ASPECTS OF SIGNAL TRANSDUCTION IN THE GASTROINTESTINAL TRACT.

BY

JULIE MARIE WILLIAMS

A thesis submitted for the
degree of
Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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This study was undertaken to increase knowledge of the mechanisms of inter- and intracellular signalling in the gastrointestinal tract. Specific aims were: to use cell lines to elucidate factors affecting growth of gastric cells, to investigate the distribution and aspects of function of isoforms of protein kinase C in a gastric cell line and in the rat gastrointestinal tract and to determine the presence and regulation of nitric oxide synthase in gastrointestinal tissues from the rat and in cell lines.

The gastric cancer cell line HGT-1 was used to investigate control of growth. Increases in cell number were found to be dependent on the seeding density of the cells. In cells plated at low density insulin, epidermal growth factor and gastrin all increased cell number. Gastrin produced a bell-shaped dose response curve with a maximum activity at 5nM. No effect of gastrin was apparent in cells plated at high density.

α and β isoforms of protein kinase C were found, by immunoblotting procedures, to be widespread in the gastrointestinal tract of the rat, but protein kinase Cε was confined to the gastric mucosa and gastrointestinal smooth muscle. HGT-1 cells contained protein kinase C α and ε but β or γ were not detected. Preincubation of HGT-1 cells for 24h with 1μM phorbol-12,13-dibutyrate down-regulated protein kinase C α but not ε. The inhibition by the activator of protein kinase C, 12-O-tetradecanoylphorbol 13-acetate (TPA) of the histamine-stimulated increase in cAMP in HGT-1 cells was down regulated by phorbol-12,13-dibutyrate. Inhibition of histamine-stimulation of adenylate cyclase by TPA was Ca^{2+}-dependent and inhibited by the addition of an antibody to protein kinase C α. A role for protein kinase C α in modulating the effect of histamine on adenylate cyclase in HGT-1 cells is suggested.

No nitric oxide synthase activity was detected in the gastrointestinal cell lines HGT-1, MKN-45 or CaCo-2. Ca^{2+}-dependent nitric oxide synthase activity was observed in the gastric mucosa and the gastrointestinal smooth muscle from stomach to colon. The gastric mucosal enzyme was soluble and showed half-maximal activity at 400nM Ca^{2+}. Pretreatment of rats with endotoxin (3mg/kg body weight) induced nitric oxide synthase activity in both jejunal, ileal and colonic mucosa and muscle. A major portion of the induced activity in ileal and colonic mucosa was Ca^{2+}-independent. Nitric oxide synthase activity in a high-density fraction of gastric mucosal cells was inhibited in a dose-dependent fashion by L-nitroarginine, N^G-monomethyl-L-arginine, trifluoperazine and L-canavanine (in descending order of potency). Preincubation with okadaic acid and addition of ATP/Mg^{2+} to the homogenisation buffer inhibited enzyme activity, which implies that phosphorylation inhibits gastric mucosal nitric oxide synthase.

Key words: HGT-1 cells. Gastrin. Protein kinase C. Nitric oxide.

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COMMONLY USED ABBREVIATIONS.

TPA	12-O-tetradecanoylphorbol 13-acetate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(b-aminoethyl ether-N,N,N',N',-tetraacetic acid
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])
CHAPS	(3-[(3- cholamidopropyl)-dimethylammonio]-1-propanesulphonate
cAMP	3',5'-cyclic adenosine monophosphate
ATP	adenosine triphosphate
GTP	guanosine triphosphate
DMSO	dimethylsulphoxide

CHAPTER 1.

GENERAL INTRODUCTION.

This thesis is concerned with the general mechanisms of inter- and intracellular signalling in the gastrointestinal tract. The functional morphology of the gastrointestinal tract will be described first, followed by the importance of gastrointestinal cell lines in the investigation of cellular signalling.

1.0 Gross Morphology of the Gastrointestinal Tract.

The gastrointestinal tract is divided up into a number of distinct regions, the oesophagus, the stomach, the small intestine and the large intestine. The stomach is a sac-like stucture between the oesophagus and the small intestine. It can be sub-divided into three regions: the fundus - proximal to the gastro-oesophageal junction, the corpus - between the gastro-oesophageal junction and the incisura angularis and the antrum - between the incisura angularis and the duodenum. In the rat the fundus is replaced by a non-glandular forestomach. The small intestine is also comprised of three separate areas: the duodenum, the jejunum and the ileum and makes up 80-90% of the entire gut length in humans. The duodenum runs from the pyloric sphincter to the ligament of Trietz. Of the remainder of the small intestine, in this thesis, the upper half is termed jejunum and the lower half ileum. The colon is divided into eight parts: the appendix, the caecum, the ascending colon, the transverse colon, the descending colon, the sigmoid colon, the rectum and the anal canal.

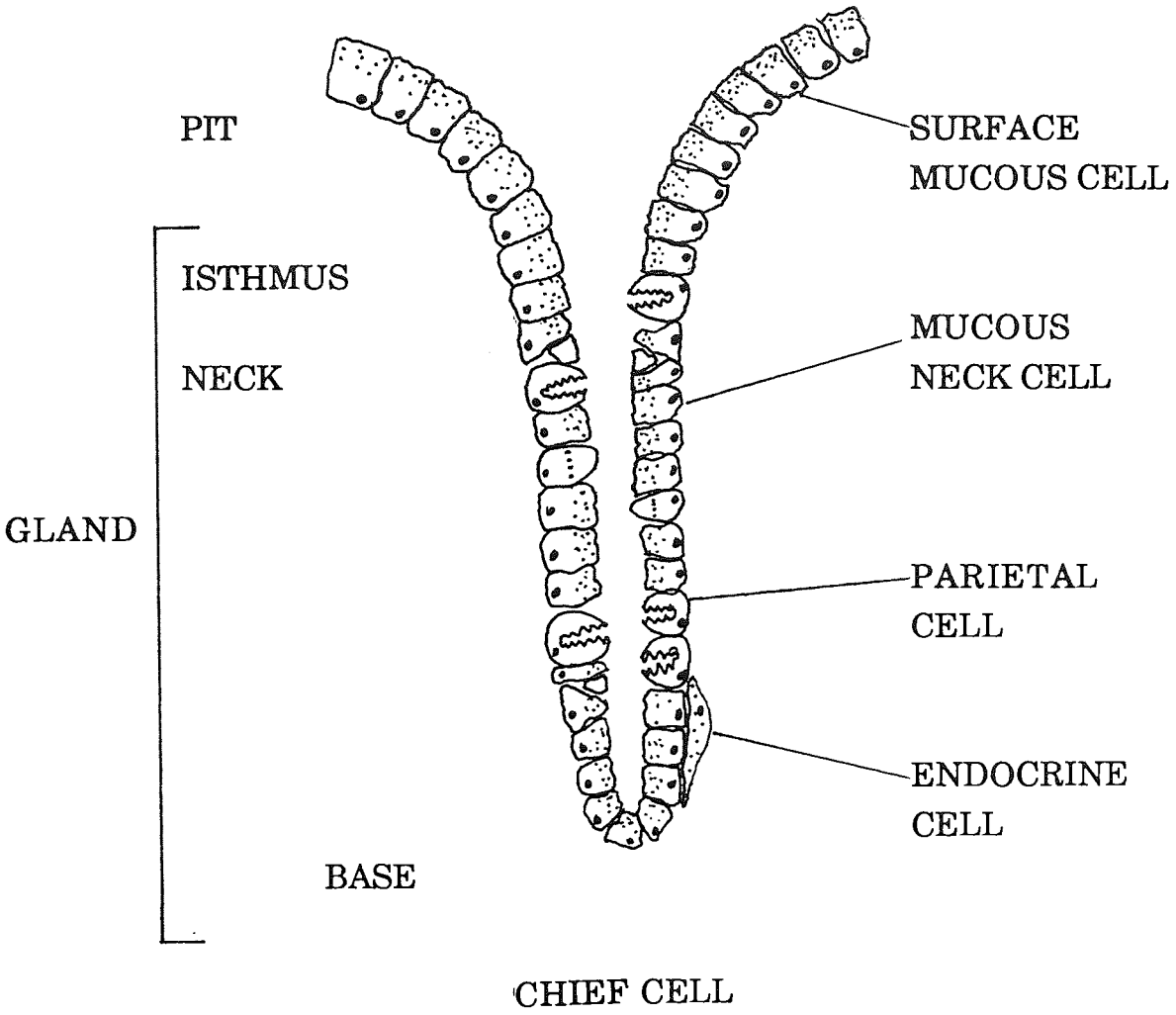
1.1 Ultrastructure of the Gastrointestinal Tract.

1.1.1 The Stomach.

In transverse section the stomach has a number of layers. Firstly the mucosa which comes into contact with the luminal contents. The mucosa contains specialised cells which serve to protect and to carry out digestive functions. The next layer is the muscularis mucosa which is a thin layer of muscle cells, arranged as two or three sublayers, and this separates the mucosa from the submucosa. The submucosa is a dense mass of connective tissue containing large blood vessels and nerves, including Meissner's plexus. The next layer is the muscularis which comprises layers of muscle cells first circular then longitudinal. The myenteric plexi lie between the circular and longitudinal muscle. The final layer is the serosa which is thin connective tissue covered with a layer of squamous epithelial cells, the mesothelium.

The gastric mucosa is not a flat layer, it is full of invaginations called gastric pits. At the base of these pits can be found the gastric glands which can be branched or simple (fig 1.1.1). Not all of the mucosa contains glands and the non-glandular region is covered by a layer of keratinised stratified squamous epithelial cells. The remainder of the gastric mucosa contains one of three types of gastric gland. The cardiac glands are found in a narrow zone near the oesophageal opening and are rich in mucus. They have short pits and branched and coiled glands. The pyloric glands are found in a slightly larger region adjacent to the duodenum and are also rich in mucus. They have deep pits and straight glands. The oxyntic (also called gastric or fundic) glands are found mostly in the fundus and corpus. They can have anything from one to seven glands to a pit and are usually straight. There are a wide variety of different cells found in the different glands (Ito, 1987). Cardiac glands

Fig 1.1.1 Diagram of a Typical Gastric Gland (not to scale).



contain predominantly surface mucous cells with a few endocrine cells. Pyloric glands house mainly mucus-secreting cells with some G cells which contain gastrin. Oxyntic glands contain a myriad of different types of cells with the main ones being parietal cells, chief cells and mucous epithelial cells plus smaller numbers of mucous neck cells, endocrine cells and undifferentiated cells. Oxyntic glands are divided up into three regions: the isthmus which contains parietal cells and surface mucous cells, the neck which houses parietal cells and mucous neck cells and the base which contains chief cells and parietal cells. Parietal cells comprise 40% of the corpus mucosa of the rat, the chief cells 20-26%, the surface mucous cells 18% and the mucous neck cells 8%. Below the epithelial layer of the gastric glands there is a thin layer of connective tissue called the lamina propria which contains blood vessels, nerves, smooth muscle and white blood cells.

Mucus is formed in surface mucous cells and secreted to provide lubrication and to protect the stomach from damage by ingested foodstuffs, acid, digestive enzymes and refluxed contents of the duodenum. Protection against the digestive enzyme, pepsin, occurs because mucus acts as a permeability barrier which prevents access of pepsin to the epithelial cells. The mucus also forms a continuous, visoelastic unstirred layer in which hydrogen ions diffusing towards the epithelium from the lumen are neutralised by bicarbonate secreted from epithelial cells. The surface mucous cells are columnar epithelium with an apical cytoplasm, containing mucous granules, and an indistinct basal nucleus. The apical membrane has short microvilli and an external glycocalyx. Surface mucous cells are renewed approximately every three days. The mucous neck cells are similar to the surface mucous neck cells but they contain larger mucus granules in their paranuclear region. Parietal cells are often found in close apposition to the mucous neck cells.

Parietal cells are the source of hydrochloric acid in the stomach. They are only rarely found in the gastric pits or the surface of the mucosa. They are the largest cells of the stomach at 25μm diameter and are oval or pyramidal in shape. They contain a high proportion of mitochondria which provide the ATP to power acid secretion. Parietal cells contain unique structures known as secretory canaliculi. These are a series on canals running into the cell often extending into the basal cytoplasm and around the nucleus. These canaliculi are continuous with the lumen of the gland and increase the cells surface area. The canaliculi have been found to contain a proton pump on their membrane for the extrusion of hydrogen ions into the lumen. Parietal cells have numerous microvilli and no glycocalyx.

Chief, or peptic, cells are responsible for the synthesis, storage and secretion of pepsinogen and are a typical protein-secreting exocrine cell. They are predominantly found in the corpus, rarely in the pyloric glands and never in the cardiac glands. The pepsinogen is contained in zymogen granules, in the apical region of the cell and is released by exocytosis. Chief cells have an abundant endoplasmic reticulum, a few stubby microvilli and a thin glycocalyx.

Enteroendocrine cells comprise only a small part of the gastric glands. They are of mixed population and can produce several hormones from the same cell. There are at least nine different types of these cells in the oxyntic and pyloric glands, with some lower in the gastrointestinal tract (Grube and Forssmann, 1979). Examples of these cells are; the endocrine-like cell which secretes histamine, the endocrine cell which secretes serotonin, the G cell which secretes gastrin and ACTH-related peptides, the D cell which secretes somatostatin, the H cell which secretes VIP, the A cell which secretes glucagon and the P cell which possibly secretes bombesin in frogs or gastrin-releasing peptide in mammals. In the

pyloric antrum the predominant endocrine cell is the G cell which comprises around 2% of the whole of the glands. It is a medium sized cell which has long microvilli and granules of gastrin which are released either by exocytosis or emiocytosis.

There are a number of other cells in the gastric mucosa. Both intraepithelial lymphocytes and globular leukocytes are known to move between cells in the glands.

In this thesis a preparation of isolated gastric mucosal cells is employed. These were prepared by digestion with pronase and intermittent Ca^{2+}-chelation (Hatt and Hanson, 1989). The resulting preparation contained approximately 20% parietal cells and 30% mucous cells with the remainder being predominantly chief cells. Parietal cells contain a high proportion of membranes and are therefore less dense than other gastric cells. This property was used to separate the cells using differential centrifugation on a Percoll™ gradient.

The lamina propria was mentioned briefly above as an area of loose connective tissue. The mucosal epithelium covers a continuous basement membrane, or lamina, under which lies collagen, small reticular fibres and some elastic fibres. The vascular supply to the mucosa is found here in the form of primary mucosal arteries and capillaries from the submucosa. There are also a few lymphatic vessels. The lamina propria also contains smooth muscle, lymphocytes, plasma cells, eosinophils, polymorphonuclear leukocytes, mast cells, fibroblasts, nerves and occasionally endocrine cells and globular leukocytes.

The stomach comprises three muscle layers; the outer layer is longitudinal muscle and this predominates on the greater curvature. The middle layer is circular muscle and this is found throughout the

stomach. The inner layer is oblique muscle and this is especially prominent on the lesser curvature.

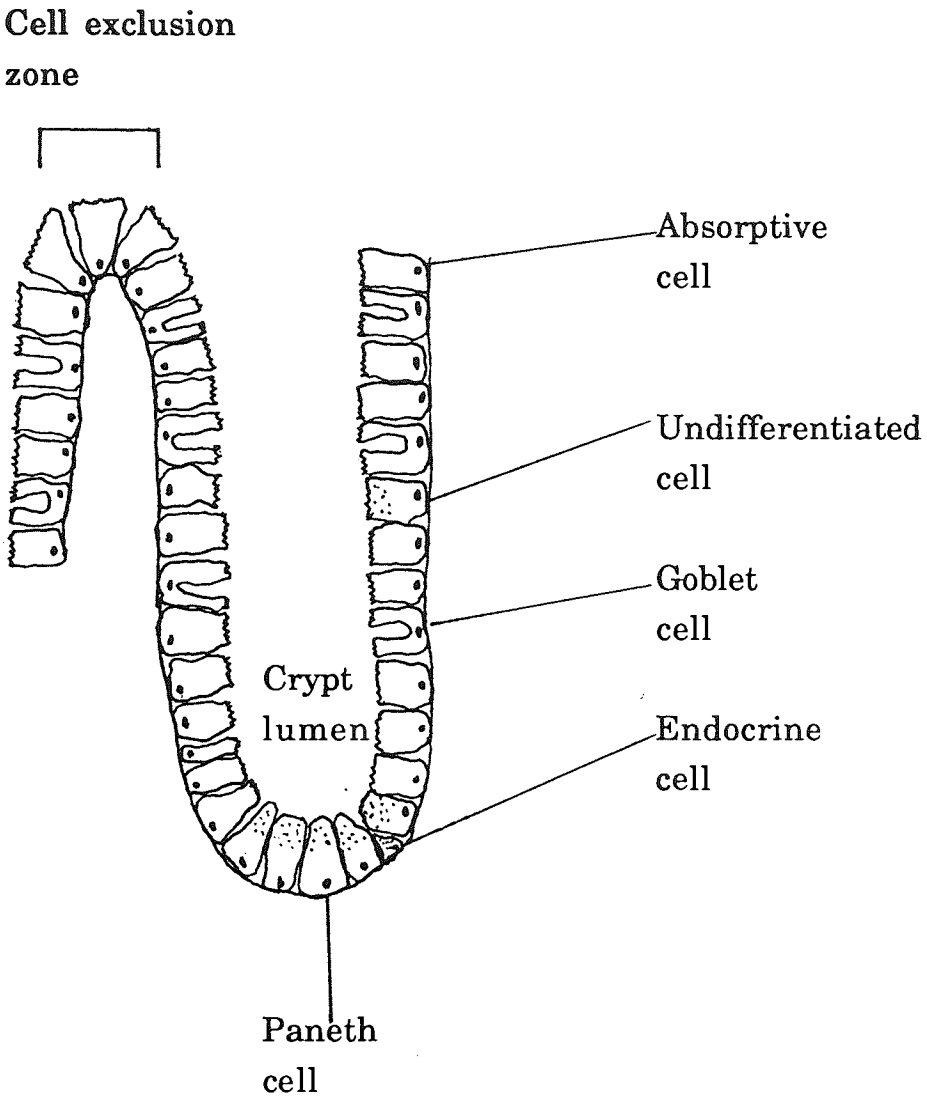
The stomach is innervated predominantly by parasympathetic nerves from the vagus. The vagus also carries some adrenergic fibres. When low threshold fibres of the vagus are stimulated contraction occurs but when high threshold fibres are stimulated relaxation is the result. The low threshold fibres are known to be cholinergic but the transmitter in high threshold fibres is non-cholinergic and non-adrenergic (NANC) (see chapter 5). There are also sympathetic nerves which inhibit contraction and this is probably via modulation of the myenteric plexus (Meyer, 1987).

1.1.2 The Small Intestine.

The small intestine is highly differentiated as it has a number of diverse functions. The overall surface area is increased by the structure being folded to form plicae, these are most dense in the distal duodenum and the proximal jejunum. In addition to this the small intestine contains numerous mucosal villi and crypts which increase the surface area seven to fourteen fold (fig 1.1.2). The proximal duodenum contains elaborately branched acinar glands called Brunner's glands and these contain serous and mucous cells. These glands are found largely in the submucosa but often penetrate the mucosa. The Brunner's glands have ducts into the base of the crypts which empty bicarbonate, epidermal growth factor and glycoprotein-rich secretions into the gut lumen.

There are three distinct layers of the small intestinal mucosa. The muscularis mucosa is a continuous sheet of muscle three to ten cells thick which separates the submucosa from the mucosa. It is possible that it contributes to the movement of the villi. The lamina propria is comprised of connective tissue. This substance forms the core of the villi

Fig. 1.1.2 Diagram of a Typical Villus (not to scale).



and surrounds the crypt epithelium. The lamina propria contains blood vessels and a central arteriole runs up the core of each villus and then branches into capillaries to run back down. There is also a lymphatic vessel in the core and this is responsible for the transport of lipids. The third layer is a layer of epithelial cells which line the villi and the crypts. This is one cell thick and has a thin continuous basement membrane. The crypts and the villi have entirely different functions. The villi are solely involved in the process of absorption, whereas the crypts have a number of functions including; cell renewal, exocrine secretion, electrolyte and water secretion and endocrine secretion. This means that the villi and crypts have to contain different types of cells. The crypt epithelium is formed by undifferentiated proliferating cells, goblet cells endocrine cells, caveolated cells (rare) and Paneth cells. The villus epithelium is composed of absorptive cells (enterocytes), goblet cells, a few endocrine cells, caveolated cells (rare) and a few cup cells. The morphology and functions of these cells will now be outlined.

The absorptive cells are tall columnar epithelial cells with a basal nucleus. They have a terminal web at their apex, closely packed microvilli to increase the surface area and a glycocalyx. Disaccharidase and peptidase enzymes are present on the membrane of the microvilli along with transporters for Na^+/D-glucose, Na^+/amino acids and possibly fatty acids. Receptors for cobalamine plus the intrinsic factor and bile salts are present in ileal cells.

The undifferentiated cells are most abundant in the crypts of Lieberkühn and are concerned with proliferation. They are columnar with a basal nucleus, a few short microvilli and a less well developed glycocalyx. Cells divide and the daughter cells migrate up the crypt and differentiate. By the time that they reach the crypt mouth they have an appearance like that of the absorptive cells. As well as being the

precursors of absorptive cells they also form Paneth cells, endocrine cells, goblet cells and possibly M cells and cup cells. There is substantial evidence that these undifferentiated cells may play an important role in the secretion of water and ions into the lumen (Madara and Trier, 1987).

Goblets cells are polarised mucus-secreting cells which are prominent throughout the intestine but show an increase in frequency from the proximal jejunum to the distal ileum. They have few microvilli and a poor glycocalyx. The apical two thirds is usually distended with mucin granules which are surrounded by a membrane. The apical membrane is often cup-shaped and full of mucin.

Paneth cells partly form the base of the crypts of Lieberkühn and they are also found in the lower half of the sides. They are zymogenic in nature and resemble a truncated pyramid, which is widest at its base, and there is a basal nucleus. The cells have rudimentary microvilli and granules are found between the nucleus and the apex. The Paneth cells have an elaborate Golgi apparatus and lots of rough endoplasmic reticulum. Their role it still uncertain but it is possible that they secrete digestive enzymes (Madara and Trier, 1987).

The ileal surface has large patches of lymphoid tissue known as Peyer's patches. These are predominantly in the submucosa but they extent into the mucosa. Overlying the apex of these patches are absorptive cells called M cells which are possibly involved in the transport of intraluminal antigens to the lymphoid tissue of the Peyer's patches.

Caveolated cells are often called tuft cells, brush cells, multivesicular cells or fibrovesicular cells. They are found in the crypts of the entire small intestine. Caveolated cells are pear-shaped with a narrow apex. Interspersed between bundles of microfilaments in the apex are caveoli

which are vesicular in appearance. The functional significance of calveolated cells is not known but it is possible that they could be chemoreceptors.

Cup cells comprise only a minor part of the villus epithelium (and are very rare in the rat). The have a very short brush border compared to the surrounding absorptive cells and therefore give the impression of a cup-like indentation in the villus brush border. The apical cytoplasm of the cup cells is also slightly indented further adding to the effect. At the moment their function is unknown but they do pinocytose small amounts of protein.

In this work cells were isolated from ileal mucosa and this preparation consisted predominantly of enterocytes (villus absorptive cells) plus a few goblet cells. Jejunal and ileal mucosal cells were also prepared by scraping the intestine with a microscope slide.

Underneath the mucosal layer is the submucosa which is composed of connective tissue, lymphatic tissue and vascular tissue. Next to the submucosal layer is the submucosal plexus, or Meissner's plexus.

Like the stomach the small intestine is also surrounded by muscle. There are two muscle layers; the outer longitudinal muscle which is thin and the inner circular layer which is thick. The two layers are separated by the myenteric plexus (Auerbach's plexus) and linked by bridges. Overlying the muscle layer is the serosa which is a thin sheet of epithelial cells and connective tissue.

1.1.3 The Colon.

The colon has an extremely thick muscle wall. The longitudinal muscle is grouped into bands with only a thin layer in-between. The circular muscle is arranged with tight bands at regular distances. There is a layer of muscle in the mucosa which consists of inner circular muscle and outer longitudinal muscle tightly fixed together by the intertwining of the bundles at the interface of the two layers.

The colonic mucosa has both a protective and an absorptive function. Its primary functions are to recover water from the liquid that enters from the small intestine and to provide propulsion for the faeces. It does the latter by producing mucus for lubrication and using the large muscular wall to push the faeces forward. The crypts of the large intestine are more closely packed than anywhere in the whole gastrointestinal tract giving the surface a flat appearance. The colon contains three types of cells; absorptive cells (specialised for water absorption), mucus-secreting goblet cells and enteroendocrine cells. There are several types of enteroendocrine cells; H cells which secrete VIP, X cells which secrete an unknown substance and PP cells which secrete pancreatic polypeptide and possibly met-enkephalin. There are also a few other types of cells in the colon including numerous leukocytes and immune cells, the latter of which aggregate in the lamina propria and submucosa.

Colonocytes, prepared by disaggregation of colonic mucosa, were used in one part of this thesis (Chapter 5).

1.2 Gastrointestinal Cell Lines.

1.2.1 Major Characteristics of Cell Lines.

Animal tissue was first grown in culture in 1907 and this constituted a major advance in the investigation into cellular physiology. Primary cultures are initiated by dissociating the relevant tissue with proteolytic enzymes and then seeding it into a dish with liquid medium. Healthy cells will attach and divide. Once enough cells have been achieved the cells are detached from the surface, diluted and reseeded: this is the secondary culture. When cells are multiplying at a constant rate over successive transfers a cell strain has been achieved. Unfortunately these cell strains can only undergo a finite number of divisions after which they die and in humans this number of divisions is between 50 and 100. Sometimes not all of the cells die, a few become altered so that they have a different morphology, they grow faster and they can be cultured in smaller numbers. Those cells with an unlimited life form cell lines. During repeated passaging the cell line may change, for example clumps, rather than monolayers, may form. These cells are said to be transformed and are generally neoplastic. Transformed cells can also be obtained by infecting normal cells with oncogenic viruses or by exposing them to carcinogenic chemicals. There are no human cell lines which have been shown to have originated from normal tissue but there are a multitude derived from tumours. It would appear from this that the possession of a cancerous phenotype allows for easier adaptation to culture and this is possible due to the aneuploidy exhibited by most malignant cells. Transformed malignant cells have a number of characteristics which are covered in table 1.2.1.

