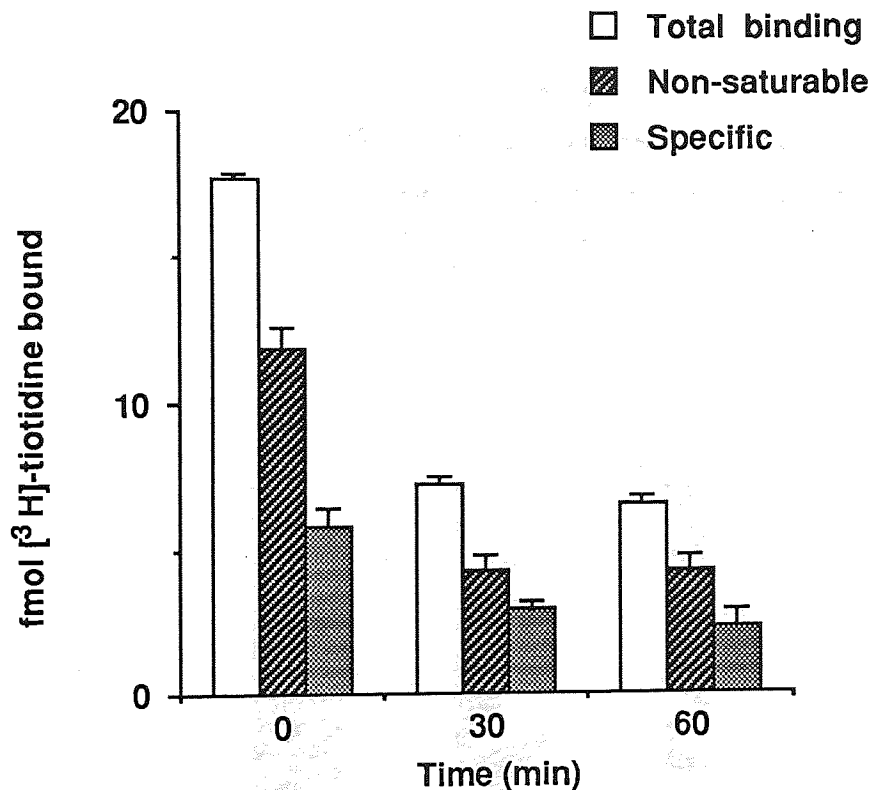
Cells were incubated with 6.7 nM [^{3}H]-tiotidine in the presence and absence of 6.7 µM cold tiotidine for 1 hour at 4°C and then washed three times in ice-cold B´. 6.7 µM cold tiotidine was then added and further incubations carried out for either 30 min or 60 min. The cells were again washed three times before solubilising in 1M NaOH prior to counting. Results are means ± SEM of triplicate determinations from a single lot of cells.

Figure 3.17
Comparison of the reduction in the amount of [^{3}H]-tiotidine bound to cells in the presence of either histamine, cimetidine or pyrilamine.

☐ Total binding.

▨ Binding in presence of agent.

▨ Non-specific binding assessed in the presence of 6.7 μM tiotidine.
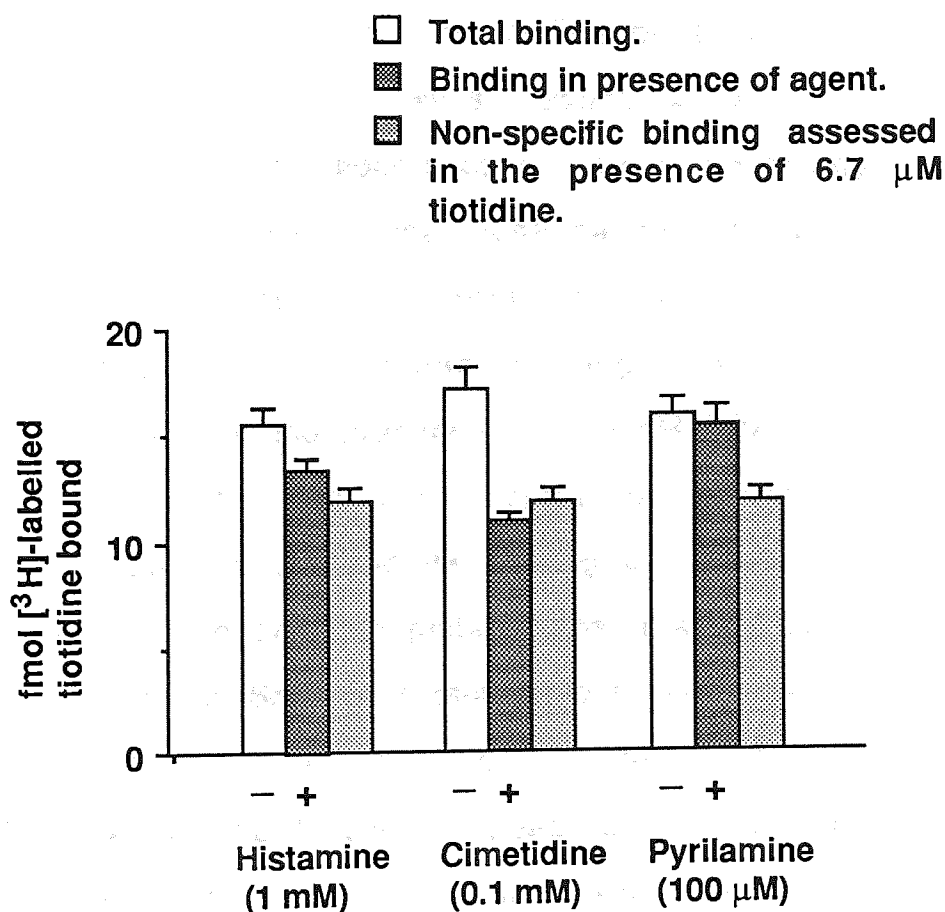
Cells were incubated with 6.7 nM [^{3}H]-tiotidine in the presence and absence of either, histamine (1 mM), cimetidine (0.1 mM) or pyrilamine (100 μM) for 1 hour at 4°C. Non-specific binding assessed in the presence of unlabelled tiotidine (6.7 μM) is included for comparison. The cells were washed three times before solubilizing in 1M NaOH prior to counting. Results are means ± SEM from two experiments with triplicate determinations in each experiment.

3.3.7.3   Effect of preincubation with TPA on specific binding of [³H]-tiotidine.

Binding curves were constructed by examining the effect of various concentrations of unlabelled tiotidine on tracer binding (Fig. 3.18). For each plate of six wells, results were normalized by expressing the specifically bound counts as a percentage of the counts bound specifically in the absence of added cold label. Examination of the first component of the competition curve representing high-affinity tiotidine binding sites showed that preincubation of cells in the presence of 100 nM TPA for 10 min at 37°C significantly shifted the binding curve to the right (Fig. 3.18). The inability to perform a complete binding experiment on a single plate and the variation between plates made analysis of the number of binding sites by a Scatchard plot difficult. However the binding data could be fitted to a two-site binding model 2SITEINHIB (Barlow, 1983) which yielded the following values for the concentrations of added unlabelled tiotidine causing half-maximal inhibition of tracer binding (IC_{50}) at each site:

High affinity site; 5.55 nM control, 16.63 nM TPA.

Low affinity site; 372 nM control, 507 nM TPA.

Although a fit was obtained with this model, the results clearly show the limitations of [³H]-tiotidine in such binding studies because the IC_{50} for the high affinity site in control cells (5.55 nM) falls below the tracer concentration of tiotidine which was 6.7 nM.

These figures would seem to suggest an effect of protein kinase C, either directly upon the H_2 receptor or on a protein closely associated with it. However, the change although significant was

Figure 3.18
Effect of added unlabelled tiotidine on the binding of [^{3}H]-tiotidine to cells.
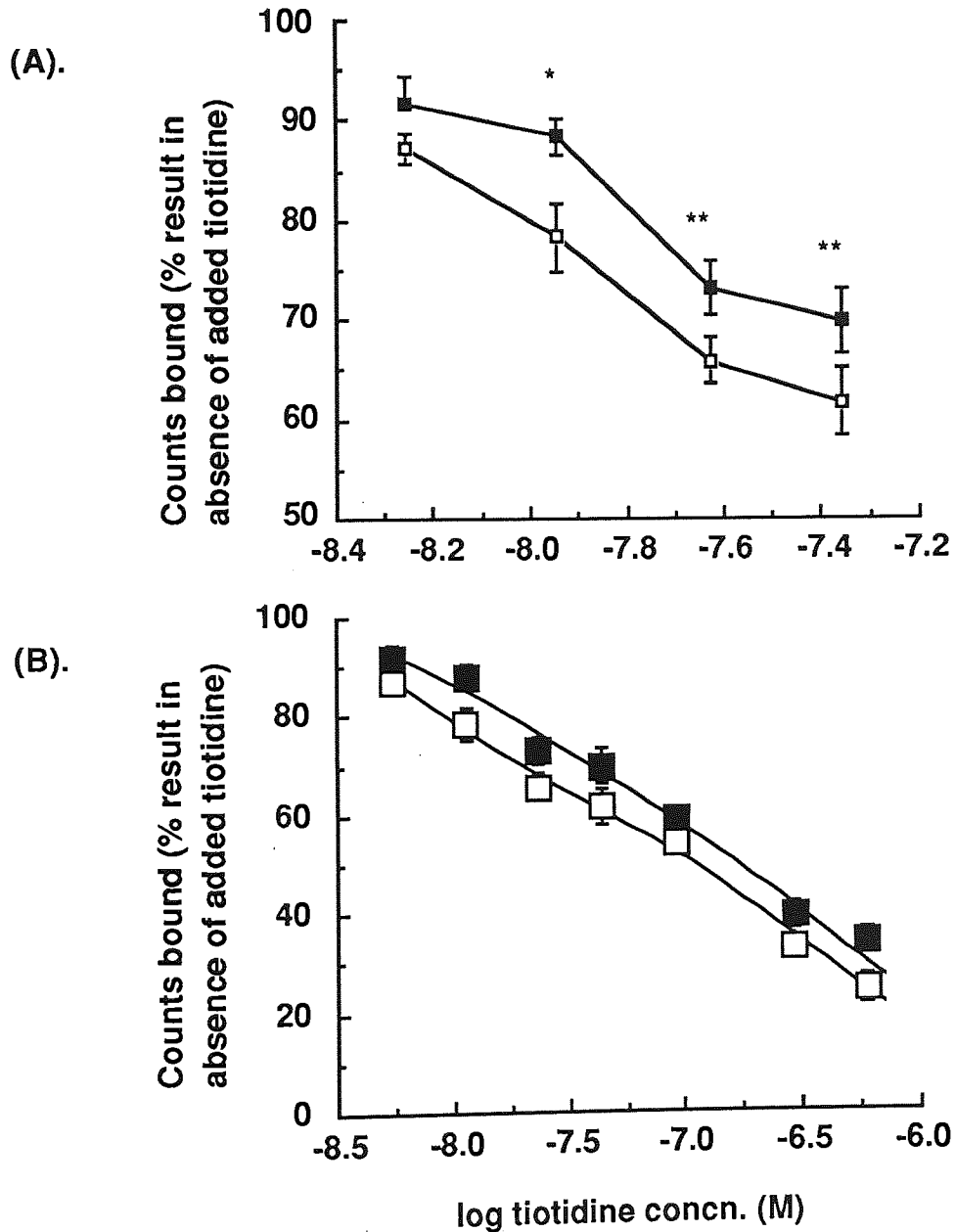
Preincubation was with 100 nM TPA (■) or vehicle (□) for 10 min at 37°C. Binding was assessed at 4°C for 1 h. Results are means ± SEM from three experiments, with the results at each concentration being determined in quadruplicate in each experiment. (A); enlargement of the high affinity section of the binding curve. (B); All of the data fitted to the two-site binding model, 2SITEINHIB (Barlow, 1983). Error bars where not evident are masked by the symbols. * P < 0.05; ** P < 0.01 for a significant effect of TPA determined by ANOVAR (Appendix A.5.1).

small and difficult to measure and consequently further experiments to assess the specificity of the action of TPA were not performed.

3.4   GENERAL DISCUSSION.

There is clear evidence that receptors coupled to the stimulation of adenylate cyclase can be phosphorylated, not only by protein kinase C but also by kinases of the β-ARK (β-adrenoceptor kinase) family (Bouvier et al., 1987), and by protein kinase A. Phosphorylation by all of these kinases has also been shown to result in an attenuation of the ability of the β2-adrenergic receptor to couple to G_s (Pitcher et al., 1992). Protein kinase C has also been proposed to uncouple a receptor for glucagon-29 from G_s by phosphorylating a form of the glucagon receptor (Houslay, 1991a). Although the receptor for TGLP-1 in the rat gastric mucosa appears to be different from that for glucagon-29 (Uttenthall and Blazquez, 1990), they might be part of a closely related family and thus be affected similarly. The argument that protein kinase C-induced phosphorylation of the TGLP-1 receptor reduces the efficiency of coupling to G_s is thus not unreasonable.

The amino acid sequence of the human histamine H2 receptor has recently been determined (Gantz et al., 1991) and the sequence of a portion of the C-terminal cytosolic region of this receptor is reproduced below (amino acids are designated using their one-letter symbols, serine residues are depicted in bold type):

Q L F C C R L A N R N S H K T S L R S N A S Q L S R T Q S R E P R.

This sequence exhibits several sites at which serines (S) are flanked by the basic residues lysine (K) arginine (R) and histidine (H). These sites could therefore represent a substrate for protein kinase C (Graff et al., 1989). An effect of protein kinase C on receptor-G_s coupling mediated by a phosphorylation of G_s also

however remains a possibility. There is now evidence that this G-protein can be phosphorylated by protein kinase C (Pyne et al., 1992), however the effect of these phosphorylations upon the regulation of adenylate cyclase has yet to be determined.

In conclusion, the most likely explanation of the present results is that phosphorylation of the histamine H_2 receptor by protein kinase C partially uncouples the receptor from G_s. The reduction of receptor-G_s coupling then reduces the ability of histamine to activate adenylate cyclase. However this uncoupling cannot be responsible for the change in tiotidine-binding as the extent of receptor-G_s coupling does not affect binding of antagonists. Therefore the change in tiotidine binding induced by TPA, and probably the action of protein kinase C, may be due to a conformational change in the receptor binding site caused by phosphorylation of the receptor on the carboxy-terminal sequence.

3.5   SUMMARY.

1.   A 10 min preincubation of HGT-1 cells with 100 nM TPA significantly reduced the cyclic AMP responses to 0.5 mM histamine, and 100 nM TGLP-1, and increased the response to 100 nM GIP. These effects were not apparent in the presence of 200 nM staurosporine and thus indicate that the effects of TPA were in fact mediated through activation of protein kinase C. The differential effects of TPA are not consistent with a single action involving activation of a cyclic AMP phosphodiesterase.

2.   Preincubation for 20 min with 200 nM staurosporine significantly increased the cyclic AMP responses to both 0.5 mM histamine and 100 nM GIP but did not significantly affect the response to 100 nM TGLP-1. Pertussis toxin prevented the increase in the response to GIP induced by TPA but did not affect the action of TPA on histamine stimulation of cyclic AMP. This suggests that in these cells, an activated form of G_i possibly regulates adenylate cyclase activity stimulated by GIP, and that this mechanism is different from that involved in the regulation of adenylate cyclase activity stimulated by histamine.

3.   No effects of TPA on the cyclic AMP responses to either 5 μM forskolin or 500 ng/ml cholera toxin were detected. This suggests that the effects of TPA on the cyclic AMP response to histamine are not mediated at the catalytic subunit of adenylate cyclase or at at the level of interaction between G_s and the catalytic subunit. Thus, the site of action of protein kinase C is most likely to be at the level of, or close to the H_2 receptor.

4. Preincubation of cells in the presence of 100 nM TPA for 10 min at 37°C significantly affected the ability of the H$_2$ receptor antagonist tiotidine to bind to the H$_2$ receptor thus suggesting an effect of protein kinase C upon the receptor or on a protein closely associated with it.

5. The most likely explanation of the above results is that a protein kinase C-mediated phosphorylation of the H$_2$ receptor causes a reduction in the efficiency of coupling of the receptor to G$_s$, and this is then observed as a reduction in the cyclic AMP response to histamine.

CHAPTER 4

THE ISOFORM OF PROTEIN KINASE C RESPONSIBLE FOR INHIBITION OF ADENYLATE CYCLASE ACTIVITY IN HGT-1 CELLS.

4.1 INTRODUCTION.

4.1.1 Downregulation of protein kinase C.

Two approaches which can be used to study the effects of a particular enzyme in a signalling pathway are the use of specific inhibitors of the enzyme or to downregulate the enzyme by affecting its synthesis or degradation. Downregulation of protein kinase C will occur (Blackshear, 1988) if phorbol ester-sensitive forms of protein kinase C are present in the cells, and if the rate of degradation can be made higher than the rate of synthesis (Fig. 4.1). Under these circumstances, protein kinase C should be depleted from the cell and the actions of agonists, whose effects are mediated through protein kinase C-dependent pathways, should be diminished.

Long-term treatment with high concentrations of phorbol ester causes protein kinase C to bind to membranes where a conformational change increases the susceptibility of the enzyme to cleavage by Ca^{2+}-activated neutral proteases (Melloni et al., 1986). These enzymes initiate the degradation of protein kinase C via specific proteolytic attack at basic residues located within the hinge region (Melloni et al., 1986). The primary products of degradation are the regulatory and catalytic domain fragments (Young et al., 1987) and these are eventually completely degraded by proteases. The rate of downregulation varies according to the isoform of protein kinase C and the tissue type and is dependent upon the rate constants (K) associated with the individual steps. The steady state levels of the holoenzyme become low if $K_2 >> K_0$ and constitutively active fragments will remain in the cell if $K_2 >>$

Figure 4.1
The mechanism of downregulation of protein kinase C.

Synthesis

K_0

Hinge
region

K_1

K_{-1}

Holoenzyme
(80 kDa)

Ca^{2+}
dependent
protease

K_2

K_3

Proteases

Catalytic
subunit
(50 kDa)

Regulatory
subunit
(30 kDa)

Degraded
fragments
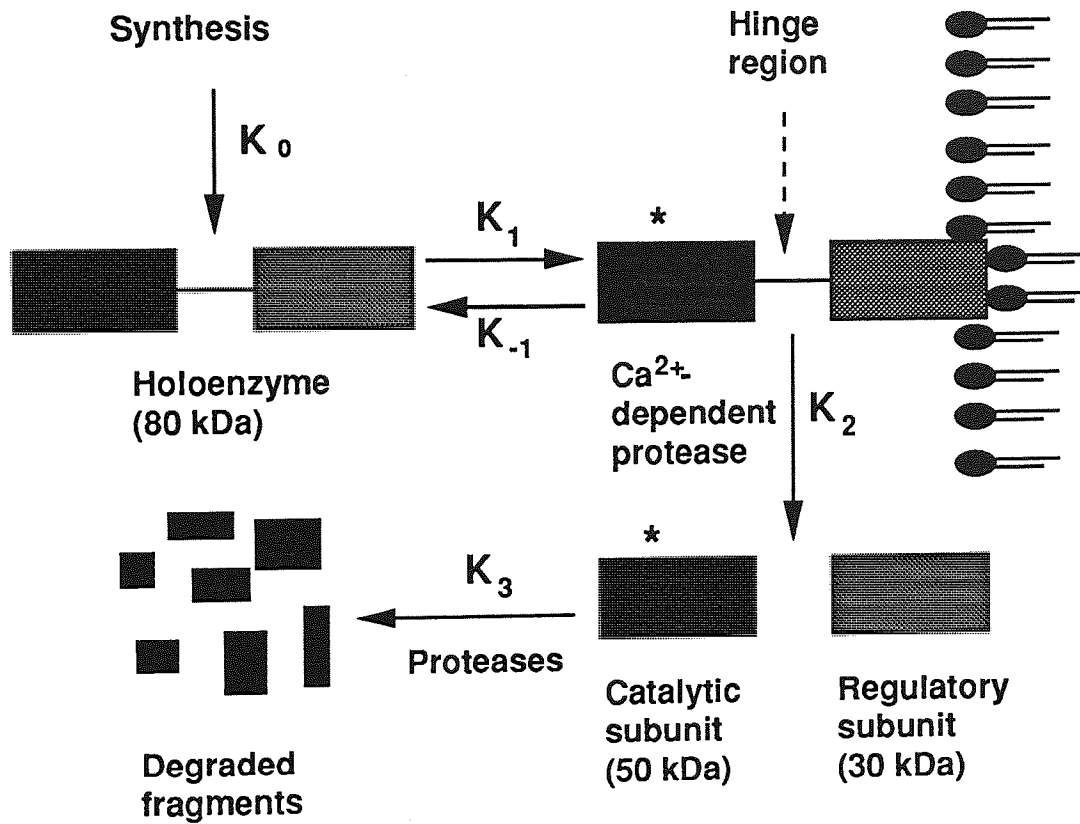
* Denotes activated forms of protein kinase C. K_0, K_1, K_{-1}, K_2, and K_3 represent the rate constants for the associated steps. Certain phorbol esters increase K_1.

K_3 (Fig. 4.1). Phorbol esters increase K_1, however, differences in K_2 between isoforms of protein kinase C may explain different rates of downregulation of isoforms (Parker et al., 1989).

The advantage of downregulation, by contrast with the use of most inhibitors of protein kinase C, is the possibility of selectivity towards particular isoforms of the enzyme. The disadvantage is that downregulation is not associated with significant changes in the rate of synthesis of protein kinase C (Young et al., 1987), and therefore complete removal of activity may never occur (Blackshear, 1988).

In rat parietal cells, TPA caused an inhibition of histamine-stimulated aminopyrine accumulation that correlated with a reduction in histamine-stimulated cyclic AMP content of the cells (Hatt and Hanson, 1989). A similar effect of TPA on histamine-stimulated cyclic AMP content was also found in HGT-1 cells (Chapter 3). In order to investigate further the mechanism whereby TPA elicits these effects, and to attempt to identify the isoform of protein kinase C responsible, downregulation of protein kinase C was attempted.

4.1.2 Effects of activation of protein kinase C on adenylate cyclase activity stimulated by histamine in isolated membranes.

Activation of protein kinase C by TPA in HGT-1 cells results in an inhibition of histamine-stimulated cyclic AMP content (Chapter 3). To prove that adenylate cyclase was the site of action of

TPA/protein kinase C, it was necessary to demonstrate that the activity of this enzyme was inhibited by TPA. Pilot experiments in which intact cells were pretreated with TPA and then homogenized and assayed for adenylate cyclase activity did not give clearcut results. Recent work (Chakravarthy et al., 1991; 1992) has suggested that in some cultured cells protein kinase C may be found in a membrane-associated but inactive form. The possibility that TPA could inhibit adenylate cyclase activity in isolated membranes was therefore investigated. An advantage of this approach was that, if successful, the ability of isoform-specific anti-protein kinase C antibodies to block effects of TPA could be investigated, as could the requirements for Ca^{2+}.

4.2   METHODOLOGY.

4.2.1   Preparation of reagents.

TPA, forskolin, cholera toxin and staurosporine were all prepared as described previously (Chapter 3).   Phorbol 12,13-dibutyrate was dissolved in DMSO and was always prepared from a concentrated stock solution on the day of use. [^{3}H]-labelled phorbol 12,13-dibutyrate was supplied already dissolved in ethanol and an appropriate dilution of this stock or vehicle was added to cultured cells 1 h prior to their detachment.   Phosphatidylserine was stored dissolved in ethanol at a concentration of 10 mg/ml.   13.3 µl of this stock was evaporated under nitrogen, 250 µl of incubation buffer (Appendix A.2.3) was added to the residue and the mixture sonicated at 30 W for 1 min on ice (MSE Soniprep).   The sonicate was added to 6 ml of incubation buffer to give a final phosphatidylserine concentration of 20 µg/ml.   Antibodies to the α- and ε-isoforms of protein kinase C were dissolved in phosphate-buffered saline (Appendix A.2.2) at a concentration of 1 mg/ml and stored at -20°C. Anti-protein kinase C α anti-peptide antibody was generated in rabbit against a unique amino acid sequence in the V$_3$ hinge region of the enzyme corresponding to amino acids 313-326 (Makowske et al., 1988).   Anti-protein kinase C ε anti-peptide antibody was also generated in rabbits using a unique peptide from the same hinge region (residues 313-326).   These stocks were further diluted with phosphate-buffered saline as required and appropriate quantities of antibody or vehicle added to the adenylate cyclase assay mixture. Peptide against which the α-isoform was generated was also dissolved in phosphate-buffered saline.

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4.2.2   Downregulation of protein kinase C.

HGT-1 cells were grown in two flasks until almost confluent. 24 h prior to use, the culture medium was removed and replaced with 20 ml of fresh culture medium to which either 20 µl of DMSO (0.1 % $^v/_v$) or 20 µl of a 1 mM stock solution of phorbol 12,13-dibutyrate had been added.   The final phorbol 12,13-dibutyrate concentration in the medium was 1 µM. Cells were then incubated for 24 h in a CO_2 incubator maintained at a temperature of 37°C. The medium was removed and the cells washed and detached as described previously (Chapter 2).   The cells were washed a further three times in medium B′ (Appendix A.2.1) before use.   Effects of histamine on the cyclic AMP response were determined as described previously.   This protocol (C), plus two other protocols that were employed are summarized in Table 4.1.

4.2.3   Effects of TPA on adenylate cyclase activity stimulated by histamine.

4.2.3.1   Preparation of an isolated membrane fraction derived from a suspension of HGT-1 cells.

Cells were cultured and detached from flasks as described previously (Chapter 2) then washed twice prior to resuspension at a concentration of 2 x 10^7 cells/ml in homogenization buffer (pH 7.5) consisting of 10 mM Tris HCl, 1 mM EDTA, 30 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride and 10 µg/ml of each of leupeptin, pepstatin, aprotinin and soybean trypsin inhibitor. The above suspension was homogenised on ice using a hand-held

Table 4.1

Experimental protocols used to investigate effects of exposure to phorbol 12,13-dibutyrate on protein kinase C.

In all cases cells were challenged with 0.5 mM histamine at t = 0 and cyclic AMP content measured after 5 min of incubation at 20°C.

Protocol A.

Treatment:	Detach and wash	Suspension + 0.1 mM IBMX	Suspension + 1 μM phorbol 12,13-dibutyrate or DMSO
Time:		-20 min	-10 min
Temperature:		20°C	20°C

Protocol B.

Treatment:	Detach and wash	Suspension + 1 μM phorbol 12, 13-dibutyrate or DMSO	Wash	Suspension + 0.1 mM IBMX + 200 nM staurosporine
Time:		10 min period		-20 min
Temperature:		20°C		20°C

Protocol C.

Treatment:	Attached cells + 1 μM phorbol 12,13-dibutyrate or DMSO	Detach and wash	Suspension + 0.1 mM IBMX ± 200 nM staurosporine
Time:	24 h		-20 min
Temperature:	37°C		20°C

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teflon-glass homogeniser (30 strokes/min for 2 min). Cell counts were performed before and after homogenization in order to determine the number of cells either broken or permeable to trypan blue. The homogenate was then centrifuged at 40,000 x g for 90 min at 4°C in an MSE superspeed 50 centrifuge. The supernatant was discarded and the surface of the pellet washed twice with ice-cold homogenization buffer. The pellet was then resuspended in a volume of ice-cold homogenization buffer equivalent to the original volume used for homogenization of cells. 0.25 ml aliquots of this homogenate were frozen to -80°C and stored overnight prior to use.

4.2.3.2 Determination of the protein content of an isolated membrane fraction.

The protein content of the membrane fraction was determined using a modification of the Lowry method (Markwell et al., 1978). Stock solutions of reagent A containing 2.5 % ($^w/_v$) Na_2CO_3, 0.5 % ($^w/_v$) NaOH, 0.2 % ($^w/_v$) sodium tartrate and 1.25 % ($^w/_v$) sodium dodecylsulphate, and reagent B containing 5 % ($^w/_v$) $CuSO_4.5H_2O$ were prepared and mixed together in the ratio of 100 parts of reagent A to 1 part of reagent B to form reagent C. An isolated membrane fraction prepared as described above (section 4.2.3.1) but without the final addition of homogenization buffer was solubilized in 1 ml of 1 % ($^w/_v$) sodium dodecylsulphate in NaOH (0.1 M). 4 µl samples of this mixture were then added to a further 396 µl of 1 % ($^w/_v$) sodium dodecylsulphate in NaOH (0.1 M) and the entire sample then added to 600 µl of reagent C and incubated at room temperature for 10 min. 75 µl of Folin-Ciocalteu's phenol reagent diluted 1:1 with distilled water was then added to the above and the samples mixed gently by

inversion. These samples were incubated at room temperature for 45 min before measurement of absorbance at 750 nM. Blanks were prepared as described above for samples but without the addition of homogenate. Bovine serum albumin was dissolved in 1 % ($^w/_v$) sodium dodecylsulphate in NaOH (0.1 M) and used as standard.

<u>4.2.3.3</u>   <u>Assay of adenylate cyclase activity.</u>

The protein content of the homogenate was determined as described above and the homogenate then diluted to give a final concentration of 0.67 μg protein/μl homogenate. 15 μl aliquots of the diluted homogenate were then added to 235 μl of incubation buffer (Appendix A.2.3) to give the following final concentrations in the assay mixture (pH 7.5): 25 mM Tris HCl, 1 mM EGTA, 0.06 mM EDTA, 0.06 mM dithiothreitol, 1.8 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 20 mM creatine phosphate, 35 U/ml creatine kinase, 0.2 mM IBMX, 1 mg/ml bovine serum albumin, 200 μg/ml bacitracin and 200 μM GTP. Phosphatidylserine (20 μg/ml) and Ca^{2+} (free concentration, 10^{-5} M) were sometimes added to the assay system. The free Ca^{2+} concentration was calculated as described by Portzehl et al., (1964). 10 mM EGTA was present in the incubation medium when experiments were performed in the presence of negligible free Ca^{2+}. Histamine (0.5 mM) and TPA (100 nM) were also added where appropriate. Homogenate was added to the prewarmed incubation buffer, the mixture vortexed and then incubated for 10 min at 30°C. The reaction was terminated by addition of 250 μl of ice-cold ethanol to the assay mixture and immediately vortexing. The assay mixture was then spun at 10,000 x g for 1 min in a Beckman microfuge and the supernatant collected and frozen at -20°C.