Table 1.2.1 Phenotypic Characteristics of Transformed Malignant Cells.

CYTOLOGICAL CHARACTERISTICS

- Increased basophilia
- Increased number and size of nuclei
- Increased nuclear : cytoplasm ratio
- Formation of clusters/cords of cells

ALTERATION OF GROWTH CHARACTERISTICS

- "Immortality"
- Decreased density-dependent inhibition/loss contact inhibition
- Decreased serum requirement
- Loss of anchorage dependence and acquisition of ability to grow in soft agar
- Loss of "restriction point" control - fail to stop at G1/S boundary

CHANGES IN CELL MEMBRANE STRUCTURE AND FUNCTION

- Increased agglutinability by plant lectins
- Alteration of surface glycoproteins and glycolipids
- Loss of surface fibronectin
- Increased uptake of amino acids, hexoses, nucleosides
- Appearance of tumour-associated antigens

ALTERATION OF ENZYME PATTERNS

- Increased levels of enzymes for nucleic acid synthesis
- Increased levels of proteases

PRODUCTION OF GROWTH FACTORS

- Tumour angiogenesis factor
- Transforming growth factor

PRODUCTION OF ONCODEVELOPMENTAL GENE PRODUCTS

- Increased carcinoembryonic antigen
- Increased placental hormones
- Increased placental-foetal type isoenzymes

1.2.2 Cell lines.

There a number of gastrointestinal cell lines available with many different properties (table 1.2.2). The predominant area of the tract cultured is the colon and many well-differentiated, well-characterised cell lines have been isolated from this region. Although quite a few gastric cell lines appear to be available the problem is that relatively few are well-differentiated, well-characterised (either by cytogenetics, immunology or isoenzyme markers), isolated from the original tumour as opposed to a metastasis (to minimise contamination) or have been cultured long-term. In this study the predominant cell line used was the HGT-1 line.

1.2.3 The HGT-1 Cell Line.

The HGT-1 cell line was established from a poorly differentiated gastric adenocarcinoma from a 60 year old Moroccan male, of blood group O Rh^-, with no metastases. The tumour was situated on the posterior wall of the body of the stomach, it did not involve the antrum. It was an ulcerating infiltrating tumour which penetrated the entire thickness of the wall. The tumour was removed, disaggregated with collagenase, and plated in 0.3% agarose over a 0.5% agarose layer. After 10 days colonies had appeared and these were cultured for 15 days. The colonies were reseeded into liquid medium over a feeder layer of human fibroblasts. The cells grew in suspension and when the resulting cells were reseeded into plastic flasks they adhered to the surface and grew (Laboisse et al., 1982).

HGT-1 cells exhibited a typical epithelial cell appearance (plates 1.2.1). They exhibited no gland formation (but occasionally did show a pseudoacinar pattern), no mucin granules, and had no secretory products. The cells had a high nucleus : cytoplasm ratio and junctions between the cells. HGT-1 cells had large indented nuclei with prominent nucleoli and most were mononucleic although a few were multinucleic.

Table 1.2.2 Major Gastrointestinal Cell Lines.

Cell Line	Origin of tumour	Species
HGT-1	stomach	human
TMK-1	stomach	human
MKN-45	stomach	human
MNK-45G	stomach	human
KATO III	stomach	human
GTL-16	stomach	human
MKN-1	stomach	human
MKN-7	stomach	human
MKN-28	stomach	human
MKN-74	stomach	human
IEC-18	small intestine	human
HUTU 80	small intestine	human
IEC-6	small intestine	human
CaCo-2	colon	human
HT-29	colon	human
LoVo	colon	human
SNU-C1	colon	human
SW 403	colon	human
WiDr	colon	human
SW-480	colon	human
SW-620	colon	human
MIA-PaCa 2	pancreas	human
AR4-2J	pancreas	rat

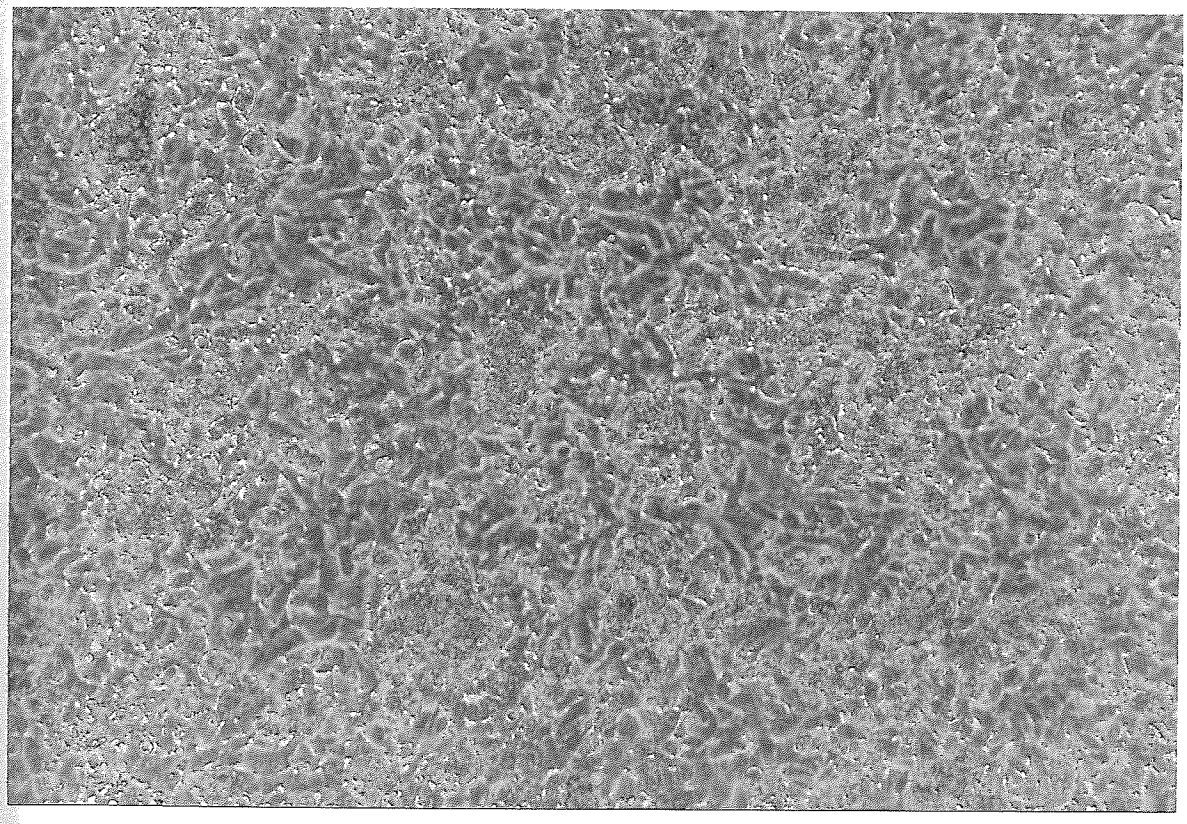


Plate 1.2.1 HGT-1 cells in culture (x 156.25).

The intercellular junctions were mostly tight but some were gap junctions. The cells showed numerous microvilli with no secretory vacuoles although there were some empty ones. There were numerous mitochondria present as well as polysomes but the Golgi apparatus was not prominent and lysosomes were rare. HGT-1 cells exhibited the ability to produce tumours *in vivo* with same morphology as the initial growth. The tumours did occasionally have a glandular pattern. The cells had a modal chromosome number of 57 (varied between 55 and 60) and in 66% of metaphases the Y chromosome was absent. A number of chromosomes were able to be used as markers. HGT-1 cells did not express carcinoembryonic antigen (Laboisse et al., 1982).

100μM histamine was able to increase cAMP levels in the cells 5.7 fold and this is comparable with values in the fundic glands. Cimetidine inhibited the increase and therefore H_2-receptors are involved. The adenylate cyclase activity in HGT-1 cells was increased three fold by 1mM histamine and cimetidine also reversed this effect (Laboisse et al., 1982). HGT-1 cells also have receptors for vasoactive intestinal peptide (Emami et al., 1983), gastric inhibitory peptide and pancreatic glucagon (Emami et al., 1986) all of which elevate cAMP. The somatostatin receptor is present and there appear to be two forms (as there is in parietal cells). This receptor is negatively coupled to adenylate cyclase via an inhibitory GTP-subunit (Reyl-Desmars et al., 1986). A receptor for truncated glucagon-like peptide exists on the cell and this also activates adenylate cyclase (Hansen et al., 1988). The histamine H_2 receptor on HGT-1 cells is pharmacologically indistinguishable from that on human gastric glands (Gespach et al., 1988). The histamine receptor undergoes desensitisation after exposure to histamine (Prost et al., 1984; Emami et al., 1986b). This desensitisation does not appear to be due to a decrease in the receptor's affinity but to an uncoupling from adenylate cyclase and it has a half-life of 20 min. The vasoactive intestinal peptide receptor also undergoes desensitisation.

of 20min. The vasoactive intestinal peptide receptor also undergoes desensitisation (Gespach et al., 1984).

HGT-1 cells have also been shown to possess ion channels. Fraser et al. (1990) discovered a high conductance K^+ channel with properties similar to the channel in the basolateral membrane of parietal cells. Sandle et al. (1990) showed that an increase in cAMP levels could activated a Cl^- channel in HGT-1 cells.

1.2.4 Other Cell Lines Used.

MKN-45 cells were also used in this study. This is a poorly differentiated adenocarcinoma isolated from the germinal region of the human gastric gland. The cells exhibit intracellular canaliculi, receptors for histamine (H_2) and EGF and they secrete EGF and TGFα. There has also been a clonal variant of MKN-45 formed called MKN-45G which produces gastrin (Watson et al., 1991).

The colonic cell line CaCo-2 was used in chapter 5. CaCo-2 cells have the ability to spontaneously differentiate into small intestinal epithelial cells. These cells are highly polarised with a typical brush-border membrane containing high levels of membrane-associated hydrolases. In culture CaCo-2 cells form domes and exhibit tight junctions (Pinto et al., 1983).

Alkaline phosphatase is a cell-surface glycoprotein present in varying amounts through all stages of cellular developement. The activity of the enzyme in a cell is frequently used as a measure of the amount of differentiation a cell has undergone (Garen et al., 1960). CaCo-2 cells show an increase in enzyme activity over time (Rousset et al., 1985; Matsumoto et al., 1990).

1.3 Aims and structure of this thesis.

The availability of gastrointestinal cells lines enables specific physiological processes to be observed in isolation from the rest of the mucosa. Primary cultures are useful but are subject to a high degree of contamination with other cells and there is a constant problem of de-differentiation. Results obtained with cell lines are usually highly repeatable, and their preparation does not involve the traumas associated with disaggregation of fresh tissue to produce isolated cells.

The initial aim of this work was to use gastrointestinal cell lines, HGT-1 especially, to examine aspects of the regulation of growth and inter- and intracellular signalling in the gastrointestinal tract. Factors involved with growth are introduced in Chapter 3 which also contains the results of this aspect of the work.

Intracellular signalling involving isoforms of protein kinase C and regulation of histamine-stimulated adenylate cyclase was studied, and this topic is covered in Chapter 4. To put the work into context the distribution of some isoforms of protein kinase C in the rat gastrointestinal tract was examined.

Nitric oxide is a novel signalling molecule which has functions in the gastrointestinal tract (Chapter 5). It was decided to search for the presence of the enzyme responsible for its synthesis, nitric oxide synthase, in gastrointestinal cell lines with a view to using such cells to investigate the regulation of its activity. Comparative studies were to be performed on the presence and properties of nitric oxide synthase in the rat gastrointestinal tract. The results of this section of the work are described in Chapters 5 and 6.

Initially this project was intended to deal solely with cultured cell lines. The author therefore did not hold a Home Office Licence. Certain procedures on animals were in the event required. These were always performed by licenced personel, but only up to the point where the author could take over.

CHAPTER 2.

GENERAL METHODOLOGY.

2.0 CELL CULTURE.

All cell culture media, supplements and enzymes were from Gibco BRL Ltd (Paisley, Scotland). Disposable cell culture equipment was from Northumbria Biologicals.

2.0.1 HGT-1 Cells.

HGT-1 cells (passages 95 - 142) were routinely cultured in sterile conditions using a Gelair laminar flow hood and were grown in $80cm^2$ cell culture flasks. Cells grew in Dulbecco's modified Eagle's minimum essential medium (DMEM) without sodium pyruvate and with 4500mg/l glucose, supplemented with 5% foetal calf serum (FCS), 2mM L-glutamine, 100IU/ml penicillin and 100µg/ml streptomycin. The flasks were kept in a LEEC CO_2 incubator at 36.5°C in 10% carbon dioxide / 90% air (v/v) and the medium was changed every second day.

Cells were passaged every seven days. For detachment cells were exposed to a solution of 0.05% (w/v) trypsin / 0.02% (w/v) EDTA for approximately two minutes at 37°C. Then an equal volume of fresh medium was added and the suspension was seeded by adding 1ml (2×10^6 cells) to 20ml of medium in a clean flask.

For storage HGT-1 cells were detached and slowly frozen at -140°C in culture medium containing 10% FCS and 10% dimethylsulphoxide (DMSO) at a cell concentration of 1×10^7 cells/ml. Cells were reseeded by rapidly defrosting at 37°C in a water bath and diluting 1ml of suspension

into 20ml culture medium containing 10% FCS. The medium was changed the following day to remove any DMSO.

2.0.2 MKN-45 Cells.

MKN-45 cells were routinely cultured in DMEM supplemented with 10% FCS, 2mM L-glutamine, 100IU/ml penicillin and 100μg/ml streptomycin at 37°C in 5% carbon dioxide / 95% air (v/v). Cells were passaged and frozen as for HGT-1 cells.

2.0.3 A549 Cells.

A549 cells were cultured in nutrient mixture F12 (Ham's) supplemented with 10% FCS, 2mM L-glutamine, 100IU/ml penicillin and 100μg/ml streptomycin at 37°C in 5% carbon dioxide / 95% air (v/v). Cells were passaged and frozen as for HGT-1 cells.

2.0.4 CaCo-2 Cells.

CaCo-2 cells were routinely cultured in DMEM supplemented with 10% FCS, 2mM L-glutamine and 1% MEM non-essential amino acids (final concentration in mg/l) L-alanine 8.9, L-asparagine.H_2O 15.0, L-aspartic acid 13.3, L-glutamic acid 14.7, glycine 7.5, L-proline 11.5 and L-serine 10.5) at 37°C in 10% carbon dioxide / 95% air (v/v). CaCo-2 cells were passaged every seven days (unless they were to be used in experiments where differentiation was necessary) by using a 0.25% (w/v) trypsin / 0.1% (w/v) EDTA solution to detach the cells. The cell suspension was reseeded at a 1 in 4 dilution into normal culture medium. Cells were frozen as for HGT-1 cells.

2.0.5 Use of the Haemocytometer.

A cell suspension was prepared in balanced salt solution. This was diluted 1:1 with trypan blue (4mg/ml in 0.9% w/v saline). Using a small amount of moisture the coverslip was placed on the counting chamber (E. Leitz, Wetzlar) until "Newton's rings" could be seen. The cell suspension was taken up into a pasteur pipette by capillary action and applied to the edge of the coverslip. The chambers were allowed to fill without the suspension overflowing. The cell in the middle and four corner 1mm squares were counted in both chambers. At least 200 cells were counted to be accurate. The cell concentration was calculated by the following equation:

Average count/square x dilution factor x 10^4 = cells/ml

Viability was assessed by the inability of viable cells to take up trypan blue.

2.1 ISOLATION AND ENRICHMENT OF RAT GASTRIC MUCOSAL CELLS.

2.1.1 Crude Cell Preparation.

Fed rats of body weight approximately 200 - 250g were anaesthetised by peritoneal injection of sodium pentabarbitone (Sagatal™) at 60mg/kg body weight. A mid-line incision was made, the stomach exposed and the gastrosplenic and hepatogastric ligaments cut. The oesophagus was ligated and cut through above the ligature and the duodenum was ligated and severed below the ligature. The animal was then killed by rupturing the diaphragm (removal of the tissues carried out by Mr J. F. Brown, who held the necessary Home Office Licence, subsequent procedures performed by the author).

The removed stomach was rinsed in ice-cold 0.9% (w/v) saline and ligated along it's fundic-antral border (this lies one third of the way between the pylorus and the non-glandular region). The non-glandular region was removed the stomach everted and rinsed in 0.9% (w/v) saline. The open end was then ligated to make a stomach sac. The sac was then filled with medium A (Table 2.1.2) containing pronase (isolated from Streptomyces griseus) at 1000PUK/ml (1 PUK unit is that which produces after hydrolysis of casein at 40°C in phosphate buffer pH7.4 for 10min, and precipitation of protein by trichloroacetic acid, an absorbance of 1.0 at 660nm with Folin and Ciocalteau's reagent) using a 26-gauge hypodermic syringe.

Sacs were incubated in a sealed container with 40ml of medium A prewarmed to 37°C for 30min in a shaking water bath (60cycles/min) and gassed with 5% CO_2 / 95% O_2 (v/v). Sacs were removed, blotted to remove any residual medium and placed into 20ml medium B (Table 2.1.2). The

sacs were incubated for 30min at room temperature whilst gassing with 5% CO_2 / 95% O_2 (v/v) with gentle stirring with a magnetic follower. The harvested cells were filtered through nylon mesh (150μm pore - Sericol Group Ltd, London) into centrifuge tubes and subjected to centrifugation at 100g for 5min at 15°C. The pellet was resuspended with a plastic transfer pipette into 10ml fresh medium B and stored with shaking at 60 cycles/min at 37°C whilst being gassed as above. Sacs were incubated for a further 2h with alternate changes of medium A and medium B every 30 min. The cells obtained were pooled and underwent centrifugation at 100 g for 5min at 15°C. The pellet was then resuspended in medium C (Table 2.1.2) at 4 - 5 x 10^6 cells/ml.

2.1.2 Enrichment.

Enrichment of the crude cell preparation made use of the differences in density of the different types of cells in the gastric mucosa (Chapter 1). 20ml of a solution of iso-osmotic Percoll™ was produced by adding 25mM $NaHCO_3$ (final concentration) and 2ml of 10 x Eagles minimum essential medium to 18ml of Percoll™. This solution was gassed for 5min at room temperature whilst being slowly stirred and then adjusted to pH7.4. 3ml aliquots of this solution was placed in polycarbonate centrifuge tubes and to this 4.5ml of the crude cell suspension in medium C was added to give a 36% v/v Percoll solution. The tubes were capped and inverted twice to ensure adequate mixing. The cell suspension underwent centrifugation at 30,000g_{av} (20° angle rotor) for 15min at 4°C on a MSE Superspeed 50 centrifuge. The top 1.5ml represented the low density parietal cell-rich fraction whereas the band at the bottom of the tube was a high density parietal cell depleted fraction. The high density fraction, which was routinely used as a source of nitric oxide synthase (Chapters 5 and 6), was diluted to 10ml with medium B' (Table 2.1.2) and subjected to

centrifugation at 100g for 5min at 15°C. Resuspension and centrifugation was repeated twice to remove any trace of Percoll™ from the cells.

2.2 ISOLATION OF ILEAL MUCOSAL CELLS.

The preparation follows the method described by Vidal et al. (1988). The ileum was removed from an adult male Wistar rat and washed with ice-cold 0.9% (w/v) saline. Both ends of the ileum were ligated and buffer containing: 120mM NaCl, 25mM $NaHCO_3$, 4.5mM KCl, 1.8mM Na_2HPO_4, 0.2mM NaH_2PO_4, 10mM HEPES, 5mM EDTA, 1mM dithiothreitol and 0.25g/100ml bovine serum albumin, pH7.4 gassed with 95% O_2 / 5% CO_2 (v/v) was injected into the lumen with a syringe. The sac was placed in a beaker containing 100ml of buffer A which was composed of: 120mM NaCl, 25mM $NaHCO_3$, 4.5mM KCl, 1.8mM Na_2HPO_4, 0.2mM NaH_2PO_4, 10mM HEPES, pH7.4, gassed with 95% O_2 / 5% CO_2 (v/v) and gently agitated in a water bath at 37°C for 30min. Next the ileum was drained and the contents kept on ice. The sac was then tied off and refilled with buffer A, placed on a glass plate over ice and gently massaged. The solution was drained out and the contents mixed with the first batch. The pooled batches were subjected to centrifugation for 10min at 200g. A portion of the cells were removed for the estimation of the wet and dry weights and to the rest was added 3 volumes of homogenisation buffer. The cells were freeze fractured as described for section 5.1.1.1 and the homogenate then underwent centrifugation at 12,000g for 20min at 4°C. The dry weight was estimated by adding the cells to a preweighed vial. drying at 80°C until constant weight was reached and then subtracting the final weight of the vial. Dry weight, rather than cell number, was used as an estimate of the quantity of cells because clumping of cells precluded counting on a haemocytometer.

2.3 ISOLATION OF COLONIC MUCOSAL CELLS.

The procedure used is essentially that of Roediger and Truelove (1979). Two animals were killed by cervical dislocation and the colons removed (from the caecum to the rectum). Each colon was flushed through with ice-cold 0.9% (w/v) saline, ligated at one end and everted. The colons were filled with medium B (Table 2.1.3) and the other end ligated. The colons were placed in a flask containing medium C (Table 2.1.3) and incubated at 37°C, under 95% O_2 / 5% CO_2 (v/v), in a shaking water bath (at 60-70 oscillations/min) for 30min. The colons were rinsed in medium A (Table 2.1.3) to remove any EDTA and placed in a beaker of medium B. The solution was stirred with a magnetic follower for 2min to detach the cells and the cell suspension then split into four equal parts and subjected to centrifugation for 5min at 295g. One of the pellets was retained for the estimation of wet and dry weight and the other three pellets were resuspended in 10ml of medium D (Table 2.1.3). The suspension was subjected to centrifugation for 5min at 295g and the pellet resuspended in homogenisation buffer at 5ml of buffer for every 1g wet weight of tissue. The suspension was the freeze-thawed three times in liquid nitrogen and spun at 12,000g for 20min at 4°C.

Table 2.1.1 Composition of Eagle's Minimum Esential Medium.

The medium was purchased in powder form (Sigma) and had the following composition:

L-Arginine	0.7mM
L-Cysteine	0.23mM
L-Glutamine	2.0mM
L-Histidine	0.27mM
L-Isoleucine	0.4mM
L-Leucine	0.4mM
L-Lysine	0.5mM
L-Methionine	0.09mM
L-Phenylalanine	0.2mM
L-Threonine	0.4mM
L-Tryptophan	0.05mM
L-Tryrosine	0.25mM
L-Valine	0.4mM
Choline chloride	7.0μM
Folic acid	3.0μM
Myo-inositol	0.01mM
Niacinamide	8.0μM
D-Pantothenic acid Ca	2.1μM
Pyridoxal HCl	4.86μM
Riboflavin	0.3μM
Thiamine HCl	3.0μM
$CaCl_2$	1.8mM
KCl	5.4mM
$MgSO_4$	0.8mM
NaCl	116.4mM
NaH_2PO_4	1.0mM
D-Glucose	5.6mM
Phenol Red Na	0.001% w/v

Powdered medium was dissolved in 1l of double distilled water with 25mM $NaHCO_3$ and 20mM HEPES (final concentration). The medium was stirred for 30min at room temperature whilst gassing with 5% CO2 / 95% O_2 (v/v) and the pH was then adjusted to 7.4.

Table 2.1.2 Composition of Media for Isolation and Enrichment of Gastric Mucosal Cells.

To EMEM plus 20mM HEPES (Table 2.1.1) the following additions were made:

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

Table 2.1.3 Composition of media used in the Isolation of Colonocytes.

Medium A

NaCl	120mM
KCl	4.5mM
$MgSO_4$	1.0mM
Na_2HPO_4	1.8mM
NaH_2PO_4	0.2mM
$NaHCO_3$	25mM

Dissolve in 0.9% (w/v) saline.

Medium B

As medium A, except:

Bovine Serum Albumin	0.25% (w/v)

Medium C

As medium B, except:

EDTA	5mM

Medium D

As medium D, except:

$CaCl_2$	1.25mM

All media gassed with 5% CO_2 / 95% O_2 (v/v) and pH adjusted to 7.4.

2.4 GEL ELECTROPHORESIS.

2.4.1 Gel preparation and electrophoresis.

Proteins were resolved on an 8% sodium dodecylsulphate-polyacrylamide separating gel and a 3% stacking gel (Table 2.4.1). The separating gel was introduced between two 20cm x 20cm glass plates 0.75cm apart and poured to a height of 12cm. 1cm of a mixture of distilled water and methanol (1:1) was layered over the top and the gel left to polymerise. The overlay was removed, the stacking gel poured on top and a comb inserted to produce the wells. This too was left to polymerise and then the comb removed. Wells were filled with precooled running buffer (Table 2.4.1) and 10µl of "rainbow" molecular mass marker, diluted 1:1 with 2 x sample buffer (Table 2.4.1) and then boiled for 2min and cooled, was placed in the end well. 50µl of each sample (in sample buffer) was then loaded into each remaining well. The upper reservoir was placed on top of the glass plates and filled with running buffer. The whole assembly was then placed into the tank of running buffer at 10°C and electrophresis performed at a constant current of 25mA with a maximum voltage of 500V until the bromophenol blue marker was approximately 1cm from the bottom of the gel.

2.4.1 Electrophoretic Transfer.

Proteins were transferred from the SDS-polyacrylamide gel onto Hybond C-Super nitrocellulose paper by electrophoretic transfer. A transfer unit was set up consisting of nine filter papers, the nitrocellulose, the gel and three more filter papers. The whole assembly was soaked completely in electrode solution (Table 2.4.1) and placed on the graphite anode which had been soaked with distilled water. The cathode plate (which had also been soaked with distilled water) was placed on top

of the pile and the current set at a constant 0.8mA/cm^2 of gel. The transfer unit was left to run for 1h after which the nitrocellulose paper was removed. Efficiency of transfer was estimated visually by the movement of the "rainbow" molecular mass markers from the gel onto the nitrocellulose.

Table 2.4.1 Stock Solution for Electrophoresis.

Electrophoresis Sample Buffer (x2).

0.125M	Tris base
20% v/v	Glycerol
4%	Sodium dodecyl sulphate
0.2M	Dithiothreitol
0.025mg/ml	Bromophenol blue

Separating Gel.

0.375M	Tris base pH 8.8, 4°C
0.1% w/v	Sodium dodecyl sulphate
8% w/v	Methylenebisacrylamide/acrylamide (1:37.5)
0.5mg/ml	Ammonium persulphate
0.0375% v/v	TEMED

Stacking Gel.

0.125M	Tris base pH 6.8, 4°C
0.1% w/v	Sodium dodecyl sulphate
3% w/v	Methylenebisacrylamide/acrylamide (1:37.5)
0.5mg/ml	Ammonium persulphate
0.05% v/v	TEMED

Running Buffer.

3.03g/l	Tris base
14.4g/l	Glycine
1g/l	Sodium dodecylsulphate

Electrode Solution.

48mM	Tris base pH 7.5, 4°C
39mM	Glycine
0.0375% w/v	Sodium dodecylsulphate
20% v/v	Methanol

2.5 MODIFIED FOLIN-LOWRY PROTEIN ASSAY.

Homogenates were diluted in 1% (w/v) sodium dodecylsulphate / 0.4% (w/v) sodium hydroxide. 100 volumes of reagent A (2.5% (w/v) sodium carbonate, 0.5% (w/v) sodium hydroxide, 0.2% (w/v) sodium tartrate and 1.25% (w/v) sodium dodecylsulphate) was mixed with 1 volume of reagent B (5% (w/v) copper sulphate) to form reagent C. 0.4ml of diluted homogenate was added to 0.6ml of reagent C, mixed and left for 10min. 75µl of Folin-Ciocalteu's reagent (diluted 1:1 with distilled water) was added, the solution mixed immediately and left for 45min at room temperature. Absorbance of solutions was determined at 750nm using distilled water as a reference. The blank contained 0.4ml of a mixture of homogenisation buffer and 1% (w/v) sodium dodecylsulphate / 0.4% (w/v) sodium hydroxide and was subtracted from all sample and standard results. A standard curve was generated each time by making up solutions of bovine serum albumin from 0 - 20µg/ml and assaying them as above.

CHAPTER 3.

THE REGULATION OF GROWTH IN HGT-1 CELLS.

3.0 INTRODUCTION.

3.0.1 General considerations for survival of cells in culture.

There are four main influences on the survival of cells in culture: a) the material on which the cells grow, b) the gaseous phase, c) the temperature and d) the culture medium (Freshney, 1987). Cells normally grow on a solid matrix, although there are some in which growth is anchorage independent There are a variety of substrates used for cell adhesion with the most popular being polystyrene. The density at which cells are seeded onto their support also influences their growth rates. If seeding densities are too high then overcrowding occurs and this can inhibit cell growth, on the other hand if the density is too low then cells often grow poorly, probably due to a low concentration of autocrine growth stimulants.

The oxygen requirement of different cell lines varies but usually a partial pressure close to atmospheric values is adequate. The temperature at which a cell culture is grown is usually close to the body temperature of the animal from which it was derived. For human cells normally a temperature around 36.5°C is used as this allows for small rises in the incubator temperature, and this was the temperature used with the HGT-1 cells. Elevations above 37°C are less well tolerated than a decrease in temperature.

The composition of the medium is probably the single most important factor in cell culture. Cell lines vary quite dramatically in their

requirements for growth. Most need a pH of around 7.4 and this is usually buffered to mop-up carbon dioxide and lactic acid. Cells have a fairly wide tolerance to the osmolarity of the medium and viscosity also has little effect on growth. The vital factor is the actual composition of the medium. A medium can be simple, which means that it just contains amino acids, vitamins and salts (eg. Eagle's minimum essential medium) or complex with extra metabolites and minerals (eg. RPMI 1640). Dulbecco's modified Eagle's medium (DMEM) was used to culture HGT-1 cells, as this was the medium used by Laboisse (1982), and in this medium nutrient concentrations are high. The concentration and balance of amino acids can limit the growth rate and 2mM L-glutamine is present in the medium used to culture HGT-1 cells. The salts used commonly in different media include the ions, Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}, PO_4^{3-} and HCO_3^-. These are all present in DMEM but it has especially high HCO_3^- requiring gassing with 10% CO_2. Glucose is often added to provide energy for the cells and in DMEM it is at a concentration of 4500mg/l. Nucleosides and lipids are sometimes added and DMEM has linoleic acid. Serum, in the case of HGT-1 cells foetal calf serum, is usually required for attachment and growth. Serum contains a wide range of substances including proteins, polypeptides and hormones, metabolites, nutrients and minerals, such as iron, copper, zinc and selenium. There are obvious disadvantages in using serum in that it has an undefined composition which can vary from batch to batch and over time. The alternative is to use a serum-free medium with supplements such as adhesion factors, protease inhibitors, hormones, growth factors, nutrients, proteins and polyamines, but this does not contain the undefined entities in the serum which can exert an influence over growth.

3.0.2 Growth Factors.

Growth factors are responsible for proliferation, differentiation, development, inflammation and repair. Their actions can be classed as one of two types: either autocrine or paracrine. Paracrine agents diffuse to targets on cells close to the producing cell, whilst autocrine agents affect the producing cell itself. Once at their sites of action trophic agents exert three effects: i) they promote amino acid uptake, ii) they increase protein, RNA and DNA synthesis and, iii) they decrease protein catabolism (Johnson, 1987). There are a large family of these polypeptide growth factors and many of them are structurally related, for example, transforming growth factor (TGF) shows a marked homology with epidermal growth factor (EGF).

Growth factors play a very important role in neoplastic growth as well as in that of normal cells. There are several places in the growth factor cascade where an aberration could lead to neoplasm. For example, an increased number of growth factor receptors or a change in their affinity for agonists could lead to a greater intracellular stimulus. A change in the receptor structure itself could lead to a receptor being permanently "switched on". The v-*erb* B oncogene is a good example of this as it codes for a truncated, and consequently constantly active, EGF receptor (Sorrentino, 1989). Another reason for excessive growth could be the over-expression of the growth factor itself when the cell receptors would be constantly saturated with the peptide.

Many growth factors activate receptors with tyrosine kinase activity. The molecular mechanism by which these agents act is now close to being established (fig 3.1). When a ligand binds to a receptor with tyrosine kinase activity it promotes its interaction with another receptor to form a dimer. This dimerisation causes the stimulation of tyrosine kinase

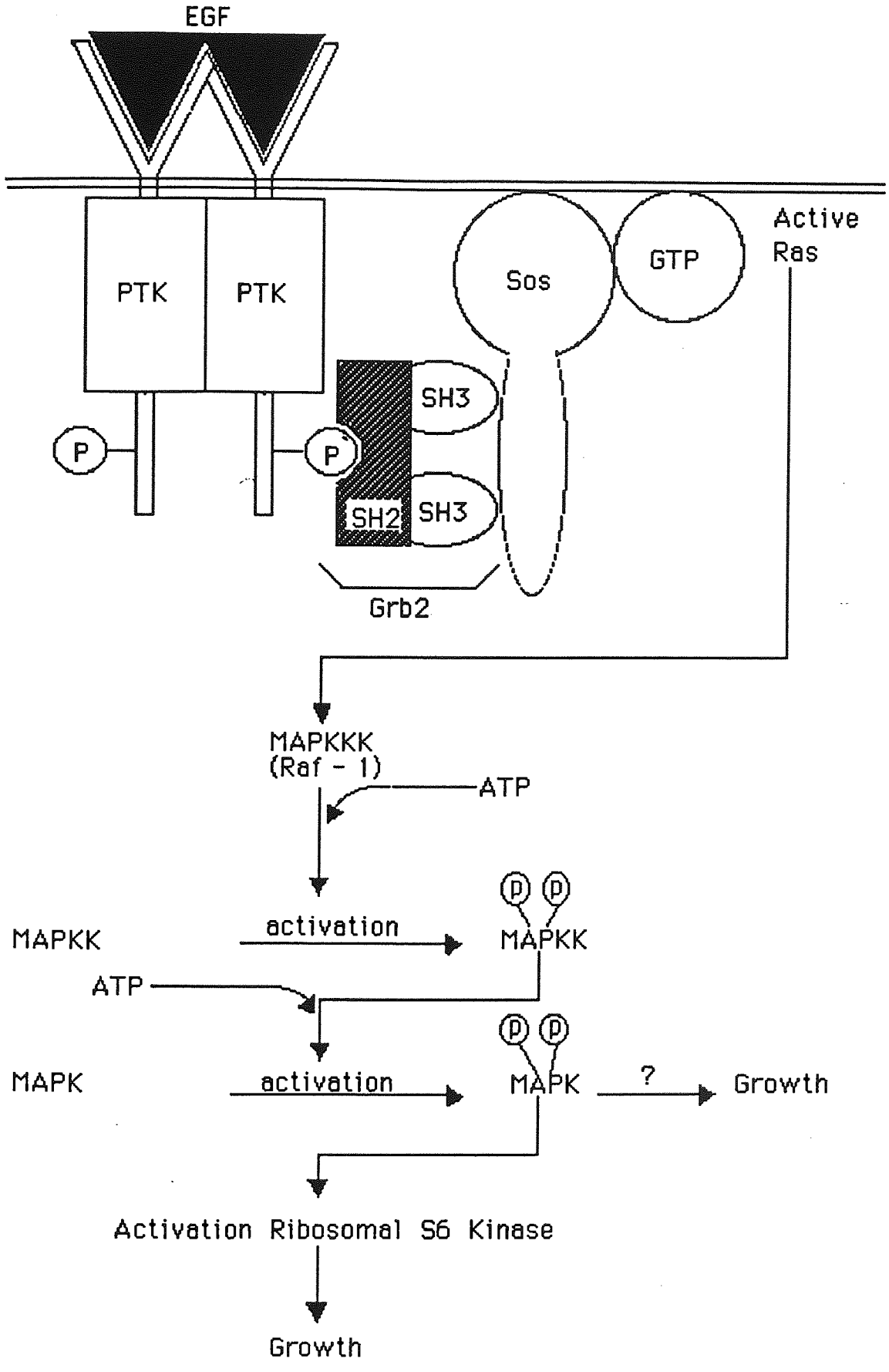


Fig. 3.1 A model of the EGF receptor second messenger pathway as an example of signalling through tyrosine kinase receptors (Schlessinger,1993 and Nishida & Gotoh, 1993).

activity and subsequent transphosphorylation of the receptor complex. The phosphorylation takes place on a number of tyrosines (in the EGF receptor there are at least five, one of which is tyrosine residue 1068) and one of these is contained in an area which is an high affinity binding site for the SH2 (*src* homology) domain of signalling molecules. The specificity of this binding site depends on the amino acids situated around the relevant tyrosine and in human cells the site is specific for an adapter protein known as Grb2. The Grb2 protein contains two other regions known as SH3 domains which it uses to form a complex with another molecule called Sos1. Sos1 is a guanine nucleotide exchange factor and the formation of the receptor/Grb2/Sos1 complex brings Sos1 into close contact with the plasma membrane. In the membrane is found the Ras protein, this is only active when in its GTP-bound form and this requires the presence of a guanine nucleotide exchange factor such as Sos1 (Schlessinger, 1993). Once stimulated Ras promotes a further cascade whereby it actives the enzyme MAP kinase kinase kinase (Raf-1), this activates MAP kinase kinase and this in turn activates MAP kinase which is either responsible directly for growth or it goes on to activate ribosomal S6 kinase which promotes growth. MAP kinases require a dual phosphorylation with a threonine residue being phosphorylated as well as a tyrosine (Nishida & Gotoh, 1993). The insulin receptor cascade shows a slightly different pathway in that there are two extra proteins involved. These are termed Shc and tyrosine phosphorylated insulin receptor substrate 1 and these interact with Grb2 (Schlessinger, 1993).

3.0.3 Growth factors in the stomach.

There are a wide variety of factors which influence trophic activity in the gastrointestinal tract (table 3.1). Two growth factors which appear to be particularly important in the stomach are EGF and gastrin.

TABLE 3.1 Major Growth Factors in the Gastrointestinal Tract (Johnson, 1987).

Mitogen	Trophic effect in:-
Growth hormone	Gastric mucosa Crypt cells of duodenum
Thyroxine	Crypt cells of duodenum
Gastrin	Oxyntic gland stem cells
Cholecystokinin	Pancreas
Secretin	Pancreas
Enteroglucagon	Small intestinal mucosa
Glucagon	Oxyntic gland Colonic mucosa
Bombesin	Oxyntic gland Colonic mucosa
EGF	Oxyntic gland Duodenal crypt cells Ileal crypt cells Colonic crypt cells

EGF was first discovered for its ability to induce premature eyelid opening in newborn mice (Cohen, 1962) and was later shown to be a strong mitogen for both epithelial and mesenchymal cells (Carpenter, 1987). It is a polypeptide of 53 amino acids, molecular weight 6045, (Savage et al., 1972) which is released from cells after cleavage from a precursor (Sorrentino, 1989). It is synthesised at several anatomical sites in the body and in the gastrointestinal tract is found in the salivary glands and Brunner's glands of the duodenum (Lemoine et al., 1992). EGF receptors are expressed in normal epithelial cells of the gastrointestinal tract and are found in the orthopharynx, the ducts of the salivary glands, the oesophagus and the mucous neck cells and parietal cells of the gastric glands. EGF receptors are often over-expressed in gastric cancers but it also requires the presence of an activating factor to make cells malignant (Lemoine et al., 1992).

Partially homologous with EGF is transforming growth factor α (TGFα) which was first found in the medium of murine 3T3 fibroblasts that had been transformed with Moloney murine sarcoma virus. TGFα was found to have the ability to bind to the EGF receptor and cause cell growth. TGFα was later discovered to be present in many normal tissues and significant amounts in the gastrointestinal tract were suggested to be involved with self-renewal (Burgess, 1989). TGFα is more often produced by tumour cells than EGF (Burgess, 1989) and, as they utilise the same receptor, any effects seen with exogenous EGF in transformed cells may be the result of TGFα under normal circumstances.

Gastrin was first discovered by Edkins (1905) who produced extracts from cat antral mucosa which when administered intravenously stimulated acid secretion. Gregory and Tracy (1964) isolated pure hog gastrin, determined its structure and synthesised it. Gastrin is produced in the G cells in the pyloric glands of the antral mucosa and in the

proximal duodenum. Circulating gastrin is predominantly made up of two types; the heptadecapeptide little gastrin and big gastrin (34 amino acids). There is also a small amount of minigastrin (13 amino acids). Gastrin is synthesised initially as big gastrin and then proteolytically cleaved in the G cell at a trypsin-sensitive site to form little gastrin. Gastrin's biological action arises from the carboxy-terminal moiety and the final pentapeptide is identical to that of cholecystokinin (CCK). Gastrin release is stimulated by acetylcholine, the vagus nerve, stomach distension, ingestion of large amounts of calcium, and the breakdown products of protein digestion, as well as a peptide called gastrin releasing peptide. A negative feedback loop is involved in the regulation of release whereby a reduction in pH inhibits the release of gastrin. Other inhibitors of gastrin release include somatostatin, secretin, glucagon, vasoactive intestinal peptide, gastric inhibitory peptide and calcitonin. Gastrin can have a number of actions in the stomach including increasing cell proliferation, controlling water, electrolyte and enzyme secretion, stimulating smooth muscle, increasing blood flow and increasing the release somatostatin. Gastrin receptors are found predominantly on parietal cells but are also present on other cells of the fundic mucosa (D-cells and histamine-containing cells). Stimulation of the gastrin receptor leads to an increase in the intracellular free calcium concentration. This is now thought to be due to an increase in phosphoinositide turnover (Roche et al., 1989). Gastrin may play an important part in the growth of gastric cancers as it both increases the growth rate of primary gastric cell cultures (Watson et al., 1989) and has a trophic effect on established cell lines such as MKN45 (Watson et al., 1989b) and AGS-P (Ishizuka et al., 1992). MKN45G, a clonal variant of MKN45 which is insensitive to stimulation by gastrin, has been shown to possess intracellular gastrin in the heptadecapeptide form (Watson et al., 1991). The case for gastrin as an autocrine growth factor is enhanced by the fact that its gene is expressed at the peptide level in several other cell types, such as the TG cells of the small intestine. In addition gastrin is found in malignant

types, such as the TG cells of the small intestine. In addition gastrin is found in malignant growths such as pituitary adenomas, bronchogenic carcinomas and colorectal carcinomas (Rehfeld et al., 1992). Although the concentrations of gastrin in these cells is too small to ever be detected in the plasma it is possible that the hormone has an autocrine function.

The aim of this section was to determine what factors were important for the growth of the HGT-1 cell line, and to establish whether the line might be useful for examining the mechanisms involved in the regulation of the growth of gastric cells.

3.1 METHODOLOGY.

3.1.1 Preparation of Multiwell Culture Plates.

HGT-1 cells were grown as described in section 2.0.1 until confluent when they were detached from the flask using the aforementioned trypsin/EDTA solution (see section 2.0.1). Cells were diluted with culture medium and 3ml of this solution was placed into each well of a 6 well multiwell plate. For growth studies the medium was changed every 48h.

3.1.2 Preparation of Conditioned Medium.

HGT-1 cells were grown as described in section 2.0.1 for 48h. The spent medium was removed from the flask and frozen at -20°C until required. Before using, conditioned medium was diluted with fresh culture medium in the ratios 20ml conditioned medium : 80ml fresh medium and 50ml conditioned medium : 50ml fresh medium. HGT-1 cells were diluted directly in the different concentrations of conditioned medium before seeding into multiwell plates as described above.

3.1.3 Addition of Peptides.

Multiwell culture plates were seeded as described above and the cells left to adhere for 4h. After this time the medium was removed and fresh medium containing either 1.65nM EGF (receptor grade, from mouse submaxillary glands), 0 - 1.74μM insulin (from bovine pancreas) or 0 - 100nM [Leu^{15}]-Gastrin I (human sequence) was added.

3.1.4 Cell Counting.

Cells were detached from their multiwell culture plates using trypsin/EDTA solution (section 2.0) and gentle agitation. Cells were then diluted 1:1 in trypan blue solution and counted in an Haemocytometer (section 2.0.5). The number of cells in the medium after 4h of incubation (section 3.1.3) was counted to give a measurement of how many cells had attached.

3.2 RESULTS.

3.2.1 The effect of seeding density on the increase in cell number of HGT-1 cells.

When HGT-1 cells were at seeded at $1.35x10^5$ cells/well an increase in cell number with time was evident (fig. 3.2.1). There was a lag phase which lasted for 24h and this was followed by a period of rapid increase in cell number. During the increase a population doubling time of 22.5h was measured. When the cells were plated at $3.6x10^4$ cells/well no increase in cell number was evident, even after 170h.

3.2.2 The effect of serum concentration on the increase in cell number of HGT-1 cells seeded at low density.

When HGT-1 cells were plated at low density ($3x10^4$ cells/well), increasing the concentration of FCS from 5% to 10% did not result in an increase in cell number (fig. 3.2.2).

3.2.3 The effect of conditioned medium on the increase in cell number of HGT-1 cells seeded at low density.

The addition of conditioned medium to HGT-1 cells plated at low density ($2.1x10^4$ cells/well) caused an increase in cell number. The cells plated in a mixture of 50% conditioned medium with 50% fresh medium exhibited the greatest increase in cell number ($P<0.01$) whereas the cells in the 20% conditioned medium:80% fresh medium mixture showed a lesser but still significant increase ($P<0.05$, data analysed by one way analysis of variance plus a Dunnett's test) (fig. 3.2.3).

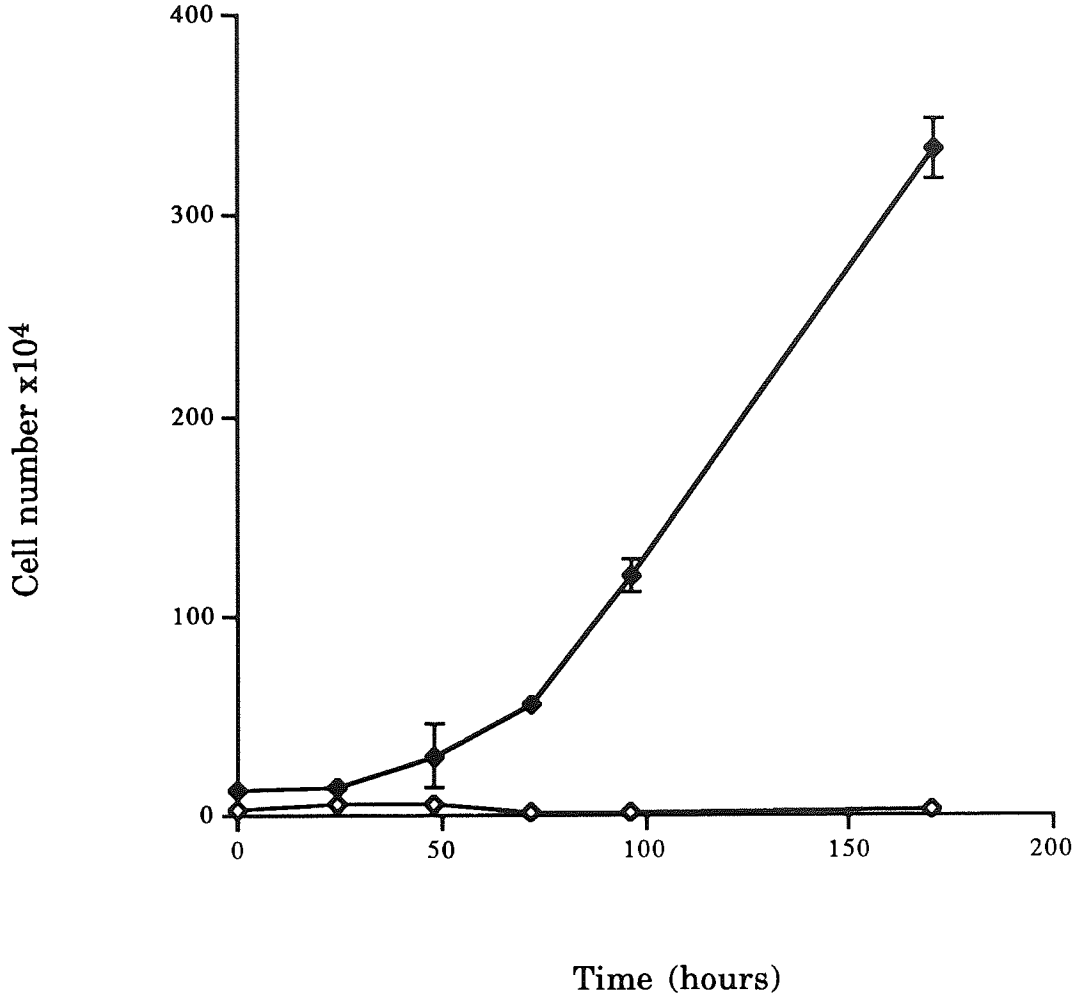


Fig 3.2.1 The effect of plating density on the growth of HGT-1 cells. (—◆—) cells plated at 1.35 x 10^5 cells/well, (—◇—) cells plated at 3.6 x 10^4 cells/well. Results expressed as mean± SEM of triplicate determinations for a single experiment which was typical of two others. Population doubling time was 22.5±2.3h, calculated by using the values at 72h and 96h.

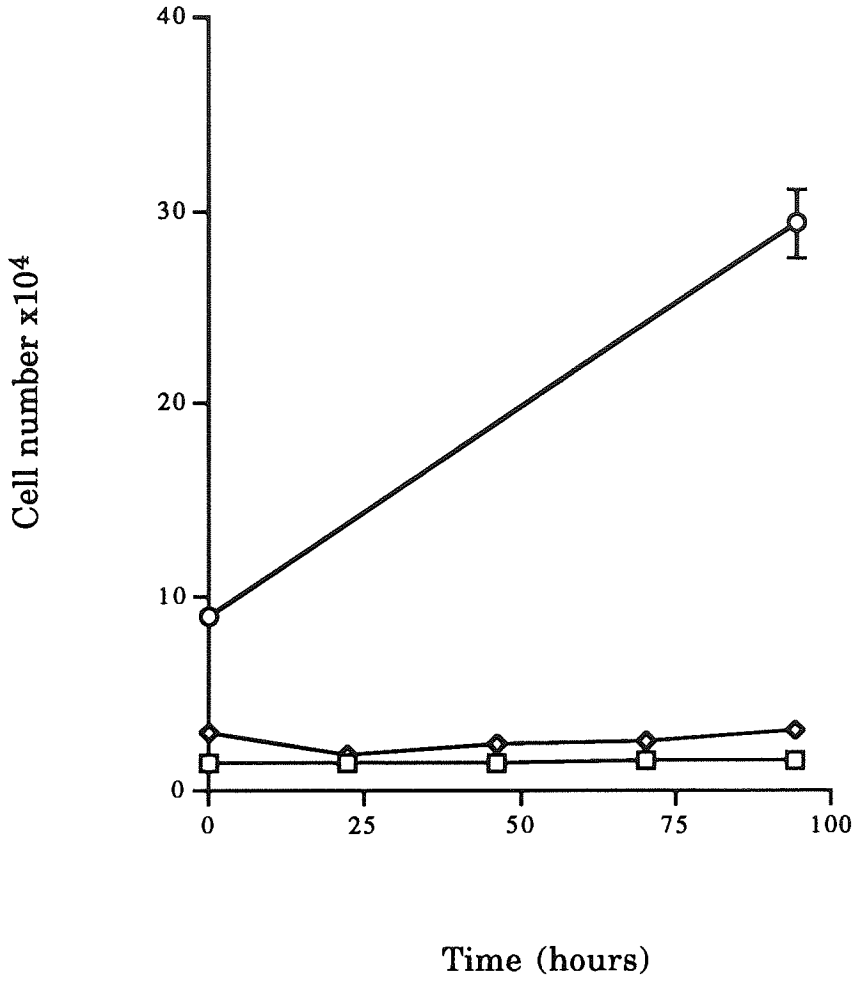


Fig 3.2.2 The effect of serum concentration on the growth of HGT-1 cells plated at low density.