Samples were evaporated to dryness in a vacuum oven at a temperature of 40°C and the cyclic AMP content of the residue then determined as described previously (Chapter 2).

4.3 <u>RESULTS AND DISCUSSION.</u>

4.3.1 <u>Downregulation of protein kinase C.</u>

4.3.1.1 <u>Effect of preincubation for 24 h with phorbol esters on the response of HGT-1 cells to histamine.</u>

The protocol can be summarized as follows: (see also table 4.1).

Treatment	Attached cells + phorbol 12,13-dibutyrate	Detachment + washing	Suspension + 0.1 mM IBMX	0.5 mM histamine	Assay of cyclic AMP
Time	-24 h		-20 min	0	5 min

No effect of preincubation with phorbol 12,13-dibutyrate was detected on the basal cyclic AMP content of the cell suspensions (Fig. 4.2). Preincubation of cells for 24 h in the presence of 1 μM phorbol 12,13-dibutyrate produced a depression in the cyclic AMP response to stimulation with histamine (0.5 mM) that was not observed in cells preincubated with DMSO (Table 4.2). The possibility existed that the depressed response was caused by the presence of activated protein kinase C during challenge by histamine, perhaps because phorbol 12,13-dibutyrate was not removed by washing of the cells and protein kinase C had not been downregulated. 0.45 μCi of [3H]-labelled phorbol 12,13-dibutyrate was added to the culture medium 1 h prior to detachment of cells. The activity recovered after washing represented 0.006 $\pm$ 0.001 % (n = 3) of the total added, which would indicate a final unlabelled

Figure 4.2
Effect of time of preincubation with phorbol 12,13-dibutyrate on the subsequent effect of staurosporine on the cyclic AMP content of cells stimulated with histamine.
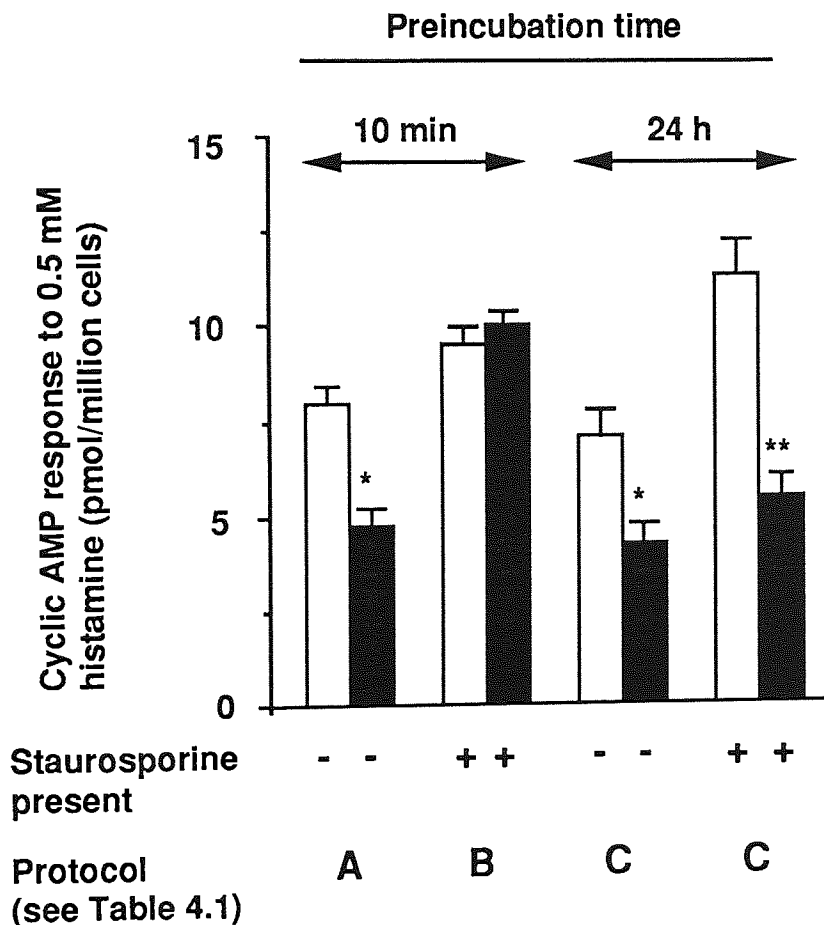
Results are means ± SEM from 3-6 experiments and are expressed as stimulation above basal by 0.5 mM histamine. Preincubation was for the time indicated in the presence (■) of 1 µM phorbol 12,13-dibutyrate (PDBu) or vehicle (□). Challenge with histamine was for 5 min at 20°C. For further details of protocols A, B and C see Table 4.1. There was no effect of preincubation time or staurosporine on basal cyclic AMP content of the cell suspensions which were, pmol/million cells: 10 min preincubation, 1.88 ± 0.56 (control), 1.91 ± 0.59 (PDBu) for cells incubated in the absence of staurosporine and 1.73 ± 0.43 (control), 1.92 ± 0.57 (PDBu) for cells incubated in the presence of staurosporine. 24 h preincubation, 1.28 ± 0.16 (control), 1.23 ± 0.25 (PDBu)for cells incubated in the absence of staurosporine, and 1.54 ± 0.24 (control), 1.61 ± 0.27 (PDBu) for cells incubated in the presence of staurosporine. * $P < 0.05$; ** $P < 0.01$ for effects of phorbol 12,13-dibutyrate analysed by paired t-test.

Table 4.2
Effect of preincubation with 1 μM phorbol 12,13-dibutyrate (PDBu) for 24 h on the cyclic AMP response to agents that stimulate adenylate cyclase and the lack of effect of a recovery period on this response.

Agent	Number of experiments	Recovery period prior to stimulation with agonist (h)	Pretreatment	
			Control	1 μM PDBu
Histamine (0.5 mM)	6	0	7.11 ± 0.70	4.26 ± 0.26**
Histamine (0.5 mM)	3	4	8.01 ± 0.91	4.96 ± 0.38*
forskolin (5 μM)	3	0	5.61 ± 0.62	7.01 ± 0.22
cholera toxin (500 ng/ml)	3	0	54.18 ± 2.14	51.07 ± 3.04

Cells were preincubated for 24h in the presence of 1μM phorbol 12,13-dibutyrate or with vehicle before detachment and washing. Subsequent incubations in the presence of agonist were for 5 min at 20°C (histamine and forskolin) or for 1h at 37°C (cholera toxin). Results are means ± SEM from the indicated number of experiments. They are expressed as pmol/10^6 cells and represent stimulation of cyclic AMP content above basal by agonist. Preincubation with phorbol 12,13-dibutyrate had no effect on the basal cyclic AMP content of the cell suspensions which was, pmol/10^6 cells: 2.08 ± 0.60 (control), and 1.94 ± 0.37 (phorbol 12,13-dibutyrate). ** $P < 0.01$, * $P < 0.05$ for effect of preincubation with phorbol 12,13 dibutyrate analysed by paired t-test.

phorbol 12,13-dibutyrate concentration in the incubation medium, assuming an even distribution, of 60 pM. A similar concentration of TPA was shown to have a negligible effect on the cyclic AMP response to histamine (Chapter 3), and since phorbol 12,13-dibutyrate is known to be a 3-fold less potent activator of protein kinase C than TPA, it seems unlikely that the depressed response to histamine was due to the activation of protein kinase C by residual phorbol 12,13-dibutyrate.

Long-term treatment with phorbol esters has been shown to lead not only to downregulation but also to constitutive activation of the remaining protein kinase C (Blackshear, 1988). Thus, retention of an activated form of protein kinase C after washing of the cells to remove phorbol 12,13-dibutyrate was also considered a possible explanation of the decreased cyclic AMP response to histamine. Staurosporine is a potent inhibitor of protein kinase C that binds to its catalytic domain both in the holoenzyme (Tamaoki and Nakano, 1990) and in proteolytically generated fragments (Gescher and Dale, 1989), and would therefore be expected to inhibit the activity of both intact and constitutively active fragments of protein kinase C. The effects of staurosporine on cells which had been preincubated over both short and long-time periods with phorbol 12,13-dibutyrate was therefore investigated using protocols B and C mentioned previously (Table 4.1). In all cases, histamine was added at time zero to cell suspensions incubated at 20°C and the suspension removed for assay of cyclic AMP 5 min later.

Addition of 1 µM phorbol 12,13-dibutyrate to cells 10 min before challenge with histamine reduced the effect of histamine on

cyclic AMP content (Fig. 4.2, protocol A). If the cells were similarly treated with 1 μM phorbol 12,13-dibutyrate but washed and treated with staurosporine (200 nM) before challenge with histamine then this effect of 1 μM phorbol 12,13-dibutyrate was abolished (Fig. 4.2, protocol B). This means that washing plus staurosporine can reverse the effects of short-term incubation with 1 μM phorbol 12,13-dibutyrate on the response to histamine. The same treatment did not however reverse the effects of long-term (24 h) preincubation with phorbol 12,13-dibutyrate (Fig. 4.2). Nor did a 4 h recovery period in the absence of phorbol ester return the cyclic AMP response to histamine to control levels (Table 4.2) . It seems unlikely that the reduced histamine response of cells, which had been treated for 24 h with phorbol 12,13-dibutyrate was due to the presence of a constitutively active protein kinase C.

The cyclic AMP responses to forskolin (5 μM) and cholera toxin (500 ng/ml) were unaffected by preincubation of cells with 1 μM phorbol 12,13-dibutyrate (Table 4.2). This result implies that the effect of preincubation for 24 h with 1 μM phorbol 12,13-dibutyrate did not result in a modulation of the adenylate cyclase system at or distal to G_s, or indeed to a general loss of cellular function. Sustained activation of protein kinase C could cause a loss of histamine H_2 receptors but this was not tested due to the unsatisfactory nature of [3H]-tiotidine for precise binding studies with these cells (Chapter 3).

In conclusion, the reduced response to histamine in HGT-1 cells preincubated with 1 μM phorbol 12,13-dibutyrate for 24 h was caused by a different mechanism from that involved in short-term

regulation of the histamine response by phorbol esters principally because it was not reversed by washing of the cells and adding staurosporine. Such pretreated cells could therefore be used to investigate whether the short-term inhibitory response to TPA was still retained under conditions causing potential downregulation of protein kinase C.

4.3.1.2 Effect of preincubation with phorbol 12,13-dibutyrate on the subsequent effect of TPA on histamine-stimulated cyclic AMP content.

An inhibitory effect of TPA on the response to histamine was apparent ($P < 0.01$) in the cells pretreated with DMSO (Fig. 4.3, Fig. 4.4). The cells exposed to phorbol ester for 24 h exhibited a marked decrease in the cyclic AMP response to subsequent challenge with histamine (see above), however this level of stimulation was not further reduced by the presence of TPA (Fig. 4.3, Fig. 4.4). Thus prolonged incubation with phorbol ester caused a loss of antagonism of the cyclic AMP response to histamine by TPA. This result may have been a consequence of downregulation of protein kinase C because both protein kinase C α and protein kinase C ϵ have been detected in HGT-1 cells by protein blotting, and loss of immunologically detectable protein kinase C α but not protein kinase C ϵ has been shown following 24 h preincubation in the presence of 1 μM phorbol 12,13-dibutyrate (J. M. Williams, personal communication). The loss of the α-isoform coupled with the inability of TPA to inhibit the response to histamine (Fig. 4.3, Fig. 4.4) following preincubation for 24 h with phorbol 12,13-dibutyrate implies an involvement of protein kinase C α in inhibition of the

Figure 4.3
**Effect of TPA on the cyclic AMP response to histamine
following a 24 h preincubation in the presence and
absence of phorbol 12,13-dibutyrate.**

KEY

□ Preincubation with DMSO; incubation with DMSO

■ Preincubation with DMSO; incubation with TPA

△ Preincubation with PDBu; incubation with DMSO

▲ Preincubation with PDBu; incubation with TPA
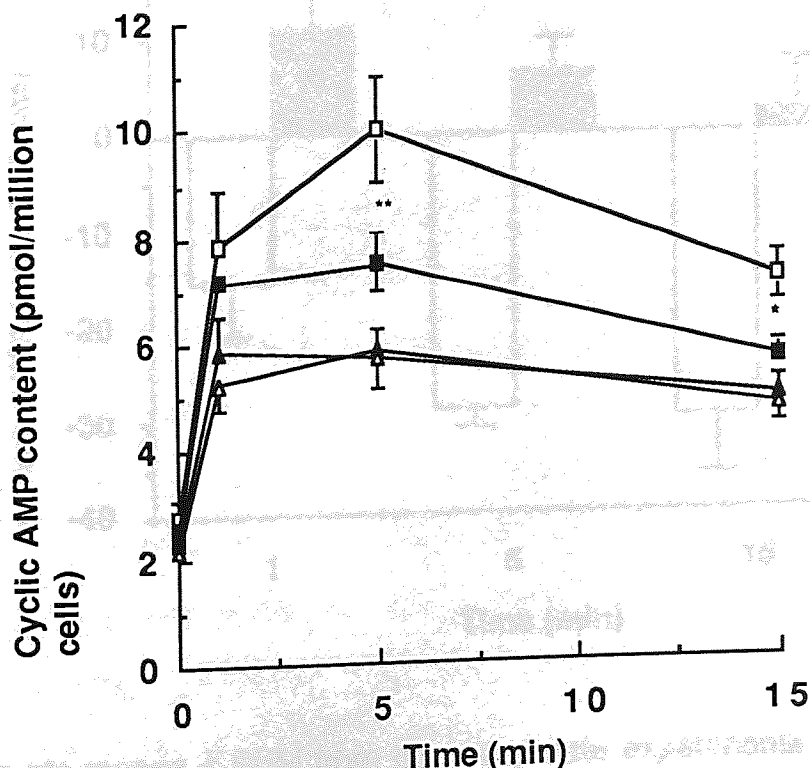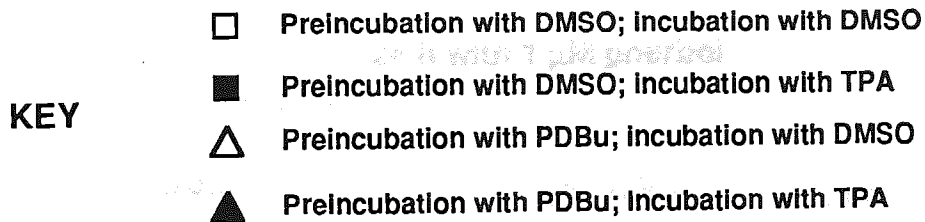
Cells were preincubated in the presence of 1 μM phorbol
12,13-dibutyrate or vehicle for 24 h and washed three times before
resuspension. TPA (100 nM) was added 10 min prior to subsequent
challenge with 0.5 mM histamine. Results are means ± SEM from
three separate experiments. Effects of TPA were determined by
ANOVAR (Appendix A.5.1) and are denoted: * P < 0.05; ** P < 0.01.

Figure 4.4
Effect of preincubation for 24 h with 1 μM phorbol 12,13-dibutyrate on the inhibitory effect of TPA on the stimulation of cyclic AMP content by histamine. Data from figure 4.3 re-expressed to show the effect of TPA on stimulation by histamine.

■ **Preincubated for 24 h with 1 μM phorbol 12,13-dibutyrate.**

☐ **Control.**
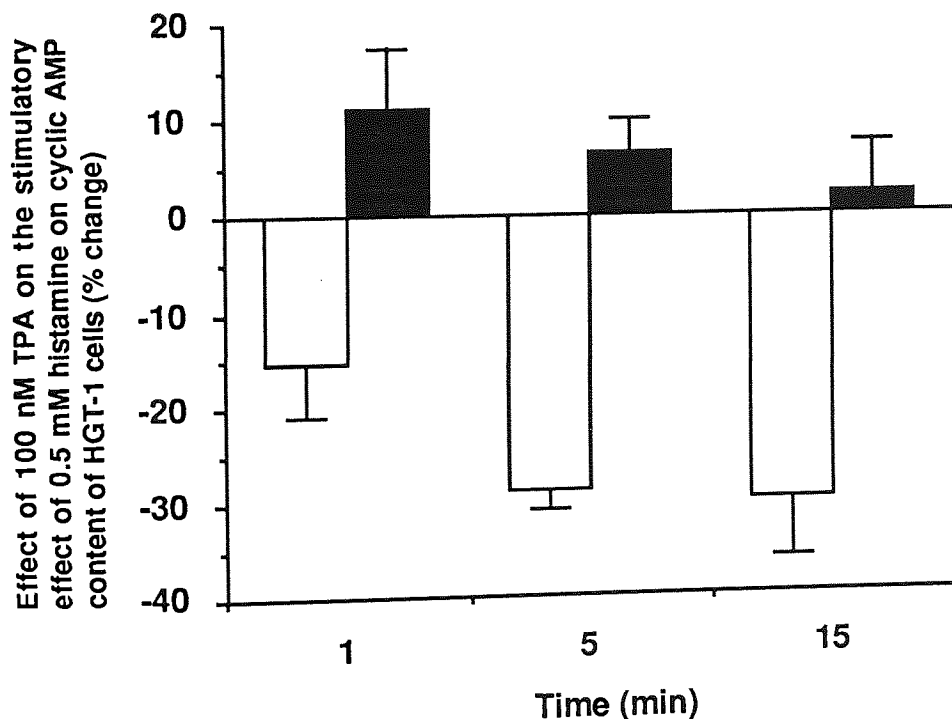
Results are means ± SEM from three separate experiments and are corrected for basal cyclic AMP contents of the cell suspensions after washing, which were: 2.17 ± 0.22 and 2.40 ± 0.32 pmol/million cells for cells preincubated in the presence of 1 μM phorbol 12,13-dibutyrate and incubated in the absence and presence of TPA (100 nM) respectively; and 2.73 ± 0.59 and 2.54 ± 0.24 pmol/million cells for cells preincubated with vehicle and incubated in the absence and presence of TPA. TPA was added to the cell suspension 10 min before challenge with histamine (0.5 mM) which was for the times indicated.

cyclic AMP response to histamine. It must be borne in mind however that the failure of TPA to effect a response occurred against a background of reduced stimulation by histamine alone following phorbol 12,13-dibutyrate pretreatment. Reasons for thinking that this decreased response was a separate issue are discussed in section 4.3.1.1.

4.3.2 Assay of histamine-stimulated adenylate cyclase activity in isolated membranes from HGT-1 cells.

4.3.2.1 Validation of adenylate cyclase assay procedure.

In order to validate the adenylate cyclase assay procedure, it was necessary to show that: (i), increasing the amount of enzyme resulted in a proportional increase in the amount of product formed; (ii), the amount of product formed was linearly related to time, with the curve extrapolating back to the origin. The relationship between the protein concentration in the assay system and the amount of product formed was indeed linear (Fig. 4.5) and a 20 min incubation in the presence of either 20 µg or 40 µg of protein resulted in product formation that was respectively 2.24 and 2.11 times that obtained using the same protein concentrations, but incubating for only 10 min (Fig. 4.5). Both curves, if extrapolated back also intersected at the origin.

The above validation assay was carried out on a whole cell homogenate whereas subsequently, adenylate cyclase activity was assayed in isolated membrane fractions. In view of this a further validation assay was carried out on an isolated membrane fraction.

Figure 4.5
Effect of the amount of protein in homogenate derived from a suspension of HGT-1 cells on adenylate cyclase activity stimulated by 0.5 mM histamine after incubating for either 10 or 20 min.
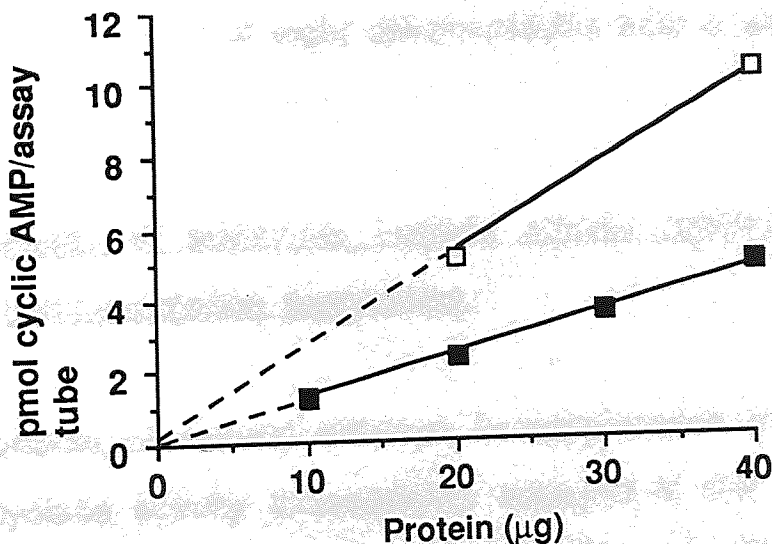
Adenylate cyclase activity was assayed in whole cell homogenate produced using a hand-held teflon glass homogeniser (20 strokes/min for 2 min). Results are means of duplicate determinations from a single experiment and are expressed as total cyclic AMP content in the assay tube. (■) Incubation for 10 min, (□) incubation for 20 min.

Adenylate cyclase activity in an isolated membrane fraction containing 30 μg of protein was 2.81 times that measured in a membrane fraction containing only 10 μg of protein (n = 1). The protein content of the above fraction was determined using a modification of the Lowry procedure (Markwell et al., 1978) and was calculated to be 114 ± 5.77 μg protein per 10^6 cells homogenized (data are means ± SEM of eight determinations from a single batch of cells).

4.3.2.2   Assay of adenylate cyclase activity following different homogenization procedures.

The effects of several different homogenization procedures on adenylate cyclase activity subsequently assayed in the absence and presence of 0.5 mM histamine were compared (Fig. 4.6. a-f). Adenylate cyclase activities assayed in the presence of 0.5 mM histamine following sonication at either 4 W (4 x 5 s bursts with 5 s intervals) or at 8 W (1 x 5 s burst) were 22.9 % and 18.9 % above basal respectively. The use of an Ultra-Turrax (3 x 5 s bursts with 5 s intervals at 60 % maximum speed) to homogenize the cells resulted in only a slight improvement in response (58.3 % above basal) as did cell breakage caused by the repeated rapid freezing and thawing of the cells (47.1 % above basal). A novel method of homogenization using microfuge tubes as mortars and with moulded plastic pestles (20 strokes/min for 1 min at 250 RPM) (Hearse, D.J., 1984). was also tried and this resulted in an increase in adenylate cyclase activity in the presence of 0.5 mM histamine of 172.6 % over basal. By far the best results were obtained however using a hand-held teflon-glass homogeniser (30 strokes/min for 2 min).

Homogenization of cells by this method resulted in a dramatic increase in adenylate cyclase activity which was 258.5 % above basal in the presence of 0.5 mM histamine. Cell counts performed before and after homogenization revealed that in all of the above procedures, the number of cells either broken or permeable to trypan blue following homogenization exceeded 90 %.

The above results indicate that more vigorous homogenization procedures such as sonication and use of an Ultra-Turrax result in a very poor adenylate cyclase response to histamine in these cells. A possible explanation for this is that one or more components of the adenylate cyclase system are liberated from the membrane during the homogenization process, or that inactivation of components occurs for example due to localized heating during homogenization. The hand-operated teflon-glass method of homogenization followed by preparation of isolated membranes was considered the most suitable procedure and was used in all subsequent experiments, with 10 µg of membrane protein incubated for 10 min.

4.3.3 Effects of Ca^{2+} on the action of TPA on histamine-stimulated adenylate cyclase activity.

Basal adenylate cyclase activity in membranes containing 10 µg protein and incubated for 10 min at 30°C in the presence of negligible free Ca^{2+} (Incubation buffer containing 10 mM EGTA, 60 µM EDTA) was 6.85 ± 0.44 pmol cyclic AMP/10^6 cells (Fig. 4.7.A). This value was not significantly changed by the presence of 100 nM TPA in the assay (5.90 ± 0.45 pmol cyclic AMP/10^6 cells) and was little different from that in the presence of 20 µg/ml

Figure 4.6

Effect of different homogenisation procedures on adenylate cyclase activity measured in homogenates derived from a suspension of HGT-1 cells.
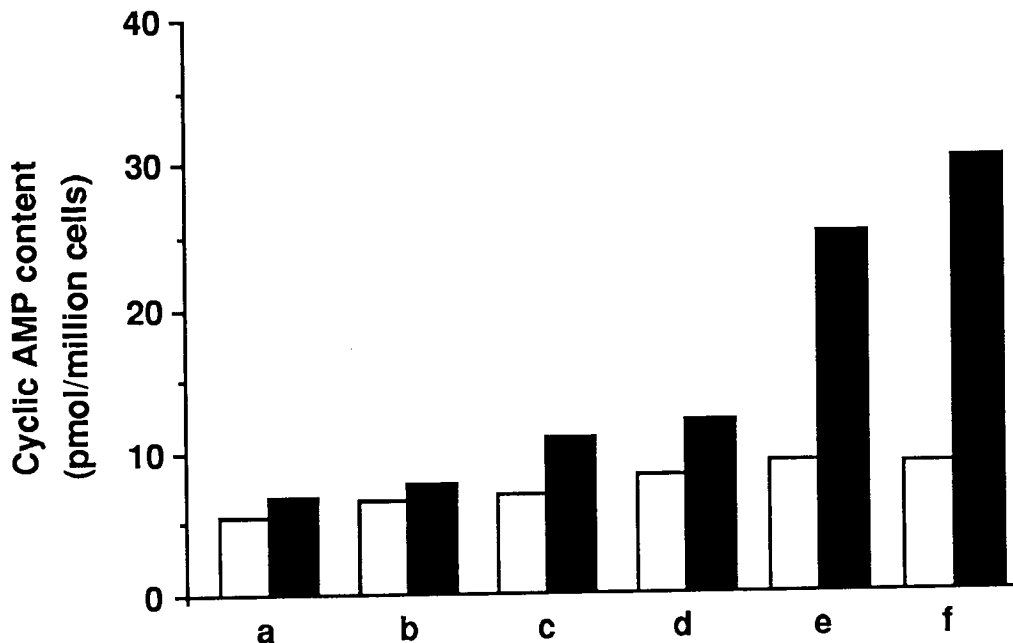
Results are means of duplicate determinations from two experiments. (□) Basal adenylate cyclase activity, (■) 0.5 mM histamine-stimulated activity. 1 ml of cell suspension was centrifuged at 10,000 x g for 15s to pellet the cells. The pellet was washed before resuspending in 1 ml ice-cold homogenisation buffer at a cell concentration of 10 million cells/ml and then treated as follows: (a) Sonication at 4W, 4 x 5s bursts with 5s intervals. (b) Sonication at 8W for 5s. (c) Ultra-turrax, 3 x 5s bursts at 60% maximum speed. (d) Freezing and thawing. The cell pellet was rapidly frozen by immersion in liquid nitrogen and then dispersed in homogenisation buffer by vortexing. This mixture was again frozen in liquid nitrogen and then thawed in a water bath at 20°C. (e) Homogenisation using microfuge tubes as mortars and with moulded plastic pestles, 20 strokes/min for 1 min at 250 RPM. (f) Hand-operated teflon-glass homogeniser, 20 strokes/min for 2 min. The above operations (with the exception of procedure 'd') were carried out on ice. To prepare an isolated membrane fraction the broken cell suspensions were vortexed and spun at 10,000 x g for 15s to sediment the nuclei. 2 x 0.4 ml aliquots of supernatant were then collected and centrifuged at 40,000 x g for 90 min at 4°C. The pellet was washed once and resuspended in homogenisation buffer at a concentration equivalent to 20 million cells/ml. This homogenate was dispensed to microfuge tubes and stored at -80°C until required. Adenylate cyclase activity was assayed in either whole cell homogenates (procedures a, b and e) or in isolated membrane fractions (procedures c, d and f).

phosphatidylserine (6.07 $\pm$ 0.59 and 6.03 $\pm$ 0.72 pmol cyclic AMP/10^6 cells in the absence and presence of 100 nM TPA respectively; Fig. 4.7.B).

No significant effect of 100 nM TPA on adenylate cyclase activity stimulated by 0.5 mM histamine in the absence of free Ca^{2+} could be detected (Fig. 4.7.A). Most isoforms of protein kinase C so far identified are known to require the presence of phospholipid, normally phosphatidylserine for activation (Table 3.3), thus a possible explanation for the above lack of effect of TPA was that protein kinase C was non-functional without the addition of extra phosphatidylserine to supplement that already present in the membranes. However no significant effect of TPA on histamine-stimulated adenylate cyclase activity in the presence of phosphatidylserine (20 μg/ml) was found (Fig. 4.7.B).

In the presence of 10^{-5} M free Ca^{2+} and 20 μg/ml phosphatidylserine, 100 nM TPA inhibited adenylate cyclase activity stimulated by 0.5 mM histamine by 30.6 $\pm$ 3.8 % ($P < 0.02$ for effect of TPA analysed by paired t-test, Fig. 4.8). The presence of 10^{-5} M free Ca^{2+} in the incubation medium did not affect adenylate cyclase activity assayed in the presence of 0.5 mM histamine alone which was 30.87 $\pm$ 3.41 and 31.0 $\pm$ 2.12 pmol cyclic AMP/10^6 cells in the presence and absence of 10^{-5} M free Ca^{2+} respectively. The adenylate cyclase isoform in HGT-1 cells appears therefore not to be type II or type IV (Table 3.4). Finally no effect of 100 nM TPA on basal adenylate cyclase activity was found when activity was assayed in the presence of both 10^{-5} M free Ca^{2+} and 20 μg/ml phosphatidylserine (4.61 $\pm$ 0.89 and 4.62 $\pm$ 0.70 pmol cyclic AMP/10^6

Figure 4.7

Comparison of the effects of 100 nM TPA on adenylate cyclase activity in the absence of free Ca^{2+} and in the presence and absence of 20 μg/ml phosphatidylserine.