(—□—) cells grown in 5% FCS and plated at 1.5 x 10^4 cells/well.

(—◇—) cells grown at 10% FCS and plated at 3 x 10^4 cells/well.

(—○—) cells seeded at 9 x 10^4 cells/well and grown in 5% FCS.

Results expressed as mean ± SEM of three determinations from a single experiment.

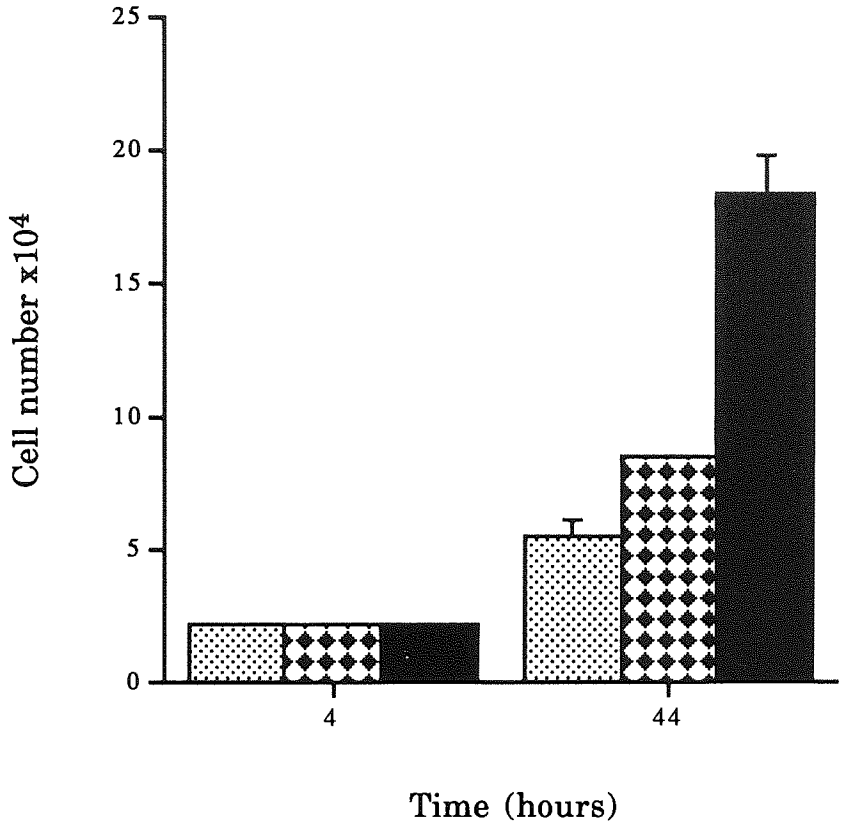


Fig 3.2.3 The effect of the concentration of conditioned medium on the growth of HGT-1 cells.

Control cells (⊡), cells grown in conditioned medium diluted 20:80 with normal culture medium (⊠) and cells grown in conditioned medium diluted 50:50 with normal culture medium (■) were all plated at 2.1×10^4 cells/well. Results are means±SEM (where visible) of triplicate determinations from a single experiment.

3.2.4 The effect of peptides on the increase in cell number of HGT-1 cells seeded at low density.

10nM gastrin, 1.65nM epidermal growth factor and 1.74μM insulin all produced a significant increase in cell number ($P<0.01$, analysed by one way analysis of variance and a Dunnett's test) over control cells (fig. 3.2.4).

3.2.5 The effect of gastrin on the increase in cell number of HGT-1 cells seeded at low density.

HGT-1 cells plated at low density ($2.1x10^4$ cells/well) showed a bell-shaped dose-response curve to the concentrations of gastrin used (fig. 3.2.5). The peak appeared at 5nM gastrin and concentrations above 25nM showed no increase in cell number.

3.2.6 The effect of gastrin on the increase in cell number of HGT-1 cells seeded at high density.

HGT-1 cells plated at high density (greater than 1×10^5 cells/well) did not show an increase in cell number with increasing concentrations of gastrin (fig. 3.2.6).

3.2.7 The effect of insulin on the increase in cell number of HGT-1 cells seeded at low density.

HGT-1 cells plated at low density (2.7×10^4 cells/well) showed an increase in cell number with increasing concentrations of insulin (fig. 3.2.7). Cell number remained unchanged after 174nM insulin.

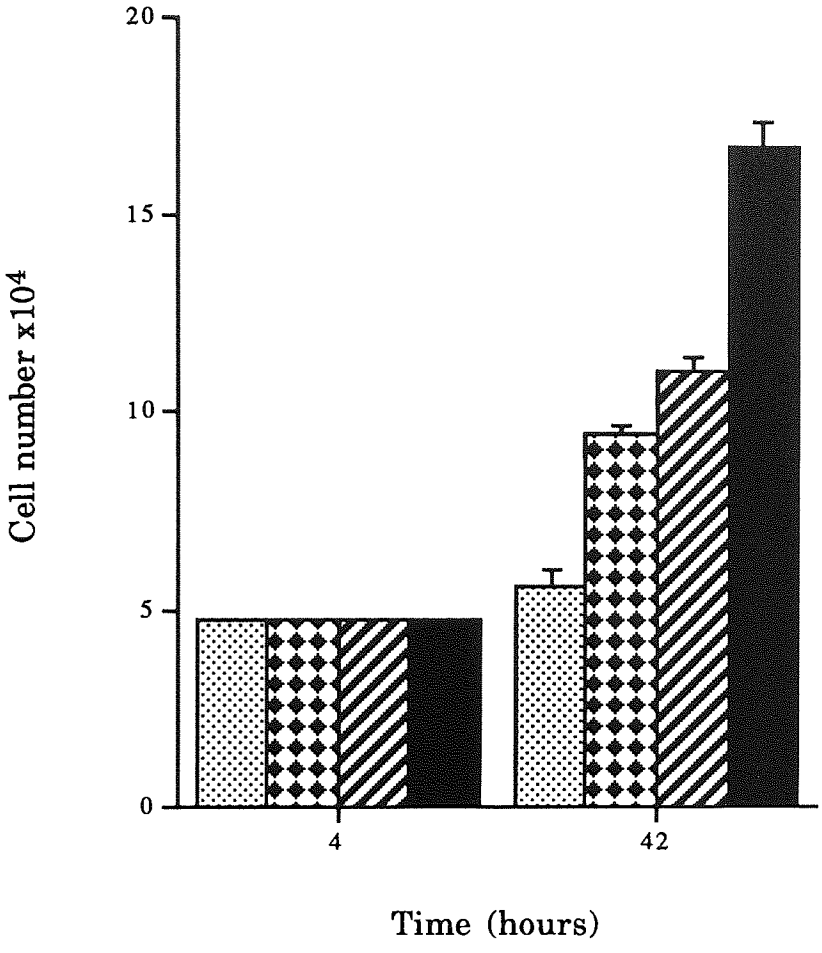


Fig 3.2.4 The effect of different peptides on the growth of HGT-1 cells. Control cells (⬚), cells grown with 10nM gastrin (⬚), cells grown with 1.65nM EGF (⬚) and cells grown with 1.74nM insulin (■) were all plated at 4.8×10^4 cells/well. Results expressed as mean ± SEM (where visible) of triplicate determinations from a single experiment.

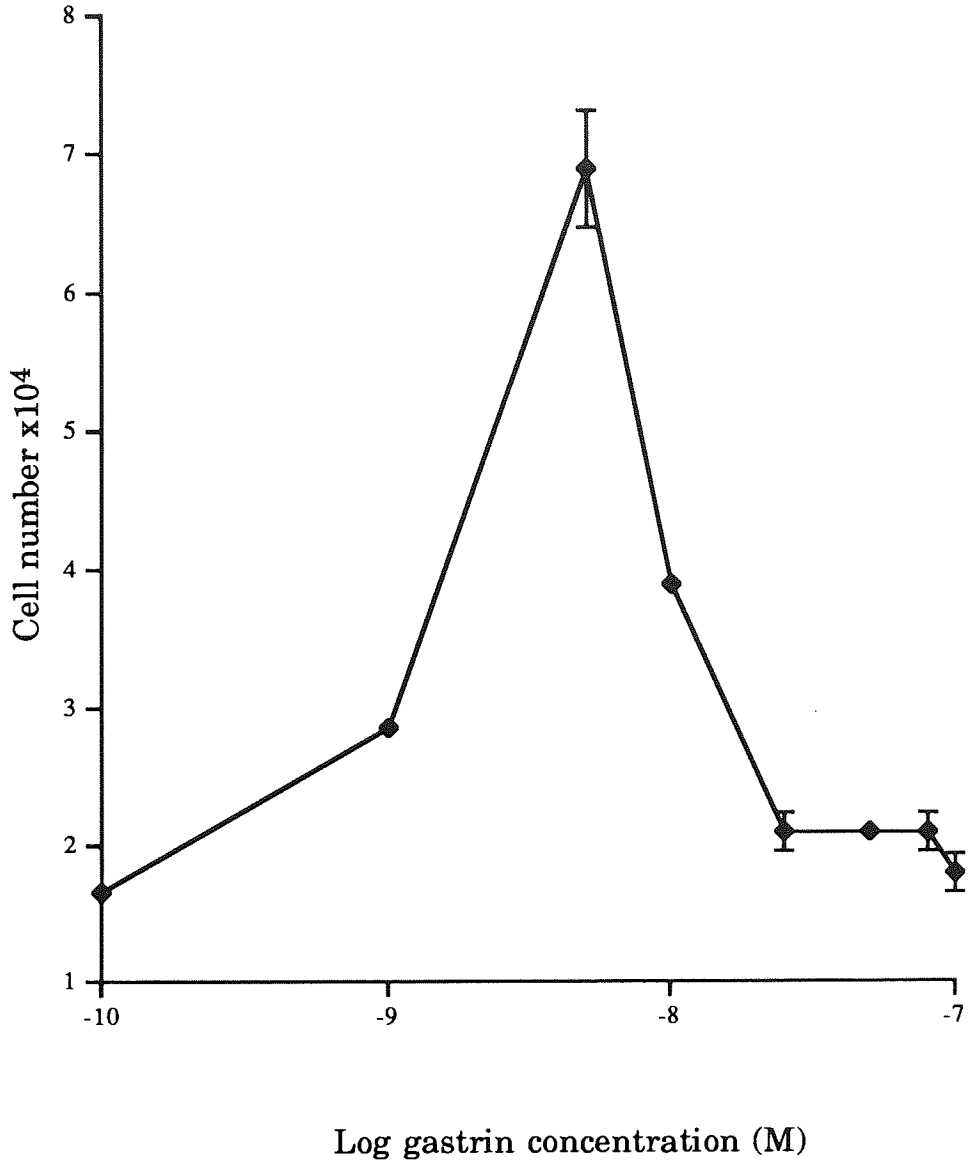


Fig 3.2.5 The effect of varying concentrations of gastrin on the growth of HGT-1 cells.

Cells were plated at 2.1×10^4 cells/well and grown for 48h. Results expressed as mean ± SEM of triplicate determinations for a single experiment which was typical of three others. Control values in the absence of gastrin were $2.1 \pm 0.14 \times 10^4$ cells/well.

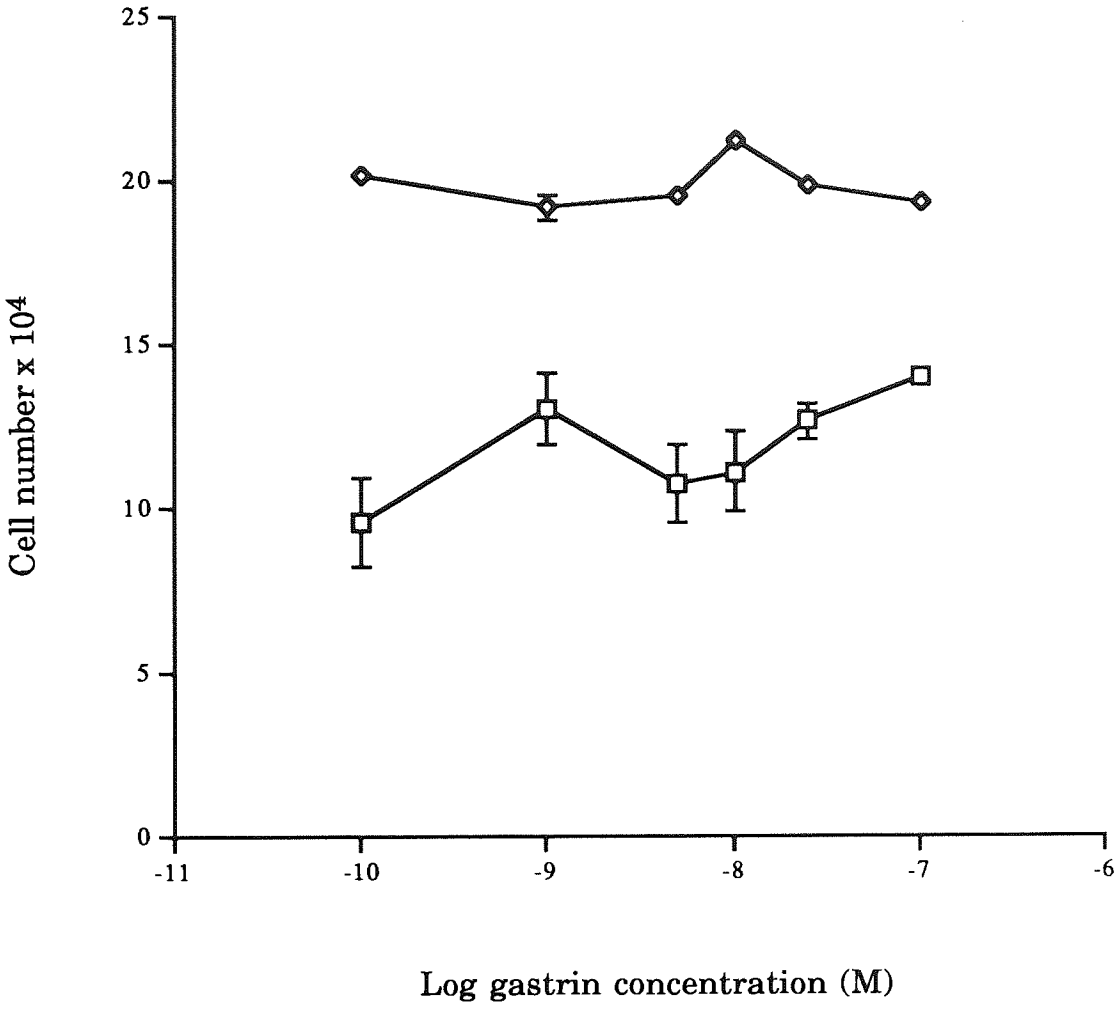


Fig 3.2.6 The effect of varying concentrations of gastrin on the growth of HGT-1 cells plated at high density.

(—□—) cells plated at 9.99 x 104 cells/well. Control value in the absence of gastrin was 1.19 x 105 cells/well. (—◇—) cells plated at 1.71 x 105 cells/well. Control value in the absence of gastrin was 2.26 x 105 cells/well. All cells were grown for 48h. Results expressed as mean± SEM of triplicate determinations for two separate experiments.

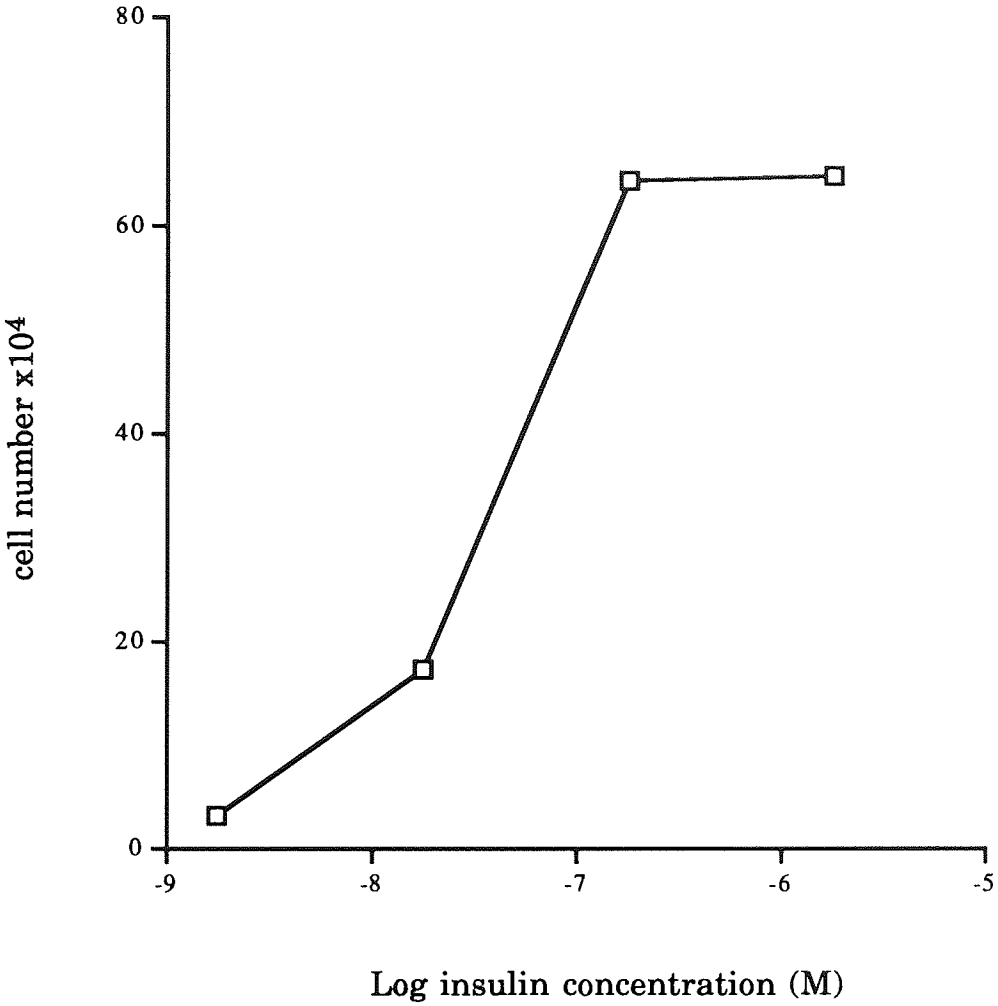


Fig 3.2.7 The effect of varying concentrations of insulin on the growth of HGT-1 cells plated at low density.

Cells were plated at 2.7 x 10⁴ cells/well and grown for 48h. Results expressed as mean ± SEM of triplicate determinations from a single experiment. Control value in the absence of insulin was 6.1 ± 0.91 x 10⁴ cells/well.

3.3 DISCUSSION.

The population doubling time of HGT-1 cells plated at 1.35×10^5 cells/well was 22.5h. This is comparable with the time estimated by Laboisse (1982) who found that cells at passage 40 doubled in 27.86 ± 2.71hr and cells at passage 59 did so in 19.48 ± 1.2hr. A normal cell cycle from G1 through S, G2 and M can take anything from 10hr to 30hr (Sorrentino, 1989) and so HGT-1 cells are well within this range.

HGT-1 cells did not appear to increase in number when plated at low density. Many cultures fail to grow when plated below a certain density (Torado et al., 1963) reverting to a quiescent phase of the cell cycle. Whether cells become quiescent is dependent on the availability of nutrients, cell size, density and the presence of growth factors (Sorrentino,, 1989).

HGT-1 cells seeded at low density did not appear to increase in cell number when the concentration of FCS was raised to 10%. Hassell et al. (1977) showed that growth of Vero cells was dependent on the concentration of serum present rather than the area available for growth. This does not seem to be the case for HGT-1 cells. HGT-1 cells were established in a medium containing 5% FCS so it is possible that only those cells capable of dividing at this concentration have been selected and therefore increasing the serum concentration will have no effect as it is already at optimal. Whatever growth stimulus is missing from HGT-1 cells grown at low density does not appear to be in serum in sufficient concentration at 10% FCS to increase cell number.

HGT-1 cells show an increase in cell number with increasing concentrations of conditioned medium. Rein (1971) put this phenomenon down to what was termed a "conditioning factor". The factor was possibly

a macromolecule produced by the cell and because of it's size was slow to diffuse away. Therefore, if the cells are close together the concentration of this factor becomes high enough to support growth, but if the cells are far apart the factor takes time to reach them. Alternatively it might be the overall concentration of the factor rather than its diffusion that was limiting. Transformed cells produce a wide variety of growth factors and so a number of peptides were investigated.

Insulin has a trophic effect on HGT-1 cells plated at low density. Insulin is a well known mitogen in the gastrointestinal tract and a number of cell lines have been used to investigate its action. Cezard (1981) found that the duodenal cell line HUTU 80 and the colonic cell line HT 29 both showed a significant growth response at physiological concentrations of insulin (0.1nM) with a maximum effect at 100nM. The maximum effect of insulin on HGT-1 cells was seen at 174nM (fig 3.2.7). Specific binding sites were also shown to be present on both of Cezard's cell lines. Whilst receptors for insulin have never been reported on HGT-1 cells this can not be ruled out. On the other hand the insulin might be binding to the receptor for insulin-like growth factor I (IGF-I). Van Wyk (1975) noted for the first time that high concentrations of insulin could act via the IGF receptor on small cell lung carcinoma. Thompson (1990) found that the gastric cancer cell line LIM-1839 possessed both IGF-I and IGF-II receptors as well as secreting IGF-II and its necessary binding proteins. Upon further investigation it was found that the IGF-I receptor was producing the pleiotrophic response. The human pancreatic cell line MIA-PaCa2 secretes IGF-I (Ohmura et al., 1990) into the medium and in this case it is this peptide that provides the trophic response. Nakanishi (1988) found that in small cell lung carcinoma cells IGF-I was two orders of magnitude more potent than insulin.

EGF also stimulated an increase in cell number in HGT-1 cells grown at low density. Sakai (1986) found that out of fifteen gastric cancers nine had EGF receptors. Parietal cells and mucous neck cells exhibit EGF receptors in a normal stomach (Johnson, 1987). A number of gastric cell lines including TMK-1 and MKN-28 have been shown to produce mRNA for EGF and there is often an amplification of the gene for the EGF receptor (Yoshida et al., 1990). Once operative the EGF receptor activates the enzyme ornithine decarboxylase, via tyrosine kinase, which, in turn, activates RNA polymerase (Johnson, 1987).

The trophic effect of gastrin can be seen in TMK-1 gastric cancer cells at 10nM (Ochiai et al., 1985), MKN45 gastric cancer cells at 0.2μg/l (Watson et al., 1989), AGS-P gastric cancer cells at 1μM (Ishizuka et al., 1992), LoVo colonic cancer cells at 0.72nM (Kusyk et al., 1986) and MC-26 colonic cancer cells at 0.5nM (Guo et al., 1990). In MC-26 cells gastrin produced a significant increase in the number of gastrin receptors (Guo et al., 1990). In TMK-1 10nM gastrin increased in the cAMP level and activated cAMP-dependent protein kinase (Ochiai et al., 1985). In isolated parietal cells gastrin increased intracellular free Ca^{2+} levels (Cabero et al., 1989) and the association of protein kinase C with membranes. These changes are probably linked to phosphatidylinositol breakdown and an increase in IP_3 and DAG has been noted within 5s of gastrin being added to parietal cells (Roche et al., 1989). Kobori (1982) found a bell-shaped dose response curve to tetragastrin in BV9 rat gastric carcinoma cells. High concentrations of gastrin may down-regulate the gastrin receptor or cause excessive increases in Ca^{2+} or even the diacylglycerol produced may negatively modulate cell division.

Remy Heintz et al. (1993) found that concentrations of gastrin from 0.1 - 100nM did not stimulate the growth of HGT-1 cells which were plated at a much higher cell density ($2x10^5$ cells/well in a 12 well plate) than in this

thesis ($2.1x10^4$ cells/well in a 6 well plate). This lack of effect was reproduced in cells plated at a density of around 1×10^5 cells/well in a 6 well plate. This result could be due to the production of sufficient gastrin by the cells themselves when grown at higher densities, however no immunoreactive gastrin was found in medium from HGT-1 cells grown at high density (G. J. Dockray, personal communication). On the other hand the gastrin receptor could be similar to the EGF receptor in that the number decreases with increasing cell density (Holley, 1977). There is additional evidence for an autocrine involvement of gastrin in the growth of HGT-1 cells. 2mM proglumide (a glutaramic acid derivative that binds to the gastrin receptor) inhibited growth of HGT-1 cells plated at high density by 22%, 10nM L365,260 (a CCK B/gastrin type receptor antagonist derived from a benzodiazepine) inhibited growth by 72% but 10nM L364,718 (a CCK A receptor antagonist) had no effect. In both of the inhibitions a biphasic response was seen which indicates that the hormone is acting on binding sites of more than one affinity. A peptide detected by a rabbit antiserum specific for the C-terminal of gastrin was also found in the cells at a concentration of 1.66 ± 0.32 fmol/10^6 cells along with mRNA for a gastrin-like substance, but in agreement with our results nothing was found in the medium. It is possible that these agents are not secreted or they may remain attached to the cell exterior. No specific high affinity binding sites were found for gastrin (Remy- Heintz et al., 1993).

In conclusion, although some growth promoting agents were identified by this preliminary survey the HGT-1 cell line was found to be unsuitable as a model for the investigation of the regulation of growth of gastric cells. Effects of exogenous agents could only be found at low cell densities which would yield insufficient material for biochemical analysis of the intracellular effects of exogenous agents unless very large culture plates were used. The HGT-1 cell line is however a useful cell line for the investigation of the pathways involved in the functioning of the human

histamine H2-receptor, which is pharmacologically indistinguishable from that on human gastrin glands (Gespach et al., 1988). This feature of HGT-1 cells was therefore used in the next series of experiments (Chapter 4).

3.4 SUMMARY.

In summary a number of discoveries were made concerning the growth of the HGT-1 gastric cancer cell line. Firstly increases in cell number were dependent on the density at which the cells were seeded and this was true even at increased serum concentrations. Secondly HGT-1 cells appeared to be producing a factor, or factors, at high density which, when applied to cells at low density, encouraged growth. Insulin, EGF and gastrin all produced an increase in cell number in cells plated at low density. The dose response curve for gastrin was found to be bell-shaped with the maximum effect observed at 5nM. No effect was seen when gastrin was applied to cells plated at high density. The dose response curve with insulin reached a plateau at 174nM.

The cell line was considered unsuitable as a model for investigation of the regulation of gastric cell growth because positive results were only obtained at low cell density, when insufficient cells were available for biochemical analysis.

CHAPTER 4.

CHARACTERISATION AND FUNCTIONS OF ISOFORMS OF PROTEIN KINASE C IN GASTROINTESTINAL CELLS.

4.0 INTRODUCTION.

4.0.1 Basic Principles of Signal Transduction.

Many intercellular messengers interact at the cell surface with a receptor molecule. Once this connection has been made the message must be relayed into the cell interior and this happens in one of three ways. Firstly, the messenger can interact with a receptor that is also a channel, causing it to open and allow through certain ions. Secondly, the messenger can attach to a receptor with an intracellular domain which exhibits altered enzyme activity as a consequence of binding. Thirdly, the messenger can exert its effect via a receptor coupled to a specific membrane-bound G protein (guanine nucleotide binding protein). These G proteins serve as tranducers which can activate enzymes or ion channels. There are two main groups of target enzymes: membrane-bound adenylate cyclases which form cyclic adenosine monophosphate (cAMP) and which are affected by Gs and Gi, and the phospholipases an example of which is the phospholipase C which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to the second messengers 1,2-*sn*-diacylglycerol (subsequently refered to as diacylglycerol) and inositol-1,4,5-trisphosphate (IP_3). This enzyme appears to be activated by Gq and G_{11}.

cAMP binds to the regulatory domain of the enzyme cAMP-dependent protein kinase and thereby liberates an active catalytic region. cAMP-dependent protein kinase phosphorylates its substrate proteins thereby

activating or deactivating them. IP_3 binds to a receptor protein on part of the endoplasmic reticulum causing the release of Ca^{2+}. Ca^{2+} is responsible for activating other protein kinases, such as Ca^{2+}/calmodulin-dependent protein kinase II. Diacylglycerol produced by hydrolysis of phospholipids remains in the membrane and is also a second messenger. It binds to the enzyme protein kinase C and, along with other co-factors, activates it.

4.0.2 Protein Kinase C - General Characteristics.

Protein kinase C was identified in 1977 as a proteolytically activated protein kinase (Takai et al., 1977). It is a family of serine/threonine kinases with single polypeptide chains of molecular mass 77 - 90 kDa (Azzi et al., 1992). For activation to occur protein kinase C has to form a complex with phospholipid, typically phosphatidylserine and, for some isoforms, with Ca^{2+}. Diacylglycerol is an activator and increases the affinity of some isoforms for Ca^{2+} (Nishizuka, 1986).

There are two basic groups of protein kinase C enzymes: conventional and novel (Osada et al., 1990). The conventional group require Ca^{2+} for activation and there are four isoforms: α, βI, βII and γ. The novel forms of protein kinase C do not require Ca^{2+} and there are currently five isoforms: δ, ε (and ε' its truncated form), ζ, η (L in humans) and θ (Hug, 1993). These isoforms come from separate genes except for the βI and βII forms which arise from the differential splicing of the RNA from a single gene (Azzi et al., 1992).

Structurally protein kinase C is composed of conserved (C) and variable (V) regions (fig. 4.0.1). The conventional forms of the enzyme have four C regions and five V regions, whereas the novel forms have one less C region. Each region of the enzyme has a different function. C1, C2, V1,

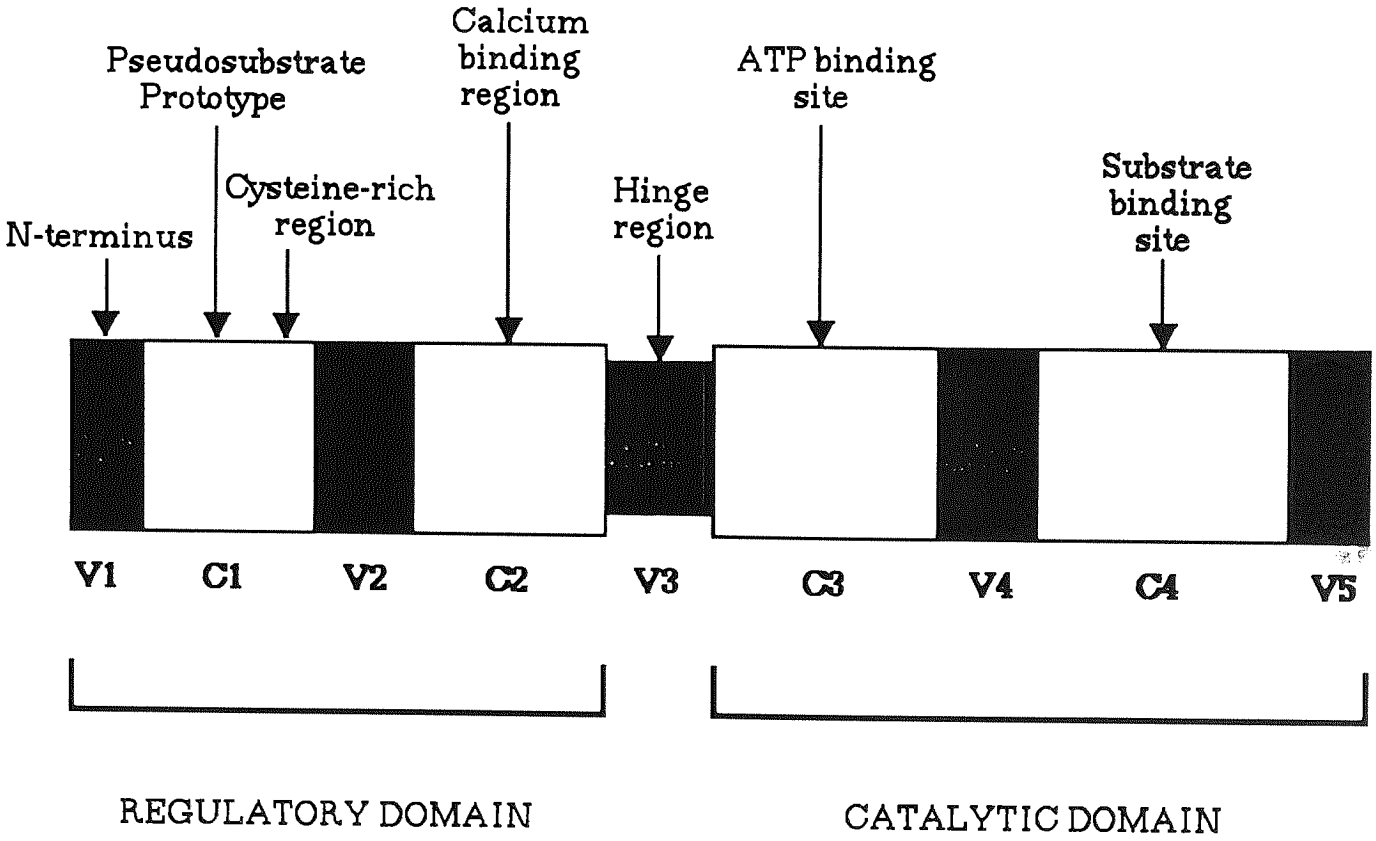


Fig. 4.0.1 The Domain Structure of Protein Kinase C
(after O'Brian and Ward 1989).

and part of the V3 domains comprise the regulatory region of protein kinase C and this is the part which interacts with Ca^{2+}, phosphatidylserine and DAG (Azzi et al., 1992). The catalytic portion of protein kinase C encompases the C3 and C4 regions. The C2 region is responsible for binding Ca^{2+}. The V3 region comprises a hinge linking the regulatory and catalytic portions and is the site of proteolysis by Ca^{2+}-activated proteases. C3 has an ATP binding site and the C4 region houses the substrate binding site and the phosphate transfer region. A cysteine rich area can be found in the C1 region and this has two zinc finger motifs. These zinc fingers are repeats of approximately 30 amino acids with a zinc atom tetrahedrally co-ordinated between pairs of invariant cysteine and histidine residues (Gschwendt et al., 1991). This area is necessary for binding of diacylglycerol and phorbol esters (Hug, 1993). Protein kinase Cζ has one of its zinc fingers missing and is therefore unable to bind diacylglycerol. It is possible that *in vivo* this isoform may be constitutively active (Hug, 1993). C1 also contains a pseudosubstrate site which has an alanine residue where, in the substrate, a serine should be (Hug, 1993). It is thought that this region may occupy the substrate binding site in the inactive enzyme.

Protein kinase C activity is ubiquitously distributed throughout the body. However, different isoforms are found in different cells, often with more than one being expressed at a time (Table 4.1).

With the conventional isoforms of protein kinase C activation in intact cells is relatively simple. IP_3 produces a rise in intracellular Ca^{2+} and this binds to the inactive protein kinase C. Once the Ca^{2+} has bound it causes the protein kinase C to be translocated to the membrane where the enzyme comes into contact with diacylglycerol and phosphatidylserine. The novel forms of protein kinase C are not dependent on Ca^{2+}, only phosphatidylserine and diacylglycerol. *In vitro* diacylglycerol binds in a

Table 4.1 The distribution of Protein Kinase C Isoforms in Tissues.

Isoform	Tissue
α	Universal.
βI	Some, not kidney.
βII	Many, not kidney.
γ	Central nervous system.
δ	Many.
ε	Brain and others, not liver.
ζ	Brain, liver.
η	Skin, lung, some in brain and spleen.
θ	Skeletal muscle, some in lung, spleen, skin and brain.

one to one relationship with protein kinase C but between 4 and 10 phosphatidylserine molecules can attach to an enzyme (Nishizuka, 1986). Breakdown of PIP_2 is not the only source of diaclglycerol. Thus agonist induced hydrolysis of other membrane phospholipids, especially phosphatidylcholine, by phospholipase D may play an important role (Nishizuka, 1992). This pathway is often activated after PIP_2 breakdown and may help to maintain activation of protein kinase C when Ca^{2+} has returned to resting levels.

Protein kinase C operates its own feedback system termed down-regulation. After activation protein kinase C is proteolytically cleaved by Ca^{2+}-dependent neutral proteases I and II (calpains) (Hug, 1993). Cleavage occurs in the V3 region and gives rise to two fragments, one being the catalytic portion (termed protein kinase M) which is now constitutively active due to the removal of the regulatory part. It is not yet clear whether protein kinase M then undergoes further proteolytic cleavage or whether it carries on as an activator-independent kinase activity in the cytosol. Constant stimulation can cause the complete disappearance of protein kinase C in some cell lines (down-regulation) (Hug, 1993).

It appears that there is no one substrate for protein kinase C but a whole host of them. There are three basic classes of protein kinase C substrate: i) proteins involved in signal transduction and protein kinase C activation, eg. the EGF receptor which is phosphorylated on threonine 654 causing a decrease in its affinity for EGF, ii) proteins involved in metabolic pathways, eg. channels and pumps and iii) proteins involved in regulatory functions concerning gene expression such as transcription and translation factors (Hug, 1993). Phosphorylation by protein kinase C confers a conformational change on the substrate allowing a change in its properties. To be classed as a physiological substrate for protein kinase C

a molecule must be rapidly phosphorylated by purified protein kinase C in a cell-free assay system and the protein should also be phosphorylated at the same site in response to physiological activators and to phorbol esters (see below) in intact cells (Hardie, 1991).

4.0.3 Protein Kinase C and Phorbol Esters.

Phorbol esters are derived from the *Euphorbaciae* family of higher plants. They are among the most potent tumour promoters and induce numerous biochemical and cellular responses which can lead to cellular proliferation and hyperplasia. The high potency of these phorbol esters has suggested that they have a specific receptor and it is now known to be protein kinase C. Phorbol esters intercalate into the plasma membrane of a cell and cause the rapid translocation of protein kinase C from the cytosol to the particulate fraction (Kraft and Anderson, 1983).

When the amount of phorbol ester binding is monitored it is found that it rapidly reaches a peak, after which it decreases. The amount of time taken for this decrease varies between cell types. In V79 lung fibroblasts 100% of binding disappears within 5h but Swiss 3T3 cells take 30h. In addition not all of the binding may disappear, in MDBK bovine kidney cells only 30% of binding could be removed with phorbol ester (Adams and Gullick, 1989). The decrease in binding seen with all of these cell lines was paralleled by a decrease in protein kinase C and this down-regulation is due to enhanced proteolytic degradation. The only isoform of protein kinase C which does not down-regulate is ζ and this is because it only has one zinc finger and therefore cannot bind phorbol esters (Liyanage et al., 1992).

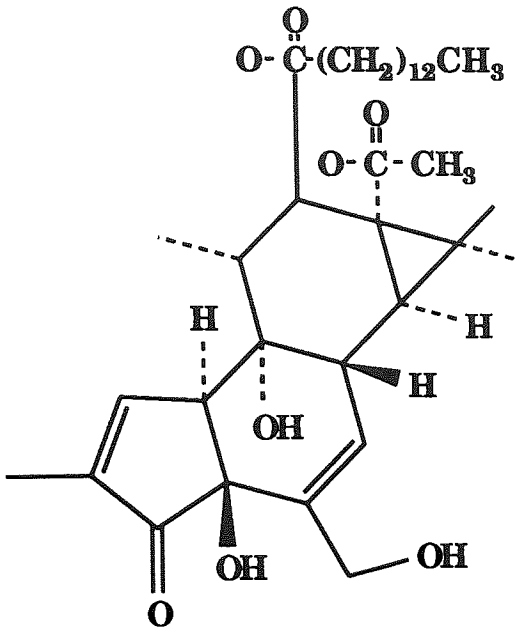
The action of phorbol esters does not have an absolute requirement for Ca^{2+} but the presence of Ca^{2+} lowers the concentration of phorbol ester

necessary for an effect. The effect observed with a phorbol ester is not completely physiological as, unlike diacylglycerol, the compounds are not metabolised in the cell and therefore there may be some distortion of normal cellular responses. It is also possible that protein kinase C is not the sole target for phorbol esters. Phorbol esters mimic the action of diacylglycerol and consequently it would follow that they had similar structural moiety. The two most widely used phorbol esters are (TPA) and phorbol dibutyrate (PDBu) (see fig 4.0.2). The latter is less potent but more easily removed from the cells by washing. The phorbol ester TPA has two possible areas where it can bind to protein kinase C and both of these are essential for activity. The probable sequence of binding is: the C3 acts as an hydrogen acceptor and the C9 and C20 act as hydrogen donors. TPA actually has a higher affinity for protein kinase C than DAG itself (Gschwendt et al., 1991). In this work binding of phorbol-12,13-dibutyrate was used as an estimate of protein kinase C.

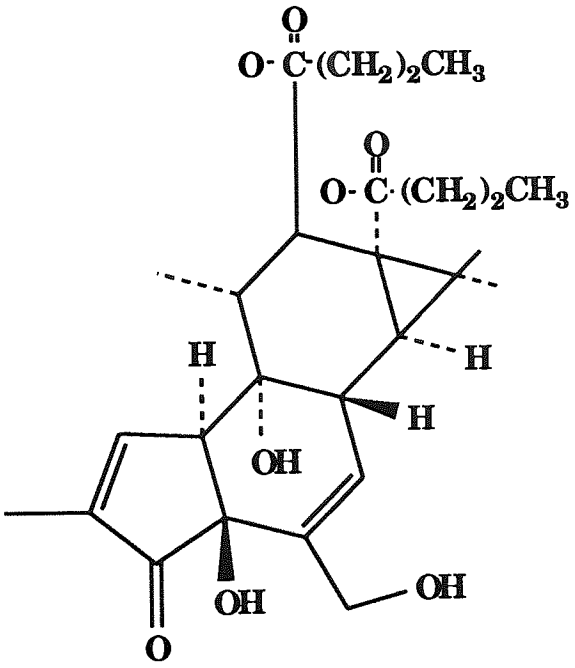
4.0.4 Intracellular "Cross-talk".

Second messenger pathways in cells are often involved in "cross-talk" with other pathways. Thus phorbol esters, and therefore probably protein kinase C, have effects on the production of second messengers. Adenylate cyclase can be both inhibited and activated by protein kinase C with the effect being dependent on cell type (Houslay, 1990). Attenuation of adenylate cyclase activity is brought about by phosphorylation by the kinase and this causes the functional uncoupling of the enzyme from the Gs subunit. Increased activity of adenylate cyclase via protein kinase C is probably due to a deactivation of the Gi subunit or an inactivation of cAMP phosphodiesterase (Houslay, 1990). Protein kinase C can also modify other pathways, for example, guanylate cyclase is phosphorylated to give increased activity. In addition protein kinase C can also activate phospholipase D leading to the hydrolysis of phosphatidylcholine to

A



B



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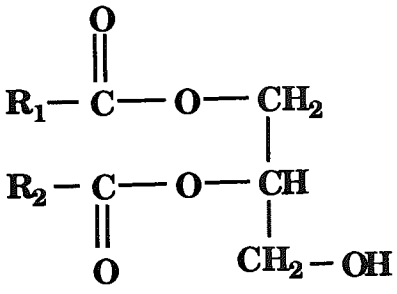


Fig. 4.0.2 Chemical structure of (A) TPA, (B) phorbol-12,13-dibutyrate and (C) diacylglycerol (the R groups represent fatty acid chains).

produce diacylglycerol. When tyrosine kinase-type receptors are phosphorylated by protein kinase C there is an attenuation of ligand binding. Phosphorylation of other types of receptor by protein kinase C, for example transferrin receptors, is known to be a signal for their internalisation (Houslay, 1990).

4.0.5 Protein Kinase C and Gastric Function.

4.0.5.1 Gastric Acid Secretion.

Protein kinase C plays a modulatory role in acid secretion. Both pentagastrin and carbachol increase the translocation of protein kinase C to the membrane and, therefore, activate it (Hanson and Hatt, 1989). However experiments with TPA suggest that activation of protein kinase C may have a negative effect on secretory activity. Thus the accumulation of aminopyrine (a weak base whose accumulation by parietal cells is used as an index of acid secretion) in response to the secretagogue carbachol was decreased by TPA (Hanson and Hatt, 1989). Also activation of protein kinase C inhibited histamine-stimulated accumulation of aminopyrine and the increase in cAMP in parietal cells. An aim of this work was to try to use the HGT-1 cell line to investigate the isoforms of protein kinase C involved in the regulation of histamine-stimulated adenylate cyclase.

4.0.5.2 Protein Kinase C and Gastric Cancer.

Protein kinase C is probably involved in some forms of malignancy but the exact mechanisms are not known. A large number of the cell lines have an increased amount of protein kinase C present (Hirai et al., 1989) and some even have abnormal forms, such as Swiss 3T3 cells which have an α isoform lacking a regulatory domain that is translocated to the

nucleus (Hug, 1993). It is possible that the presence of a certain isoform could have an unusual effect on cells. Certain clones of the gastric adenoma cell line SK-GT contain isoform β whereas others do not. Only the cells showing this isoform exhibit invasive behaviour, and this is probably due to the stimulation of production of collagenase which degrades the basement membrane (Schwartz et al., 1993). Numerous primary carcinomas have been investigated and found to have altered amounts of protein kinase C. Hashimoto et al. (1989) discovered that protein kinase C was increased in the cytosol in cases of gastric cancer, but Lim et al. (1987) found that it was also raised in the membrane fraction. Yasui et al. (1985) found increased protein kinase C in the cytosol in gastric cancer and in addition discovered decreased amounts of cAMP-dependent protein kinase.

4.0.6 Antipeptide Antibodies to Protein Kinase C.

Rabbit anti-peptide antibodies were purchased and used to probe for isoforms of protein kinase C in cell lines and in the rat gastrointestinal tract. The synthetic peptides used for immunisation were derived from the variable sequence of the hinge region of each protein (Makowske et al., 1988). Anti-protein kinase C α was raised against the peptide: ala-gly-asn-lys-val-ile-ser-pro-ser-glu-asp-arg-arg-gln. This sequence corresponds to amino acids 313-326 of protein kinase C α and the antibody gave an 80kDa band on a Western blot using total rat brain extract (Gibco data sheet). Anti-protein kinase C β was generated against the peptide: gly-pro-lys-thr-pro-glu-glu-lys-thr-ala-asn-thr-ile-ser-lys-phe-asp. This sequence corresponds to amino acids 313-329 of protein kinase C β and the antibody gave an 80kDa band on a Western blot using total rat brain extract (Gibco data sheet). This antibody is unable to distinguish between the βI and βII subtypes as they differ only at their C-terminal regions. Anti-protein kinase C ε was generated against the peptide: lys-gly-phe-

ser-tyr-phe-gly-glu-asp-leu-met-pro. This sequence corresponds to amino acids 313-326 of protein kinase C ε and the antibody gave an 90kDa band on a Western blot using total rat brain extract (Gibco data sheet). The antisera were purified by passing them over protein G agarose and eluting them with immunoglobulin G. All antibodies were provided with a separate sample of their peptide which were stated to completely block all binding when added in a 1:2 ratio to the antibody.

The secondary antibody used in blotting procedures was an affinity purified, biotinylated antibody raised against rabbit immunoglobulin and prepared from donkey polyclonal antisera. The technique for detection of secondary antibody makes use of the extremely strong bond formed between biotin and avidin. Avidin is conjugated to the enzyme alkaline phosphatase which is capable of converting the dye nitroblue tetrazolium to an insoluble formazan product.

4.0.6 Aims of this Section.

Due to the possible links of protein kinase C with cancer, especially that of the gastrointestinal tract, it was decided to investigate the presence of various isoforms within the gut and in the gastric cancer cell line HGT-1. Previous work with HGT-1 cells had shown that they contained an histamine H_2-receptor pharmacologically identical to that on human parietal cells (Emami et al., 1983). The stimulation of adenylate cyclase by this receptor appeared to be inhibited by phorbol esters (McKenna et al., 1993). The subcellular localisation and isoforms of protein kinase C in HGT-1 cells together with their involvement in inhibition of histamine-stimulated adenylate cyclase was therefore to be investigated.

4.1 METHODOLOGY.

4.1.1 Sample Preparation.

4.1.1.1 Cultured Cells.

Confluent HGT-1 and A549 cells were detached with a 0.02% EDTA solution with and without trypsin (respectively) and washed with ice-cold Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (1 phosphate buffer tablet dissolved in 100ml of distilled water gave a solution containing: 0.2g/l KCl, 8g/l NaCl, 0.2g/l KH_2PO_4 [anhydrous] and 1.15g/l Na_2HPO_4, pH7.3) twice. The pellet was resuspended at $8x10^6$ cells/ml in boiling 1x electrophoresis sample buffer (Table 2.4.1), sonicated for six x 5s with 5s intervals on a Soniprep 150 sonicator (MSE, Croydon) at setting 6μm, and then boiled for 5min.

4.1.1.2 Brain.

The brain was removed from an adult male Wistar rat (200 - 250g) killed by terminal anaesthesia with Sagatal™ (procedure performed by Dr. J. P. McKenna) and washed with ice-cold physiological saline. The tissue was minced roughly and 20ml of homogenisation buffer (Table 4.1.1) added per gram of tissue. The brain was homogenised for 1min on ice at maximum using the Ultra Turrax tissue homogeniser. Samples were diluted with an equal volume of boiling 2x electrophoresis sample buffer and boiled for 5min.

4.1.1.3 Gastrointestinal Mucosa.

The gastrointestinal tract was removed from an adult male Wistar rat under anaesthesia with Sagatal™ (procedure performed by Dr P. J.

Hanson) and rinsed with ice-cold 0.9% (w/v) saline. The tract was cut along it's length, the mucosa blotted with filter paper, scraped gently with a microscope slide and transferred to homogenisation buffer (Table 4.1.1) containing 1% Triton X-100. The samples were homogenised on ice as for the brain and left to settle for 5min. Homogenates were subjected to centrifugation at 12,000g for 5min at 4°C to remove any large particles (the protein kinase C remains in the supernatant as it has been solubilised by the detergent). The samples were diluted with homogenisation buffer until they had a protein concentration of 9.5mg/ml (determined by the modified Folin-Lowry protein assay - section 2.5), and equal volume of boiling 2x electrophoresis sample buffer added and boiled for 5min.

4.1.2 Subcellular Fractionation by Differential Centrifugation.

Cultured cells were detached and washed as previously described in section 4.1.1.1 and resuspended at $1.6x10^7$ cells/ml in homogenisation buffer (Table 4.1.1). The suspension was sonicated as before and then subjected to centrifugation on an MSE superspeed cenrifuge at 100,000g for 30min at 4°C. The supernatant was removed and an equal volume of 2x electrophoresis sample buffer added to it. This was designated the cytosolic or soluble fraction. The tubes were drained and homogenisation buffer containing 1% Triton X-100 added. The pellet was resuspended by sonication at 20μm as described in section 4.1.1.1 at 4°C and an equal volume of 2x electrophoresis sample buffer added such that the total volume in the tube equalled that of the supernatant extract. This was deemed the membrane or particulate fraction. Both fractions were boiled for 5min.