☐ **Basal.**
■ **Basal + 100 nM TPA.**
▦ **0.5 mM histamine.**
▨ **0.5 mM histamine + 100 nM TPA.**
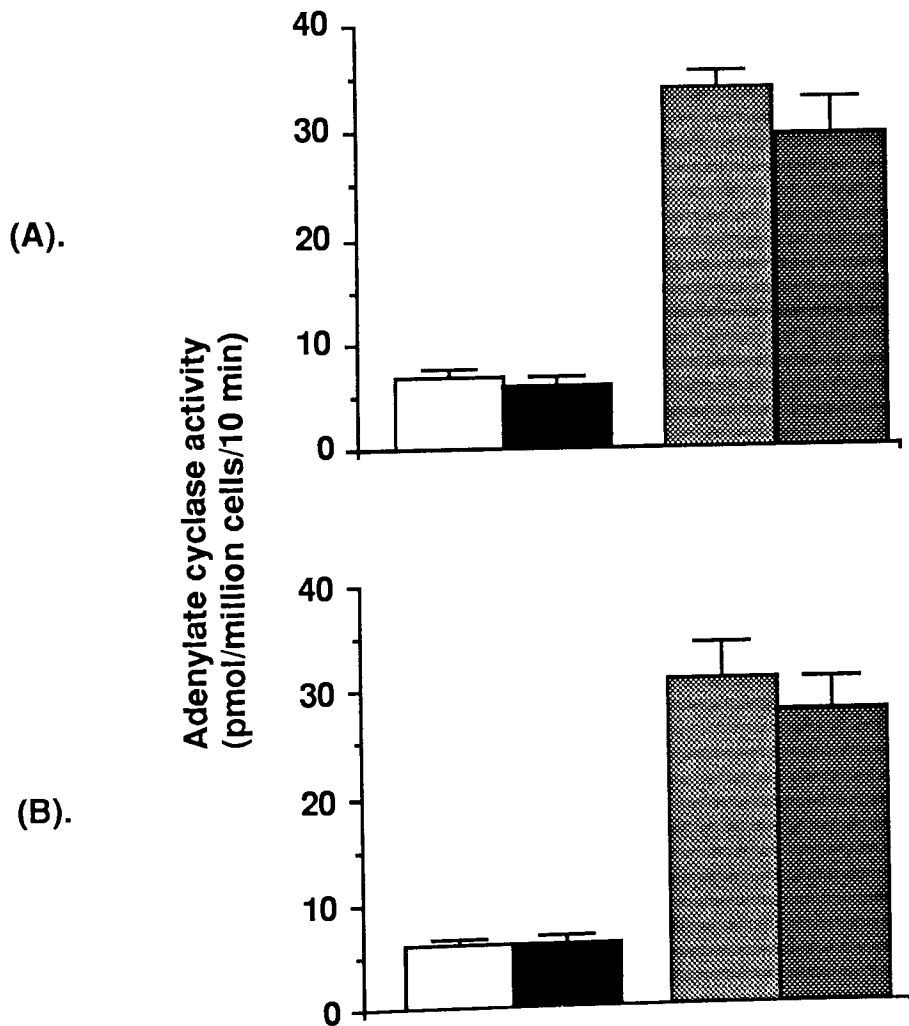
Results are means ± SEM from four membrane preparations in each case. (A), effect of 100 nM TPA in the absence of both phosphatidylserine and free Ca^{2+}. (B), effect of 100 nM TPA in the presence of phosphatidylserine (20 μg/ml) but in the absence of free Ca^{2+}. No significant effects of TPA on either basal or histamine-stimulated adenylate cyclase activity were apparent (data analysed by paired t-test).

Figure 4.8

Effect of 100 nM TPA on adenylate cyclase activity in the presence of both 10 μM free Ca^{2+} and 20 μg/ml phosphatidylserine.

☐ **Basal.**
■ **Basal + 100 nM TPA.**
▨ **0.5 mM histamine.**
▨ **0.5 mM histamine + 100 nM TPA.**
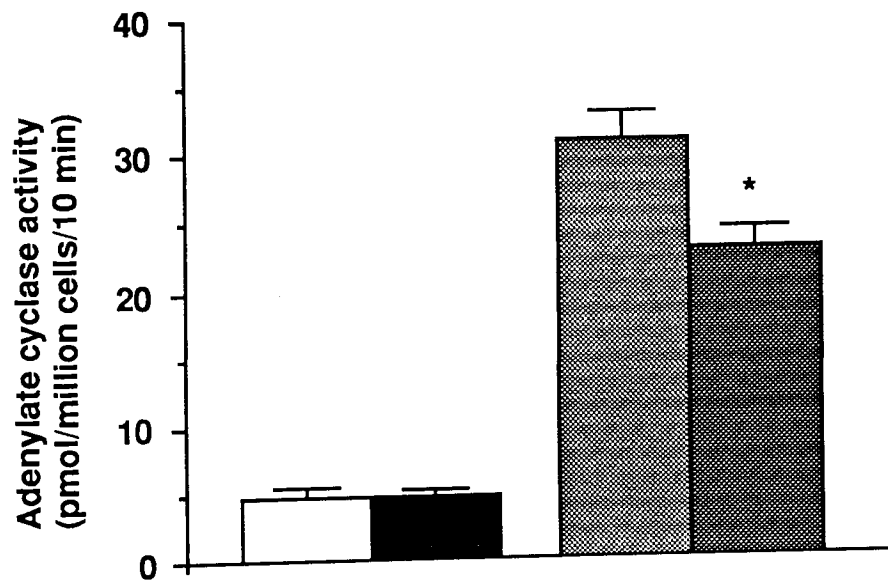
Results are means ± SEM from four membrane preparations. * P < 0.02 for effect of 100 nM TPA on adenylate cyclase activity stimulated by 0.5 mM histamine (data analysed by paired t-test).

cells for activity assayed in the absence and presence of 100 nM TPA respectively, Fig. 4.8).

The above findings indicate that effects of TPA on cyclic AMP content in intact cells stimulated with histamine were most probably due to an effect on adenylate cyclase activity, and that TPA could activate protein kinase C in isolated membranes. The inhibitory effect of TPA on adenylate cyclase was dependent upon the presence of Ca^{2+} which suggests the involvement of an isoform of protein kinase C that requires Ca^{2+} for activation. The Ca^{2+}-activated isoforms of protein kinase C all belong to the cPKC group of isoforms (Table 3.3) and include the α, β and γ subspecies (Asaoka et al., 1992). The α-isoform of protein kinase C but not the β-isoform has been detected by protein blotting in the particulate subcellular fraction derived from HGT-1 cells (J.M. Williams, personal communication). The γ-isoform is also known to be absent (Dr. M.G. Rumsby, personal communication). Thus these results imply that it is the α-isoform of protein kinase C that is responsible for inhibition of adenylate cyclase activity stimulated by histamine in these cells.

4.3.4   Effect of specific antibodies to isoforms of protein kinase C on the TPA-induced inhibition of adenylate cyclase activity.

To investigate further the possibility of involvement of protein kinase C α in the inhibition of histamine-stimulated adenylate cyclase activity, an antibody to the above isoform was added to the assay medium just prior to the addition of homogenate.

A range of concentrations of antibody to protein kinase C α (4 - 400 ng/ml) were used and this produced a dose-related antagonism of the effect of TPA on histamine-stimulated adenylate cyclase activity. Inhibition of the effects of 100 nM TPA were, 95.99 ± 11.96 %, 22.09 ± 5.67 % and -8.26 ± 6.14 % in the presence of 400 ng/ml, 40 ng/ml and 4 ng/ml antibody respectively (Fig. 4.9). The effects of the maximum concentration of antibody used (400 ng/ml) were prevented by the co-administration of 200 ng/ml of the peptide against which the antibody was raised (Fig. 4.9). The effects of an antibody to the ε-isoform of protein kinase C (400 ng/ml) were also investigated as this isoform too has been detected in these cells (J.M. Williams, personal communication). The presence of antibody to the ε-isoform of protein kinase C did not prevent the inhibition by TPA of histamine-stimulated adenylate cyclase activity (Fig. 4.9).

These results again support the premise that it is the α-isoform of protein kinase C that was responsible for the inhibition of adenylate cyclase activity stimulated by histamine. However, demonstration that the antibodies can specifically inhibit TPA-induced phosphorylation of exogenous peptide substrates by the membrane preparation is required to fully substantiate that the anti-protein kinase C α antibody is exerting its effect through inhibition of the activity of this enzyme.

Figure 4.9

Comparison of the effects of antibodies to the α- and ε- isoforms of protein kinase C on the inhibition by TPA of histamine-stimulated adenylate cyclase activity.
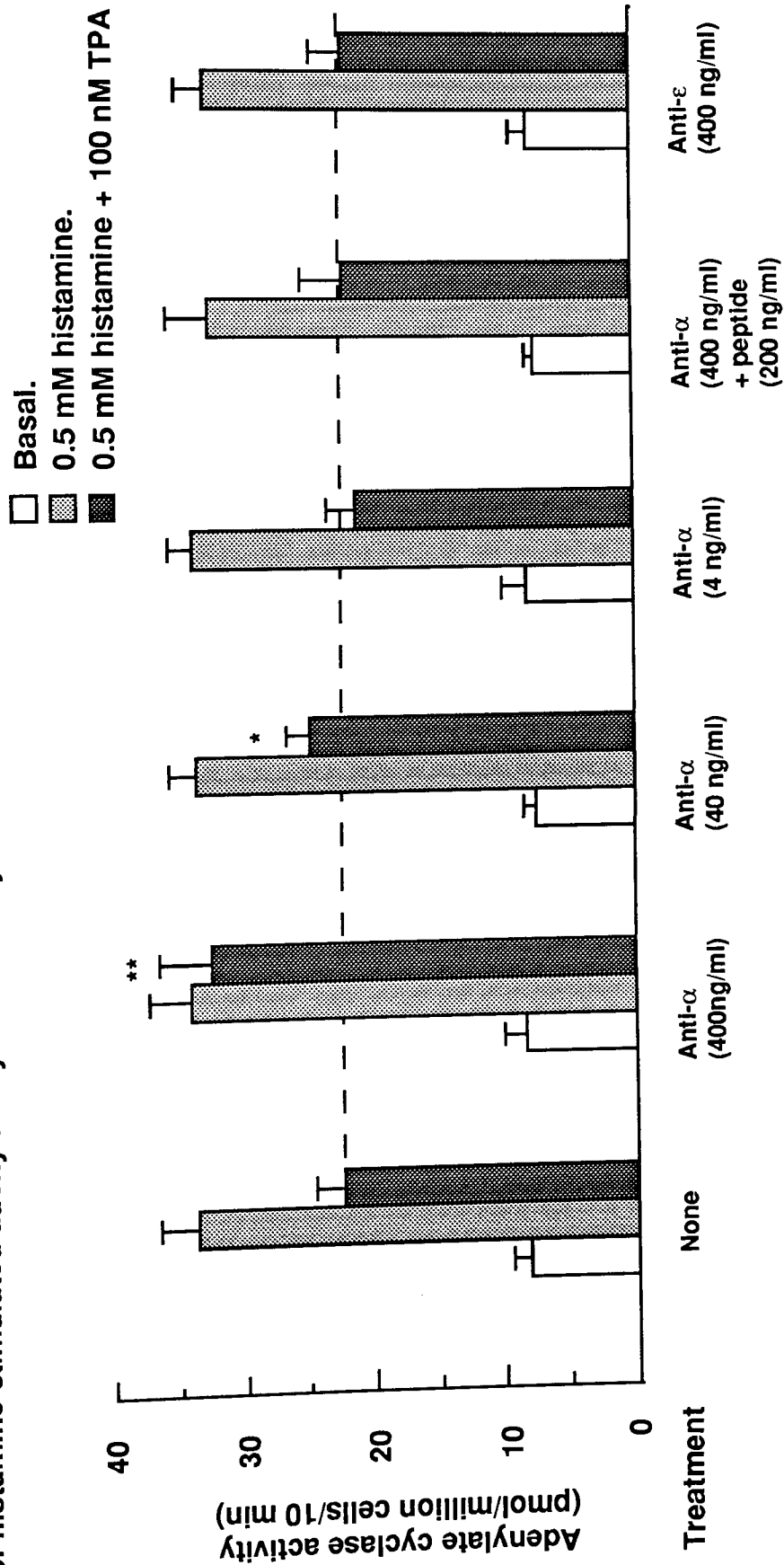
Results are means ± SEM from four experiments. Incubations were in the presence of 20 µg/ml phosphatidylserine and 10 µM free Ca^{2+}. * $P < 0.01$, * $P < 0.05$ for difference from result with 0.5 mM histamine and 100 nM TPA alone by ANOVAR (Appendix A.5.1) and Dunnett's test (Appendix A.5.2).

4.4　　SUMMARY.

1.　Culture with 1μM phorbol 12,13-dibutyrate for 24 h prevented the effect of TPA on the stimulation of the cyclic AMP content of HGT-1 cells by histamine. Data of J.M. Williams show that protein kinase C isoforms α and ε were detected by protein blotting in the particulate subcellular fraction from HGT-1 cells and that culture with 1 μM phorbol 12,13-dibutyrate for 24 h downregulated protein kinase C α but not ε.

2.　Effects of TPA on histamine stimulation of adenylate cyclase were found with isolated membranes. These effects were dependent on Ca^{2+}, and were specifically abolished by antibody to protein kinase C α.

3.　Taken together the results suggest that protein kinase C α is the isoform responsible for inhibiting the action of the histamine H_2 receptor on adenylate cyclase.

CHAPTER 5

EFFECT OF THE SPECIFIC INHIBITOR OF PROTEIN KINASE C, RO 31-8220, ON STIMULATION OF SECRETORY ACTIVITY BY CARBACHOL IN RAT PARIETAL CELLS.

5.1 __INTRODUCTION.__

5.1.1 __Activation of acid secretion by mobilization of__ Ca^{2+} __in parietal cells.__

This topic is reviewed in Chapter 1.

5.1.2 __Involvement of protein kinase C in the response of parietal cells to muscarinic cholinergic receptor stimulation.__

Acetylcholine activates a muscarinic receptor on the parietal cell which is of the M_3 type (Pfeiffer et al., 1990; Kajimura et al., 1992). Stimulation of this receptor by carbachol induces a rapid breakdown of phosphatidylinositol 4,5-bisphosphate and consequently, the production of inositol 1,4,5-trisphosphate (Pfeiffer et al., 1990; Chiba et al., 1989; Puurunen et al., 1987) and diacylglycerol (Pfeiffer et al., 1989). The rise in inositol 1,4,5-trisphosphate is coupled to an elevation of intracellular Ca^{2+} which appears to be obligatory for stimulation of acid secretion by carbachol (Brown and Chew, 1989; Leonard et al., 1990; Negulescu et al., 1989). The production of diacylglycerol is normally associated with activation of protein kinase C (Nishizuka, 1988) and this enzyme has been detected in parietal cells isolated from the rat (Anderson and Hanson, 1985), rabbit (Chew, 1985), guinea-pig (Beil et al., 1987) and dog (Chiba et al., 1989). Both TPA and carbachol have been shown to induce an increase in the membrane-associated form of protein kinase C in canine parietal cells (Park et al., 1987; Chiba et al., 1989) and thus can be presumed to activate it.

The role of protein kinase C in the response of parietal cells to carbachol is, however, not clear. Some results imply a stimulatory role for protein kinase C in the response to carbachol. Thus, thapsigargin, a tumour promoter that elevates $[Ca^{2+}]_i$ without increasing inositol 1,4,5-trisphosphate concentration or activating protein kinase C, generates a level of secretory activity (aminopyrine accumulation) well below that produced by carbachol, but which is enhanced by the addition of TPA to activate protein kinase C (Chew and Petropoulos, 1991). In contrast, an inhibition of carbachol-stimulated aminopyrine accumulation in the presence of either TPA or OAG has also been shown (Schepp et al., 1989), although this may have been due to an already maximal activation by carbachol of phosphatidylinositol 4,5-bisphosphate breakdown and therefore a maximal stimulation of protein kinase C (Schepp et al., 1989). Stimulatory effects of TPA on basal aminopyrine accumulation have been detected in rabbit (Brown and Chew, 1987), and rat (Schepp et al., 1989) parietal cells, and stimulation by TPA of the response of rabbit gastric glands to submaximal concentrations of dibutyryl cyclic AMP has also been shown (Brown and Chew, 1987). A similar enhancement by carbachol of the response to submaximal dibutyryl cyclic AMP was demonstrated with rat parietal cells (Pfeiffer et al., 1987). Finally, some of the parietal cell proteins that are phosphorylated in response to carbachol are also phosphorylated in response to activation of protein kinase C by TPA (Brown and Chew, 1989). The consequences of these phosphorylation events are, however, unknown.

An involvement of protein kinase C in a feedback inhibitory regulation of the muscarinic receptor has also been suggested.

Thus, when protein kinase C is activated in parietal cells by the addition of either TPA (Chiba et al., 1989; Anderson and Hanson, 1984; Brown and Chew, 1987) or OAG (Choquet et al., 1991), the secretory activity (aminopyrine accumulation) in response to carbachol is inhibited. TPA also decreased carbachol-stimulated inositol 1,4,5-trisphosphate production (Puurunen et al., 1987; Choquet et al., 1991). The effect may involve a protein kinase C-induced phosphorylation of the muscarinic receptor (Richardson et al., 1992; Kopp et al., 1990) that results in either an uncoupling of the receptor from phospholipase C (Chiba et al., 1989; Puurunen et al., 1987), or a reduction in the number of muscarinic receptors (Chiba et al., 1989). Further evidence for an inhibitory role for protein kinase C comes from the inhibition of carbachol-stimulated secretory activity in parietal cells upon elevation of diacylglycerol by the diacylglycerol lipase inhibitor RHC 80267. Interpretation of these results is however complicated by RHC 80267 itself being an activator of phospholipase C (Pfeiffer et al., 1989).

5.1.3   Inhibition of protein kinase C.

Inhibitors of protein kinase C may be classified into two groups depending upon whether they interact with the catalytic or regulatory domain of the enzyme (Table 5.1). Compounds that are targeted towards the catalytic domain tend to bind to residues that are conserved in protein kinases and as such, are not usually highly selective for protein kinase C. Thus, staurosporine, a microbial alkaloid derived from Streptomyces and the most potent inhibitor of protein kinase C known exhibits some, but not complete selectivity for this kinase (Tamaoki and Nakano, 1990), while other inhibitors

Table 5.1
Characteristics of inhibitors of protein kinase C.

Target site	Inhibitors	Source	IC$_{50}$ (µM)		
			PKC	PKA	PKG
Catalytic domain	staurosporine	actinomyces	0.0007	0.007	0.0085
	K252a	actinomyces	0.025	0.016	0.015
	H-7	synthetic	6	3	5.8
	aminoacridine	synthetic	40		
	sangivamycin	actinomyces	10		
Regulatory domain	calphostin C	fungi	0.05	>50	>25
	cercosporin	fungi	0.37	22.4	
	chlorpromazine	synthetic	40		
	polymyxin B	bacteria	10		
	adriamycin	actinomyces	50		

(Tamaoki and Nakano, 1990; L. C. Laboratories Signal Transduction Handbook, 1993).

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such as H-7 (Tamaoki et al., 1986) and K252a (Davis et al., 1989) are non-selective and exert significant inhibition against both cyclic AMP-dependent protein kinase (Table 5.1) and Ca^{2+}/calmodulin-dependent protein kinase (Davis et al., 1989) at the concentrations required to inhibit protein kinase C. In contrast, inhibitors targeted towards the regulatory domain of protein kinase C which is not conserved in protein kinases show higher selectivity. The usefulness of these compounds is however generally limited by their reduced potency (Table 5.1).

5.1.3.1 Ro 31-8220.

Recently, a series of highly specific bisindolylmaleimide inhibitors of protein kinase C which are based on the structure of staurosporine (Figure 5.1.A) and K252a have been developed (Davis et al., 1989). These compounds compete with ATP for binding to protein kinase C and are therefore targeted towards the catalytic domain of the enzyme. The most potent of these inhibitors, Ro 31-8220 (Figure 5.1.B) has been shown not only to be equiactive with staurosporine against protein kinase C (Davis et al., 1989; Dieter and Fitzke, 1991), but also to exhibit a 100-fold lower inhibitory potency against cyclic AMP- and Ca^{2+}/calmodulin-dependent protein kinases (Davis et al., 1989). The increased selectivity of this compound, when compared to staurosporine, makes it an improved and useful tool with which to investigate the involvement of protein kinase C in cellular responses. Ro 31-8220 was therefore considered a suitable alternative to staurosporine for use during investigations of the role of protein kinase C in the stimulation of acid secretion by carbachol in rat parietal cells. This compound was

Figure 5.1
Comparison of the structures of staurosporine and Ro 31-8220.

A. Staurosporine.
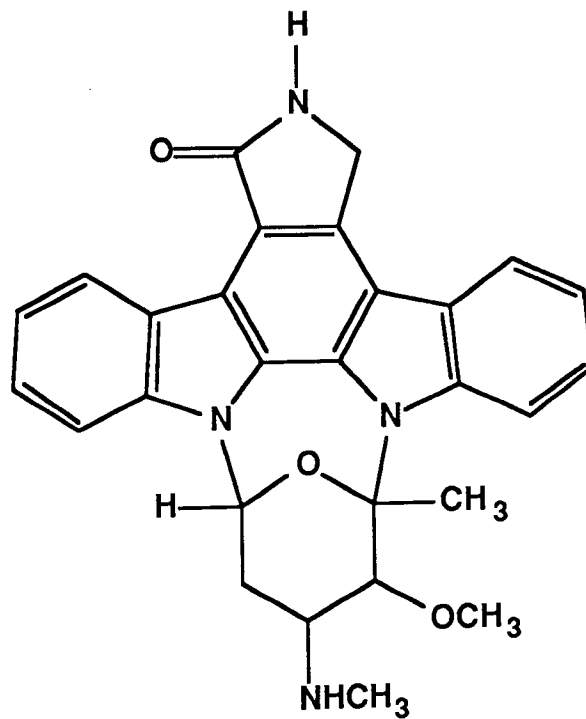
B. Ro 31-8220.
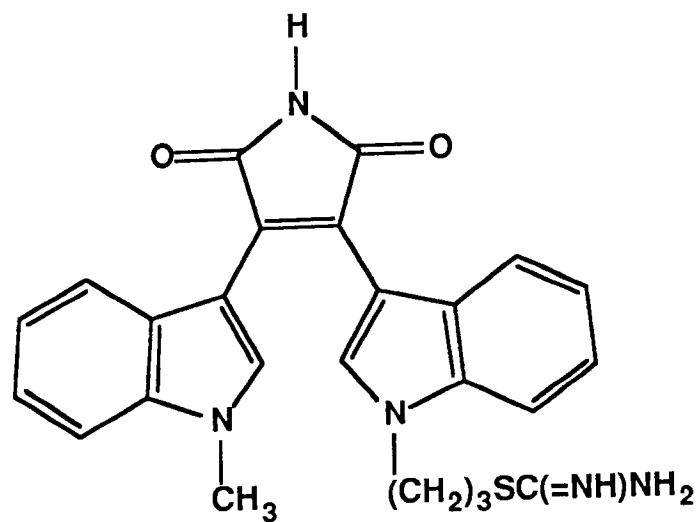
unavailable when earlier experiments (Chapters 3 and 4) with HGT-1 cells were performed.

5.1.4 Fura-2 and measurement of intracellular Ca^{2+}.

The fluorescent Ca^{2+} chelator, Fura-2 is derived from the Ca^{2+} chelator BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid) which is a double aromatic analogue of EGTA (Cobbold and Rink, 1987). It was developed by Grynkiewicz et al. (1985) and combines a stilbene fluorophore with an 8-coordinate tetracarboxylate pattern of liganding groups (Fig. 5.2) that is characteristic of EGTA. The lipophilic membrane-permeant derivative, Fura-2 acetylmethoxy ester is used to load the dye into cells whereupon cleavage by cytosolic esterases generates the active form which is then trapped in the cytosol.

Fura-2 binds Ca^{2+} with a simple 1 : 1 stoichiometry and thus the relationship between fluorescence and [Ca^{2+}] in the region of the K_d is approximately linear (Cobbold and Rink, 1987). The dye exhibits excitation peaks at 380 nm and 340 nm in the Ca^{2+}-free and bound forms respectively (Grynkiewicz et al., 1985). Thus, emission at 510 nm from excitation at 340 nm increases 3-fold with Ca^{2+}-saturation while the 380 nm excitation signal decreases 10-fold. Consequently the signal from these two wavelength pairs is uniquely determined by the ratio of the free and bound dye, and is very sensitive to changes in free [Ca^{2+}]. The sensitivity of Fura-2 to small changes in [Ca^{2+}], coupled with the quantum yield of Fura-2 being such that it can be used at intracellular concentrations where buffering by Fura-2 is negligible, now make it the preferred dye for

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Figure 5.2
The structure of Fura-2.
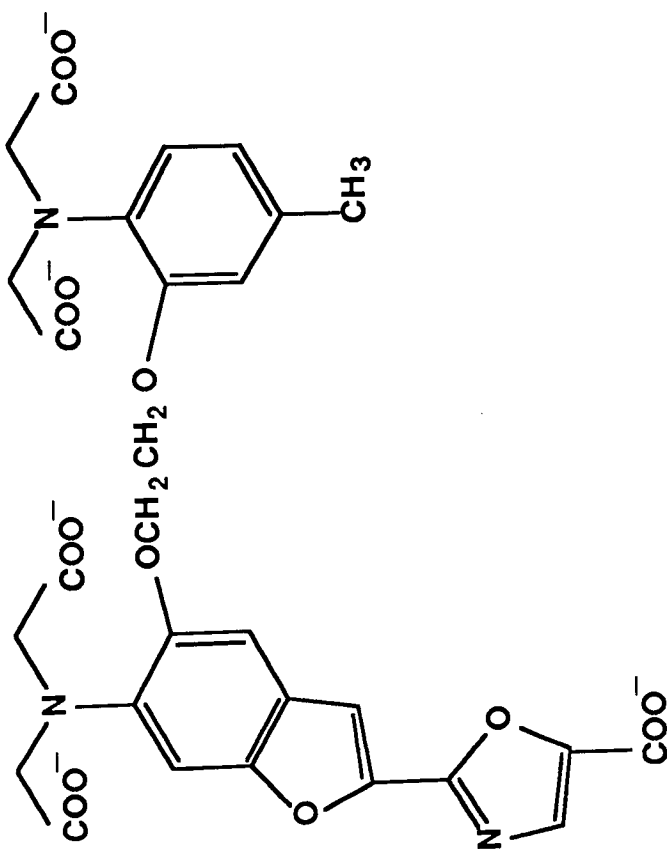
measurements of intracellular Ca^{2+} in a wide range of cells
(Grynkiewicz et al., 1985).

5.2   METHODOLOGY.

5.2.1   Preparation of reagents.

TPA, staurosporine, histamine and IBMX were all prepared as described previously (Chapters 3 and 4). Fura-2 acetoxymethylester (Fura-2AM), Ro 31-8220 and ionomycin were dissolved in dry DMSO and aliquots stored frozen as 2 mM, 2.14 mM and 1 mM stock solutions respectively. A volume (1 μl) of these stocks, or of DMSO in controls, was added to 1 ml of cell suspension. Carbachol was dissolved in saline to a concentration of 0.01 M, and 1,2-Di(2-aminoethoxy)ethane-N,N,N,'N'-tetra-acetic acid (EGTA) and atropine were dissolved in distilled water to concentrations of 1 M and 5 mM respectively. These stocks were made fresh prior to use. A 10 % solution of Triton-X-100 was prepared by adding 1 ml of Triton-X-100 to 9 ml of distilled water and then heating to 37°C in a shaking water bath. This stock then remained stable for several weeks at room temperature.

5.2.2   Preparation and incubation of parietal cells.

The preparation of unenriched and enriched suspensions of parietal cells and measurement of the aminopyrine accumulation ratio is described in detail in Chapter 2.

5.2.3   Measurement of intracellular Ca^{2+}.

5.2.3.1   Loading of Fura-2AM into parietal cells.

An enriched fraction of parietal cells was suspended at 2.5 x 10^6 cells/ml in Eagle's minimum essential medium containing

20 mM   N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic   acid
(HEPES), 1 mg/ml bovine serum albumin and 2 μM Fura-2AM and then
incubated for 20 min at 37°C.   This incubation time was selected
because time-course studies have shown that loading of this probe
into rat parietal cells takes 20 min to reach equilibrium (Black et
al., 1989).   Loading and formation of Fura-2 from the acetoxymethyl
ester were monitored by the shift in the cellular fluorescence
emission peak from 488 to 502 nm which is indicative of the
hydrolysis of the esterified form of the dye and formation of the
intracellular Ca^{2+}-sensitive form of the probe.   The cells were
washed once and then resuspended at a concentration of 0.5 x10^6
cells/ml in a balanced salt solution (pH 7.4) (Black et al., 1989)
consisting of 0.5 mM NaH_2PO_4, 1 mM Na_2HPO_4, 20 mM $NaHCO_3$, 70 mM
NaCl, 5 mM KCl, 1 mM $CaCl_2$, 50 mM HEPES, 10 mM glucose and 0.1
mM isoleucine which had been gassed with 95% O_2/5% CO_2. This
preparation was stored in the dark at room temperature until
required.

5.2.3.2   Measurement of intracellular Ca^{2+}.

For each experiment, 2 ml of cell suspension loaded with Fura-
2 as described above was added to a disposable plastic fluorescence
cuvette and the suspension left to equilibrate in a Perkin-Elmer LS-
50 spectrofluorimeter with the stirrer on low for 3 min at 37°C.
Recording was initiated and 30 s later, Ro 31-8220, atropine or
vehicle was added, followed by either carbachol (0.1 mM) or
ionomycin (1 μM) after a further 120 s. The response to carbachol
was recorded for 150 s. Measurements, made in the Intracellular
Biochemistry Mode, of the emission ratio (R) at 510 nm from

alternating excitation at 340 and 380 nm were made, and the intracellular Ca^{2+} concentration then calculated by computer program (Perkin-Elmer) from the equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}] = K_d \, [(R-Rmin)/(Rmax-R)] \, (S_{f2}/S_{b2}).$$

Where K_d =224 nM, Rmin = emission ratio in the presence of Triton-X-100 (0.2 % v/v) and 20 mM EGTA, Rmax = emission ratio in the presence of Triton-X-100 (0.2 % v/v) alone, S_{f2}/S_{b2} = the ratio of the emission intensities of the free and bound probe with excitation at 380 nm.

5.2.4   Presentation of results.

Results are presented as means $\pm$ SEM from n cell preparations. Data on aminopyrine accumulation are presented in normalized form to reduce the effect of variation between cell batches as is performed by other workers (Soll, 1980a). All statistical analyses were performed on data before normalization.

5.3   RESULTS AND DISCUSSION.

Pilot experiments with staurosporine, which demonstrated the unsuitability of this compound, are described first followed by data obtained using Ro 31-8220.

5.3.1   Effect of staurosporine on the aminopyrine accumulation ratio stimulated by histamine in the presence and absence of TPA.

A 92.4 % inhibition by 100 nM TPA of parietal cell secretory activity stimulated by 0.5 mM histamine was detected in a cell suspension containing approximately 20% parietal cells (aminopyrine accumulation ratios in the presence of histamine (0.5 mM) and in the absence and presence of TPA respectively, were, 35.96 ± 0.68 and 2.72 ± 1.22, $P < 0.001$ for an effect of 100 nM TPA by unpaired t-test. Data are means $\pm$ SEM of 4 determinations in each case from a single batch of cells). Addition of 200 nM staurosporine to the incubation medium however resulted in only a 25.1 % antagonism of the inhibitory effect of 100 nM TPA (aminopyrine accumulation ratios in the presence of both 0.5 mM histamine and 200 nM staurosporine and in the absence and presence of 100 nM TPA respectively, were, 27.45 ± 0.52 and 8.97 ± 0.52, $P < 0.001$ for an effect of 100 nM TPA by unpaired t-test. Data are means $\pm$ SEM of 4 determinations from a single batch of cells). An increase in the concentration of staurosporine above 200 nM was prevented because this same concentration of staurosporine also caused a significant ($P < 0.01$) inhibition of the aminopyrine accumulation ratio measured in the presence of 0.5 mM histamine alone (Table 5.2).

Table 5.2

Effect of Staurosporine on the aminopyrine accumulation ratio stimulated by histamine.

Concentration of Staurosporine (nM)	Stimulation of aminopyrine accumulation by 0.5 mM histamine	inhibition by staurosporine (%)
0	67.14 ± 0.88	
2	69.53 ± 0.68	- 3.56
20	69.27 ± 0.14	- 3.17
200	51.08 ± 0.76	23.92 **

Results are expressed as means ± SEM of quadruplicate determinations from a single batch of cells containing 18.8 % parietal cells. ** P < 0.01 for an effect of staurosporine on the aminopyrine accumulation ratio stimulated by histamine (0.5 mM) analysed by ANOVAR (Appendix A.5.1) and Dunnett's test (Appendix A.5.2).

Staurosporine is known to inhibit the activity of the catalytic domains of both protein kinase C and protein kinase A with IC_{50} values of 0.7 nM and 7 nM respectively (Tamaoki and Nakano, 1990, Table 5.1), and thus, the inhibition of histamine-stimulated aminopyrine accumulation by 200 nM staurosporine was therefore most likely caused by an inhibition of cyclic AMP-dependent protein kinase. The inhibitory effect of TPA on secretory activity in parietal cells has already been established (Anderson and Hanson, 1984), and is most likely mediated via an activation of protein kinase C (reviewed by Hanson and Hatt, 1989). An inability of staurosporine to completely antagonise the effects of TPA/protein kinase C, coupled with the fact that simultaneous inhibition of both protein kinase C and protein kinase A activities would unneccessarily complicate interpretation of results, made staurosporine unsuitable for use as an inhibitor of protein kinase C in rat parietal cells.

5.3.2 **Antagonism of the effects of TPA on histamine-stimulated aminopyrine accumulation by Ro 31-8220.**

Before using Ro 31-8220 to investigate the involvement of protein kinase C in the action of carbachol, it was necessary to show that Ro 31-8220 could inhibit protein kinase C activity in parietal cells. In an unenriched suspension of rat parietal cells, Ro 31-8220 produced a concentration-dependent reduction in the inhibitory effect of 100 nM TPA on aminopyrine accumulation stimulated by 0.5 mM histamine (Fig. 5.3.A). The half-maximally effective concentration of Ro 31-8220 was estimated using the computer

Figure 5.3.A
Antagonism by Ro 31-8220 of the inhibitory effect of TPA on histamine-stimulated acid secretion.
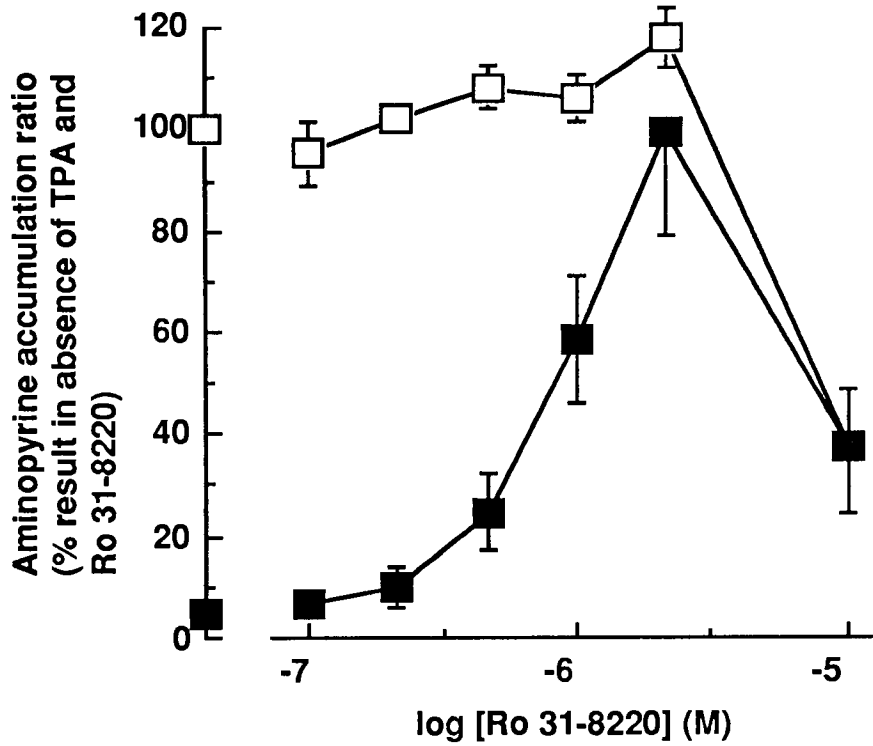
Results are presented as means ± SEM from four batches of unenriched cells and have been normalized to the stimulation obtained in the presence of 0.5 mM histamine plus 0.1 mM IBMX which was 25 ± 5.4. (□) control, (■) addition of 100 nM TPA.

Figure 5.3.B
Antagonism by Ro 31-8220 of the inhibitory effect of TPA on histamine-stimulated acid secretion.
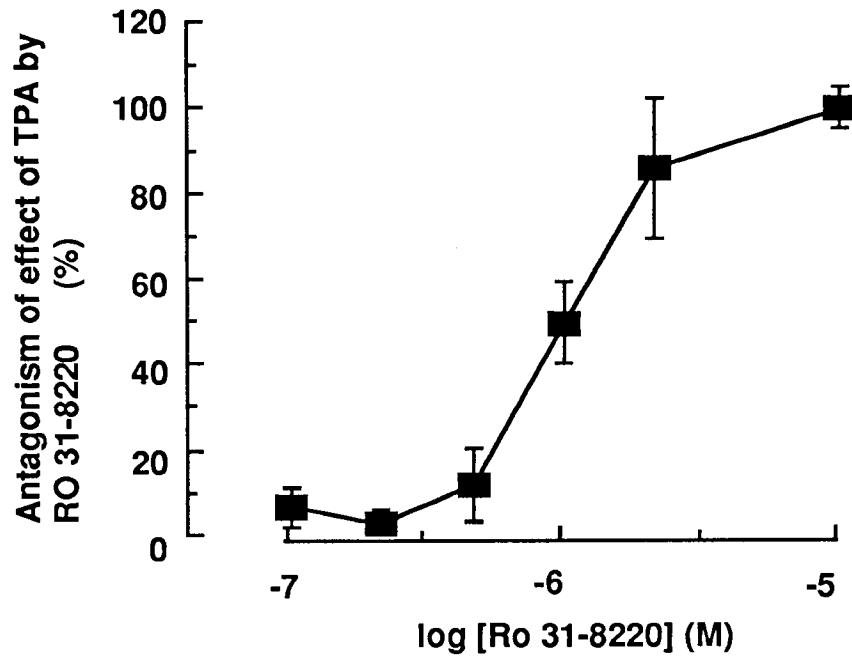
The antagonism of the effect of TPA by Ro 31-8220 was calculated from data presented in Figure 5.1.A as [1-(effect TPA at a particular concentration of Ro 31-8220/effect TPA in absence of Ro 31-8220)] x100. The half maximally effective concentration of Ro 31-8220 was estimated by using the computer program FIT to be 1 μM (Barlow, 1983).

program FIT (Barlow, 1983) to be 1 μM (Fig. 5.3.B), and this value was close to that obtained for the inhibition of the effects of TPA by Ro 31-8220 in other cell types (Davis et al., 1989). Inhibition of the effect of 100 nM TPA in the presence of 2.14 μM Ro 31-8220 was determined to be 85.62 ± 16.63 % (Fig. 5.3.B). Use of Ro 31-8220 at concentrations above 2.14 μM was prevented by substantial inhibitory effects of Ro 31-8220 on aminopyrine accumulation stimulated by 0.5 mM histamine alone becoming apparent (Fig. 5.3.A, Section 5.3.3.4).

5.3.3   Comparison of the effects of Ro 31-8220 on the aminopyrine accumulation ratio in unenriched and enriched suspensions of parietal cells.

5.3.3.1   Lack of effect of Ro 31-8220 on basal aminopyrine accumulation.

Basal aminopyrine accumulation in both unenriched and enriched suspensions of parietal cells was unaffected by the presence of Ro 31-8220 (Table 5.3 and legends to Figures 5.4 and 5.6).

5.3.3.2   Enhancement of aminopyrine accumulation stimulated by histamine or dibutyryl cyclic AMP in unenriched cells by Ro 31-8220 (0.1 - 2.14 μM).

In an unenriched suspension of rat parietal cells Ro 31-8220, over the concentration range 0.1 to 2.14 μM produced a slight but significant enhancement of the aminopyrine accumulation ratio

stimulated by 0.5 mM histamine (P < 0.05 by analysis of variance, Fig. 5.3.A). A significant effect of 2.14 μM Ro 31-8220 on the dose-response curve for histamine-stimulated aminopyrine accumulation was however only apparent at the highest concentration (0.5 mM) of histamine used (P < 0.05 by paired t-test, Fig. 5.4, Table 5.3.A), although a similar enhancement by 2.14 μM Ro 31-8220 on the aminopyrine accumulation ratio stimulated by 1 mM dibutyryl cyclic AMP was also observed (Table 5.3.A). These results could suggest an antagonism by Ro 31-8220 of an inhibitory modulation of the histamine response by basal protein kinase C activity (Hatt and Hanson, 1989).

5.3.3.3 Lack of effect of Ro 31-8220 (2.14 μM) on aminopyrine accumulation stimulated by histamine or dibutyryl cyclic AMP in enriched cells.

In contrast to the above, no effect of 2.14 μM Ro 31-8220 on aminopyrine accumulation stimulated by either histamine (0.5 mM) or dibutyryl cyclic AMP (1 mM) in an enriched suspension of parietal cells was found (Table 5.3.B). This lack of an effect of Ro 31-8220 in enriched cells may be due to an effect of the enrichment procedure on basal protein kinase C activity, although the possibility of the results obtained with unenriched cells being due to interactions between different cell types cannot be ruled out. More experiments are required to clarify these effects but such information is not essential to the interpretation of the results presented in this Chapter on the action of protein kinase C in the response of parietal cells to carbachol.

Table 5.3.A

Effect of 2.14 µM Ro 31-8220 on aminopyrine accumulation stimulated by carbachol, dibutyryl cyclic AMP or histamine in suspensions of cells containing approximately 20 % parietal cells.

Agent	n	Basal aminopyrine accumulation		Stimulation of aminopyrine accumulation		Inhibition of response to agonist by Ro 31-8220 (%)
		Control	2.14 µM Ro 31-8220	Control	2.14 µM Ro 31-8220	
0.1 mM carbachol	4	1.72 ± 0.14	1.64 ± 0.14	2.36 ± 0.21	1.45 ± 0.26	39.90 ± 5.52**
1 mM dibutyryl cyclic AMP	3	1.75 ± 0.07	1.68 ± 0.10	18.52 ± 0.79	24.44 ± 1.69	-31.76 ± 4.44*
0.5 mM histamine + 0.1 mM IBMX	6	1.87 ± 0.18	1.77 ± 0.19	18.84 ± 2.77	24.78 ± 3.32	-33.56 ± 8.64*

Results are expressed as means ± SEM of duplicate determinations from each experiment. ** $P < 0.01$, * $P < 0.05$ for effects of 2.14 µM Ro 31-8220 analysed by paired t-test.

Table 5.3.A

Effect of 2.14 µM Ro 31-8220 on aminopyrine accumulation stimulated by carbachol, dibutyryl cyclic AMP or histamine in suspensions of cells containing approximately 20 % parietal cells.

Agent	n	Basal aminopyrine accumulation		Stimulation of aminopyrine accumulation		Inhibition of response to agonist by Ro 31-8220 (%)
		Control	2.14 µM Ro 31-8220	Control	2.14 µM Ro 31-8220	
0.1 mM carbachol	4	1.72 ± 0.14	1.64 ± 0.14	2.36 ± 0.21	1.45 ± 0.26	39.90 ± 5.52**
1 mM dibutyryl cyclic AMP	3	1.75 ± 0.07	1.68 ± 0.10	18.52 ± 0.79	24.44 ± 1.69	-31.76 ± 4.44*
0.5 mM histamine + 0.1 mM IBMX	6	1.87 ± 0.18	1.77 ± 0.19	18.84 ± 2.77	24.78 ± 3.32	-33.56 ± 8.64*

Results are expressed as means $\pm$ SEM of duplicate determinations from each experiment. ** $P < 0.01$, * $P < 0.05$ for effects of 2.14 µM Ro 31-8220 analysed by paired t-test.

Figure 5.4
Effect of Ro 31-8220 on the secretory response of unenriched parietal cells to different concentrations of histamine.
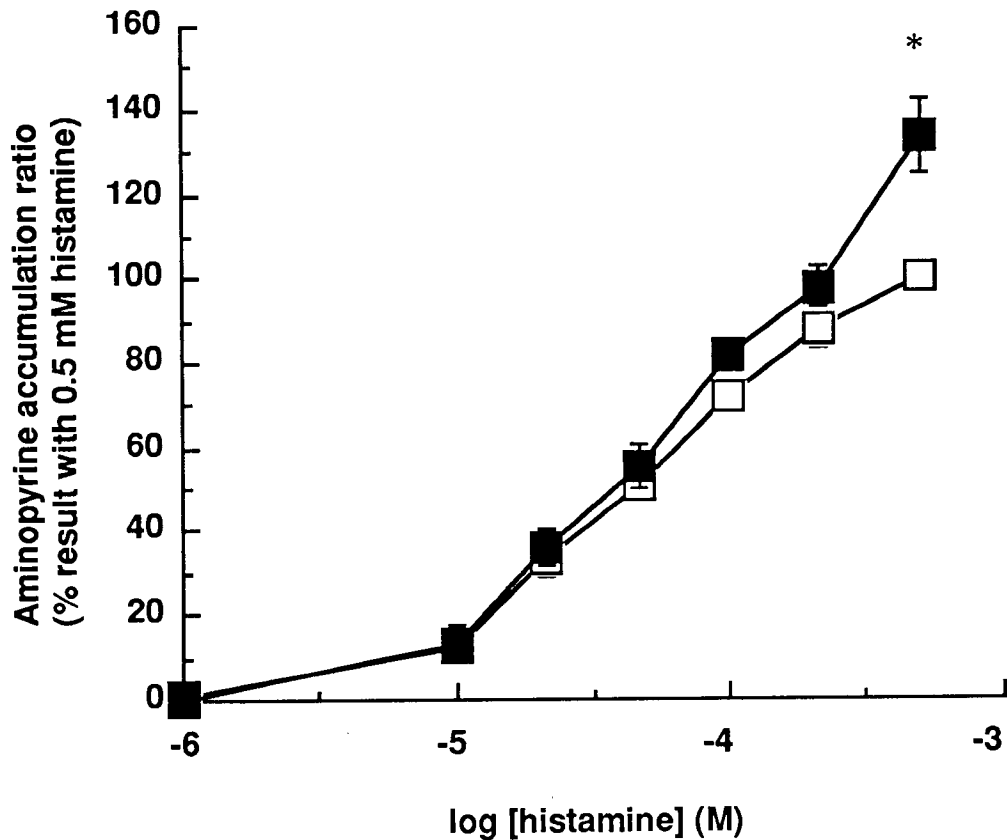
Results are presented as means ± SEM from six batches of unenriched cells and have been normalized to the stimulation of the aminopyrine accumulation ratio obtained with 0.5 mM histamine plus 0.1 mM IBMX which was 18.8 ± 2.8. Basal aminopyrine accumulation was unaffected by the addition of 2.14 µM Ro 31-8220 being 1.9 ± 0.18 and 1.8 ± 0.19 respectively in the absence and presence of Ro 31-8220. (□) control, (■) 2.14 µM Ro 31-8220. * P < 0.05 for effect of TPA by paired t-test.

Ro 31 - 8220

<u>5.3.3.4   Conclusion: selectivity of Ro 31-8220.</u>

An inhibition of the aminopyrine accumulation ratio stimulated by 0.5 mM histamine became evident when the concentration of Ro 31-8220 was raised to 10 μM (Fig. 5.3.A). This result might be explained by inhibition of cyclic AMP-dependent protein kinase, on which the action of histamine depends (Chew, 1985), for at high concentrations Ro 31-8220 has been shown to inhibit this enzyme (Davis et al., 1989). Nevertheless, the lack of an inhibitory effect of Ro 31-8220 at concentrations of 2.14 μM and below on the acid secretory response to histamine (see above), coupled with a clear antagonism by Ro 31-8220 of the effect of TPA on this response suggests that, at concentrations of 2.14 μM and below, Ro 31-8220 exerts a selective inhibition of protein kinase C with negligible inhibitory effect on protein kinase A. These results accord with experiments using isolated cyclic AMP-dependent protein kinase and protein kinase C where the IC_{50} for inhibition of the former enzyme by Ro 31-8220 was 150 times higher than that for protein kinase C (Davis et al., 1989).

5.3.4   Effect of incubation time in the presence of carbachol on the magnitude of the acid secretory response.

The aminopyrine response to carbachol in parietal cells is significantly less than the response to histamine plus 3-isobutyl-1-methylxanthine (Schepp et al., 1990; Kajimura et al., 1990). The maximal response to carbachol has been reported to occur after either 30 min (Ecknauer et al., 1981; Chiba et al., 1988) or 60 min

(Schepp et al., 1989) of incubation. To investigate this, and in an attempt to maximize this response, the effect of incubation time in the presence of a maximally stimulatory dose of carbachol (0.1 mM, Negulescu et al., 1989) was examined. A significant reduction in the aminopyrine accumulation ratio was observed after 45 min and 60 min of incubation in the presence of 0.1 mM carbachol compared to 30 min of incubation (P < 0.01 by Newman-Keuls's test, Appendix A.5.3) (Fig. 5.5), whereas no significant difference between 45 min and 60 min incubation periods was found. A similar time-course of aminopyrine accumulation following stimulation by carbachol has been shown in parietal cells isolated from the rabbit (Leonard et al., 1990). These results may be explained by an increased rate of leakage of protons and/or aminopyrine from the secretory canaliculi into the medium after 30 min, or alternatively may reflect either an earlier reduction in the initial $[Ca^{2+}]_i$ peak (Negulescu et al., 1989), or desensitization of the M_3 receptor. A 30 min incubation period was used in all subsequent experiments measuring the acid secretory response to carbachol.

5.3.5   Inhibition by Ro 31-8220 of aminopyrine accumulation stimulated by carbachol.

In an unenriched suspension of parietal cells, the presence of 1 μM or 2.14 μM Ro 31-8220 produced significant and progressive shifts in the dose-response curve for the stimulation of aminopyrine accumulation by carbachol downwards and to the right (Fig. 5.6, P < 0.05 by factorial analysis of variance). An identical concentration of Ro 31-8220 (2.14 μM) in a similar cell preparation failed to inhibit the response to histamine, even when the level of

Figure 5.5
Effect of incubation time in the presence of 0.1 mM carbachol on the magnitude of the acid secretory response.
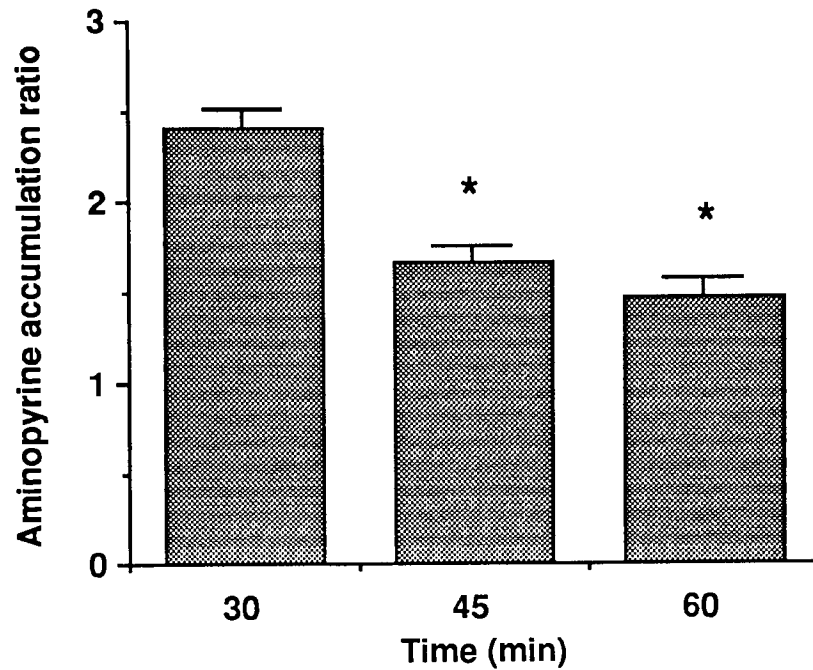
Results are means ± SEM of triplicate determinations from a single batch of unenriched cells and are expressed as stimulation above basal aminopyrine accumulation which was 1.87 ± 0.11. A significant reduction in the aminopyrine accumulation ratio after either 45 min or 60 min of incubation in the presence of 0.1 mM carbachol compared to 30 min of incubation was apparent. * P < 0.01 by ANOVAR (Appendix A.5.1) and Newman-Keul's test (Appendix A.5.3).

secretory activity was similar to that induced by carbachol (Fig. 5.4). Thus, the above inhibitory action of Ro 31-8220 on the response to carbachol could not have been due to a non-specific effect on either aminopyrine accumulation or on the acid secretory process.

The half-maximally effective concentration of Ro 31-8220 for the inhibition of secretory activity stimulated by 0.1 mM carbachol in the above cell suspensions was 0.78 μM (Figure 5.7). This figure was similar to that found for antagonism of the effects of TPA in parietal (Section 5.3.2), and other (Davis et al., 1989) cells. Thus, the effect of Ro 31-8220 on carbachol-stimulated secretory activity was most likely mediated via an inhibition of protein kinase C. Extrapolation of the dose-response curve for inhibition of 0.1 mM carbachol-stimulated secretory activity by Ro 31-8220, suggests that Ro 31-8220, at a maximally effective concentration would not completely abolish the response to carbachol. Consequently, the role of protein kinase C is more facilitatory than essential to the action of carbachol.

5.3.6   Lack of effect of Ro 31-8220 on mobilisation of Ca^{2+} by carbachol in the parietal cell.

5.3.6.1   Check on methodology.

In order to test the Fura-2 system it was necessary to show that entry of Ca^{2+} into cells resulted in a changed fluorescence output. To this end, the calcium ionophore, ionomycin, which does not fluoresce in the ultra-violet spectrum and which displays a high

Figure 5.6
Effect of Ro 31-8220 on the secretory response of unenriched parietal cells to different concentrations of carbachol.
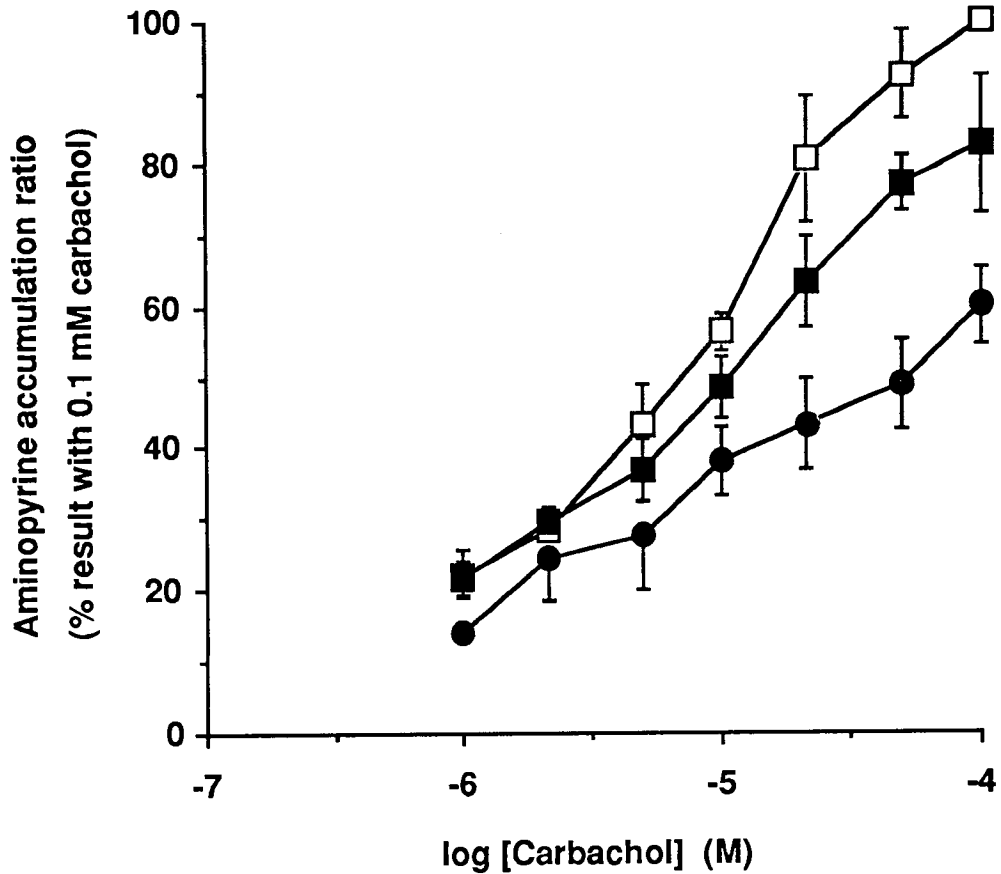
Results are presented as means ± SEM. from four batches of unenriched cells. Symbols denote the effect of the concentration of carbachol in the absence (□), and presence, of 1 µM (■) and 2.14 µM (●) Ro 31-8220. Results have been normalized to the stimulation of aminopyrine accumulation obtained with 0.1 mM carbachol alone which was 4.1 ± 0.3. The basal aminopyrine accumulation ratio was 1.7 ± 0.14, and results in the presence of 1 µM and 2.14 µM Ro 31-8220 were 1.6 ± 0.14 and 1.6 ± 0.14 respectively.

Figure 5.7
Effect of the concentration of Ro 31-8220 on the secretory response to 0.1 mM carbachol.
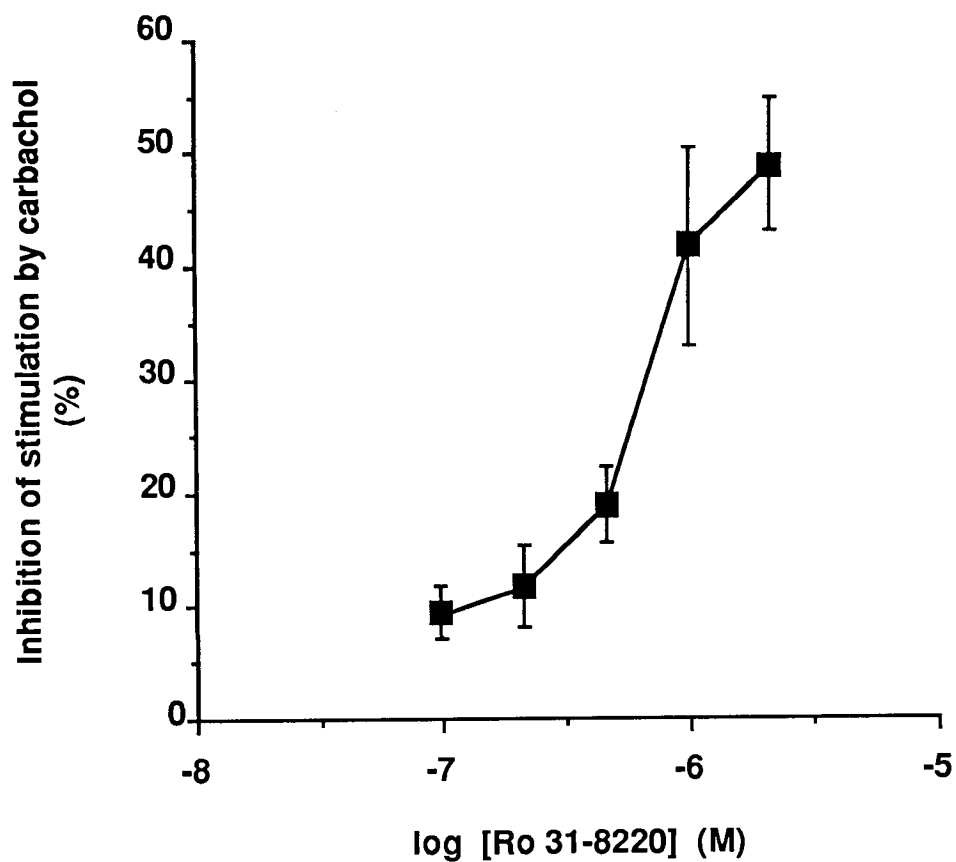
Results are means ± SEM. from four experiments and were performed with a suspension containing unenriched parietal cells. Basal aminopyrine accumulation was 1.8 ± 0.10, and that in the presence of 0.1 mM carbachol was 3.36 ± 0.38.

selectivity for Ca^{2+} over other divalent cations (Williams and Fay, 1990) was chosen. Addition of 1 μM ionomycin produced an increase in the cytosolic Ca^{2+} concentration that was approximately 4.3-fold above basal (Fig. 5.8). The response reached a plateau which did not decline during the course of the experiment, thus indicating that a steady state had occurred.