4.1.3 Enrichment by DE-52 Chromatography.

Brain was vortexed with homogenisation buffer (Table 4.1.1) containing 1% Triton X-100 and sonicated 5 x 5s with 5s intervals at 6μm on ice. The homogenate was left for 1h at 4°C with agitation every 15min, after which it underwent centrifugation at 100,000g for 30min at 4°C on the MSE Superspeed centrifuge. The resulting supernatant was loaded onto a 1ml DE-52 cellulose column at 4°C (the DE-52 resin had been swollen overnight in homogenisation buffer) and washed with 5ml of homogenisation buffer. Fractions were eluted by adding homogenisation buffer containing 125mM or 450mM NaCl to the homogenisation buffer with the former eluting protein kinase C α and the latter protein kinase C ε (Homan et al., 1991). The protein present in the eluate was precipitated by the addition of ice-cold trichloracetic acid to a final concentration of 10% (w/v) and left for 10min. The sample was subjected to centrifugation at 2000g for 5min and washed once with diethyl ether. Samples were dried under nitrogen and solubilised in 1x boiling electrophoresis sample buffer.

HGT-1 cells were grown to confluence, detached as previously described (section 2.0.1) and resuspended in homogenisation buffer containing 1% Triton X-100 at 10^7 cells/ml. The cell suspension was then treated as for the brain preparation above.

4.1.4 Immunodetection.

Nitrocellulose membranes were blocked overnight at 4°C in a solution of tris-buffered saline - 0.1% (v/v) Tween (Table 4.1.1) containing 5% dried milk powder. This solution was also used in all of the following washing and diluting procedures. All of the immunodetection procedures, except the final developement, were carried out at room temperature in plastic

jars on rollers. The primary rabbit anti-protein kinase C isoform antibody was diluted 1:400 and applied for 1h after which the membrane was washed for twice for 1min and thrice for 5min. The secondary anti-rabbit biotinylated antibody was diluted at 1:500 and added for 20min after which the membrane was washed as above. The streptavidin-alkaline phosphatase conjugate was diluted 1:3000 and applied for 20min before the membrane was washed again. The diethanolamine buffer containing the phosphate substrate of the following composition: 10.5mg/ml diethanolamine (pH9.5, 4°C) and 1mg/ml $MgCl_2.6H_2O$ was added for 15min in a plastic trough and the membrane washed with distilled water for twice for 1min then thrice for 5min to arrest developement. Developed membranes were stored desiccated at 4°C.

4.1.5 Preincubation of HGT-1 cells with Phorbol Dibutyrate.

HGT-1 cells were grown in flasks until confluent. Phorbol-12,13 dibutyrate, dissolved in dimethyl sulphoxide, was added to the flasks to a concentration of 1μM in fresh culture medium and left for 24h. Dimethyl sulphoxide was added to control flasks to give the same concentration as in the experimental flasks (0.05% v/v). HGT-1 cells were washed with phosphate buffered saline, detached using a 0.02% (w/v) solution of EDTA in phosphate buffered saline for 2min and subjected to centrifugation at 100g for 5min at 15°C. The cells were resuspended in phosphate buffered saline and washed twice. The resulting pellet was resuspended in phosphate buffered saline at 6×10^6 cells/ml. Aliquots of 0.5ml were subjected to centrifugation at 10,000g for 10s and the pellet resuspended in 0.5ml of boiling 1x electrophoresis sample buffer. The solution was sonicated 6 x 5s at 5s intervals at a setting of 6μm and samples were then boiled for 5min.

HGT-1 cells were grown until almost confluent as described in section 2.0.1 except that large petri dishes were used. Phorbol-12,13-dibutyrate was added to one petri dish at a concentration of 1μM for 24h. To the second petri dish an equivalent concentration (0.05% v/v) of its solvent DMSO was added. The cells, still attached, were washed for five times for 5min periods in Dulbecco's modified Eagle's minimum essential medium plus 20mM HEPES and 4mg/ml bovine serum albumin at 37°C, followed by Ca^{2+}-, Mg^{2+}-free phosphate buffered saline for 5min at 37°C. The cells were scraped off the dish into 1ml homogenisation buffer (Table 4.1.1) and sonicated for 6 x 5s with 5s intervals. 60μl of the homogenate was added to a reaction mixture with or with out Ca^{2+} (Table 4.1.1) and incubated overnight at 4°C. Glass-fibre filters (Whatman) were soaked in fresh 0.3% (w/v) polyethlenimine, pH10, for 1h to lower the nonspecific binding of cationic radioligands and placed at the base of a funnel in a vacuum filtration system. Incubated samples were mixed gently and 190μl pipetted onto the filter. The filter was washed through with 5ml of ice-cold 20mM tris HCl, pH7.5. The filters were transfered to scintillation vials with 1ml 1M NaOH and left at 37°C for 2h. Optiphase Hisafe II scintillation fliud was added and samples counted on a Tri-carb liquid scintillation analyser, model 1900TR (Packard-Hewlett).

Non-specific binding was determined by the addition of 10μM phorbol-12,13-dibutyrate to both the Ca^{2+}-containing and the Ca^{2+}-free mixtures before incubation of homogenate overnight.

To ascertain if the washing procedure had removed all of the phorbol-12,13-dibutyrate from the preincubation step 10^6 d.p.m of [^{3}H] phorbol-12,13-dibutyrate were added to attached HGT-1 cells for 1h at 37°C. The cells were then washed with DMEM as described above (section 4.1.5) and

the cells detached by solubilisation in 1M NaOH for 2h at 37°C. Radioactivity was counted in Optiphase Hisafe II as above.

4.1.7 Preparation of isolated membrane fractions from HGT-1 cells.

HGT-1 cells were cultured as previously described (section 2.0.1) and detached using a 0.02% (w/v) solution of EDTA in phosphate buffered saline. They were washed twice with phosphate buffered saline and resuspended at a concentration of $2x10^7$ cells/ml in homogenisation buffer (10mM Tris pH7.5, 1mM EDTA, 30mM NaCl, 1mM dithiothreitol, 1mM phenylmethylsulphonylfluoride, 10μg/ml leupeptin, 10μg/ml pepstatin, 10μg/ml aprotinin and 10μg/ml soybean trypsin inhibitor. The cell suspension was homogenised on ice using a hand-held teflon-glass homogeniser (30 up and down stokes/min for 2min). Cell viability was estimated by the ability to take up trypan blue (section 2.0.5). The homogenate was subjected to centrifugation at 40,000g for 90min at 4°C using a MSE superspeed 50 centrifuge. Ice-cold homogenisation buffer was used to wash the surface of the pellet twice and finally to resuspend it in a volume equivalent to the original amount used for the homogenisation of the cells. 0.25ml aliquots were stored at -80°C overnight.

4.1.8 Assay of Adenylate Cyclase Activity.

The protein content of the homogenate was determined by the modified Folin-Lowry procedure (see section 2.5) and the homogenate diluted to give a final concentration of 0.67μg protein/μl. 15μl aliquots of the diluted membrane homogenate were added to 235μl of incubation buffer to give the following final concentrations in the assay mixture: 25mM Tris HCl pH 7.5, 1mM EGTA, 0.06mM EDTA, 0.06mM dithiothrietol, 1.8mM NaCl, 5mM $MgCl_2$, 1mM ATP, 20mM creatine phosphate, 35U/ml creatine kinase, 0.2mM IBMX, 1mg/ml bovine serum albumin, 200 μg/ml

bacitracin, 200μM GTP and 20μg/ml phosphatidylserine. In some experiments $CaCl_2$ to give 10^{-5}M free Ca^{2+} or 10mM EGTA was added to the system. 0.5mM histamine or 100nM TPA were also added where appropriate. Homogenate of HGT-1 cells was added to prewarmed incubation buffer, vortexed and incubated for 10min at 30°C. The reaction was terminated by the addition of 250μl of ice-cold ethanol followed by immediate mixing. 0.4ml aliquots of the mixture were transfered to microfuge tubes and subjected to centrifugation at 10,000g for 1min at room temperature to remove cellular debris. 350μl aliquots of the supernatant were evaporated in a vacuum oven at 40°C and the cAMP content of the residue determined.

4.1.9 Determination of the cAMP content of homogenates (procedure performed by Dr. J. P. McKenna).

The residue obtained in section 4.1.7 was reconstituted in 100μl of sodium acetate buffer (pH6.2), mixed by vortexing with 5μl of acetylation reagent (2 volumes of triethylamine mixed with 1 volume of acetic anhydride) and incubated for 3min at room temperature. 900μl of sodium acetate buffer was added and the sample mixed.

A 40pmol/ml cAMP standard was prepared in sodium acetate buffer. 10μl of acetylating reagent was added to 200μl of the standard and incubated for 3min at room temperature. A further 1.8ml of sodium acetate buffer was added to produce a 4pmol/ml standard. A series of standards (2.0, 1.0, 0.5, 0.25, and 0.1pmol/ml) in modified assay buffer (50μl of acetylating reagent in 10ml sodium acetate buffer) was made from this solution on the same day as the samples were prepared. The cAMP [^{125}I] tracer (succinyl cAMP tyrosine methyl ester) was diluted 1:1 (v/v) with the reconstituted cAMP carrier serum immediately before use. Additions were made to the samples and the standards as shown in Table

4.1.2 and the contents mixed. The mixtures were incubated for 16-18h at 2-8°C. 0.5ml cAMP precipitator (at 2-8°C) was added to all tubes except the total counts, the tubes were mixed and subjected to centrifugation at 1250g for 15min at 4°C. The supernatant was discarded and the tubes were left to drain at an angle of 30° for 30s. The radioactivity remaining in the precipitate was assessed by a compu-gamma gamma counter (LKB Instruments Ltd., Sweden) with a counting efficiency of 82%. The standard curve with spline function (cpm vs log concentration) was plotted and the concentration of cAMP in the samples calculated using a curve fitting package associated with the gamma counter.

Table 4.1.1 Stock Solutions.

Electrophoresis Homogenisation Buffer.

20mM	Tris base pH7.5, 4°C
2mM	EDTA
10mM	EGTA
2mM	Dithiothreitol
2mM	Phenylmethylsulphonyl fluoride
10µg/ml	Pepstatin
10µg/ml	Leupeptin
10µg/ml	Aprotinin
10µg/ml	Soybean trypsin inhibitor

Tris-Buffered Saline plus 0.1% Tween.

2.42g/l	Tris base pH 7.5, 4°C
8g/l	Sodium chloride
1% v/v	Tween

Reaction Mixture for Phorbol-12,13-Dibutyrate Binding.

20mM	Tris HCl pH7.4
14.28mM	$MgCl_2$
71.43nM	^{3}H-Phorbol-12,13-dibutyrate (3174666 counts)
3.43mM	$CaCl_2$

Ca^{2+}-free Reaction Mixture for Phorbol-12,13-Dibutyrate Binding.

20mM	Tris HCl pH7.4
14.28mM	$MgCl_2$
71.38nM	^{3}H-Phorbol-12,13-dibutyrate
5mM	EDTA

Table 4.1.2 Summary of reagent additions used in the cAMP radioimmunoassay.

Tube contents	Volume (µl). Modified assay buffer	Standards	Sample	Working tracer	Anti-serum
Total counts	-	-	-	100	-
Blank	200	-	-	100	-
0.1pmol/ml standard	-	100	-	100	100
0.25pmol/ml standard	-	100	-	100	100
0.5pmol/ml standard	-	100	-	100	100
1.0pmol/ml standard	-	100	-	100	100
2.0pmol/ml standard	-	100	-	100	100
4.0pmol/ml standard	-	100	-	100	100
Samples	-	-	100	100	100

4.2 RESULTS.

4.2.1 Detection of Isoforms of Protein Kinase C by Protein Blotting.

All photographs of blots are representative of two or three experiments.

4.2.1.1 Protein Kinase C α.

A band of molecular mass 85kDa could be seen in each sample when the blot was exposed to anti-protein kinase C α (plate 4.1). The band in lane 2 (stomach) appeared to be very faint. The bands disappeared when the antibody was preabsorbed with the peptide used for immunisation (plate 4.2).

A band of molecular mass 85kDa could be seen in lanes containing HGT-1 whole cell homogenate, A549 whole cell homogenate, HGT-1 particulate fraction, A549 particulate fraction, HGT-1 cell homogenate purified on DEAE cellulose and eluted at 125mM NaCl, and rat brain tissue when the blot was exposed to anti-protein kinase C α (plate 4.3). No band was detected when the same antibody was preabsorbed with the peptide used for immunisation (plate 4.4).

A band of molecular mass 85kDa could be seen in all of the HGT-1 control lanes, the A549 whole cell homogenate and the rat brain tissue when the blot was exposed to anti-protein kinase C α (plate 4.5). The band obtained with HGT-1 cells appeared to be a doublet with the upper half being present in the soluble fraction and the lower in the particulate fraction. The appearance of a band in the cytosolic fraction was varable (plate 4.3) No protein kinase C α was detected in HGT-1 cells or A549 cells treated with 1μM phorbol-12,13-dibutyrate for 24h. A band of the same molecular mass could be seen in lanes containing rat brain tissue, control

HGT-1 cells after exposure to DMSO for 24h and HGT-1 cells treated with phorbol-12,13-dibutyrate for 0h, 0.25h, 1h, 2h and 4h (plate 4.6). A very faint band could be seen in cells treated with phorbol-12,13-dibutyrate for 8h, but no band was visible after 24h exposure.

4.2.1.2 Protein kinase C β.

A band of molecular mass 85kDa could be seen in lanes containing samples of duodenum, jejunum, ileum, caecum, colon, gastrointestinal smooth muscle and rat brain when the blot was exposed to anti-protein kinase C β (plate 4.7). The stomach sample appeared to show a very faint band but HGT-1 cells did not have any detectable β isoform. As there was no protein kinase C β present in the stomach or HGT-1 cells no preabsorbed control was performed.

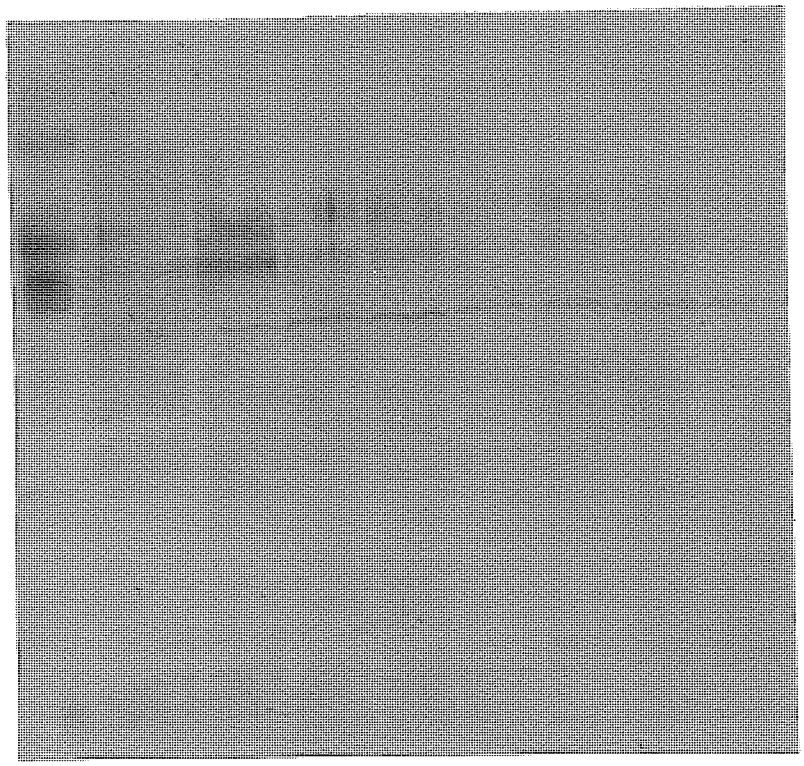
4.2.1.3 Protein kinase C ε.

A band of molecular mass 95kDa could be seen in samples containing rat brain, HGT-1 cells, gastrointestinal smooth muscle and stomach when the blot was exposed to anti-protein kinase C ε (plate 4.8).

A band of molecular mass 95kDa could be seen in lanes containing samples of HGT-1 particulate fraction, HGT-1 whole cell homogenate (but not the soluble fraction) and rat brain tissue purified on DE-52 cellulose and eluted at 450mM NaCl when blots were exposed to anti-protein kinase C ε (plate 4.9). The protein kinase C ε band appears to be a doublet. The top half of the doublet disappeared and the bottom band partially so when the same antibody was preabsorbed with the peptide which had been used for immunisation (plate 4.10).

A band of molecular mass 95kDa could be seen in lanes containing samples of rat brain tissue purified on DEAE cellulose and eluted at 450mM NaCl, HGT-1 whole cell homogenate from control and HGT-1 whole cell homogenate treated with 1μM phorbol-12,13-dibutyrate for 24h when blots were exposed to anti-protein kinase C ε (plate 4.11).

Measurements of molecular mass in the rainbow markers are given in kDa in the following plates.



Lane.

1 Rainbow marker

2 Stomach

3 Duodenum

4 Jejunum

5 Ileum

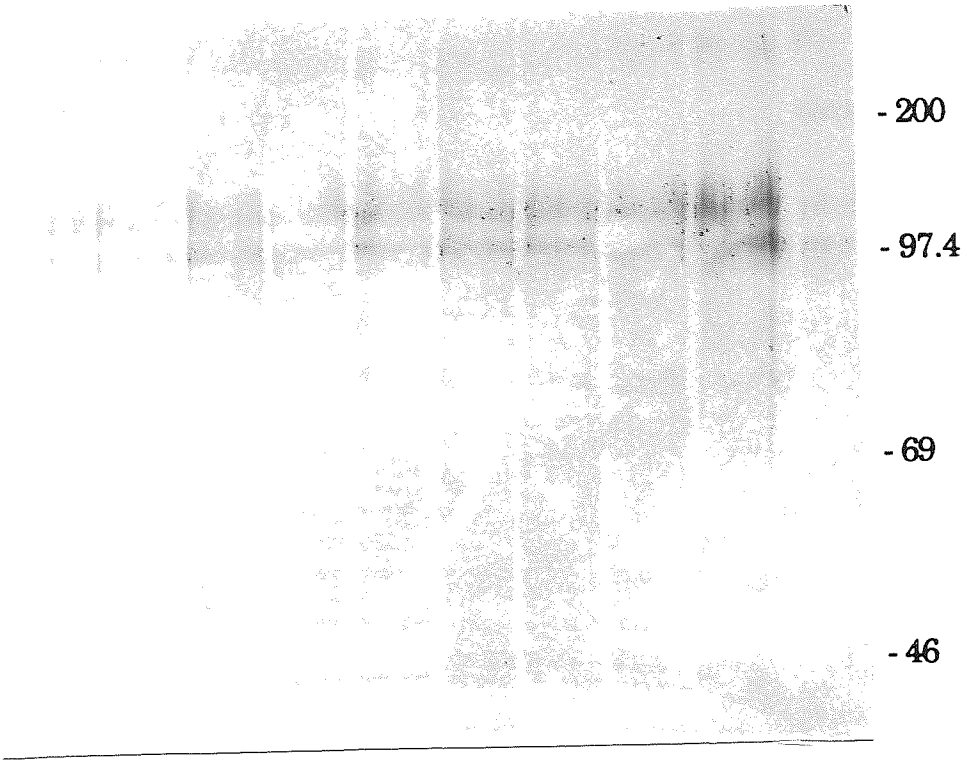
6 Caecum

7 Colon

8 Gastrointestinal smooth muscle

9 Brain

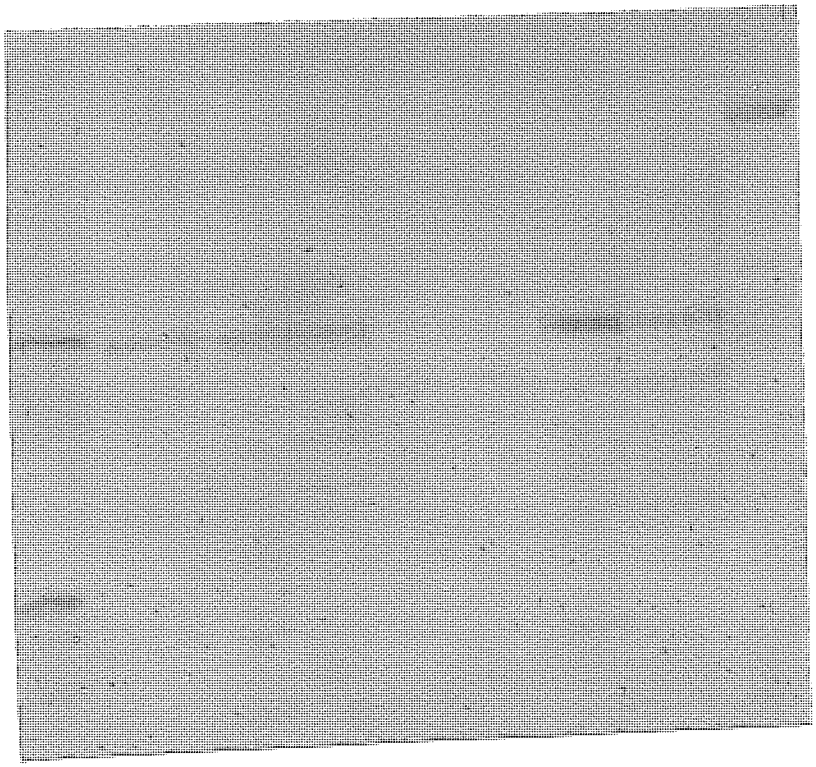
Plate 4.1 Immunodetection of Protein Kinase C α in Rat Gastrointestinal Mucosa.



Lane.

1	Rainbow marker
2	Brain
3	Gastrointestinal smooth muscle
4	Colon
5	Caecum
6	Ileum
7	Jejunum
8	Duodenum
9	Stomach
10	Rainbow marker

Plate 4.2 Immunodetection of Protein Kinase C α in Rat Gastro-intestinal Mucosa using a Preabsorbed Antibody.



- 200

- 97.4

- 69

- 46

Lane.

1 Brain

2 HGT-1 DEAE fraction

3 A549 membrane fraction

4 HGT-1 membrane fraction

5 A549 cytosolic fraction

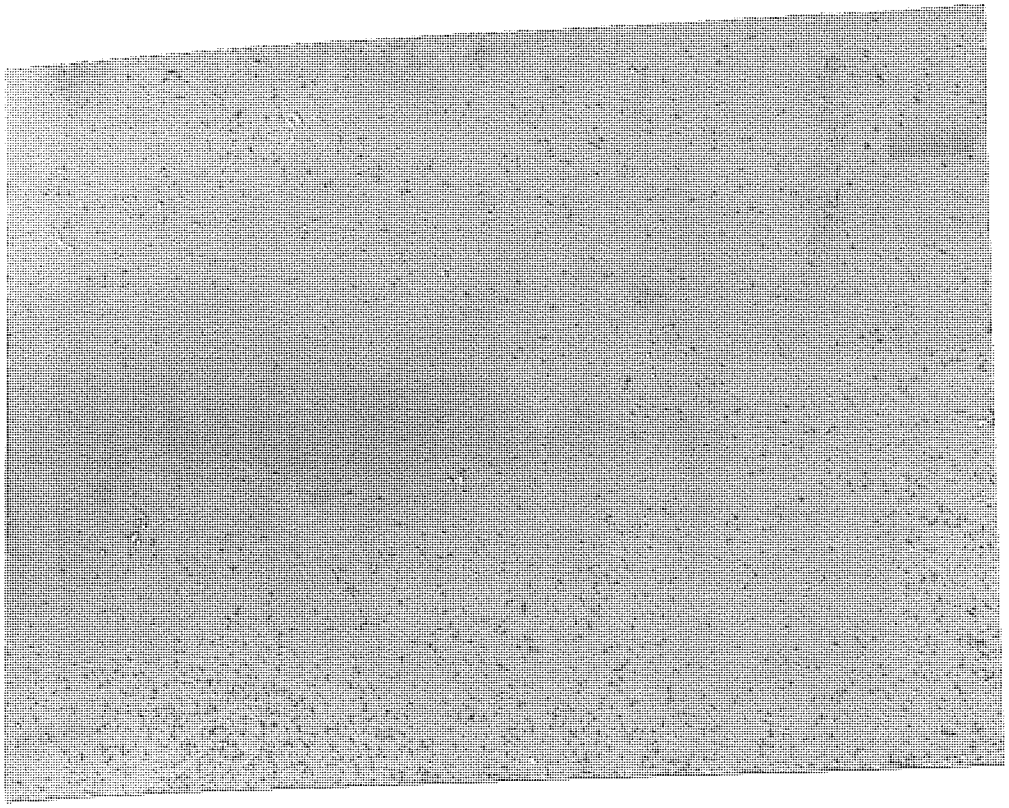
6 HGT-1 cytosolic fraction

7 A549 whole cell homogenate

8 HGT-1 whole cell homogenate

9 Rainbow marker

Plate 4.3 Immunodetection of Protein Kinase C α in HGT-1 and A549 cells.



- 200

- 97.4

- 69

- 46

Lane.

1 Brain

2 HGT-1 DEAE fraction

3 A549 membrane

4 HGT-1 membrane fraction

5 A549 cytosolic fraction

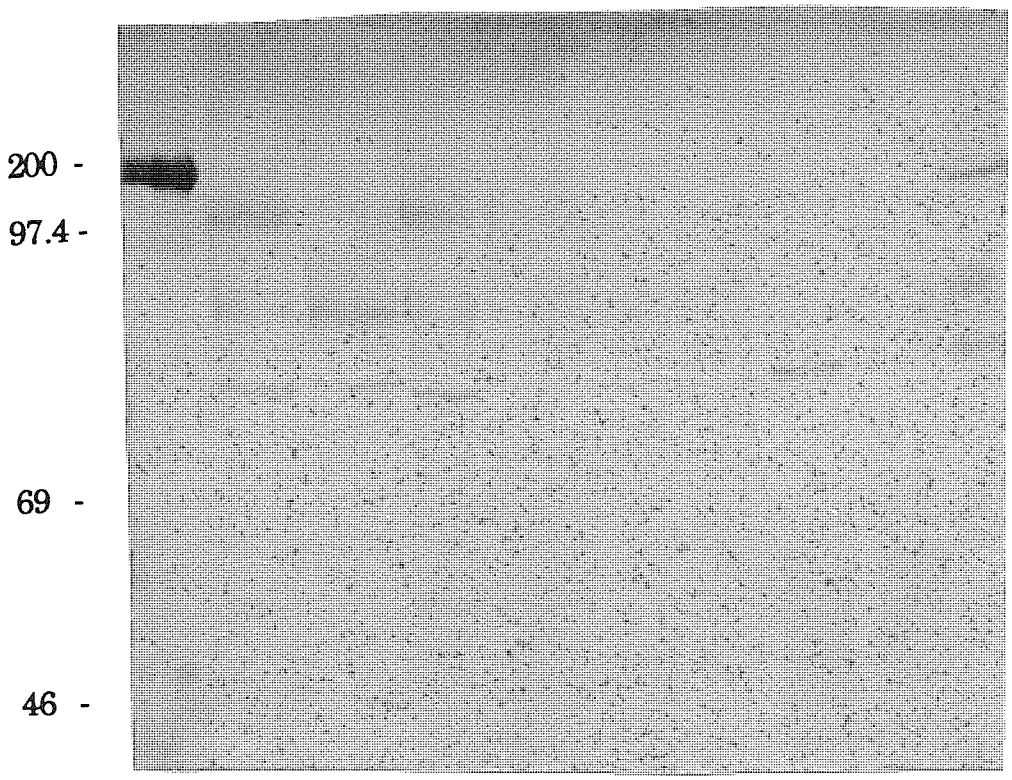
6 HGT-1 cytosolic fraction

7 A549 whole cell homogenate

8 HGT-1 whole cell homogenate

9 Rainbow marker

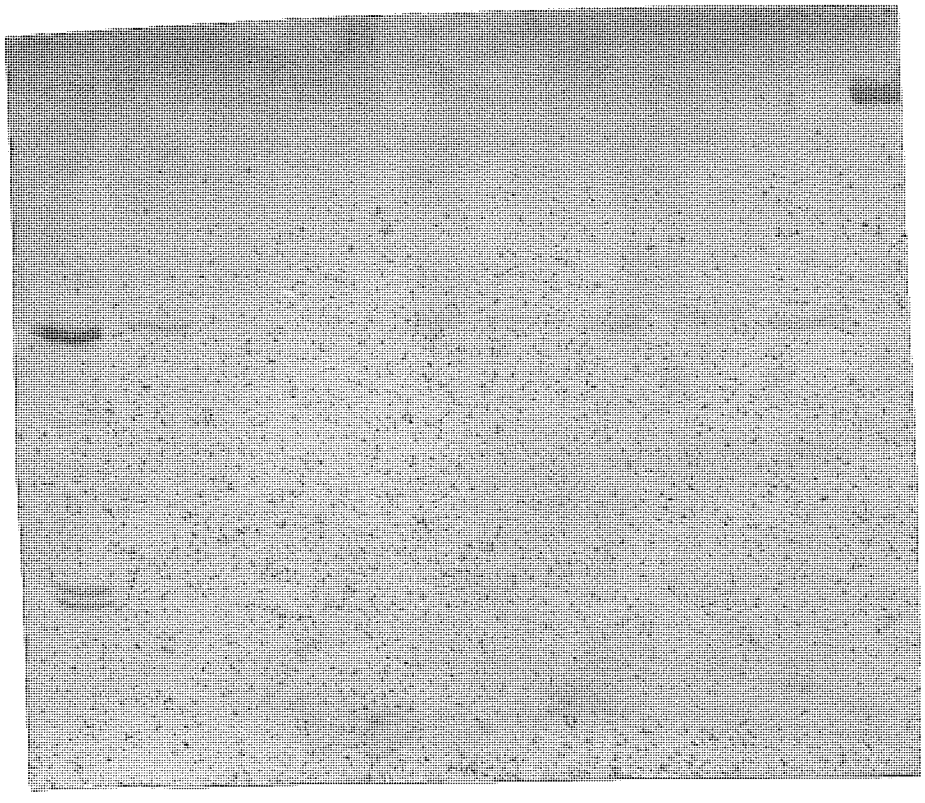
Plate 4.4 Immunodetection of Protein Kinase C α in HGT-1 and A549 cells using a Preabsorbed Antibody.



Lane.

1	Rainbow marker
2	Control HGT-1 whole cell homogenate
3	Control HGT-1 cytosolic fraction
4	Control HGT-1 membrane fraction
5	HGT-1 whole cell homogenate preincubated with phorbol ester
6	HGT-1 cytosolic fraction preincubated with phorbol ester
7	HGT-1 membrane fraction preincubated with phorbol ester
8	Control A549 whole cell homogenate
9	A549 whole cell homogenate preincubated with phorbol ester
10	Brain

Plate 4.5 Immunodetection of Protein Kinase C α in HGT-1 and A549 Cells after Treatment With 1μM Phorbol-12,13-dibutyrate.



Lane.

1 Brain

2 Control HGT-1 cells incubated with DMSO for 24h

3 HGT-1 cells pretreated for 24h with phorbol ester

4 HGT-1 cells pretreated for 8h with phorbol ester

5 HGT-1 cells pretreated for 4h with phorbol ester

6 HGT-1 cells pretreated for 2h with phorbol ester

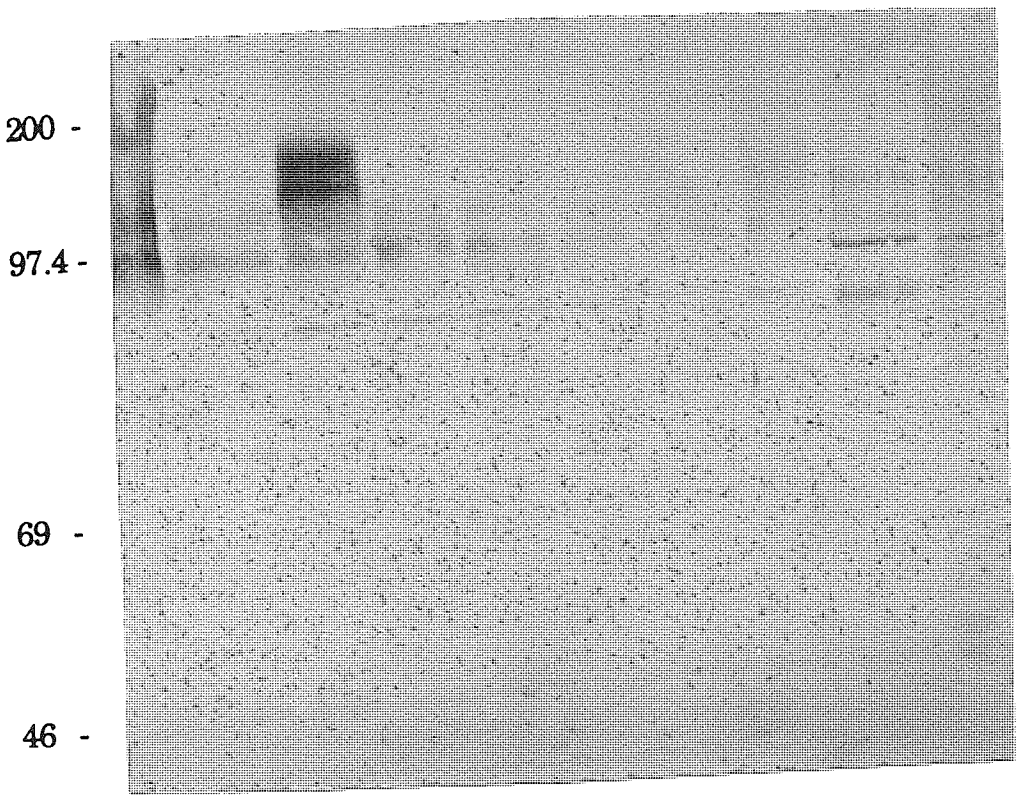
7 HGT-1 cells pretreated for 1h with phorbol ester

8 HGT-1 cells pretreated for 0.25h with phorbol ester

9 HGT-1 cells at 0h

10 Rainbow marker

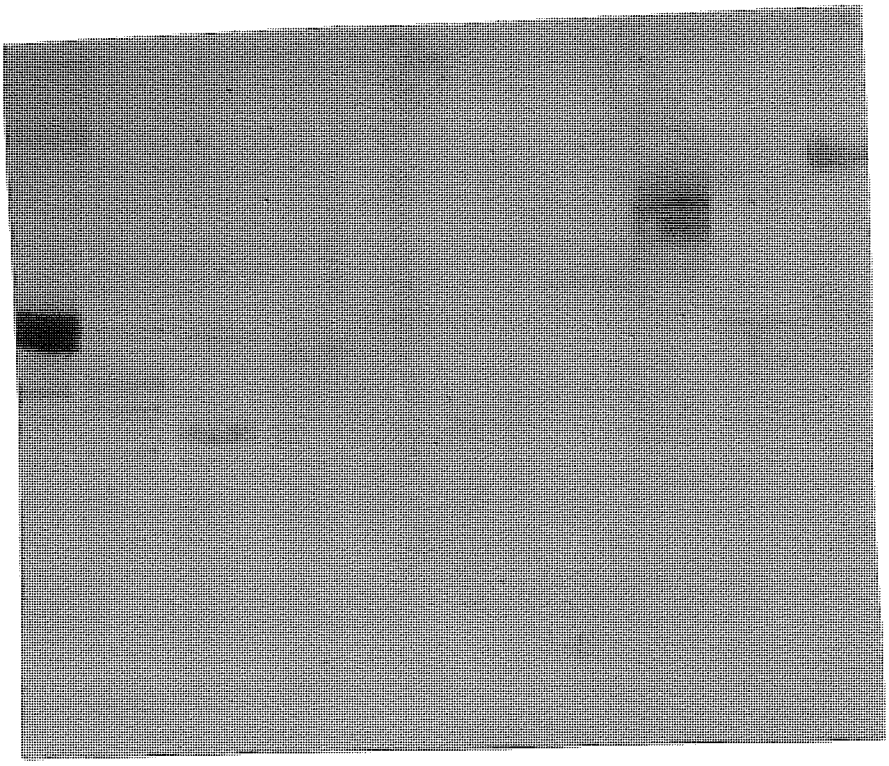
Plate 4.6 Immunodetection of Protein Kinase C α in HGT-1 Cells After Treatment with 1μM Phorbol-12,13-Dibutyrate for Varying Amounts of Time.



Lane.

1	Rainbow marker
2	Stomach
3	Duodenum
4	Jejunum
5	Ileum
6	Caecum
7	Colon
8	Gastrointestinal smooth muscle
9	Brain
10	HGT-1 cells

Plate 4.7 Immunodetection of Protein Kinase C β in Rat Gastrointestinal Mucosa and HGT-1 Cells.



Lane.

1 Brain

2 HGT-1 whole cell homogenate

3 Gastrointestinal smooth muscle

4 Colon

5 Caecum

6 Ileum

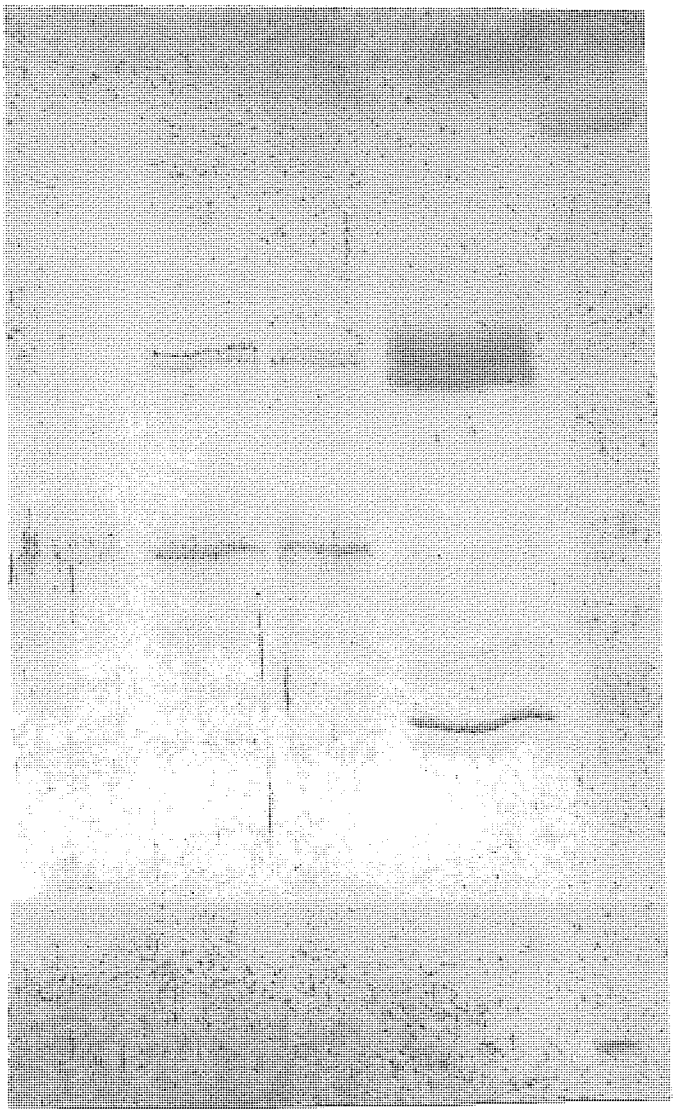
7 Jejunum

8 Duodenum

9 Stomach

10 Rainbow marker

Plate 4.8 Immunodetection of Protein Kinase C ε in Rat Gastrointestinal Mucosa and HGT-1 Cells.



Lane.

1 HGT-1 cell cytosolic fraction

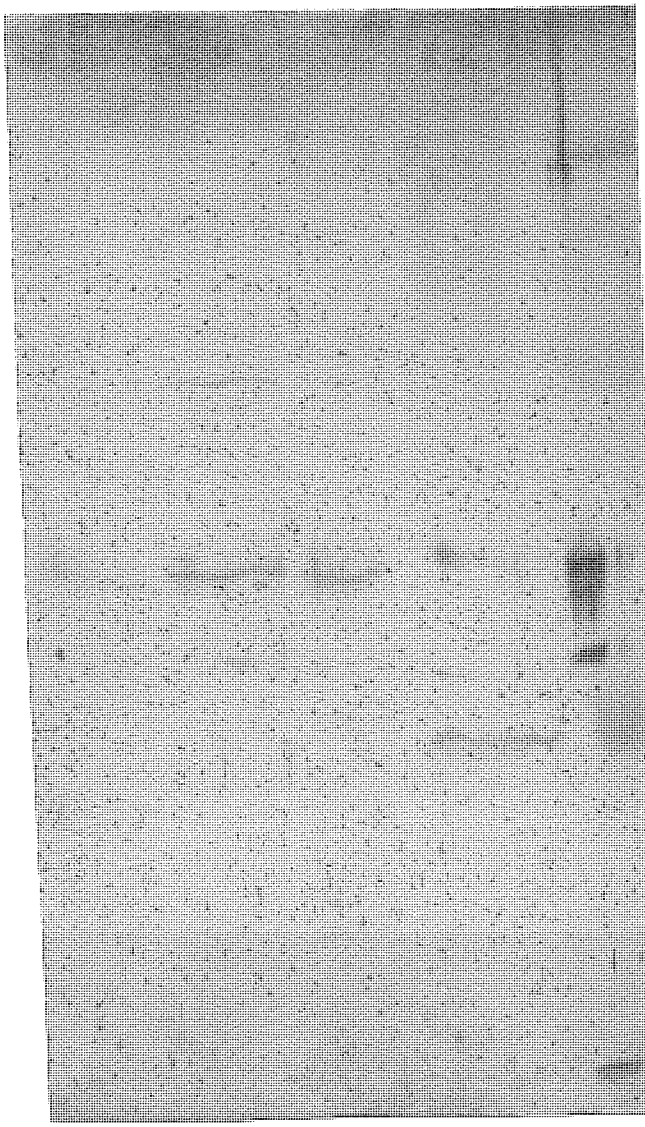
2 HGT-1 cell membrane fraction

3 HGT-1 whole cell homogenate

4 Brain DEAE 450mM fraction

5 Rainbow marker

Plate 4.9 Immunodetection of Protein Kinase C ε in HGT-1 Cells.



Lane.

1 HGT-1 cell cytosolic fraction

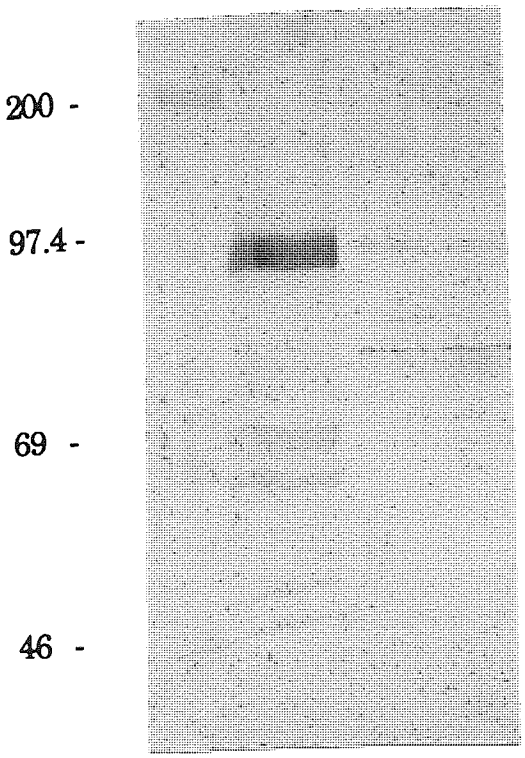
2 HGT-1 cell membrane fraction

3 HGT-1 whole cell homogenate

4 Brain DEAE 450mM fraction

5 Rainbow marker

Plate 4.10 Immunodetection of Protein Kinase C ε in HGT-1 Cells Using a Preabsorbed Antibody.



Lane.

1 Rainbow marker

2 Brain DEAE 450mM fraction

3 Control HGT-1 whole cell homogenate

4 Pretreated HGT-1 whole cell homogenate

Plate 4.11 Immunodetection of Protein Kinase C ε in HGT-1 Cells After Treatment with 1μM Phorbol-12,13-Dibutyrate for 24h.

4.2.2 The Use of Phorbol-12,13-dibutyrate Binding to Investigate Down-regulation of Protein Kinase C in HGT-1 Cells.

Phorbol-12,13-dibutyrate binding significantly above non-specific binding could be seen both in the presence and absence of Ca^{2+} (fig 4.2.1) ($P<0.001$ and $P<0.002$ respectively, paired t-test). In the experiments involving preincubation with unlabelled phorbol-12,13-dibutyrate the washing procedure probably removed the majority of phorbol ester from the cells because, in trials, only 0.11% of the counts added to the cells remained after washing (table 4.2.1). It is unlikely that this material would interfere with [^{3}H] phorbol-12,13-dibutyrate binding in homogenates of HGT-1 cells. Preincubation of cells with 1μM phorbol-12,13-dibutyrate for 24h gave an overall decrease in binding of [^{3}H] phorbol-12,13-dibutyrate in homogenates (Table 4.2.2). There was a decrease in both the Ca^{2+}-dependent and the Ca^{2+}- independent binding.

4.2.3 Effect of Ca^{2+} on the Inhibition of Adenylate Cyclase by TPA in Membranes of HGT-1 cells.

In the presence of Ca^{2+} the addition of 0.5mM histamine to an HGT-1 membrane preparation caused an increase in adenylate cyclase activity (fig 4.2.2). This activity was significantly inhibited by the presence of 100nM TPA (paired t-test, $P<0.02$). TPA did not alter the basal adenylate cyclase activity. In the absence of Ca^{2+}, TPA did not cause any significant alteration in the adenylate cyclase activity in the presence of histamine.

4.2.4 Effect of Anti-peptide Antibodies on the Inhibition of Adenylate Cyclase by TPA in membranes of HGT-1 cells.

Only the anti-protein kinase C α antibody produced a decrease in the inhibition of histamine-stimulated adenylate cyclase activity by TPA (fig

4.2.3). The effect seen was dependent on the concentration of the antibody with 400ng/ml producing a significant change ($P<0.01$), 40ng/ml producing a significant change ($P<0.05$), but with no significant effect seen at 4ng/ml using a Dunett's test after analysis of variance. Preabsorption of the anti-protein kinase C α antibody with the peptide used for immunisation led to their being no decrease in the inhibition by TPA. Likewise, the addition of anti-protein kinase C ε antibody caused no effect on the response seen with TPA.

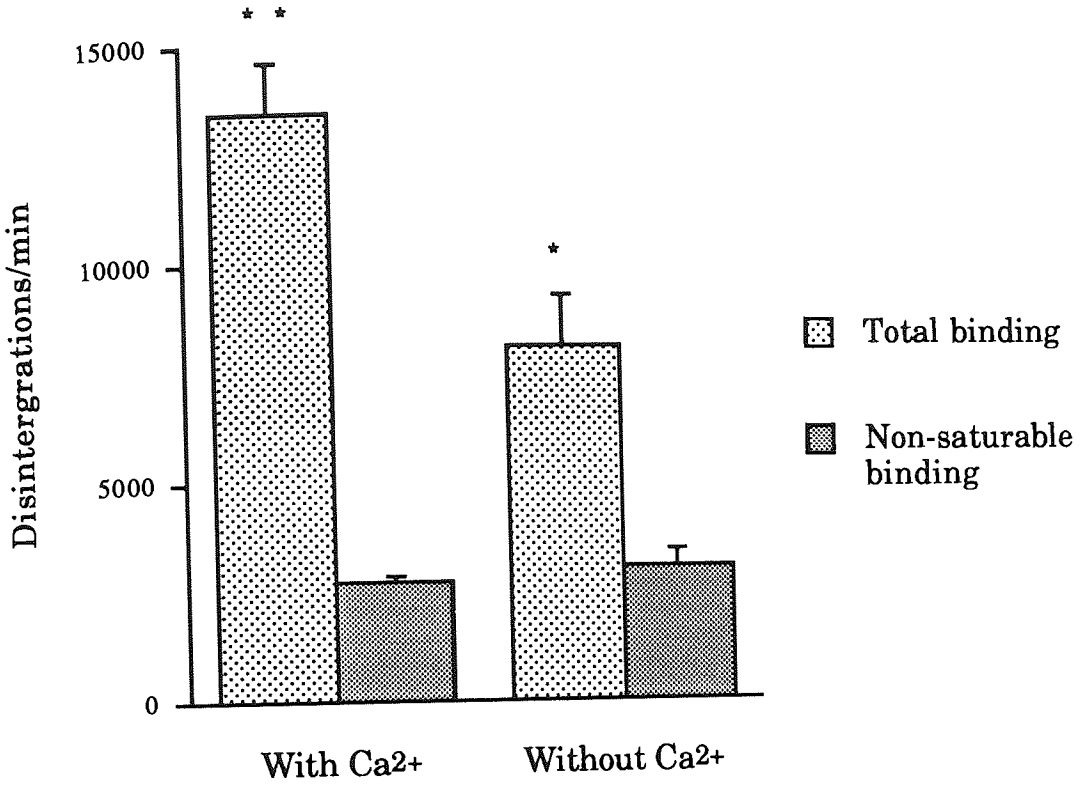


Fig. 4.2.1 3H phorbol dibutyrate binding to homogenates of HGT-1 cells in the presence and absence of Ca^{2+}.

Results are means ± SEM of three determinations on a single homogenate and are typical of three other experiments. Non-saturable binding was carried out in the presence of 10μM phorbol dibutyrate. Total binding was carried out with tracer (50nM) phorbol dibutyrate. A significant effect of 10μM phorbol dibutyrate was seen on binding (**$P<0.001$; *$P<0.02$, paired t-test).

Table 4.2.1 Validation of the Washing Procedure Used in Phorbol-12,13-Dibutyrate Binding Experiments.

Counts added/ Remaining well.	Background	Counts Attached.
10^6	22.55±0.65	1146.97±74.75

The percentage of counts remaining bound is:

$$\frac{\text{Counts remaining attached - background}}{\text{Counts added}} \times 100$$

Therefore the percentage remaining bound is 0.11%.

Results are means ± SEM for triplicate determinations from a single experiment.

Table 4.2.2 The Effect of Preincubation of HGT-1 Cells with 1μM Phorbol-12,13-Dibutyrate for 24h Prior to Binding Experiments.

	Control	Treated with Phorbol-12,13-dibutyrate.
Total binding	2.69±0.29	0.56±0.056*
Ca^{2+}-independent binding	0.77±0.34	0.14±0.054*
Ca^{2+}-dependent binding	1.91±0.21	0.41±0.105*

Specifically-bound counts are expressed as percentage of labelled phorbol-12,13-dibutyrate added (1μCi in final assay mixture). Ca^{2+}-dependent binding was obtained by subtracting the binding in the absence of Ca^{2+} from the total binding. Results (means ± SEM) are from three separate experiments with triplicate determinations in each experiment. Two-way analysis of variance and a subsequent t-test gave significance *$P<0.01$ for the effect of preincubation with phorbol-12,13-dibutyrate in all three cases.