Calibration of the response was performed using Triton-X-100 (0.2 % $^V/_v$) to lyse the cells and liberate Ca^{2+}, followed by addition of EGTA (20 mM) to chelate Ca^{2+}. This procedure allowed R_{max} and R_{min} respectively (Section 5.2.3.2) to be obtained (Fig. 5.9).

5.3.6.2   Action of Ro 31-8220.

In enriched parietal cells, which had been loaded with Fura-2, the mean cytosolic Ca^{2+} concentration in the absence of stimulatory or inhibitory agents was 187 $\pm$ 32 nM (Table 5.4). This figure agrees well with the resting Ca^{2+} concentrations measured using Fura-2 in parietal cells by other workers (Leonard et al., 1990; Tsunoda et al., 1992).

Stimulation with 0.1 mM carbachol caused a rapid transient increase in $[Ca^{2+}]_i$ which peaked after approximately 9 s and then gradually fell to a plateau which remained elevated above basal (Fig.5.10.A). The peak Ca^{2+} response was probably due to emptying of Ca^{2+} from internal stores (Negulescu et al., 1988). This is thought to be followed by an immediate efflux of Ca^{2+} from the cell rather than a resequestration into the internal stores. It is this efflux that is responsible for the fall to the plateau phase of the

Figure 5.8
Effect of 1 μM ionomycin on intracellular Ca^{2+} in a suspension containing greater than 80% parietal cells.
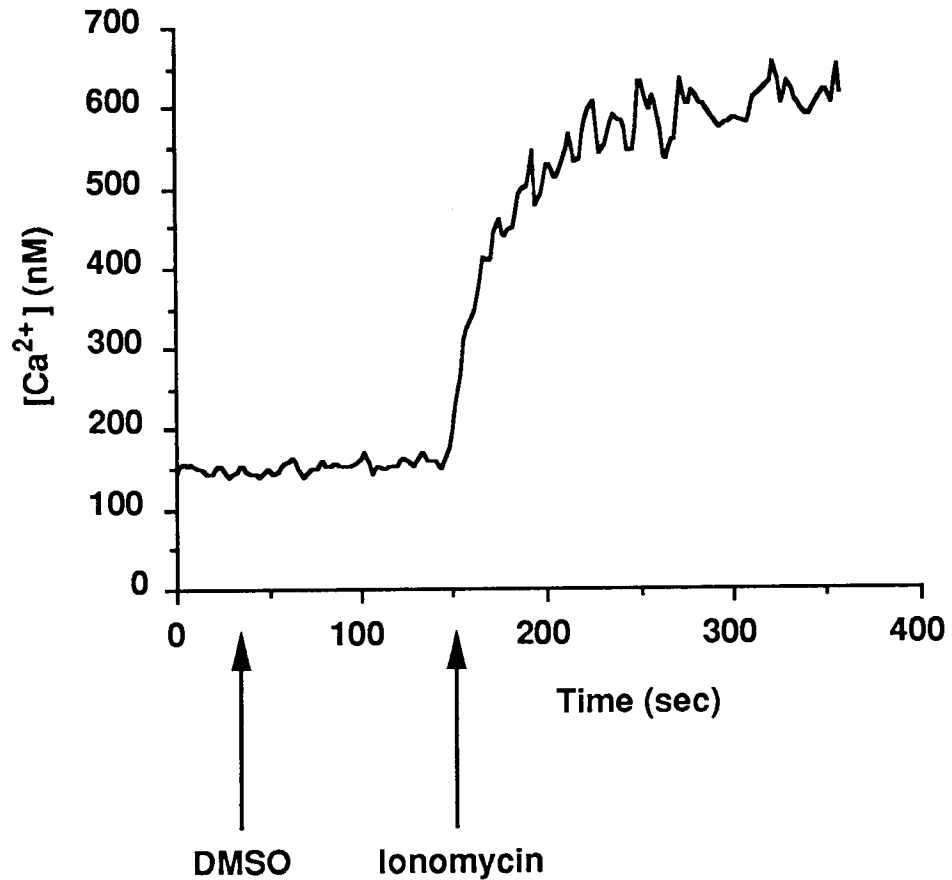
Results are from one experiment representative of three. DMSO was added at 30 s and ionomycin at 150 s. Further details are provided in the methodology section.

Figure 5.9
Calibration using Triton-X-100 and EGTA to derive R_{max} and R_{min} respectively.

A

B

Triton-X-100
(0.2% v/v)
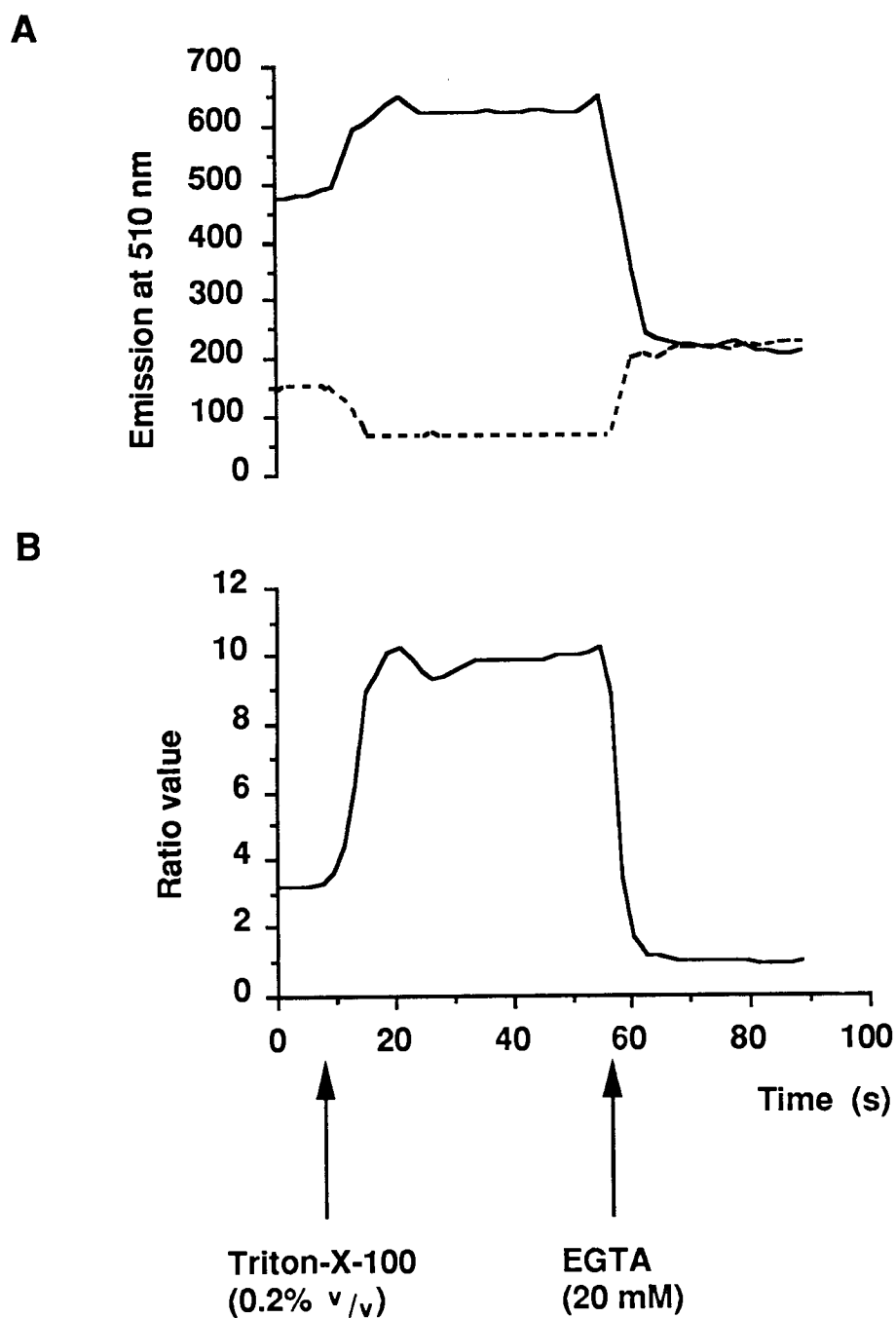
EGTA
(20 mM)

Results are from one experiment representative of three. A. Change in emission at 510 nm following addition of Triton-X-100 and EGTA. (———) Excitation at 340 nm, (----) Excitation at 380 nm. B. Emission ratio at 510 nm calculated from data in A.

Figure 5.10
Effect of 0.1 mM carbachol on intracellular Ca^{2+} in a suspension containing > 80% parietal cells was prevented by the prior addition of 0.01 mM atropine.
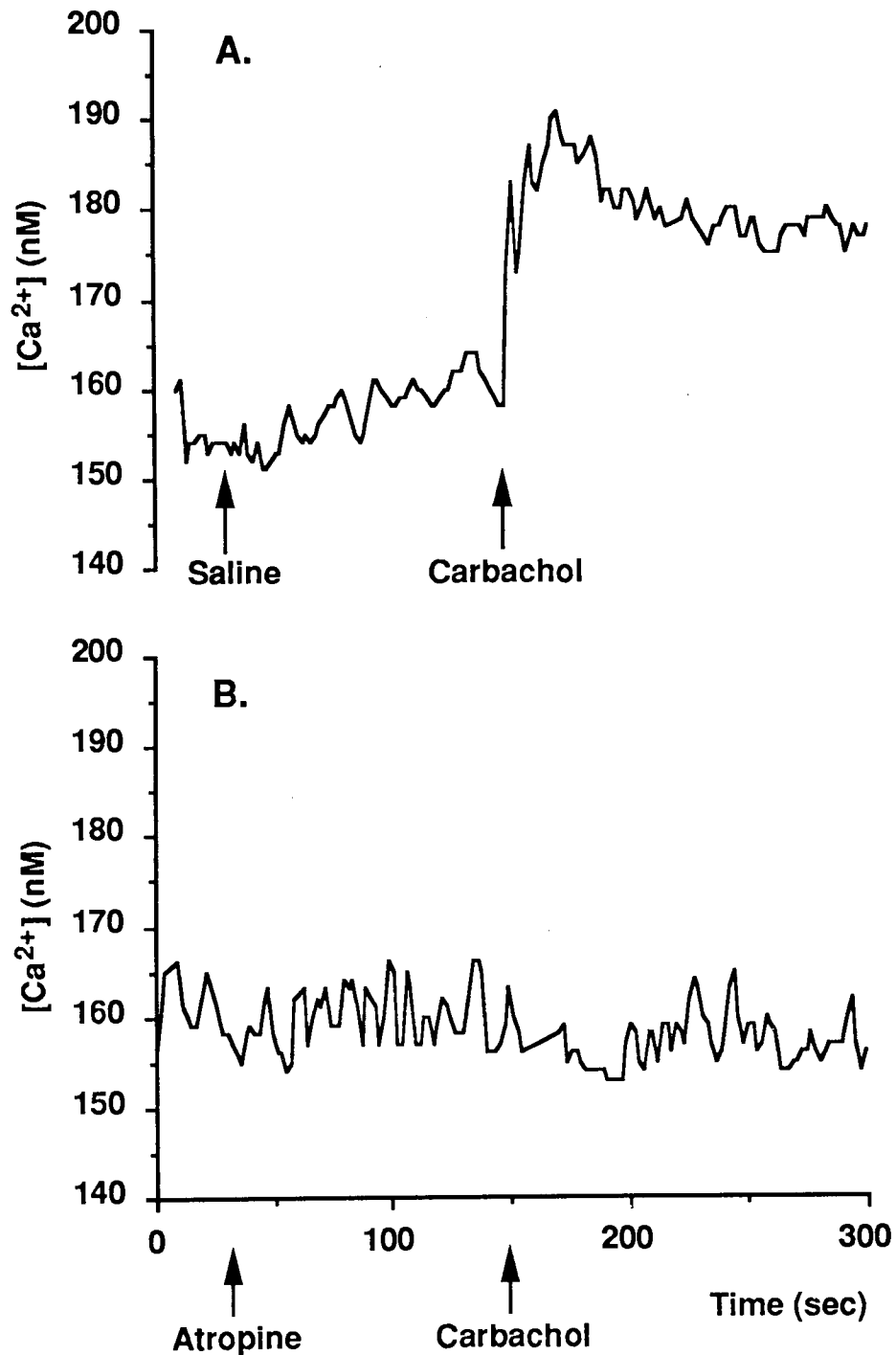
Results are from one experiment representative of three. 0.01 mM atropine or vehicle was added at t=30 s and 0.1 mM carbachol at t=150 s. Measurements were made at 1.9 s intervals.

Table 5.4

Lack of effect of Ro 31-8220 on intracellular Ca^{2+} in a suspension enriched with rat parietal cells.

Treatment	Intracellular Ca^{2+} (nM)			
	0-30 s	31-150 s	at peak response	at plateau (240-300 s)
control	187 ± 32	194 ± 33	238 ± 40	216 ± 36
Ro 31-8220	173 ± 31	181 ± 32	227 ± 42	205 ± 35

Results are means ± SEM of data from seven separate preparations enriched with parietal cells. Ro 31-8220 (2.14 μM) or vehicle was added at 30 s and carbachol (0.1 mM) at 150 s. The peak response was the highest single value for intracellular $[Ca^{2+}]$ after the addition of carbachol, other results are the average values over the time periods specified.

response (Negulescu et al., 1988). The plateau phase is maintained by an influx of Ca^{2+} across the cell membrane and will remain until the stimulation by carbachol is removed (Leonard et al., 1990). Both of the above phases have been shown to occur in individual parietal cells (Negulescu et al., 1989) and therefore the results presented here cannot be due to cellular heterogeneity. The effect of carbachol was completely abolished by the prior addition of 10 μM atropine (Fig.5.10.B) which provides strong evidence that the effects were mediated via a muscarinic receptor.

No effect of Ro 31-8220 on intracellular Ca^{2+} under basal conditions or after stimulation with 0.1 mM carbachol was detectable (Table 5.4). Thus, the effect of Ro 31-8220 on aminopyrine accumulation stimulated by carbachol (Section 5.3.5, Fig. 5.6) must have been exerted via a signalling pathway activated by carbachol apart from that involving intracellular Ca^{2+}. The most likely such pathway is that involving protein kinase C. In parietal cells, the predominant role of protein kinase C in the initial response to carbachol must therefore be a stimulatory one.

5.4   GENERAL DISCUSSION.

The present findings, along with results on the enhancement of the response to thapsigargin by TPA (Chew and Petropoulos, 1991) suggest that the predominant role for protein kinase C is in activation of the secretory response to carbachol. However, inhibitory effects of TPA on the response to carbachol in parietal cells have been found (Anderson and Hanson, 1984; Brown and Chew, 1987; Chiba et al., 1989). These effects could be explained by TPA producing a pharmacological and irreversible activation of protein kinase C which is different from that produced by carbachol. Alternatively, protein kinase C could genuinely be involved in feedback regulation of the response to carbachol, but this action may be of less importance that the stimulatory role of protein kinase C during the 30 min incubation used in the present experiments. The fact that Ro 31-8220 is a competitive antagonist for the ATP-binding site on protein kinase C suggests that it is likely to be active against all isoforms of the enzyme (Nishizuka et al., 1988) and therefore the particular isoform involved in the response cannot be identified using the above inhibitor. Different isoforms of protein kinase C could however, be involved in the multiplicity of actions of protein kinase C in parietal cells.

5.5 SUMMARY.

1. Ro 31-8220 antagonised the inhibitory effect of 100 nM 12-O-tetradecanoylphorbol 13-acetate on histamine-stimulated acid secretion with an IC_{50} of 1 μM.

2. Ro 31-8220 inhibited the stimulatory effect of a near-maximally effective concentration of carbachol (0.1 mM) on acid secretory activity in an unenriched preparation of parietal cells with an IC_{50} of 0.78 μM. This inhibitory effect was also demonstrable in a parietal cell-enriched cell suspension.

3. No effect of Ro 31-8220 on intracellular Ca^{2+} concentration stimulated by 0.1 mM carbachol was detected.

4. The main conclusion is that Ro 31-8220 is an effective antagonist of protein kinase C in rat parietal cells, and that this enzyme is involved in the mechanism by which carbachol stimulates acid secretion.

CHAPTER 6

INTERACTIONS OF EGF WITH PATHWAYS REGULATING
SECRETORY ACTIVITY IN RAT PARIETAL CELLS.

6.1　　　INTRODUCTION.

6.1.1　　The structures of EGF and TGFα.

Epidermal growth factor (EGF) is a single chain polypeptide with a molecular weight of 6045 which was originally isolated from the submaxillary gland of the male mouse (Cohen, 1962). The same peptide isolated from the rat contains 53 amino acid residues, and disulphide bonds between residues 6 and 20, 14 and 31, and 33 and 42 produce three loops in the secondary structure (reviewed by Carpenter, 1985). An homologous polypeptide, β-urogastrone originally detected in human urine (Starkey et al., 1975) and believed to represent the human form of EGF shows 62% homology to rat EGF. Not surprisingly therefore, both molecules exhibit similar biological properties (Gregory, 1975). The three-dimensional structure of residues 1-48 of this molecule has been determined (Cook et al., 1987, Fig. 6.1). Residues 49-53 do not appear to be required for biological activity (Hollenberg and Gregory, 1980).

Transforming growth factor-alpha (TGFα) isolated from rat cells is a polypeptide structurally analogous to EGF. It contains 50 amino acid residues, has a molecular weight of 5616 and shows 32% homology in sequence to rat EGF. The three-dimensional structures of EGF and TGFα are thought to be similar (Campbell et al., 1989), which provides a molecular explanation for the mutually competitive binding of both ligands to the epidermal growth factor receptor (Lewis et al., 1990a).

Figure 6.1
Schematic representation of the secondary structural features of human EGF (Cooke et al., 1987).

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The structure was determined by two dimensional nuclear magnetic resonance. Disulphide bridges are depicted as solid lines and hydrogen bonds as dashed lines. Cysteine and glycine residues are shown as C and G respectively.

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6.1.2   The presence of EGF and TGFα in the gastrointestinal tract.

In the gastrointestinal tract, immunoreactive EGF has been detected in saliva, gastric juice and pancreatic juice (Carpenter, 1985) and has been localized to murine and human submandibular glands and feline and human Brunner's glands (Kasselberg et al., 1985). EGF messenger RNA has been found in canine gastric mucosa, but has not however been demonstrated in mucosa isolated from human, rat or guinea-pig (Beauchamp et al., 1989). This has led to the suggestion that EGF detected in the stomach by immunological means may have been produced in the salivary glands (Beauchamp et al., 1989). Epithelial ulceration in the gastrointestinal tract has recently been shown to induce the development, from gastrointestinal stem cells, of a novel cell lineage which contains and secretes abundant EGF (Wright et al., 1990).

TGFα messenger RNA (Beauchamp et al., 1989) and protein (Cartlidge and Elder, 1989) have been shown to be present in normal gastric mucosa isolated from the adult dog, guinea-pig, human and rat, with little or none found in the submucosa or gastric muscle (Beauchamp et al., 1989). TGFα messenger RNA has also been found in parietal cell-enriched fractions of guinea-pig gastric cells (Beauchamp et al., 1989). The majority of the TGFα protein was shown to be associated with the tubulovesicular membranes (Polk et al., 1991).

6.1.3   The EGF/TGFα receptor.

The mature EGF/TGFα receptor is a 170 kDa glycoprotein composed of 1186 amino acid residues arranged in a single polypeptide chain (Carpenter, 1985). The receptor contains three major structural elements (Fig. 6.2). The 621 amino acid extracellular ligand-binding domain is characterized by a large number (51) of cysteine residues and is anchored in the plasma membrane by a single transmembrane region comprising 23 hydrophobic amino acids. The 542 amino acid cytoplasmic domain contains the autophosphorylation sites and shows intrinsic tyrosine kinase activity (Ullrich et al., 1984; Schlessinger, 1986).

The EGF/TGFα receptor appears to exist in both high and low affinity states in many cell types (Livneh et al., 1986). High affinity sites usually represent 5 - 10 % of the total EGF receptor population and this correlates well with the optimal receptor occupancy required for the initiation of DNA synthesis (Schlessinger, 1989). It has therefore been suggested that the high affinity receptors play a role in the mitogenic signalling process.

Ligand binding leads to dimerization of the EGF receptor and results in activation of the receptor tyrosine kinase which phosphorylates the C-terminal end of the adjacent EGF receptor. In intact cells autophosphorylation occurs mainly on tyrosine-1173 (Schlessinger, 1986), although at least two additional tyrosine residues are autophosphorylated when EGF is added either to intact membranes or to the pure receptor.

Figure 6.2
Schematic representation of the EGF receptor.

NH$_2$ -terminal   COOH-terminal

1   621 644   940   1186
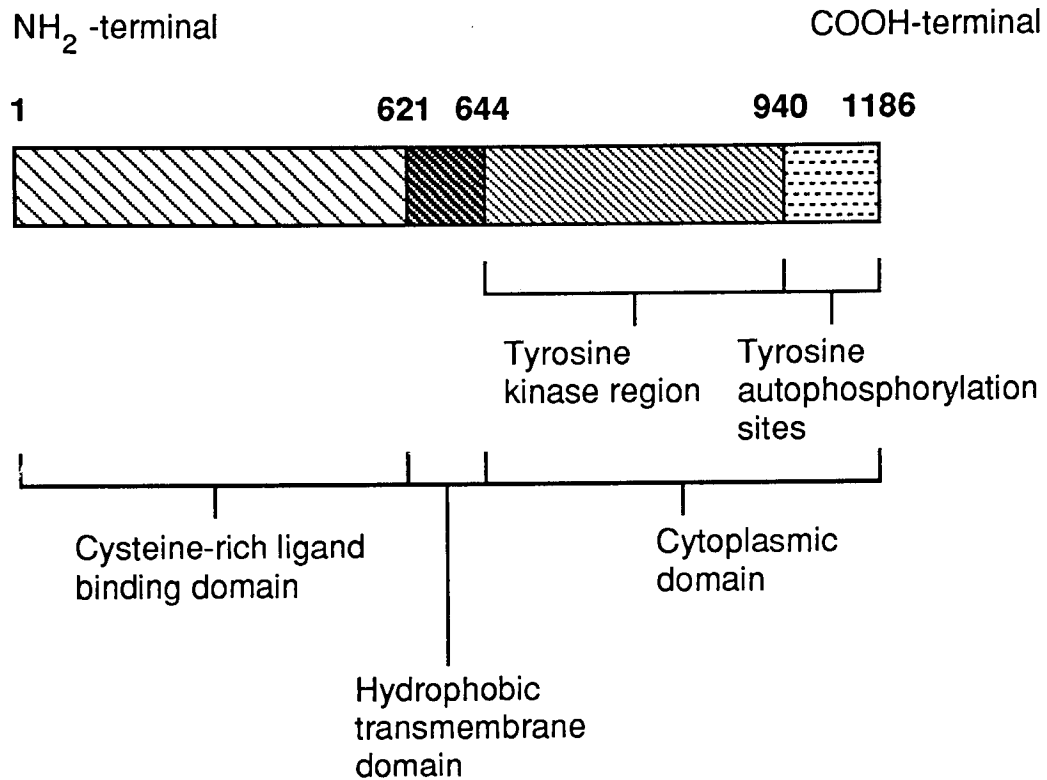
The EGF receptor is shown as a bar and the domains are derived from the amino acid sequence determined by Ullrich et al. (1984). The figures represent amino acid residue numbers.

Such receptors, autophosphorylated on tyrosine, are now known to serve as high-affinity ligands for certain *src* homology 2, (SH2) containing proteins including phospholipase C γ, Ras GTPase-activating protein (GAP) and the 85 kDa subunit of phosphatidylinositol 3-kinase (reviewed by Mayer and Baltimore, 1993). The SH2 domains in these proteins consist of small modules of approximately 100 amino acid residues. Recruitment of proteins containing SH2 domains mediates signalling by altering either their subcellular localization or phosphorylation state, or by inducing a conformational change in the SH2 protein (Mayer and Baltimore, 1993).

EGF also stimulates phosphorylation of the EGF receptor on serine and threonine residues, and these residues have been shown to be phosphorylated in response to TPA, presumably through the activation of protein kinase C (Cochet et al., 1984). Phosphorylation of the receptor on threonine-654 results in a reduced tyrosine kinase activity (King and Cooper, 1986) and abolishes the high affinity state of the receptor, (Friedman et al., 1984). It has therefore been suggested that phosphorylation at this site regulates the affinity of the extracellular domain towards the ligand and the enzymatic activity of the protein tyrosine kinase domain (Schlessinger, 1989). Following activation of the receptor, the ligand-receptor complex is internalized and both EGF and the receptor degraded in lysosomes.

High levels of EGF/TGFα receptor messenger RNA have been detected in normal gastric mucosa isolated from rat, dog, guinea-pig and human (Beauchamp et al., 1989) and significant specific binding

of [^{125}I]-human EGF to canine gastric mucosa and muscularis mucosa has also been demonstrated (Zimmerman et al., 1989). In guinea-pig gastric cells, EGF/TGFα receptor messenger RNA was localized mainly to a parietal cell enriched fraction (Beauchamp et al., 1989) which correlates with a strong EGF receptor-like immunoreactivity on canine parietal cells (Mori et al., 1987). This immunoreactivity was also demonstrated on the basolateral but not the apical membrane of human parietal cells (Mori et al., 1987). An association of EGF/TGFα receptors with the tubulovesicular apparatus in parietal cells has also been suggested (Polk et al., 1991).

6.1.4 Stimulation of prostaglandin E$_2$ (PGE$_2$) production by EGF.

6.1.4.1 Synthesis of prostaglandin E$_2$.

Membrane phospholipids, especially the phosphatidylinositol pool are known to be rich in arachidonic acid which is generally found esterified in the 2-acyl position (Irvine, 1982). Arachidonate is the precursor of all prostaglandins, and increased availability of this precursor leads to increased synthesis. Arachidonic acid may be released from phospholipids, either directly via a phospholipase A$_2$, or indirectly by a phospholipase C or D catalysed hydrolysis (Fig. 6.3). The action of phospholipase A$_2$ yields arachidonic acid and a lysophospholipid. In some cell types, this activity is regulated by pertussis toxin-sensitive G-proteins (Wang et al., 1988). Phospholipase C hydrolyses phospholipids to yield diacylglycerol (Fig. 6.3), and in the case of phosphatidylinositol 4,5-bisphosphate,

Figure 6.3
Specificity of action of phospholipases A₂, C and D on a generalised phosphoglyceride.
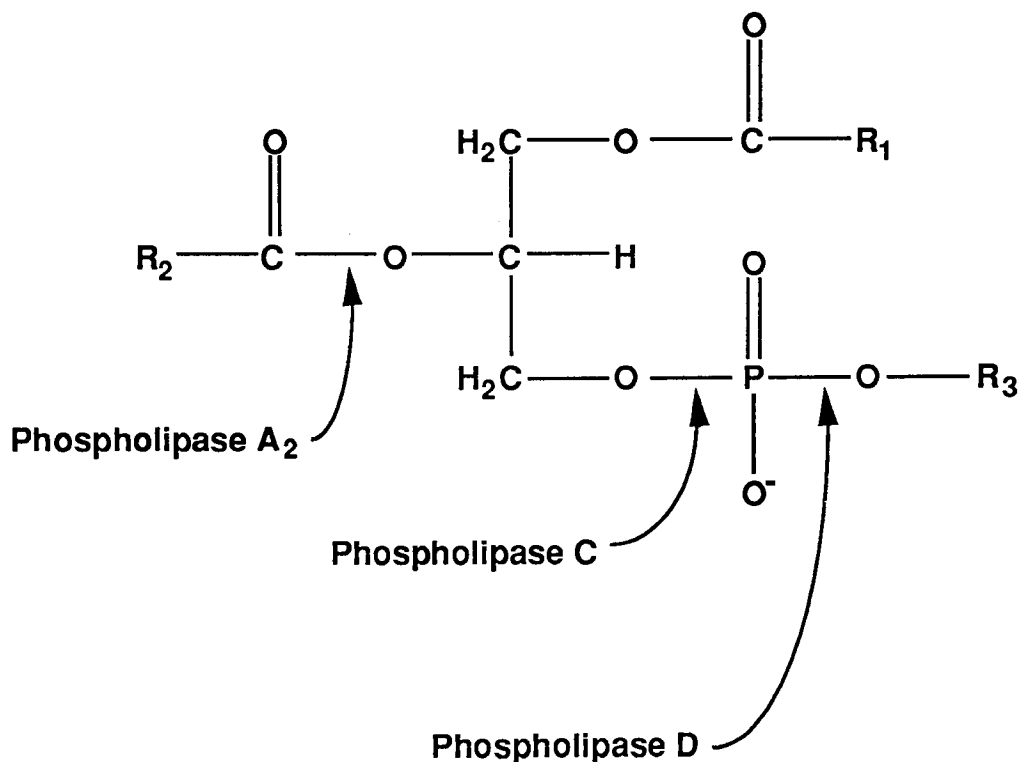
R₁ and R₂ are fatty acids esterified to the hydroxyl groups at carbon atoms 1 and 2 respectively. An unsaturated fatty acid such as arachidonic acid is normally found in the 2 position. R₃ is a polar alcohol head group which is esterified to the phosphoric acid substituent. The polar head groups differ among phospholipid classes.

inositol 1,4,5-trisphosphate. Diacylglycerol lipase, and monoacylglycerol lipase then catalyse the conversion of diacylglycerol to free arachidonic acid (reviewed by Meldrum et al., 1991). The β forms of phospholipase C are regulated by G-proteins of the type G_{11}/G_q (Hepler and Gilman, 1992). Phospholipase D yields a phosphatidic acid which can be converted to diacylglycerol and ultimately to arachidonic acid.

6.1.4.2   Effect of EGF on prostaglandin E$_2$ production.

EGF stimulates prostaglandin production in a variety of cell types (Kasai et al., 1987; Margolis et al., 1988a), and in the perfused rat stomach (Chiba et al., 1982). In a rat isolated parietal cell preparation containing more than 80 % parietal cells, EGF stimulated PGE$_2$ production with a half-maximally effective concentration of 7.5 nM (Hatt and Hanson 1988).

The above results may be thought to imply a role for PGE$_2$ in mediating the inhibitory action of EGF on acid secretory activity. However, this is unlikely as both cyclo-oxygenase and lipoxygenase inhibitors failed to prevent the inhibitory action of EGF in rat parietal cells (Shaw et al., 1987). Furthermore, the inhibitory action of PGE$_2$ on aminopyrine accumulation in canine (Soll, 1980b) and rat (Atwell and Hanson, 1988) parietal cells and in rabbit gastric glands (Levine et al., 1982) was still evident in the presence of IBMX whereas IBMX is known to block the effect of EGF (Atwell and Hanson, 1988). Finally, the stimulatory effect of EGF on PGE$_2$ release was shown to be prevented by histamine (Hatt and Hanson, 1988).

6.1.4.3 How does EGF activate prostaglandin production in gastric cells?

It is unlikely that EGF stimulates prostaglandin production by increasing the hydrolysis by phospholipase C of phosphatidylinositol 4,5-bisphosphate, because EGF, like carbachol (Chapter 1), would then be expected to stimulate acid secretion because of mobilization of intracellular Ca^{2+}. An alternative is that EGF stimulates the turnover of another phospholipid such as phosphatidylcholine, either via phospholipase C, thus generating diacylglycerol but without the production of inositol 1,4,5-trisphosphate, or via phospholipase D (Section 6.1.4.1). However, the most likely mediator of the effect of EGF on PGE_2 production is likely to be phospholipase A_2. The presence of this enzyme has been reported in the scrapings of rat gastric mucosa (Tojo et al., 1988) and significant activity has been demonstrated in rat parietal cells (Hell and Sewing, 1987). It is known to be activated in vitro by Ca^{2+} and therefore may become activated in intact cells exposed to a calcium ionophore.

In this study, the effect of A23187 on arachidonic acid production in parietal cells pre-labelled with [^{3}H]-arachidonic acid was investigated to see if a potential phospholipase A_2 activity could be detected. If this could be shown, the effect of EGF on the activity of this enzyme could then be examined. Activation of a phospholipase A_2 by EGF would produce a lysophosphatidic acid but no diacylglycerol, while activation of a phospholipase C or D would produce diacylglycerol or phosphatidic acid respectively (Fig. 6.3).

6.1.5   Effect of EGF and TGFα on gastric acid secretion.

6.1.5.1   Previous work.

The results of several studies have established both EGF and TGFα as inhibitors of acid secretion induced by a variety of stimulants (Table 6.1).   Although both acid and pepsin resistant (reviewed by Konturek, 1988), EGF and TGFα do not inhibit acid secretion stimulated by histamine when administered into the gastric lumen (Dembinski et al., 1982) in vivo.   Nor does EGF inhibit secretion when applied to the luminal surface of guinea-pig mucosal strips (Finke et al., 1985), whereas both EGF (Finke et al., 1985) and TGFα (Rhodes et al., 1986), are effective inhibitors when applied to the serosal side.   These results argue against an involvement of EGF present in the gastric lumen in the regulation of parietal cell secretory activity.

Inhibitory effects of EGF on histamine-stimulated acid secretion correlated with decreases in histamine-stimulated cyclic AMP content in parietal cells isolated from both rat (Hatt and Hanson, 1988) and rabbit (Lewis et al. 1990b).   The reversal of such effects by preincubation with pertussis toxin (Atwell and Hanson, 1988; Lewis et al., 1990b) indicates the presence in these cells of an EGF effector mechanism dependent upon activation of a guanine nucleotide binding protein sensitive to pertussis toxin.   Pertussis toxin pretreatment also prevented the inhibitory effect of TGFα on histamine-stimulated aminopyrine accumulation in rabbit parietal cells (Lewis et al., 1990a).   Genistein, a potent inhibitor of tyrosine kinase activity, reversed the effects of both EGF and TGFα in rabbit

Table 6.1

Effects of EGF and TGFα on secretagogue-stimulated acid secretory activity in gastric tissue isolated from a variety of mammalian species.

Species	Preparation	Secretagogue	Effect on secretory activity		Reference
			EGF	TGFα	
Rabbit	Isolated cells	Basal	No effect	No effect	Lewis et al., 1990a.
		Histamine	Inhibition *	Inhibition *	
		Forskolin	No effect	No effect	
		Carbachol	Inhibition	No effect	Lewis et al., 1990b.
		Histamine	Inhibition *	Inhibition *	Fras et al., 1990.
		Gastrin + IBMX	Inhibition	Inhibition	
		Carbachol + IBMX	Inhibition	Inhibition	
		Forskolin	Inhibition	Inhibition	
		Dibutyryl cyclic AMP	Inhibition	Inhibition	
Guinea-pig	Gastric mucosa	Histamine	Inhibition	Inhibition	Rhodes et al., 1986.

* Inhibitory effect fully or partially reversed by pertussis toxin.

Table 6.1 continued...

Species	Preparation	Secretagogue	Effect on secretory activity		Reference
			EGF	TGFα	
Rat	Isolated cells	GLP-1 (7-36 amide)	Inhibition *	Inhibition *	Schmidtler et al., 1991b.
		Histamine	Inhibition *	*	Atwell and Hanson, 1988.
		Histamine + IBMX	No effect		Shaw et al., 1987.
		Carbachol	No effect		
		Dibutyryl cyclic AMP	No effect		
Dog	Isolated cells	Histamine	Inhibition		Chen et al., 1984.
		Carbachol	Inhibition		
		Forskolin	Minimal effect		
		Gastrin	Minimal effect		
		Dibutyryl cyclic AMP	Minimal effect		
Human	in vivo	Pentagastrin	Inhibition		Konturek et al., 1989.
		Modified sham feeding	Inhibition		

* Inhibitory effect fully or partially reversed by pertussis toxin.

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parietal cells (Tsunoda et al., 1991) which suggests that EGF receptor tyrosine kinase activity may be involved in the signalling pathway.

EGF did not affect the initial intracellular Ca^{2+} transient, or the plateau intracellular $[Ca^{2+}]$ following stimulation by carbachol of rabbit parietal cells (Lewis et al., 1990b). These data are consistent with a site of action of EGF independent of the rise in intracellular $[Ca^{2+}]$. The mechanism by which EGF antagonizes carbachol-stimulated secretion however, is unclear.

6.1.5.2   Is a phosphatidylinositol-glycan involved in the action of EGF in parietal cells?

EGF, insulin, and insulin-like growth factor-1 have been shown to stimulate hydrolysis of a phosphatidylinositol glycan rather than phosphatidylinositol 4,5-bisphosphate in BC3H-1 myocytes via activation of a phosphatidylinositol glycan-specific phospholipase C (Farese et al. 1988, Figure 6.4). Similar results have been reported by other workers using insulin with the same (Saltiel et al. 1987; Romero et al. 1988) and different (Gaulton et al. 1988; Mato et al. 1987) cell types. One of the products of this reaction, an inositol glycan, is known to regulate the activities of several enzymes including adenylate cyclase and cyclic AMP phosphodiesterase (Low and Saltiel, 1988; Saltiel et al., 1988). The possibility of an inositol glycan acting as a second messenger for EGF in rat parietal cells was therefore investigated by determining whether EGF could increase the [^{3}H]-diacylglycerol content of suspensions of cells preloaded with [^{3}H]-myristic acid. Myristic acid is thought to be a

Figure 6.4
Phosphatidylinositol glycan-specific phospholipase C (PLC) catalysed hydrolysis of a membrane-bound phosphatidylinositol glycan.
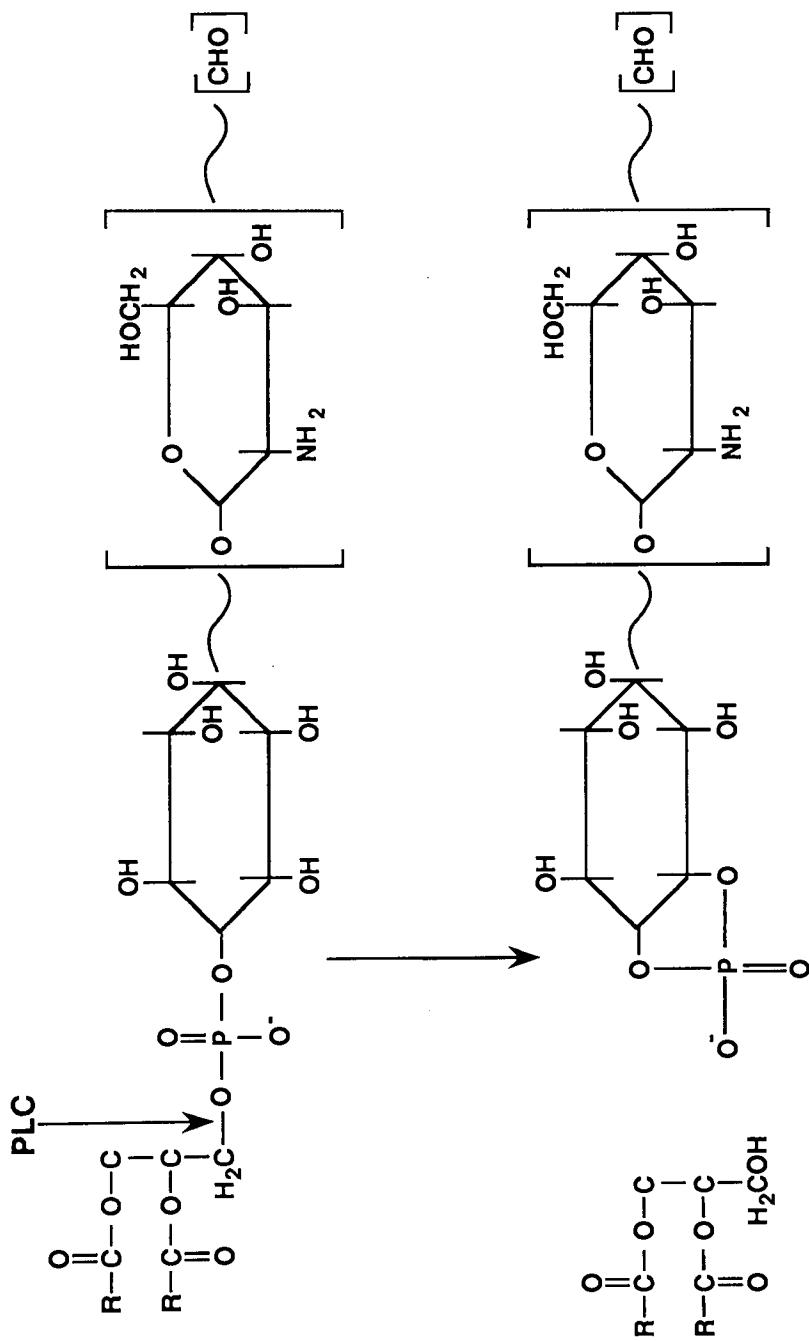
The glycophospholipid molecule consists of 1,2-diacylglycerol linked by a phosphodiester bond to inositol. Inositol is glycosidically linked to the C-1 position of glucosamine which in turn is linked to additional monosaccharides through either the C-4 or C-6 positions. Hydrolysis results in the generation of an inositol phosphate glycan and diacylglycerol.

major substituent on the glycerol backbone of the phosphatidylinositol glycan.

6.1.5.3 Does EGF activate a cyclic AMP phosphodiesterase in parietal cells?

EGF inhibits acid secretion by decreasing the cyclic AMP content of the parietal cell (Section 6.1.5.1), and this effect is blocked by the cyclic AMP phosphodiesterase inhibitor, IBMX (Hatt and Hanson, 1988). EGF could therefore decrease the cyclic AMP content by increasing cyclic AMP phosphodiesterase activity (Hanson and Hatt, 1989). Since activation of a low K_m, cyclic AMP phosphodiesterase by insulin has been demonstrated in rat fat cells (Degerman et al. 1990) and there are similarities between the receptors for EGF and insulin, it was decided to investigate whether EGF could increase cyclic AMP phosphodiesterase activity in parietal cells. The classification of cyclic AMP phosphodiesterases is shown in Table 6.2.

Table 6.2

Classification of cyclic nucleotide phosphodiesterases and inhibitors of the specific families.

Type	Description	Specific inhibitors
I	Ca^{2+}-calmodulin dependent	Phenothiazines 8-methoxymethyl-3-isobutyl-1-methylxanthine
II	Cyclic GMP-stimulated	None known
III	Cyclic GMP-inhibited	Cilostamide Milrinone
IV	Cyclic AMP-specific	Ro 20-1724 Rolipram
V	Cyclic GMP-specific	Dipyridamole Zaprinast (M&B 22948)

Common non-selective inhibitors include theophylline, papaverine and 3-isobutyl-1-methylxanthine.

6.2   METHODOLOGY.

6.2.1   Preparation of reagents.

Forskolin and A23187 were dissolved in DMSO and a small volume that was not more than 2 µl was added where appropriate to either incubation vials or assay tubes. DMSO was also added to control vials or tubes so that the final concentration did not exceed 0.133 % ($^v/_v$). EGF was stored at -20°C in aliquots at a concentration of 32 µM in saline. A fresh aliquot, or one which had been partly used and refrozen once only, was used. Snake venom (*Ophiophagus hannah*) was prepared as a 1 mg/ml stock in 40 mM Tris-HCl buffer containing 10 mM MgCl$_2$. This stock was stored at -20°C and used as described for EGF. [5,6,8,9,11,12,14,15-^{3}H]-Arachidonic acid (specific activity 200 Ci/mmol, radioactive concentration 1 mCi/ml), [9,10 (n)-^{3}H]-myristic acid (specific activity 53 Ci/mmol, radioactive concentration 1 mCi/ml) and [2,8-^{3}H]-cyclic adenosine 3′,5′-monophosphate (specific activity 41 Ci/mmol, radioactive concentration 1 mCi/ml) were all supplied dissolved in ethanol.

6.2.2   Loading of [^{3}H]-labelled arachidonic and myristic acids into cells.

The protocol used was identical for loading of both [^{3}H]-arachidonic acid and [^{3}H]-myristic acid into cells. 20 µl of the [^{3}H]-labelled fatty acid stock was evaporated to dryness under a stream of nitrogen and then sonicated on ice into 0.5 ml of medium B′ (5 x 5 s bursts with 5 s intervals at 20 W). The sonicate was added to

5.9 ml of a cell suspension containing > 80 % parietal cells that had been preincubated for 60 min. After gentle mixing, 2 x 100 μl aliquots were immediately removed and spun at 10,000 x g for 30 s to pellet the cells. 80 μl aliquots of supernatant were then transferred to scintillation vials for counting. Following a loading period of 60 min the above procedure was repeated. The counts indicated respectively the activity initially added to, and subsequently lost from the medium during the loading period. The cells were then washed twice with resuspension in medium B′ containing 1 % ($^W/_V$) fatty acid-free bovine serum albumin and were finally resuspended in 15 ml of the same medium.

6.2.3 Recovery of [^{3}H]-labelled lipid from cells.

Stimulation was carried out by exposing the cells to either EGF or A23187 for the indicated times. The experiments were terminated by the addition of 3.75 ml of lipid extraction medium (chloroform/methanol/concentrated HCl in the ratio 100 : 200 : 1 containing 0.005% ($^W/_V$) butylated hydroxytoluene as antioxidant) to 1 ml of cell suspension in the vial and vortexing. 30 μg each of monolein, diolein, triolein and either arachidonic or myristic acids were also added to the vials as carriers. The resulting mixture was left to stand on ice for 5 min at which time 1.25 ml of chloroform was added and the mixture again vortexed. Following a final addition of 1.25 ml of 2 M KCl, the mixture was again vortexed, and then centrifuged at 3000 x g for 5 min to separate the chloroform and aqueous phases. The lower chloroform phase was removed and stored while the upper aqueous phase was washed with another 2 ml of chloroform. After centrifugation at 3000 x g for 5 min, both

chloroform phases were then combined. 200 µl of the chloroform extract was transferred to a scintillation vial for counting. 1.5 ml aliquots were evaporated to dryness under nitrogen in a large volume dessicator to which a vacuum pump had been attached. The residue was finally taken up in 60 µl of chloroform/methanol (2 : 1 $^v/_v$).

6.2.3.1   Thin layer chromatography of neutral lipid.

50 µl of the lipid extract prepared as described above was added to a silica gel 60 thin layer chromatography (TLC) plate which had been preactivated by heating to 60°C for 60 min. The plate was then placed in a solvent tank containing hexane/diethyl ether/acetic acid in the ratio 65 : 35 : 4. When the solvent had risen almost to the top of the plate, the plate was removed, and after drying in air for 30 s, was transferred to a sealed tank containing iodine vapour. Iodine stained spots, identified by their co-migration with authentic standards were scraped directly into scintillation vials to which 10 ml of OptiPhase Hi-Safe II was then added for counting.

6.2.4   Determination of cyclic AMP phosphodiesterase activity.

6.2.4.1   Preparation of particulate and supernatant fractions of homogenate.

1.5 ml aliquots of a cell suspension containing > 80 % parietal cells, and resuspended at a concentration of 2.5×10^6 cells/ml, were incubated in the absence or presence of agents such as histamine and forskolin as described in Chapter 2 (General

Methodology). Following a 30 min incubation, 1 ml of the cell suspension was removed and spun at 10,000 x g for 30 s in a microfuge tube to pellet the cells. The supernatant was discarded and the surface of the pellet washed once in 200 µl of ice-cold homogenization buffer (pH 7.4) consisting of 20 mM Tris-HCl, 1 mM EDTA, 0.25 M sucrose, 2 mM benzamidine, 0.1 mM phenylmethylsulphonylfluoride, 1 µM leupeptin and 1 µM pepstatin. 200 µl of ice-cold homogenization buffer was added to the washed pellet and the suspension homogenized on ice using the microfuge tube as a mortar and with a moulded plastic pestle (30 strokes/min for 1 min at 1500 RPM) (Hearse, D. J., 1984). After centrifugation for 10 s at 10,000 x g, the supernatant was removed and stored on ice. A further 100 µl of homogenization buffer was added to the pellet and the homogenization and centrifugation procedures were repeated. The supernatants were combined (300 µl total) and stored on ice. Cell counts were performed before and after homogenization in order to determine the number of cells either broken or permeable to trypan blue. Particulate and supernatant fractions were prepared from the crude homogenate by centrifuging at 100,000 x g for 30 min at 4°C in an MSE Superspeed 50 centrifuge. The particulate fraction obtained was washed twice with, and then resuspended in 250 µl of ice-cold homogenization buffer and used fresh. As time did not allow the supernatant fraction to be used immediately, the supernatant was removed to a separate tube, frozen rapidly in liquid nitrogen, and stored at -80°C overnight.

Pilot experiments were carried out on both fresh and previously frozen supernatant samples in order to determine whether this freezing process had any effect on subsequently

assayed phosphodiesterase activity. No effect of the freezing process on samples previously incubated in either the presence or absence of EGF was found.

6.2.4.2   Determination of the protein content of homogenate fractions.

The protein content of the particulate and supernatant fractions were determined using the BIO-RAD protein assay kit which is based on the shift in absorbance maximum of Coomassie blue on binding to protein. Immunoglobulin G was used as standard. Particulate material was first solubilized by resuspending in NaOH (1 M) at 37°C prior to assay.

6.2.4.3   Assay of cyclic AMP phosphodiesterase activity.

The assay consisted of three steps. Firstly, [^{3}H]-labelled cyclic AMP was hydrolyzed to adenosine-5′-monophosphate by the phosphodiesterase reaction. Then the 5′-monophosphate was converted to adenosine by using the 5′-nucleotidase present in *Ophiophagus hannah* snake venom (Bauer and Schwabe, 1980). Finally, the uncharged adenosine was separated from the charged substrate by ion-exchange chromatography with the substrate binding to the ion-exchange resin.

The assay was carried out by adding 20 µl of either the particulate or supernatant fractions of homogenate (or preboiled homogenate for assay blanks) to 160 µl of assay buffer consisting of 40 mM Tris-HCl, 10 mM MgCl$_2$ and 8 mM 2-mercaptoethanol (pH 7.4)

and preincubating in a microfuge tube for 3 min at 30°C. 20 µl of assay buffer prewarmed to 30°C and containing 10 µM cyclic AMP (specific activity, 0.454 Ci/mmol, 10,000 dpm/µl) or for some experiments, 1 µM cyclic AMP (specific activity, 4.54 Ci/mmol, 10,000 dpm/µl) was then added to all tubes to initiate the reaction. The mixture was vortexed and the incubation allowed to proceed for 10 min. The tubes were then transferred to a boiling water bath for 2.5 min to terminate the reaction and then allowed to cool on ice. Following re-equilibration to 30°C, 50 µl of snake venom was added from a 1 mg/ml stock. After 10 min incubation at 30°C, the reaction was terminated by applying a 200 µl aliquot to a QAE A-25 Sephadex column (6 x 50 mm in Pasteur pipettes) previously equilibrated with 3 ml of 30 mM ammonium formate which had been adjusted to pH 6.0 using formic acid. The eluate was collected in scintillation vials. 2 ml of 30 mM ammonium formate, pH 6.0, was then added to the column and the eluate again collected in the same vial. 10 ml of OptiPhase Hi-Safe II was added to the eluate and the activity counted. Pilot experiments were also performed in which [3H]-substrate was removed by Dowex-1.

At the completion of each experiment the columns were regenerated by adding 2 ml of 0.2 M HCl and by washing with 3 ml of 30 mM ammonium formate (pH 6.0). Columns treated in this way, stored at room temperature without loss of binding activity for several weeks.

6.3   RESULTS AND DISCUSSION.

6.3.1   Effect of EGF on the secretory activity of isolated rat parietal cells.

The presence of a maximally effective concentration of EGF (200 nM) (Shaw et al., 1987) caused a significant inhibition of aminopyrine accumulation stimulated by histamine (0.5 mM) in both unenriched and enriched suspensions of parietal cells (Table 6.3) similar to that reported previously (Shaw et al., 1987; Hatt and Hanson, 1988). This inhibitory effect of EGF could not be ascribed to an effect on basal aminopyrine accumulation as this is unaffected by EGF (Shaw et al., 1987). Enhancement of the response to histamine by using IBMX was precluded as this compound blocks the action of EGF (Shaw et al., 1987).

6.3.2   Effects of EGF and A23187 on phospholipid metabolism in parietal cells.

All experiments were performed with an enriched fraction of cells containing greater than 80 % parietal cells.

6.3.2.1   Loading of ^{3}H into lipid and its recovery from a preparation enriched in parietal cells.

A preliminary experiment using cell-free medium indicated that the extraction procedure was successful in removing over 99 % of the [^{3}H]-arachidonic acid from the incubation medium into the organic phase of the extraction medium. Recovery of [^{3}H]-

Table 6.3

Effect of 200 nM EGF on aminopyrine accumulation stimulated by 0.5 mM histamine in unenriched and enriched suspensions of parietal cells.

Parietal cell preparation	Aminopyrine accumulation ratio		% Inhibition due to EGF
	0.5 mM Histamine	0.5 mM Histamine + 200 nM EGF	
Unenriched	6.8 ± 0.47	4.6 ± 0.20	32.4 ± 2.54 *
Enriched	7.4 ± 2.32	4.5 ± 1.64	41.9 ± 8.80 *

Results, from three unenriched and five enriched preparations, are presented as means ± SEM and are expressed as stimulation above basal aminopyrine accumulation which was 3.84 ± 0.55 and 3.92 ± 0.36 for unenriched and enriched suspensions of parietal cells respectively. * P < 0.005 for effect of 200 nM EGF analysed by paired t-test.

arachidonic acid and [^{3}H]-myristic acid from the TLC procedure was 94 % and 95 % respectively. 93 % and 97 % respectively of recovered [^{3}H]-arachidonic acid and [^{3}H]-myristic acid co-migrated with unlabelled arachidonic acid while approximately 1 % remained at the origin. The rest of the ^{3}H was distributed uniformly along the TLC plate. The fact that [^{3}H]-arachidonic acid and [^{3}H]-myristic acid co-migrated along the TLC plate meant that unlabelled arachidonic acid could be used as a marker to identify the position of [^{3}H]-myristic acid. This was necessary because the saturation of [^{3}H]-myristic acid prevented its staining by iodine.

The cell-associated label present after one hour of incubation with [^{3}H]-arachidonic acid (31 % of label initially added) was not substantially greater than that present after a 30 min incubation (25 % of label initially added, n = 1). Therefore as incubation for periods greater than one hour seemed unlikely to produce a much greater incorporation of ^{3}H, a one hour preincubation time was adopted for all further experiments.

The level of uptake of [^{3}H]-arachidonic acid by cells was approximately threefold that of [^{3}H]-myristic acid (see legend to Table 6.6). However, for experiments with both [^{3}H]-arachidonic acid (Table 6.4) and [^{3}H]-myristic acid (Table 6.5), the label extractable from the cells after a loading period of one hour was less than that which disappeared from the medium (P < 0.001 for experiments with [^{3}H]-arachidonic acid (n = 5) or [^{3}H]-myristic acid (n = 4), data analysed by paired t-test). This observation can be explained by the hydrophobic nature of these compounds resulting in their adherence to the container walls (Samples et al., 1989). The

Table 6.4

Loading of [³H]-arachidonic acid into parietal cells.

	cpm/ml	Data expressed as % of label present initially
Label present in medium at start of loading	$(1.6 \pm 0.16) \times 10^6$	
Label lost from medium during loading period	$(5.9 \pm 0.07) \times 10^5$	35.7
Label extractable from cells on completion of loading	$(2.3 \pm 0.16) \times 10^5$	13.9

6.4 ml of a cell suspension enriched in parietal cells was incubated at 37°C for 1 h in the presence of [³H]-arachidonic acid. The cell concentrations were $(3.8 \pm 0.65) \times 10^6$ cells/ml. The results are presented as means ± SEM of duplicate determinations from five different batches of cells. Label present in, and lost from the medium was determined by taking 100 µl aliquots at appropriate times and centrifuging at 10,000 x g for 1 min to sediment the cells.

Table 6.5

Loading of [^{3}H]-myristic acid into parietal cells.

	cpm/ml	Data expressed as % of label present initially
Label present in medium at start of loading	$(2.2 \pm 0.08) \times 10^6$	
Label lost from medium during loading period	$(2.3 \pm 0.27) \times 10^5$	10.4
Label extractable from cells on completion of loading	$(1.1 \pm 0.1) \times 10^5$	5.2

6.4 ml of a cell suspension containing > 80 % parietal cells was incubated at 37°C for 1 h in the presence of [^{3}H]-myristic acid. The cell concentrations were $(2.3 \pm 0.21) \times 10^6$ cells/ml. The results are presented as means ± SEM of duplicate determinations from four separate batches of cells.

use of either plasticware or siliconised glassware were previously shown to be equally ineffective in overcoming this problem (Samples et al., 1989).

6.3.2.2   Distribution of ^{3}H between different classes of lipid in parietal cells.

The incorporation of ^{3}H into various cellular lipids after a one hour preincubation in the presence of [^{3}H]-arachidonic acid or [^{3}H]-myristic acid is shown in Table 6.6.   In both cases, most of the ^{3}H recovered was in the phospholipid fraction.   The proportion incorporated into this fraction was higher for [^{3}H]-arachidonic acid than for [^{3}H]-myristic acid (P < 0.001 by paired t-test).   Arachidonic acid is thought to be incorporated predominantly into phospholipids of the plasma membrane and/or endoplasmic reticulum, and less into phospholipids of other organelles (Locher et al., 1989).   The proportionate incorporation of ^{3}H into diacylglycerol was higher from [^{3}H]-myristic acid than from [^{3}H]-arachidonic acid (P < 0.001 by paired t-test).

6.3.2.3   Effect of subsequent incubation on disposition of ^{3}H between lipid classes in cells after loading with [^{3}H]-arachidonic acid for 1 h.

The effect of subsequent incubation at 37°C on the ^{3}H content of the various cellular lipids present in a whole cell suspension after loading with [^{3}H]-arachidonic acid for 1 h and then washing is shown in Figure 6.5.   A rapid linear increase in arachidonic acid content occurred over the first 15 min of incubation, with the rate

Table 6.6

Distribution of ^3H between three classes of lipid in cells from a suspension containing > 80 % parietal cells which had been preincubated with [^3H]-arachidonic or [^3H]-myristic acid for 1h.

Lipid class:	Distribution (% of total)	
	Preincubation with arachidonic acid	Preincubation with myristic acid
Phospholipid	53.2 ± 2.42	36.6 ± 0.87
Diacylglycerol	0.3 ± 0.02	2.6 ± 0.14
Fatty acid	2.1 ± 0.13	4.4 ± 0.90

Results are presented as means ± SEM from five experiments for cells preincubated with [^3H]-arachidonic acid and from four experiments for cells preincubated with [^3H]-myristic acid. The cells were washed once and then resuspended in medium B´ (Appendix A.2.1) containing 1 % fatty acid-free bovine serum albumin prior to extraction. The total label extractable in the organic phase was $(1.6 ± 0.25) \times 10^5$ cpm/10^6 cells (preincubation with [^3H]-arachidonic acid) and $(5.2 ± 0.42) \times 10^4$ cpm/10^6 cells (preincubation with [^3H]-myristic acid).

Figure 6.5
Effect of incubation time on [^{3}H]-labelled lipids in a parietal cell enriched cell suspension after loading with [^{3}H] arachidonic acid.
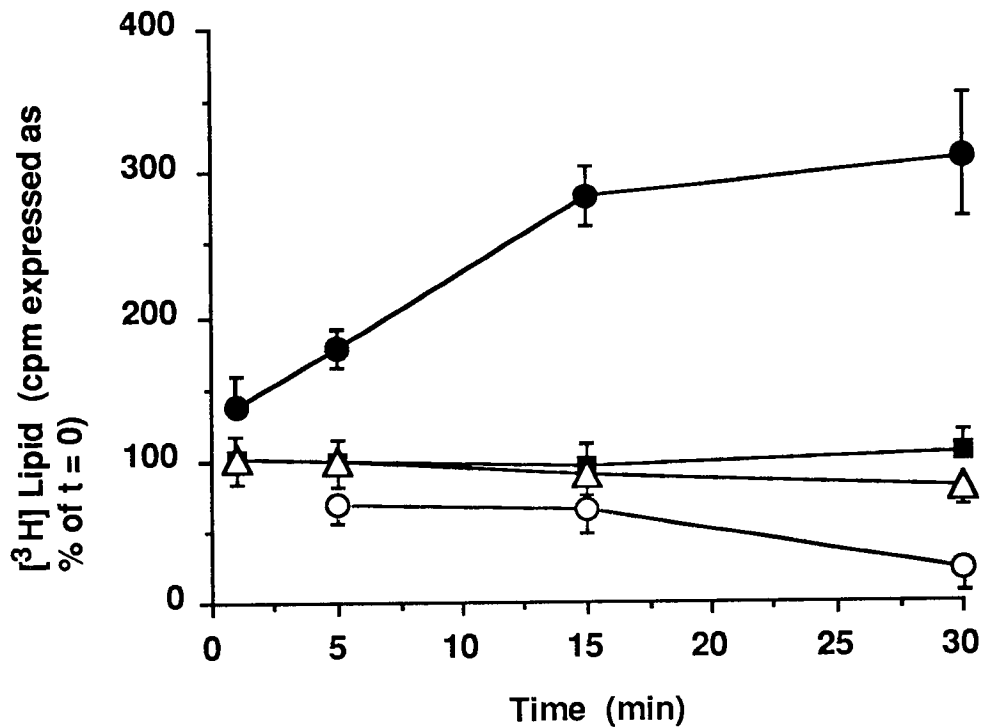
Results are expressed as % of [^{3}H] lipid present in the cell suspension at the start of incubation, and are presented as means ± SEM from 5-9 experiments (the figures in parentheses indicating the actual number of experiments). Phospholipid (■), diacylglycerol (△), arachidonic acid (●), triacylglycerol (O). At the start of incubation the tritium in the various lipid classes was (cpm/10^6 cells): Phospholipid, (6.7 ± 1.1) x 10^4 (9); diacylglycerol, 407 ± 78 (9); arachidonic acid, 2655 ± 360 (9); triacylglycerol, 8682 ± 3007 (5).

of increase declining after this time. The contents of [3H]-labelled phospholipid and diacylglycerol remained fairly constant over the 30 min period, while the content of [3H]-labelled triacylglycerol decreased. As expected, the level of [3H]-labelled total extractable lipid remained almost constant throughout the experiment (Fig. 6.7.d).

The increase in arachidonic acid content with time could reflect a release of this compound from triacylglycerol as a net loss of arachidonic acid from triacylglycerol in parietal cells was also found in a similar experiment by Locher et al. (1989), who suggested that triacylglycerols serve as a dynamic storage pool for exogenously administered fatty acids.

6.3.2.4 Effect of A23187 on arachidonic acid release from parietal cells.

Incubation of the cell suspension after loading with [3H]-arachidonic acid, with the calcium ionophore, A23187 (10 μM) produced significant increases of between 14.3 % (t = 15 min) and 34.5 % (t = 30 min) in the [3H]-arachidonic acid in the cell suspension by comparison with a control (P < 0.05, Fig. 6.6). These results suggest that activation of phospholipase A_2 by elevation of $[Ca^{2+}]_i$ is detectable in these cells, but the effect was quite small by comparison with other cell-types (Billah and Lapetina, 1982; Margolis et al., 1988b). One reason for this could be that basal release was elevated by mechanical manipulation or shear stresses imposed upon cells during incubation (Smith, 1989). Alternatively, the release could have been part of a re-equilibration process

Figure 6.6

Effect of the calcium ionophore A23187 (10 μM) on the content of [^{3}H]-arachidonic acid in a cell suspension containing > 80 % parietal cells, and previously loaded with [^{3}H]-arachidonic acid.
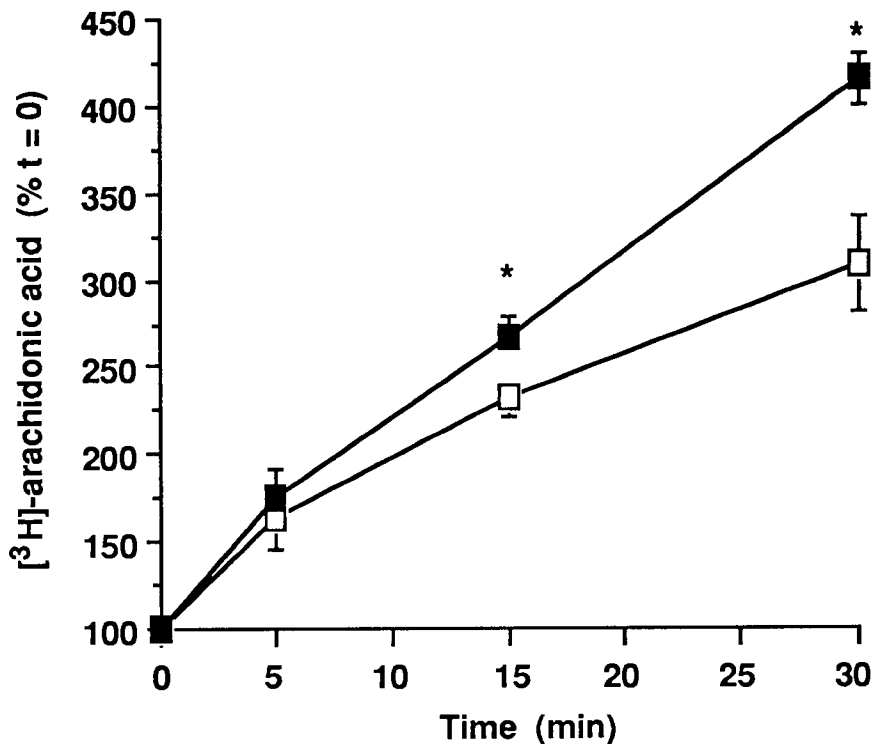
Results are presented as means ± SEM of a total of four determinations from two batches of cells and are expressed as % [^{3}H]-arachidonic acid present in the cell suspension at the start of incubation which was 2900 ± 245 cpm/10^6 cells. The cell concentration was 2 x 10^6 cells/ml in both experiments. (■) A23187 present, (□) A23187 absent. * P < 0.05 by paired t-test.

following removal of most of the free arachidonic acid by washing before resuspension and incubation. Finally it should be emphasized that the content of [^{3}H]-arachidonic acid in the whole suspension was measured so these results do not represent a wash-out of [^{3}H]-arachidonic acid from the cells.

6.3.2.5 <u>Lack of effect of EGF on [^{3}H]-labelled lipids in suspensions of cells preincubated with [^{3}H]-arachidonic acid for 1 h.</u>

No significant effect of 200 nM EGF on the level of labelled lipid classes was found in any instance (Fig. 6.7.a - 6.7.c). The lack of effect of EGF on the content of [^{3}H]-labelled diacylglycerol (Fig. 6.7.b) makes it unlikely that EGF stimulated prostaglandin production via activation of a phospholipase C. In a very similar experiment, carbachol increased [^{3}H]-diacylglycerol (Pfeiffer et al., 1989) which shows that activation of phospholipase C can be detected in this system.

The lack of effect of EGF on arachidonic acid production (Fig. 6.7.c) does not support an effect of EGF on phospholipase A$_2$ activity. This result was unexpected and is more difficult to explain. Chen et al. (1988) suggested that dog parietal, and other large cells produce little PGE$_2$ in response to EGF, and that it is the macrophages and endothelial cells that are mainly responsible for PGE$_2$ production. The cell preparation contained only 80 % parietal cells, and it is possible that the increased PGE$_2$ production measured in response to EGF (Hatt and Hanson, 1988) was derived from macrophages and/or endothelial cells present in the enriched parietal cell fraction. If a

Figure 6.7

Effect of incubation time on [^{3}H]-labelled lipids in a parietal cell enriched cell suspension preincubated in the presence of [^{3}H] arachidonic acid for 1 h and after washing, subsequently incubated in the presence (■) and absence (□) of EGF (200 nM).

(a) [^{3}H]-phospholipid

(b) [^{3}H]-diacylglycerol

(c) [^{3}H]-arachidonic acid

(d) [^{3}H]-total extractable lipid

[^{3}H]-lipid (cpm expressed as % t = 0)

Time (min)
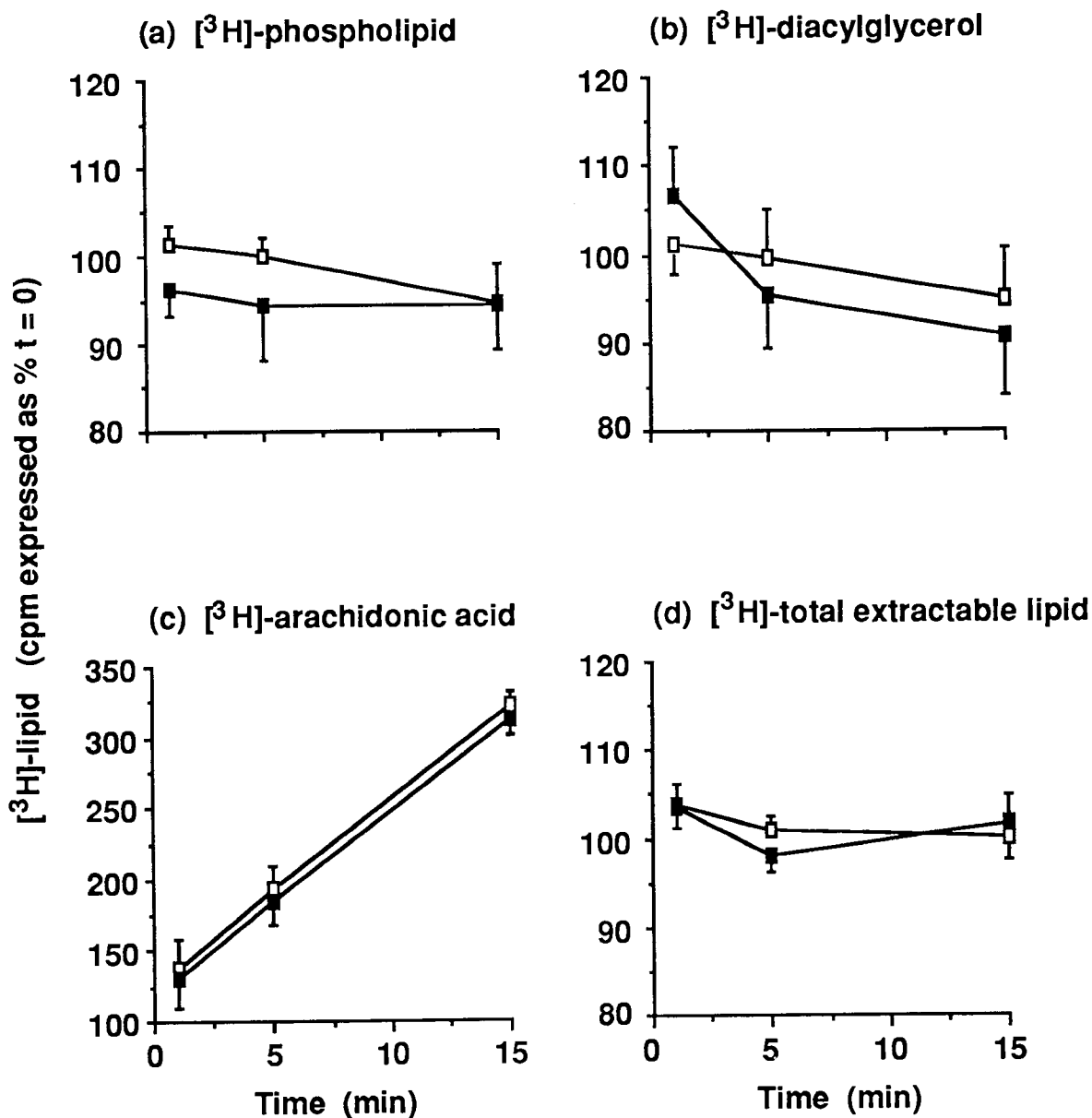
Results are expressed as % [^{3}H]-lipid present in the cell suspension at the start of incubation and are presented as means ± SEM from five separate batches of cells. At the start of incubation the [^{3}H] in the various lipid fractions was (cpm/10^6 cells): (a) phospholipid, (8.2 ± 1.4) x 10^4 ; (b) diacylglycerol, 522 ± 81; (c) arachidonic acid, 3126 ± 398; (d) total extractable lipid, (1.5 ± 0.25) x 10^5. No significant effect of EGF was found in any instance (data analysed by paired t-test).

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high proportion of the change in arachidonic acid content under basal conditions was a result of parietal cell metabolism then this may have masked an effect of EGF on arachidonic acid content in the small numbers of macrophages and endothelial cells present.

6.3.2.6 Lack of effect of EGF on [^{3}H]-labelled lipids in suspensions of cells preincubated with [^{3}H]-myristic acid for 1 h.

As found in experiments involving [^{3}H]-arachidonic acid, the cellular content of [^{3}H]-phospholipid and [^{3}H]-total extractable lipid was relatively unchanged during a 15 min incubation following loading of [^{3}H]-myristic acid (Fig. 6.8.a and 6.8.d). The amount of [^{3}H]-diacylglycerol decreased almost linearly to approximately 70 % of its original level (Fig. 6.8.b), and there was a substantial increase in the level of free [^{3}H]-myristic acid over the same period (Fig. 6.8.c). No significant effect of EGF on the level of labelled lipid present was found in any instance.

The relative increase in [^{3}H]-myristic acid production with time was similar to the effect observed under identical conditions using cells preincubated with [^{3}H]-arachidonic acid (Fig. 6.7.c), and may result from lack of equilibrium labelling of lipids with myristic acid. The lack of effect of EGF on the content of [^{3}H]-myristate labelled diacylglycerol does not provide evidence for the stimulation of a phosphatidylinositol glycan-specific phospholipase C by EGF, or the involvement of an inositol glycan as a second messenger for EGF in parietal cells. However, it should be noted that the extent of labelling of any phosphatidylinositol glycan during the 1 h

Figure 6.8
Effect of incubation time on [³H]-labelled lipids in a parietal cell enriched cell suspension preincubated in the presence of [³H] myristic acid for 1 h and after washing, subsequently incubated in the presence (■) and absence (□) of EGF (200 nM).
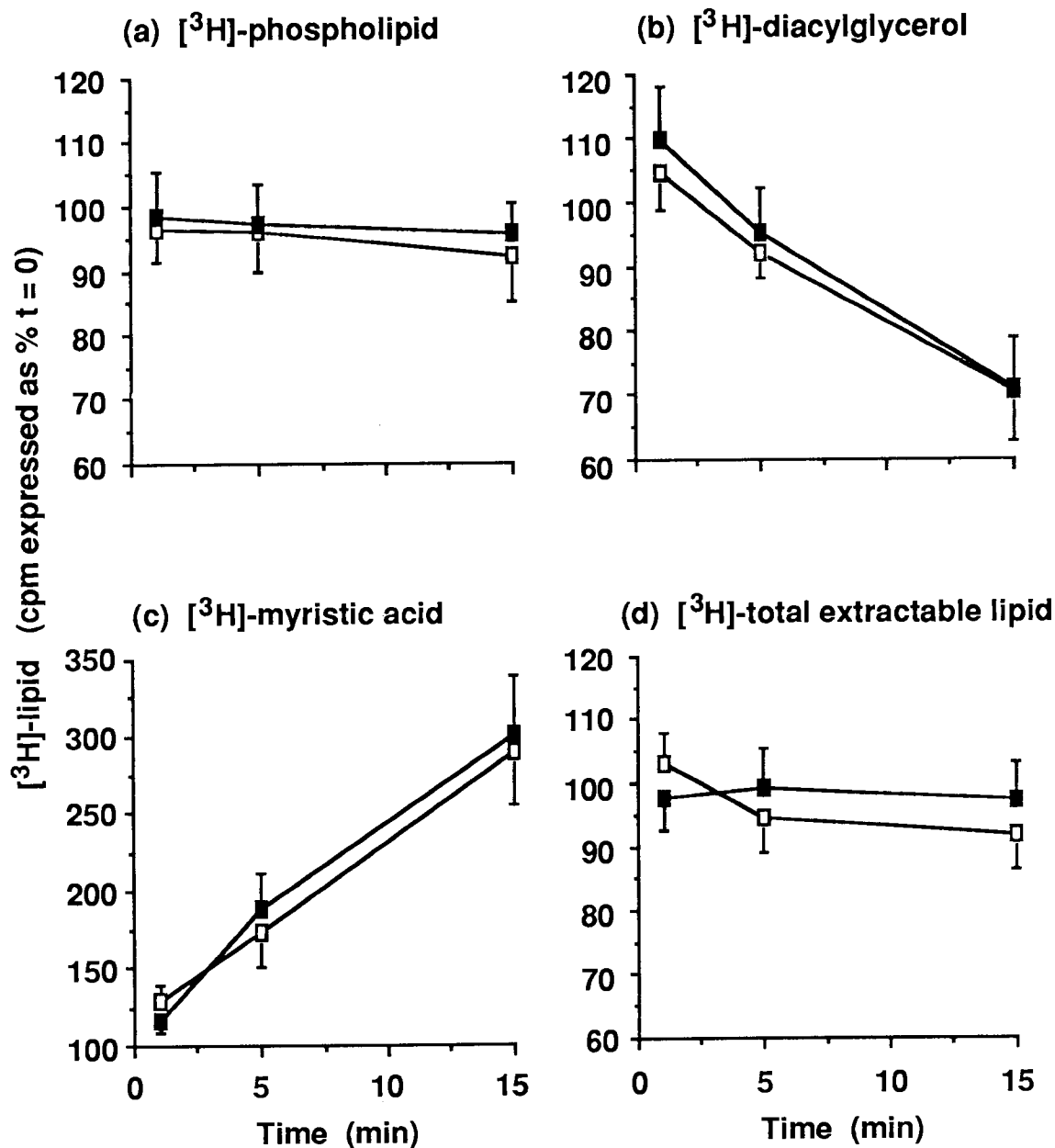
Results are expressed as % [³H]-lipid present in the cell suspension at the start of incubation and are presented as means ± SEM from four batches of cells. At the start of incubation the [³H] in the various lipid fractions was (cpm/10⁶cells): (a) phospholipid, (1.9 ± 0.19) x 10⁴; (b) diacylglycerol, 1352 ± 124; (c) myristic acid, 2315 ± 518; (d) total extractable lipid, (5.2 ± 0.42) x 10⁴. No significant effect of EGF was found in any instance (data analysed by paired t-test).

preincubation is unknown, and also, changes in labelled lipids may not necessarily accurately reflect changes in lipid mass.

6.3.3 Measurement of cyclic AMP phosphodiesterase activity in rat parietal cells.

6.3.3.1 A comparison of two methods for the isolation of products of cyclic AMP phosphodiesterase activity present in an homogenate obtained from a cell suspension enriched in parietal cells.

The two methods tested were a Dowex-1 batch procedure and an ion exchange column method using QAE-Sephadex (Table 6.7). [^{3}H]-Product obtained using Dowex-1 to remove labelled substrate was 40.75% of that using QAE-Sephadex. However if correction for the estimated binding of up to 47% [^{3}H]-adenosine to the Dowex-1 resin was made (Schwabe et al., 1972), then the product present using the Dowex-1 system was 87% of that with QAE-Sephadex. The reason for the continued discrepancy after correction of the Dowex-1 figure could be attributed to the formation, by additional enzymes present in the homogenate, of the adenosine catabolites, inosine, hypoxanthine and adenine which are adsorbed by the Dowex-1 but not by the QAE-Sephadex (Bauer & Schwabe, 1980).

The only advantage of the batch procedure over the column method was the convenience and rapidity of the technique. However, since QAE-Sephadex columns can be reused for up to six months without loss of sensitivity or increase in blank values (Bauer & Schwabe, 1980) the difference in convenience was not large. The

Table 6.7

Comparison of [³H]-products of cyclic AMP phosphodiesterase activity eluted from a QAE-Sephadex column with products not bound to Dowex-1 resin.

| | cpm | |
Sample	QAE-Sephadex	Dowex-1
None (blank)	1784 (1791, 1776)	1860 (1868, 1851)
Pre-boiled homogenate (inactive enzyme)	1095 (1074, 1116)	1440 (1506, 1374)
Homogenate	$6.98 \times 10^4 \pm 588$	$2.84 \times 10^4 \pm 358$

1 μM cyclic AMP (1.069×10^5 cpm) was incubated with 50 μl of homogenate plus 150 μl of assay buffer for 10 min before boiling. The mixture was allowed to cool and 50 μl of snake venom (50 μg) was then added, followed by a further 10 min incubation. The reaction mixture was then added either to the Sephadex, or to the Dowex. Results, from a single batch of cells are presented as either means $\pm$ SEM of four determinations, or as means of two determinations with the actual values shown in parentheses. The cell concentration prior to homogenization was 5×10^6 cells/ml.

Dowex-1 batch procedure was considered less suitable than the QAE-Sephadex column chromatographic method essentially because it gave lower sensitivity. The latter method was therefore used in all subsequent experiments.

6.3.3.2 A comparison of the effect of incubation time and dilution of the homogenate on phosphodiesterase activity.

In order to validate the phosphodiesterase assay procedure, it was necessary to show that: (i) doubling the amount of enzyme used resulted in a doubling of the amount of product formed; (ii) the amount of product formed was linearly related to time, with the curve extrapolating back to the origin. Using an homogenate dilution of 1:10, the product formed was 1.78, 2.02 and 2.01 times that obtained with a homogenate diluted 1:20 for incubation times of 5, 10 and 15 min respectively (Fig. 6.9). The two curves are linear between 5 and 15 min but if extrapolated back do not pass through the origin. Reasons for the position of the intercept could be as follows:

a) Transferring the reaction mixture to a boiling water bath did not immediately terminate the reaction as a certain amount of time is required for the temperature of the reaction mixture to be raised sufficiently to inhibit enzyme activity. This would effectively shift the curves to the left, and the intercept above 0.

Figure 6.9
Effect of incubation time on phosphodiesterase activity in dilutions of homogenate obtained from a cell suspension enriched in parietal cells.
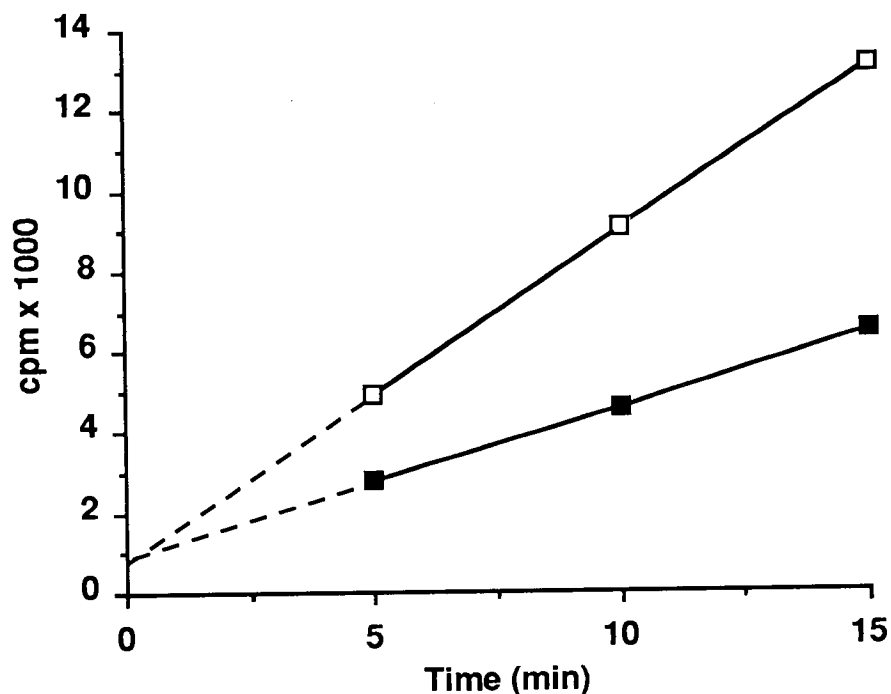
Results are expressed as the amount of [^{3}H]-material eluted from a QAE-Sephadex column (which is directly related to the phosphodiesterase activity in the original 20 μl or 10 μl sample of homogenate). The results have been corrected for the blank (a sample of preboiled homogenate) and are presented as means of duplicate determinations from a single batch of cells. (□) Homogenate diluted to 1 : 10, (■) homogenate diluted to 1 : 20 of original concentration. The cell concentration prior to homogenization was 2.6 x 10^6 cells/ml.

b)   The reaction proceeds faster at the beginning, only becoming linear after approximately five minutes. If this is the case the curves cannot be extrapolated backwards and the experiment should ideally be repeated with many more samples taken in the first five minutes of incubation to confirm that the curves really do pass through the origin. However, the essential point is that after 10 min the reaction was not slowing due to exhaustion of substrate and was directly proportional to the amount of enzyme added. A 10 min incubation period was used in subsequent experiments.

6.3.3.3   Effect of protein phosphatase inhibitors on cyclic AMP phosphodiesterase activity in particulate and supernatant fractions of homogenates.

To try to prevent reversal of potential phosphorylation of the phosphodiesterase enzyme induced by EGF, protein phosphatase inhibitors (fluoride and pyrophosphate) were added together to the homogenization buffer. However because phosphatase inhibitors might themselves inhibit low K_m cyclic AMP phosphodiesterase activity in the final assay, the effect of different concentrations of the inhibitors on phosphodiesterase activity was measured (Table 6.8). Enzyme activity in both particulate and supernatant fractions was inhibited by 50 mM-fluoride and 10 mM-pyrophosphate, with significant inhibition still apparent at 5 mM-fluoride and 1 mM-pyrophosphate. The lowest concentration of inhibitors tested (0.5 mM-fluoride and 0.1 mM-pyrophosphate) produced a negligible level of inhibition in both the particulate and supernatant fractions. The above results suggest that 5 mM-fluoride and 1 mM-pyrophosphate

Table 6.8

Effect of protein serine phosphatase inhibitors on cyclic AMP phosphodiesterase activity in particulate and supernatant fractions of homogenate obtained from a cell suspension containing > 80 % parietal cells.

| Homogenate fraction | Inhibitor concentrations (mM) | | Specific activity (pmoles/min/mg protein) | Inhibition (%) |
	Fluoride	Pyro-phosphate		
Particulate	0	0	38.0	
	0.5	0.1	38.7	-1.7
	5	1	33.7	11.3
	50	10	14.2	62.7
Supernatant	0	0	110.2	
	0.5	0.1	108.6	1.7
	5	1	102.6	7.2
	50	10	39.6	64.2

1 ml of cell suspension was centrifuged at 10,000 x g for 30 s. The pellet was washed then resuspended in 200 µl of homogenization buffer before homogenizing at 1500 RPM for 1 min. The homogenate was again centrifuged at 10,000 x g for 10 s, the supernatant removed and replaced by 100 µl of homogenization buffer, and the procedure repeated. The supernatants were combined (300 µl total) and centrifuged at 100,000 x g for 30 min to separate the particulate and supernatant fractions. The protein concentrations of the particulate and supernatant fractions were 3500 µg/ml and 2460 µg/ml respectively from a cell concentration of 3.76×10^6 cells/ml. The assay was carried out at a substrate (cyclic AMP) concentration of 1 µM. Results are presented as means of duplicate determinations and are from a single batch of cells.

could be added to the homogenization buffer without interference in the assay if the original homogenate is diluted by a factor of ten when added to the assay system.

6.3.3.4   Lack of effect of preincubation with EGF on cyclic AMP phosphodiesterase activity.

No effect of a near-maximally effective concentration of EGF (200 nM) (Shaw et al., 1987) on cyclic AMP phosphodiesterase activity measured following stimulation by 0.5 mM histamine in either particulate or supernatant fractions of homogenate obtained from an enriched preparation of parietal cells was found (Table 6.9). A possibility was that EGF might be affecting only one of the many isoenzymes of cyclic AMP phosphodiesterase.   Therefore inhibitors were used to reduce total phosphodiesterase activity in the hope that an effect of EGF might then become visible.   However a similar lack of effect of EGF on cyclic AMP phosphodiesterase activity in the presence of cyclic GMP or IBMX was also found (Table 6.10).   The idea that a cyclic AMP phosphodiesterase enzyme mediates the inhibitory action of EGF on histamine-stimulated gastric acid secretion is not confirmed by the results presented here.   The reasons for this could be as follows:

(i)   EGF does not decrease parietal cell cyclic AMP content by activation of a cyclic AMP phosphodiesterase.   The inhibitory effect of IBMX on the action of EGF on secretory activity is at a site other than a cyclic AMP phosphodiesterase.

(ii)   EGF activates a cyclic AMP phosphodiesterase but the effect is not stable to homogenization of the cells, either due to the loss of a

Table 6.9

Lack of effect of EGF (200 nM) on cyclic AMP phosphodiesterase activity in particulate and supernatant fractions of homogenate derived from a cell suspension containing > 80 % parietal cells.

Homogenate fraction	Specific activity (pmol/min/mg protein)		Inhibition by EGF (%)
	Histamine (0.5 mM)	Histamine (0.5mM) + EGF (200 nM)	
Particulate	26.0 ± 1.1	27.4 ± 1.5	-5.5 ± 4.4
Supernatant	63.7 ± 7.2	65.1 ± 7.7	-2.1 ± 3.3

Particulate and supernatant fractions were prepared as described in Section 6.2.4.1. The protein concentrations of the various fractions were, (μg/ml): Particulate fraction, 2326 ± 384 (EGF absent) and 2350 ± 374 (EGF present); Supernatant fraction, 1637 ± 291 (EGF absent) and 1627 ± 284 (EGF present). The cell concentration was (2.39 ± 0.48) x 10^6 cells/ml and the assay was carried out at a substrate (cyclic AMP) concentration of 1 μM. Results are presented as means ± SEM of duplicate determinations from six separate batches of cells.

Table 6.10

Lack of effect of EGF (200 nM) in the presence of either IBMX or cyclic GMP on cyclic AMP phosphodiesterase activity in the particulate fraction of homogenate derived from a cell suspension containing > 80 % parietal cells.

Inhibitor present	Specific activity (pmol/min/mg protein)		Inhibition by EGF (%)
	Histamine (0.5 mM)	Histamine (0.5mM) + EGF (200 nM)	
None	25.2 ± 1.26	25.1 ± 0.95	0.4
IBMX (0.1 mM)	6.7 ± 0.44	6.8 ± 0.64	-1.6
Cyclic GMP (10 μM)	13.8 ± 0.25	13.9 ± 0.57	-1.5

The protein concentration in the absence and presence of EGF respectively was 3485 and 3400 μg/ml of homogenate from a cell concentration of 3.87 x 10^6 cells/ml. The assay was carried out at a substrate (cyclic AMP) concentration of 1 μM. Results are presented as means ± SEM of a total of four determinations from two batches of cells.

mediator molecule, or because of reversal of protein phosphorylation/dephosphorylation events. The above experiments were conducted before the specific protein phosphatase inhibitor, okadaic acid, became available and should perhaps be repeated with it present.

6.4 SUMMARY.

1.   200 nM EGF produced a significant inhibition of histamine-stimulated aminopyrine accumulation in both unenriched and enriched preparations of isolated rat parietal cells.

2. Ca^{2+}-activated arachidonic acid release, probably due to phospholipase A_2 activity is detectable in parietal cells, but no effect of 200 nM EGF on arachidonic acid release from cells prelabelled with [^{3}H] arachidonic acid could be found.

3.   No effect of 200 nM EGF on the release of [^{3}H] diacylglycerol from cells prelabelled with [^{3}H] arachidonic acid or [^{3}H] myristic acid was detectable.

4.   No effects of preincubation with 200 nM EGF on low K_m cyclic AMP phosphodiesterase activity in particulate and soluble fractions of homogenates of parietal cells were detectable.

CHAPTER 7

GENERAL DISCUSSION.

This chapter aims to provide an overview of the data presented in this thesis, with some suggestions for future work.

7.1 PROTEIN KINASE C.

Much of the evidence presented in this thesis suggesting an involvement of protein kinase C in signal transduction pathways is based upon the pharmacological activation of this enzyme by phorbol esters such as TPA. It is clear however, that this ligand does not always elicit events identical to those produced by the physiological activation of protein kinase C (Lindeman and Chase, 1992; Kopp et al., 1990) and thus a degree of caution is necessary in the interpretation of results obtained solely by using TPA. Whether the differences reflect alterations in the spectrum of isoforms of protein kinase C which become activated, or the period of time over which activation is sustained remains to be established (Houslay, 1991b).

The inhibitory and stimulatory effects of protein kinase C on acid secretory activity and the possible sites of action of this enzyme in the parietal cell are discussed below.

7.1.1 Interaction of protein kinase C with the cyclic AMP pathway in parietal cells.

In isolated parietal cells, cyclic AMP production is stimulated following agonist binding to histamine H_2 (Hanson and Hatt, 1989), TGLP-1 (Schepp et al., 1992) or β_2-adrenergic (Miyazawa et al.,1992) receptors. Schepp et al. (1992), suggested that in rat

parietal cells, the secretory responses to histamine and TGLP-1 are mediated by the same signalling pathway and, as shown in this thesis, the actions of both agents are inhibited by activation of protein kinase C. Inhibition by protein kinase C of β_2-adrenergic receptor-mediated stimulation of cyclic AMP production has not been shown in parietal cells, but is likely given results with other cell types (Pitcher et al., 1992; Toews et al., 1987; Bouvier et al., 1987).

The results obtained with the HGT-1 human gastric cancer cell line suggest that the inhibitory actions of TPA on the cyclic AMP pathway are mediated through activation of protein kinase C α (Sections 4.3.3, 4.3.4), possibly via phosphorylation of the histamine H_2 receptor (Section 3.3.7.3) (Fig. 7.1). Thus, there was a reduction in the ability of the H_2 receptor to couple to G_s (Section 3.4) and a conformational change in the receptor binding site, which reduced the affinity of the receptor for an antagonist. However, the possibility of a direct effect of protein kinase C on the functional status of G_s in parietal cells cannot be discounted (Pyne et al., 1992; Beil et al., 1991). Further insight into the involvement of protein kinase C in the above pathway could be gained by investigating the effects of an anti-protein kinase C α antibody on TPA-induced protein phosphorylation in an HGT-1 cell particulate fraction, and by using [^{125}I]-iodoaminopotentidine to probe for changes in histamine H_2 receptor binding activity (Ruat et al., 1990).

7.1.2   Interaction of protein kinase C with pathways activated by carbachol and gastrin in parietal cells.

The actions of both carbachol and gastrin on acid secretion by parietal cells involve the production of diacylglycerol and the consequent activation of protein kinase C (reviewed by Hanson and Hatt, 1989).   In this work, the inhibition by Ro 31-8220 of the stimulatory effect of carbachol (Section 5.3.5) implies that, at least in rat parietal cells, protein kinase C acts as a positive mediator of muscarinic cholinergic receptor-stimulated secretory activity (Fig. 7.1).   The similarity between the pathways activated by carbachol and gastrin (Helander and Keeling, 1993; Wilkes et al., 1991) suggests that protein kinase C may also mediate the stimulatory action of gastrin.   Whether the inhibitory and stimulatory actions of protein kinase C on the histamine- and carbachol-stimulated pathways respectively are mediated through activation of the same isoform is a topic worthy of further investigation.

7.1.3   The physiological role of protein kinase C in modulation of parietal cell secretory activity.

The inhibitory actions of protein kinase C on the cyclic AMP pathway discussed above could represent a physiological mechanism whereby protein kinase C, produced as a result of muscarinic cholinergic receptor activation, could negatively modulate secretory activity induced by receptors which activate adenylate cyclase.   The cell might require such a pathway to prevent the overreaction of the acid-secreting parietal cell under the simultaneous influence of

Figure 7.1
A speculative scheme illustrating the potential sites at which protein kinase C may act to regulate acid secretion in the parietal cell.
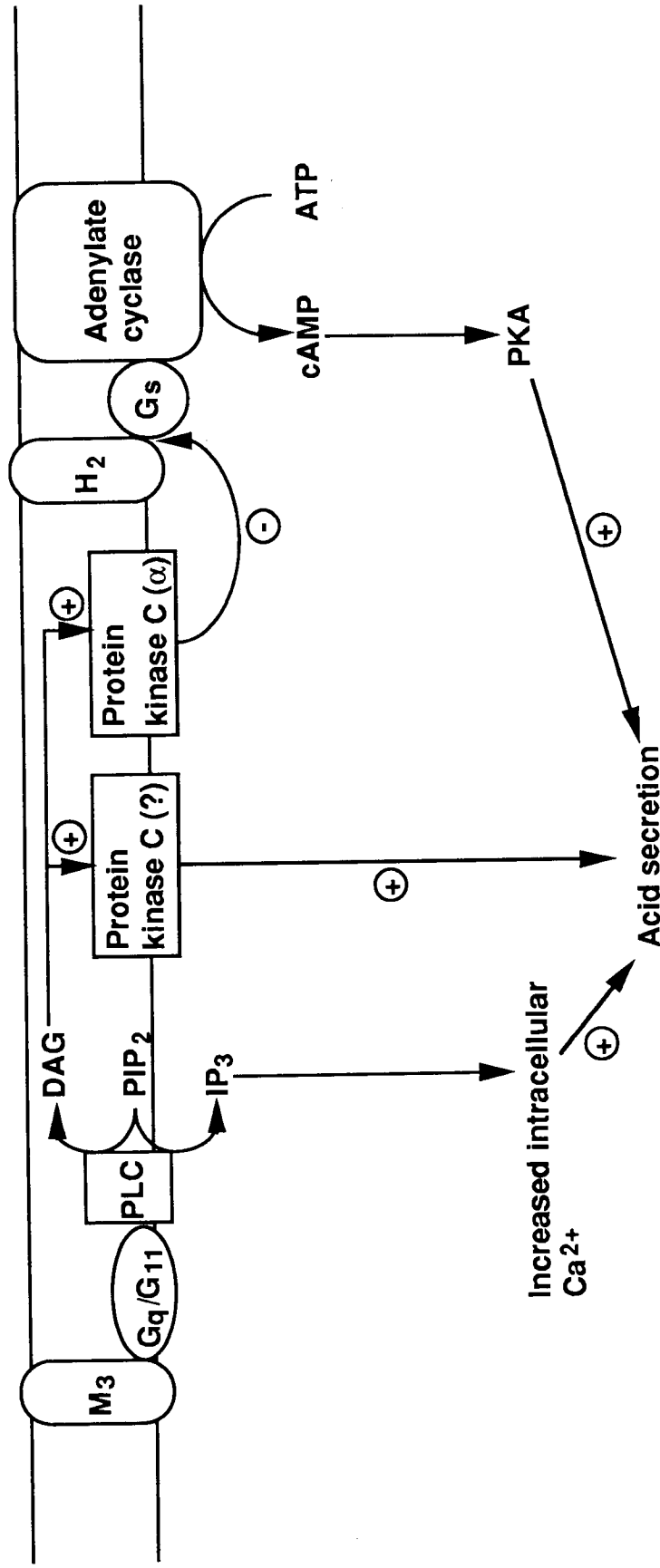
Key: + = stimulation; - = inhibition; M3, muscarinic cholinergic M3 receptor; H2, histamine H2 receptor; PLC, phospholipase C; Gq, G11, Gs, stimulatory G-proteins; DAG, diacylglycerol; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; cAMP, cyclic AMP; PKA, cyclic AMP-dependent protein kinase.

several secretagogues (Puurunen et al., 1987). In this respect, it is pertinent that elevation of intracellular cyclic AMP resulted in an attenuation of the carbachol-induced formation of inositol phospholipids (Puurunen et al., 1987), which represents another antagonistic interaction between the two secretory pathways. One problem is that in some species, such as dog (Soll, 1982), there is a clear synergism between effects of histamine and carbachol on secretory activity. However, TPA does not block histamine-stimulated aminopyrine accumulation in the dog (Chiba et al., 1989). Work in this thesis suggests that Ro 31-8220 should provide a useful tool with which to investigate interactions between carbachol- and histamine-stimulated secretory activity that involve protein kinase C.

An alternative possibility is that protein kinase C mediates the inhibitory action of an extracellular ligand. EGF, PGE$_2$ or somatostatin are unlikely to be such agents since the spectrum of inhibition by these compounds against a variety of secretagogues is different from that of TPA (Hanson and Hatt, 1989). A possible role for protein kinase C in mediating the inhibitory effects of substance P has been suggested (Hanson and Hatt, 1989) and it would be of interest to investigate the effect in parietal cells of Ro 31-8220 on the inhibitory action of substance P on aminopyrine accumulation stimulated by agents which activate the cyclic AMP pathway.

7.2   EPIDERMAL GROWTH FACTOR (EGF).

The present work did not establish the mechanisms by which EGF inhibits gastric acid secretion. Recent work on intracellular mediators of the action of EGF has shown that receptor autophosphorylation results in the binding and sometimes phosphorylation of cytosolic proteins with SH2 (*Src* homology 2) domains (reviewed by Mayer and Baltimore, 1993). If the EGF receptor signals in this manner in parietal cells then a protein with an SH2 domain must couple either directly or indirectly to a pertussis toxin-sensitive protein which in turn must affect the activity of either adenylate cyclase or, perhaps less likely given the results of this thesis, cyclic AMP phosphodiesterase.

PUBLICATIONS RESULTING FROM THIS WORK.

Full papers:

McKenna, P., Williams, J. M. and Hanson, P. J. (1993). Protein kinase C inhibits cyclic adenosine monophosphate generation by histamine and truncated glucagon-like peptide 1 in the human gastric cancer cell line HGT-1. Gut, **34**, 953-957.

McKenna, P. and Hanson, P. J. (1993). Inhibition by Ro 31-8220 of acid secretory activity induced by carbachol indicates a stimulatory role for protein kinase C in the action of muscarinic agonists on isolated rat parietal cells. Biochem. Pharmacol. (in press).

Communications:

McKenna, P. and Hanson, P. J. (1991). Lack of effect of epidermal growth factor on radiolabelled diacylglycerol in rat parietal cells. Biochem. Soc. Trans. **19**, 107S.

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APPENDICES

A.1   Source of reagents.

Reagent.   **Supplier.**

A.   General chemicals.

Reagent	Supplier
Adenosine 5′-triphosphate	Sigma
Ammonium formate	Sigma
Arachidonic acid	Sigma
Aprotinin	Sigma
Bacitracin	Sigma
Benzamidine	Sigma
Bovine serum albumin (BSA), fraction V	ICN Biomedical
Bovine serum albumin (fatty acid-free)	ICN Biomedical
Butylated hydroxytoluene	Sigma
Calcium chloride	BDH
Copper sulphate	BDH
Creatine kinase	Boehringer Mannheim
Creatine phosphate	Boehringer Mannheim
Cyclic AMP	Sigma
Dextran (M. Mass 40,000 Da)	Sigma
1,2-Di(2-aminoethoxy)ethane-N, N, N′,N′-tetra-acetic acid (EGTA)	BDH
Dimethylsulphoxide (DMSO)	Sigma
Diolein	Sigma
Di-potassium hydrogen orthophosphate	BDH
Di-sodium hydrogen orthophosphate	BDH
Dithiothreitol	Sigma
Dowex-1	Sigma
Eagle's minimum essential medium (powdered)	Sigma
Ethylenediaminetetra-acetic acid (EDTA)	Sigma
Folin and Ciocalteu's phenol reagent	Sigma
Fura-2 acetoxymethyl ester (Fura-2AM)	Sigma
Gentamicin	Sigma
Glycerol	BDH
D-glucose	BDH
L-glutamine	Sigma
Guanosine 5′-triphosphate	Sigma

N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES)	Sigma
Immunoglobulin G (bovine)	Sigma
Insulin	Sigma
Iodine	BDH
L-isoleucine	Sigma
Leupeptin	Sigma
Magnesium chloride	BDH
Magnesium sulphate	BDH
2-Mercaptoethanol	BDH
Monoolein	Sigma
Myristic acid	Sigma
Pepstatin	Sigma
Percoll	Sigma
Phenylmethylsulphonylfluoride	Sigma
Phosphatidylserine	Sigma
Potassium chloride	BDH
Potassium dihydrogen orthophosphate	BDH
QAE-Sephadex (A-25)	Sigma
Sagatal	May and Baker
Sodium chloride	BDH
Sodium dihydrogen orthophosphate	BDH
Sodium dodecylsulphate	BDH
Sodium fluoride	Sigma
Sodium hydrogen carbonate	BDH
Sodium hydroxide	BDH
Sodium pyrophosphate	BDH
Sodium tartrate	Sigma
Sodium thiocyanate	Sigma
Sucrose	Sigma
Triolein	Sigma
Tris (hydroxymethyl) methylamine	BDH
Triton-X-100	Sigma
Trypan blue	BDH
Trypsin inhibitor, lyophilized from soybean	Sigma

B.   Cell culture reagents.

Dulbecco's Modified Eagle's Medium	GIBCO
Foetal calf serum	GIBCO
Fungizone	GIBCO
L-Glutamine	GIBCO
Penicillin/streptomycin	GIBCO

C.   Enzymes and antibodies.

Antibody to protein kinase C α	GIBCO
Peptide against which antibody to protein kinase C α was raised	GIBCO
Antibody to protein kinase C ϵ	GIBCO
Peptide against which antibody to protein kinase C ϵ was raised	GIBCO
Pronase (70,000 PUK units/g)	BDH
Cholera toxin	Sigma
Pertussis toxin	Portland Down Research
Snake venom (*Ophiophagus hannah*)	Sigma

D.   Radiochemicals and scintillation counting.

[5, 6, 8, 9, 11, 12, 14, 15-^{3}H]-Arachidonic acid	Amersham International
Aminopyrine, dimethylamine-[^{14}C]	Amersham International
[2, 8-^{3}H]-Cyclic adenosine 3′, 5′-monophosphate	Amersham International
Cyclic AMP [^{125}I] radioimmunoassay kit (RIANEN™)	Du Pont
[9, 10 (n)-^{3}H]-Myristic acid	Amersham International
Optiphase Hi-Safe II	LKB Scintillation Products
[1, 2-^{3}H]-polyethylene glycol (PEG)	Amersham International
[20-^{3}H(N)]-phorbol 12, 13-dibutyrate	Du Pont
[Methyl-^{3}H]-Tiotidine	Du Pont

E   Secretagogues and agents.

A23187	Sigma

Atropine	Sigma
Carbachol	Sigma
Cimetidine	Sigma
Dibutyryl cyclic AMP, sodium salt	Sigma
Epidermal growth factor	Sigma
Forskolin	Sigma
Gastric inhibitory polypeptide (GIP)	Sigma
Histamine dihydrochloride	Sigma
3-Isobutyl-1-methylxanthine (IBMX)	Sigma
Ionomycin	Sigma
1-Oleoyl-2-acetylglycerol (OAG)	Sigma
Phorbol 12, 13-dibutyrate	Sigma
4α-phorbol 12-myristate 13-acetate	Scientific Marketing Associates
Pyrilamine	Sigma
Ro 31-8220	Roche Products
Staurosporine	Sigma
12-O-tetradecanoylphorbol 13-acetate (TPA)	Sigma
Tiotidine	ICI Pharmaceuticals
Truncated glucagon-like peptide-1 (7-36 amide)	Sigma

A.2   Preparation of media.

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

The powdered medium obtained from Sigma was dissolved at room temperature in 1000 ml of double-distilled water by stirring. The solution then contained the following components: L-arginine (0.7 mM), L-cystine (0.23 mM), L-glutamine (2 mM), L-histidine (0.27 mM), L-isoleucine (0.4 mM), L-leucine (0.4 mM), L-lysine (0.5 mM), L-methionine (0.09 mM), L-phenylalanine (0.2 mM), L-threonine (0.4 mM), L-tryptophan (0.05 mM), L-tyrosine (0.25 mM), L-valine (0.4 mM), choline chloride (7 μM), folic acid (3 μM), myo-inositol (0.01 mM), niacinamide (8 μM), D-pantothenic acid (2.1 μM), pyridoxal HCl (4.86 μM), riboflavin (0.3 μM), thiamine HCl (3 μM), $CaCl_2$ (1.8 mM), KCl (5.4 mM), $MgSO_4$ (0.8 mM), NaCl (116.4 mM), NaH_2PO_4 (1 mM), D-glucose (5.6 mM), phenol red (0.001 % $^W/_V$).

$NaHCO_3$ (25 mM) and HEPES (20 mM) were further added to the above. The medium was then covered and gassed with 95 % CO_2/5 % O_2 for 30 min with gentle stirring prior to addition of the compounds specified in Table 2.1.

A.2.2   Preparation of Ca^{2+}-, Mg^{2+}-free phosphate buffered saline.

A single tablet (Oxoid Ltd) was dissolved at room temperature in 100 ml of double-distilled water by stirring.   The solution (pH 7.3) then contained the following components: KCl (0.2 g/l), NaCl (8.0 g/l), KH_2PO_4 (anhydrous) (0.2 g/l), Na_2HPO_4 (anhydrous) (1.15 g/l).

A.2.3 Preparation of incubation buffer used during assay of adenylate cyclase activity.

The incubation buffer used during the assay of adenylate cyclase activity was prepared by first of all adding $MgCl_2$ (5.32 mM), IBMX (0.21 mM), Bovine serum albumin (1.06 mg/ml) and EGTA (1.06 mM) to 100 ml of a stock solution of Tris HCl (25 mM). The reagents were dissolved by stirring and then, to a 10 ml aliquot of this solution, ATP (1.06 mM), creatine phosphate (21.27 mM), bacitracin (212.8 μg/ml) and GTP (212.8 μM) were added. Creatine kinase (37.2 U/ml) was finally added to a 5 ml aliquot of this stock and the pH then adjusted to 7.5.

A.3   Liquid scintillation counting.

Liquid scintillation counting was used to determine the radioactivity of samples obtained following incubation of cells with either [^{14}C]-aminopyrine and [^{3}H]-polyethylene glycol, or [^{3}H]-labelled fatty acids. The basis of this technique is that radioactive emissions cause excitation of certain compounds or fluors, which then emit light (fluoresce) that can be detected by a photomultiplier tube. The light energy is subsequently converted into an electrical pulse, the magnitude of which is proportional to the original radioactive event.

Samples were counted using a TRI-CARB 2600 scintillation counter (Packard) which contains both low and high energy counting channels that allow the simultaneous determination of ^{14}C- and ^{3}H-counts. The upper and lower limits of these channels are set so that the counting efficiency of ^{3}H, the lower energy nuclide, in the higher energy channel is zero, and the counting efficiency of ^{14}C in the lower energy channel is minimised.

The efficiency of counting was determined using the external standards channel ratio (ESR) method. Thus, a quench correction curve relating counting efficiency and ESR was produced by counting a series of standard vials, each containing known amounts of both ^{3}H and ^{14}C, but with varying amounts of chloroform present as a quenching agent. The ESR for each sample counted is determined and the counts then calculated from the quench correction curve.

The activity (dpm) of the higher energy radionuclide, ^{14}C, can be calculated from the sample cpm and the counting efficiency of ^{14}C, using the equation:

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

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

The activity (dpm) of the lower energy nuclide, ^3H, can be determined from the sample cpm using the equation:

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

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

Counting of [^{14}C]-aminopyrine and [^3H]-PEG in 0.5 ml of NaOH (1 M) and 10 ml of OptiPhase Hi-Safe II resulted in the following efficiencies:

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

Reagents were reconstituted as directed in the cyclic AMP radioimmunoassay kit and were stored at a temperature of 2 - 8°C.

1.   Sodium acetate buffer: 25 ml of concentrated buffer was made up to 500 ml with double-distilled water.   The final solution (pH 6.2) contained sodium acetate (50 mM), sodium azide (0.1 %),   EDTA (0.03 %) and mannitol (0.025 %).

2.   Cyclic AMP standard: The lyophilized standard was reconstituted in exactly 2.0 ml of double-distilled water.   The final cyclic AMP concentration was 5000 pmol/ml in sodium acetate buffer, sodium azide (0.1 %) and mannitol (0.025 %).

3.   Cyclic AMP antiserum complex: The lyophilized pre-reacted primary (rabbit) and secondary (sheep) antibody was reconstituted in 21 ml of double-distilled water.   The resulting solution contained the pre-reacted antibody complex, thimerosal (0.005 %) and mannitol (0.025 %) in sodium phosphate buffer.

4.   Cyclic AMP [^{125}I]-tracer (succinyl cyclic AMP tyrosine methyl ester [^{125}I]):   The concentrated tracer in 1 ml of a 1 : 1   propanol : water solution was mixed with 5 ml of double-distilled water.   The solution normally contained 1.85 μCi on the calibration date.

5.   Cyclic AMP carrier serum: The lyophilized carrier serum was reconstituted with 6.0 ml of double-distilled water generating a solution containing carrier serum, sodium azide (0.1 %) and a stabilizer in sodium acetate buffer.

6.   Acetic anhydride and triethylamine: The separate reagents were allowed to equilibrate to room temperature before being added together in the ratio, acetic anhydride : triethylamine (1 : 2 $^V/_v$) to form the acetylation reagent.   This reagent was always made fresh just prior to use.

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

Figure A.4.1
Standard curve for the radioimmunoassay of [^{125}I]-cyclic AMP.
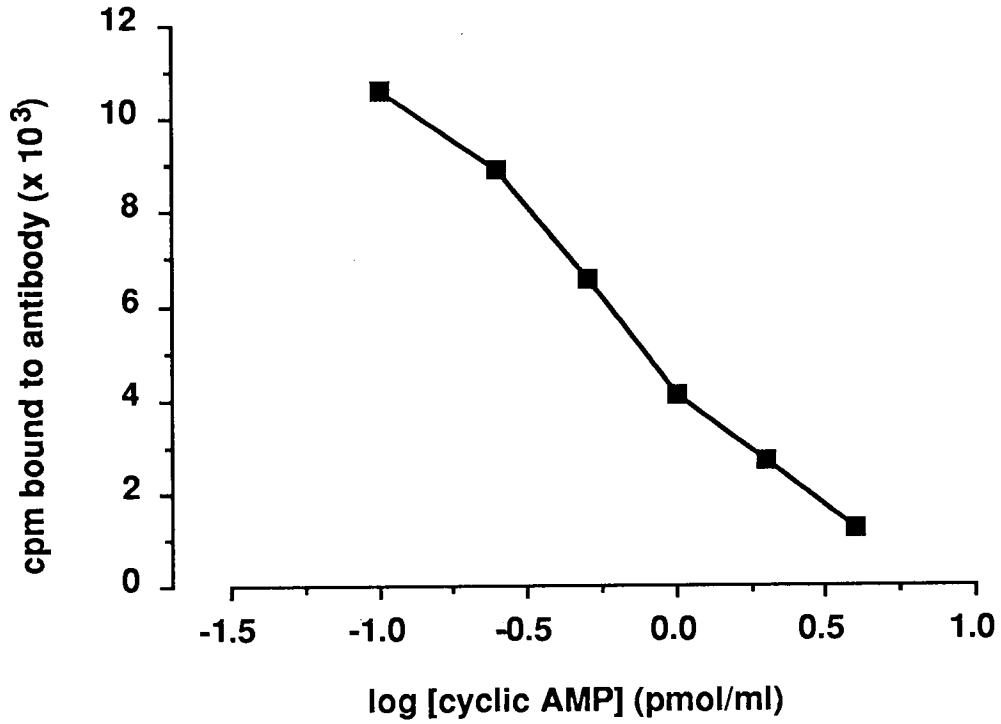
Values are from one experiment representative of ten. The total counts added to the assay tubes was 24147 cpm. The values depicted are means of duplicates and are corrected for a blank which was 842 cpm. The cyclic AMP content of the samples was calculated using a standard curve generated on the same day that the samples were assayed.

A.5   Statistical analysis.

Statistical tests used to analyse the data presented in this work are outlined below and are illustrated using specific examples from the text.

A.5.1   Analysis of variance (ANOVAR).

Data from Table 3.10 were tested using a two-way analysis of variance with a randomised block experimental design. The test examines the effects of preincubation time in the presence of TPA and of 10 min preincubation with OAG on the stimulation of the cyclic AMP content of a suspension of HGT-1 cells above basal by histamine (0.5 mM) and IBMX (0.1 mM).

DATA

Experiment No.	Control	TPA (100 nM)			OAG (100 μM)
		0 min	10 min	30 min	10 min
1	10.44	9.33	5.45	6.23	6.38
2	7.01	8.05	3.60	4.32	2.56
3	8.51	7.56	4.60	4.28	4.02

Summary of analysis of variance.

Variation	Sum of squares	Degrees of freedom	Mean squares	F-ratio
Total	74.51	14	5.32	
Treatments	54.42	4	13.61	36.01 *
Experimental	17.07	2	8.54	22.59 *
Residual	3.02	8	0.378	

The tabulated F-ratio with 4 and 8 degrees of freedom is 14.4 at a significance level of 0.001 and thus there is a significant effect of the treatments applied. * $P < 0.001$.

When a significant effect of treatments is established by ANOVAR the data can be further analysed using suitable multiple comparison tests. These tests are described below.

A.5.2 Dunnett's test.

Dunnett's test is used to determine whether a control mean differs significantly from each of the treatment means. The data analysed above by ANOVAR will now be further analysed using Dunnett's test.

Group means (ranked)

1	2	3	4	5
8.675	8.317	5.073	4.548	4.320
Control	TPA (0 min)	TPA (30 min)	TPA (10 min)	OAG (10 min)

The standard error (SE) is calculated from:

$$SE = \sqrt{\frac{2 \times \text{residual mean square}}{n}}$$

The test statistic (q′) is calculated by dividing the difference between the control and each experimental mean by the standard error. A significant difference between the means is presumed if the calculated q′ value exceeds the tabulated q′ value. The test results, outlined in table A.5.2.1, indicate significant effects of preincubation with TPA for 10 and 30 min and with OAG for 10 min on the histamine-stimulated cyclic AMP content of the cell suspension.

Table A.5.2.1
Summary of Dunnett's test.

Comparison	Difference	SE	q	p	$q'_{0.05,v,p}$	$q'_{0.01,v,p}$	Conclusion
1 vs 2	0.358	0.502	0.713	2	2.31	3.36	Not significant
1 vs 3	3.602	0.502	7.175	3	2.67	3.77	$P < 0.01$
1 vs 4	4.127	0.502	8.221	4	2.88	4.00	$P < 0.01$
1 vs 5	4.355	0.502	8.675	5	3.02	4.17	$P < 0.01$

p = number of means in the range being tested.
v = residual degrees of freedom from the ANOVAR.

A.5.3   Newman-Keuls test.

The Newman-Keuls test is used, following a significant result by ANOVAR, to compare the differences between all possible pairs of means. This test is applied to the aminopyrine ratios obtained following exposure of parietal cells to carbachol (0.1 mM) for either 30, 45 or 60 min (Fig. 5.5). Figures for the residual error degrees of freedom and mean square were calculated by ANOVAR (not shown).

Group means (ranked)

1	2	3
1.48	1.67	2.42
60 min incubation with carbachol	45 min incubation with carbachol	30 min incubation with carbachol

The standard error is calculated from:

$$SE = \sqrt{\frac{\text{residual mean square}}{n}}$$

The test statistic is calculated as for Dunnett's test. The test results outlined in table A.5.3.1 indicate that the aminopyrine ratios obtained after both 45 and 60 min of incubation with carbachol (0.1 mM) are significantly less than the results obtained following a 30 min incubation, but they do not significantly differ from each other.

Table A.5.3.1
Summary of Newman-Keuls test.

Comparison	Difference	SE	q	p	$q_{0.05,v,p}$	$q_{0.01,v,p}$	Conclusion
3 vs 1	0.94	0.087	10.81	3	4.34	6.33	$P < 0.01$
2 vs 1	0.19	0.087	2.18	2	3.46	5.24	Not significant
3 vs 2	0.75	0.087	8.62	2	3.46	5.24	$P < 0.01$

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

A.6   Animals.

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