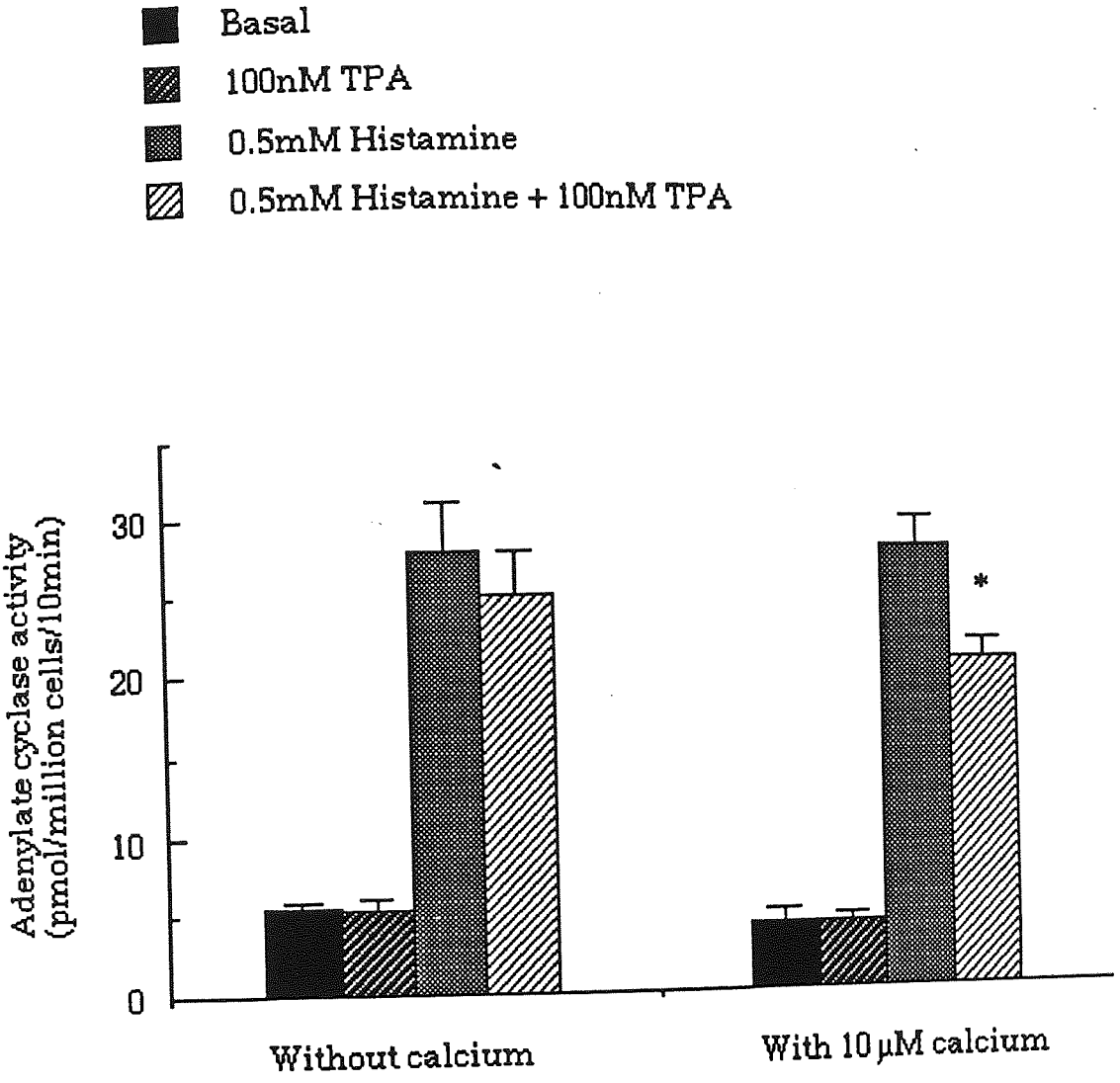
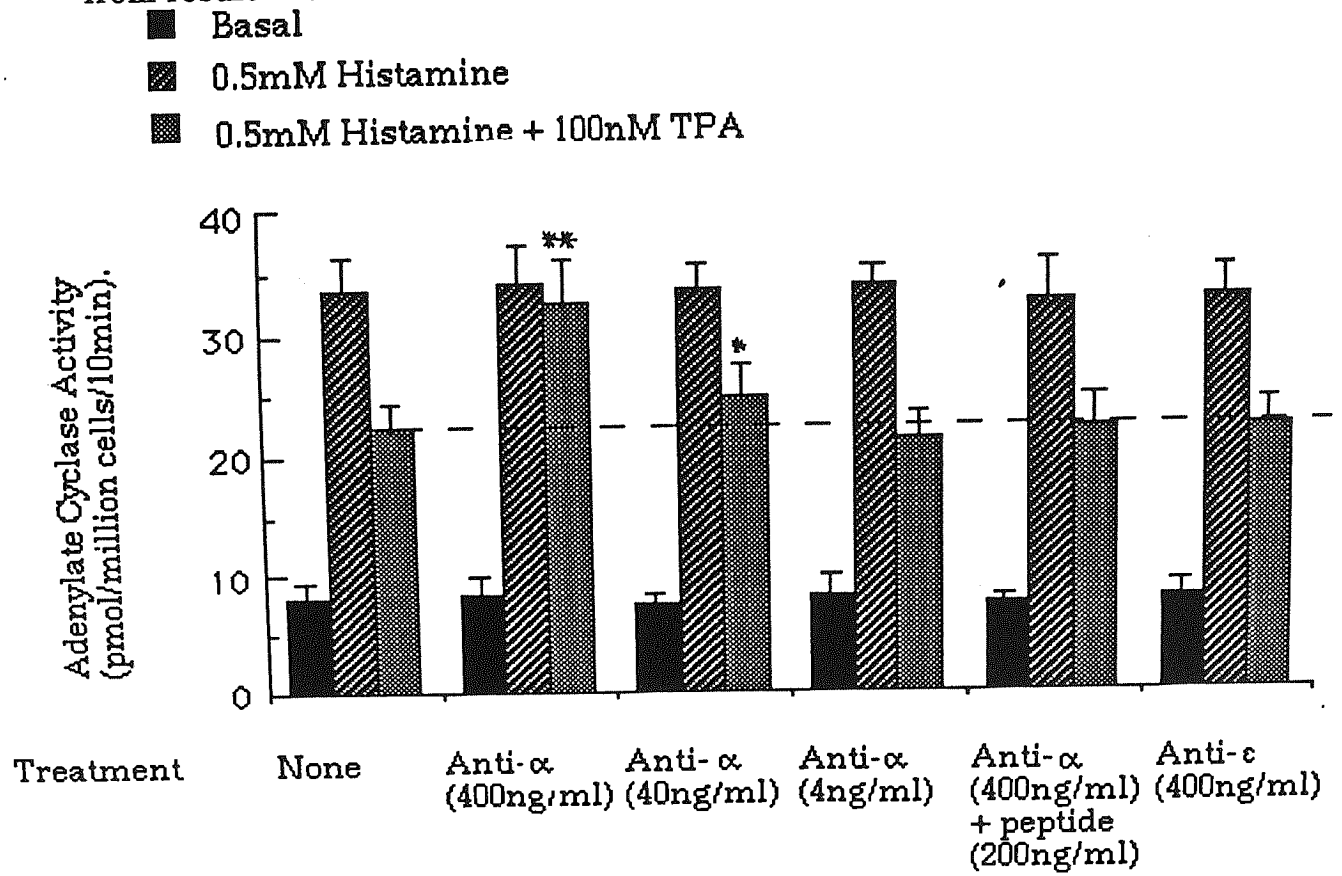


Fig. 4.2.2 Comparison of the effect of TPA on the histamine-stimulated activity of adenylate cyclase in the presence and absence of calcium.
Results are mean ± SEM of four membrane preparations.
*P<0.02 for effect of 100nM TPA on adenylate cyclase activity stimulated by 0.5mM histamine (paired t-test).

Fig. 4.2.3 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 mean ± SEM from four experiments. **P<0.01, *P<0.05 for difference from result with 0.5mM histamine and 100nM TPA (Dunnet's test).



4.3 DISCUSSION.

In order to relate the results obtained with the HGT-1 cells to the gastrointestinal tract of normal animals the distribution of species reactive anti-protein kinase C α, β and ε antibodies was investigated. Brain tissue was used as a check on the blotting procedure since it contains all known isoforms of protein kinase C. Bands of molecular mass appropriate to protein kinase C α and β were present in all regions of gastrointestinal mucosa except the stomach, the gastrointestinal smooth muscle and, as expected, the brain sample (plates 4.1 and 4.7). Bands suggestive of protein kinase Cε were present only in the gastric mucosa, the gastrointestinal smooth muscle and the brain (plate 4.8). Preabsorbtion of the antibodies with the peptides used for immunisation completely removed the above mentioned bands (except β) proving that the antibodies were recognising structures containing the peptide sequences. Although the specificty antibodies was not validated with purified isoforms of protein kinase C the competition by the peptides of antibody binding to targets of the correct molecular mass strongly suggests that protein kinase C isoforms were being recognised. In particular there was clearly no cross reaction of protein kinase C α antibody with protein kinase C ε or vice versa because otherwise bands at both 85kDa and 90kDa should have been on some blots.

The distribution of isoforms of protein kinase C had not been investigated before in the whole gastrointestinal tract. This work provides a map of the normal expression of isoforms α, β and ε for comparison with malignant tissue and cell lines.

On the same criteria as those mentioned above protein kinase C α was present in HGT-1 cells. Protein kinase C α was previously shown to be present in A549 cells (Hirai et al., 1989) which were used as a control to

check that the cell preparation procedure was not destroying the enzyme. As might be expected other gastric adenocarcinoma cell lines, such as KATO III and TMK-1 (Hirai et al., 1989) have been shown to contain protein kinase C. Various isoforms of protein kinase C have been found in cell lines taken from the gastrointestinal tract. The gastric adenocarcinoma SK-GT has been shown to express protein kinase C α, β and γ (Schwartz et al., 1993), whereas the colonic cell line HT-29 has been observed to overexpress isoform βI (Choi et al., 1990). If an homogenate of HGT-1 cells was added to a DE-52 column and was eluted with buffer containing 125mM NaCl then this fraction contained a protein of molecular mass 85kDa which bound anti-protein kinase C α. Previous work (Homan et al., 1991) had shown that protein kinase C α elutes in this fraction from a DE-52 column and the result therefore adds further support to the proposal that the 85kDa band is indeed protein kinase C α.

Bands suggestive of protein kinase C α were always seen in the particulate fraction of HGT-1 cell and sometimes in the cytosolic fraction. This variation may have occurred because the cells were not always grown for the same amount of time before use. Thus there was a suggestion that when HGT-1 cells were used at or after confluence cytosolic protein kinase C α was more prominant.

No protein kinase C β was found in HGT-1 cells and neither was there any detectable protein kinase C γ (Dr. M. G. Rumsby, personal communication).

In HGT-1 cells a band corresponding to protein kinase C ε was found in the membrane fraction. In other cell lines protein kinase C ε has been found to occur in either the cytosol, eg. neuroblastoma cells and the membrane fractions, eg. U937 cells (Hug, 1993). A doublet can be seen in the region of the protein kinase C ε band. If the blot blocked with peptide is

examined carefully it can then be seen that only the top band has disappeared completely and therefore the bottom band may not be this isoform. An alternative possibility is that the bottom band represents a form of protein kinase C ε (possibly phosphorylated) which has a very high affinity for the antibody.

Significant portions of certain isoforms are known to be permanently particulate but it is uncertain whether this means that they are permanently active. Protein kinase C α, β and ζ are predominantly cytosolic, θ is particulate, η is on the nucleus and ε is mostly cytosolic but can be particulate (Hug, 1993).

Brain tissue exhibited a dense but diffuse band in the region of protein kinase C ε. This may have been due to the partial proteolysis of the enzyme into fragments which would run down the gel slightly faster than the complete molecule.

The pattern of protein kinase C isoform expression in the gastric adenocarcinoma cell line HGT-1 is identical to that of the stomach, that is α and ε isoforms are present in both but there is little or no β. This suggests that HGT-1 cells are a reasonable model for investigating the expression and actions of gastric protein kinase C with the advantage from the clinical standpoint that they are a human cell line.

Preincubation of the cells with phorbol-12,13-dibutyrate for 24h prior to electrophoresis and protein blotting completely removed all signs of protein kinase C α. The time course for this event was such that the enzyme was present up to 8h of exposure to phorbol-12,13,dibutyrate but not after this time. The time course for down-regulation of protein kinase C by phorbol esters varies between cells. Swiss 3T3 cells take up to 30h when incubated with 100nM TPA whereas V79 chinese hamster lung

fibroblasts take only 5h with the same concentration of this agent (Adams et al., 1989) and in KM3 cells protein kinase C was removed after 15min of exposure to TPA (Ase, 1988).

Preincubation of HGT-1 cells with phorbol-12,13-dibutyrate had no effect on the presence of protein kinase C ε. Protein kinase C ε can often be resistant to the effects of phorbol esters, for example, in neither U937 cells nor murine thymocytes is protein kinase C ε down-regulated by incubation with TPA (Strulovici et al., 1991).

The decrease in specific binding of phorbol-12,13-dibutyrate seen after preincubation of HGT-1 cells with 1μM phorbol-12,13-dibutyrate for 24h strongly supports the protein blotting data to the extent that there is a reduction in the amount of protein kinase C present. The binding data did not provide clearcut information on which isoforms were being down-regulated. A problem is that some portion of the phorbol-12,13-dibutyrate binding to protein kinase C α may be Ca^{2+} independent.

HGT-1 cells contain an histamine H_2-receptor which is pharmacologically indistinguishable from that on human parietal cells (Gespach et al., 1988). Previous work with this cell line demonstrated that histamine increased adenylate cyclase activity and cAMP (McKenna et al., 1993). This response was decreased by the preincubation of the cells with TPA for 10min prior to stimulation with histamine suggesting that the activation of protein kinase C by TPA inhibited the stimulation of adenylate cyclase by histamine. Preincubation with TPA has no effect on the stimulation of adenylate cyclase by forskolin or by cholera toxin (McKenna et al., 1993). Forskolin exerts its effects by acting on the catalytic subunit of adenylate cyclase, whereas cholera toxin stimulates the Gs subunit of the G protein, so protein kinase C is not acting on either of these sites but another region closer to the histamine receptor.

Preincubation of HGT-1 cells with 1μM PDBu for 24h prevented the inhibitory effect of preincubation with TPA on histamine-stimulation of cAMP content (McKenna et al., 1993b). From the immunodetection data on the down-regulation of protein kinase C isoforms it is now possible to suggest that the isoform responsible for the effects described above is protein kinase C α which was down-regulated by phorbol-12,13-dibutyrate but not protein kinase C ε as this isoform was not altered after preincubation with phorbol-12,13-dibutyrate. Protein kinase C β and γ were absent from HGT-1 cells but the above down-regulation study cannot rule out the involvement of forms of novel protein kinase other than ε. However, work with homogenates demonstrated that the inhibitory effect of TPA on histamine-stimulated adenylate cyclase was Ca^{2+}-dependent. This mitigates against the involvement of an novel protein kinase C isoform in this effect and, since β and γ isoforms were not detected in HGT-1 cells, points to protein kinase C α. Further support for the involvment of the α isoform in mediating the action of TPA is provided by the specific blockade that was obtained with protein kinase C α antiserum. The antibody is directed against a peptide from the hinge region of the enzyme (Makowske et al., 1988). Thus it may prevent access of the enzyme to its substrate, possibly the histamine H_2-receptor (McKenna et al., 1993), rather than interfering with the catalytic site of protein kinase C α. Further work, involving measurement of protein kinase C activity in membrane fractions prepared from HGT-1 cells, is required to establish this.

4.4 SUMMARY.

Protein kinase C α and β exhibited a widespread distribution throughout the gastrointestinal tract but protein kinase C ε was found only in the gastric mucosa and gastrointestinal smooth muscle. HGT-1 cells contained protein kinase C α and ε, both of which were found in the particulate fraction. Protein kinase C α in HGT-1 cells was down regulated by incubation with phorbol-12,13-dibutyrate for 24h (with the enzyme disappearing after 8h). Protein kinase C ε was not down regulated by pretreatment with phorbol-12,13-dibutyrate for 24h. The isoform of protein kinase C involved in the inhibition by TPA of histamine-stimulated adenylate cyclase activity in HGT-1 cells was Ca^{2+}-dependent, down-regulated by pretreatment of the cells with phorbol-12,13-dibutyrate and was inhibited by an antibody to protein kinase C α. These data suggest that protein kinase C α is the isoform responsible for inhibiting histamine stimulated adenylate cyclase activity.

CHAPTER 5.

THE PRESENCE OF NITRIC OXIDE SYNTHASE ACTIVITY IN CULTURED CELLS AND IN TISSUES OF THE RAT GASTROINTESTINAL TRACT.

5.0 INTRODUCTION.

5.0.1 The Discovery of Nitric Oxide as a Messenger Molecule.

The credit for the discovery of nitric oxide as a regulator of cell function belongs to numerous individuals. The first indication of such an activity came with the discovery by Furchgott et al. (1980) that acetylcholine was unable to promote vasodilatation without the presence of vascular endothelium. The endothelium was thought to produce a factor which caused relaxation of vascular smooth muscle and this factor was termed endothelium-derived relaxing factor (EDRF) (Furchgott et al., 1980). Release of EDRF was stimulated not only by acetylcholine, but also by adenine nucleotides, thrombin, substance P, A23187 (a calcium ionophore), bradykinin, increased blood flow and electrical stimulation. EDRF had a very short half-life and its action was inhibited by haemoglobin, methylene blue and hydroquinone (Griffith et al., 1984). Physical and chemical manipulation of EDRF was used to try to determine its identity and eventually two separate groups suggested that it was nitric oxide (Furchgott, 1988; Ignarro et al., 1988). The group led by Moncada was then quick to demonstrate that nitric oxide was produced in endothelial cells by the metabolism of arginine (Palmer et al., 1987; Palmer et al., 1988) and that the biological effects of EDRF could be mimicked by nitric oxide. Thus both substances were equally unstable, bradykinin could stimulate the release of both from endothelial cell

culture and relaxation by both was inhibited by haemoglobin and enhanced by superoxide dismutase.

5.0.2 Characteristics of Nitric Oxide.

Nitric oxide is a colourless gas which is slightly soluble in aqueous solution. The molecule is formed from the terminal guanidino-nitrogen of L-arginine by a family of enzymes collectively known as nitric oxide synthases (Schmidt et al., 1993) (see below) which produce L-citrulline as a co-product. Nitric oxide can act only as a local mediator because it has a biological half-life of around 5s and is oxidised to the inactive products inorganic nitrite and nitrate (Stark et al., 1992). Nitric oxide is also converted by superoxide anions to peroxynitrite which can in turn form the •OH radical. Superoxide dismutase, which removes superoxide, can therefore prolong the half-life of nitric oxide (Stark et al., 1992).

5.0.3 Biosynthesis of Nitric Oxide.

Nitric oxide synthase enzymes require NADPH, O_2, and four cofactors: haem, FMN, FAD and tetrahydrobiopterin for activity. 1.5 molecules of NADPH are used in the reaction yielding 1.5 molecules of $NADP^+$ and three hydride atoms ($H^•$). In the first phase of the reaction one NADPH is used to form hydroxyarginine, the second phase then involves the remaining half NADPH oxidising hydroxyarginine to form nitric oxide and L-citrulline (Schmidt et al., 1993) (fig. 5.0.1).

There are a number of different forms of nitric oxide synthase, which is classified in terms of properties and location, (table 5.0.1) of which three distinct genes have been identified. The gene for the brain enzyme (type Ia) codes for a homodimer of which the subunits have a molecular mass of 160kDa (Schmidt et al., 1993). The N-terminal half of the enzyme is

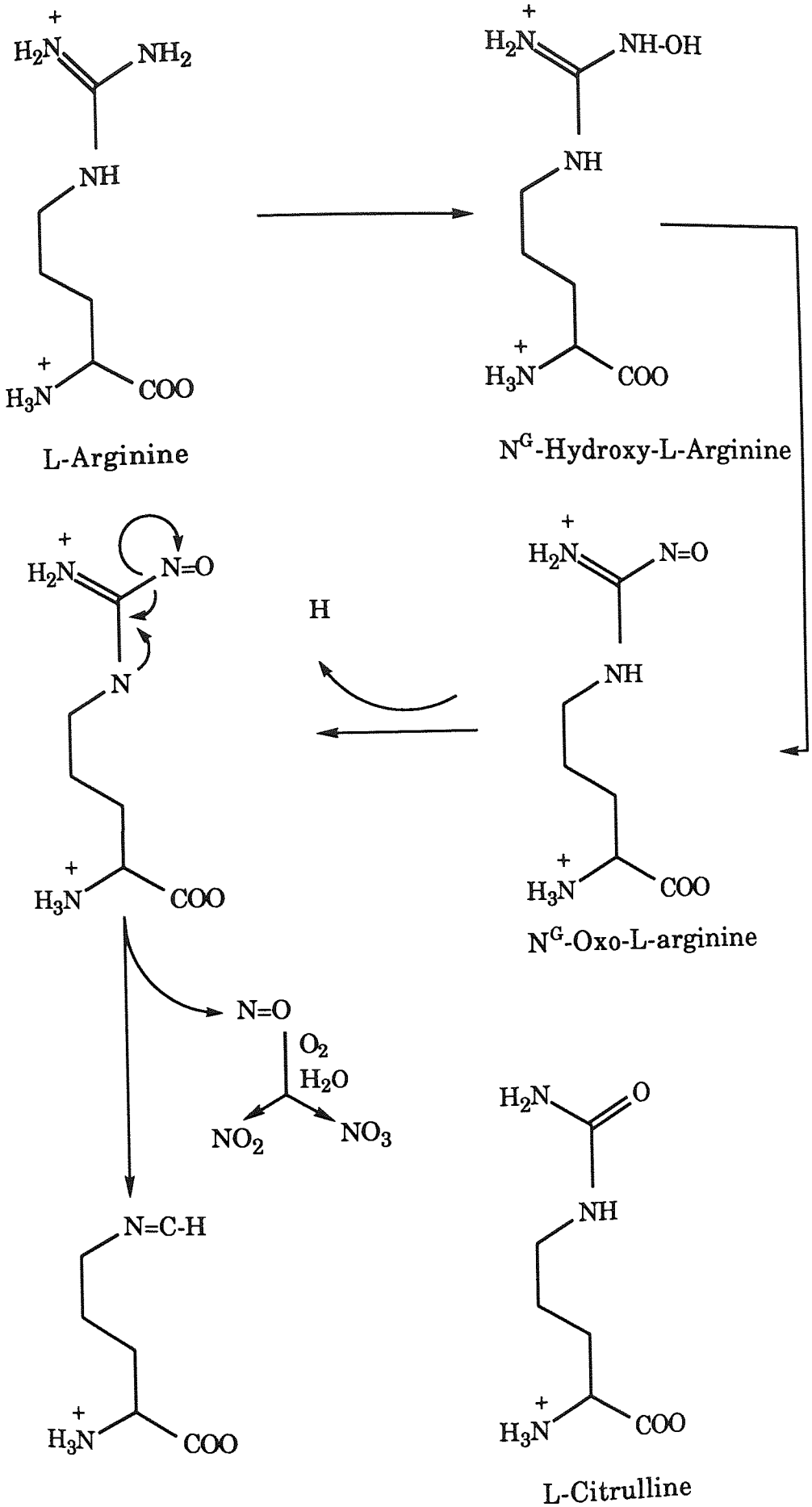


Fig. 5.0.1 The biosynthesis of nitric oxide from L-arginine (after Collier (1989).

Table 5.0.1 Characterisation of Nitric Oxide Synthases in Terms of their Properties and Localisation (after Forstermann et al., 1991).

Isoenzyme	Activation	Size (kDa)	Typical location(s)
Ia soluble	Ca^{2+} Calmodulin	155	Cerebellum
Ib soluble	Ca^{2+} Calmodulin	?	Endothelium
Ic soluble	Ca^{2+} Not calmodulin	150	Neutrophils
II soluble	Induced by cytokines and lipopolysaccharide	125-135	Macrophages Hepatocytes Kupffer cells Vascular smooth muscle Endothelium Lung
III particulate	Ca^{2+} Calmodulin	135	Endothelium
IV particulate	Induced by cytokines and lipopolysaccharide	?	Macrophages

responsible for the binding of the cofactors NADPH, FAD and FMN (fig. 5.0.2) and this region exhibits 35% homology with NADPH-cytochrome-P_{450} reductase (Schmidt et al., 1993). Binding regions for calmodulin and haem have been suggested but the actual sites of interaction of L-arginine and tetrahydrobiopterin have not been deduced as yet.

The brain enzyme is a constitutive form which means that it is always present. It is activated by receptor-mediated increases in intracellular Ca^{2+} which promote the binding of calmodulin to the enzyme. Expression of the gene for the second type of nitric oxide synthase (type II), which is found in macrophages, is induced by bacterial lipopolysaccharide and cytokines like interferon γ. Phorbol esters can also cause the induction of this form nitric oxide synthase. Hortelano et al. (1992) saw the induction of nitric oxide synthase within 45 - 60min using phorbol-12,13-dibutyrate to activate protein kinase C. The protein kinase C inhibitor Ro31-8220 was able to inhibit nitric oxide induction in macrophages (Severn et al., 1992) and so there appears to be a strong link between protein kinase C and the induction of nitric oxide synthase. Thus the activity of inducible nitric oxide synthase is regulated via its transcription, and because it binds calmodulin tightly it needs negligible Ca^{2+}. The inducible enzyme has approximately 40% amino acid homology with the constitutive form (Springall et al., 1992).

Induction of the synthesis of *de novo* mRNA for nitric oxide synthase can be inhibited by glucocorticoids such as cortisol and dexamethasone but not by progesterone. The activity of constitutive nitric oxide synthase is not affected by these substances (Knowles et al., 1990a). Some of the known anti-inflammatory effects of glutocorticoids may stem from this inhibition.

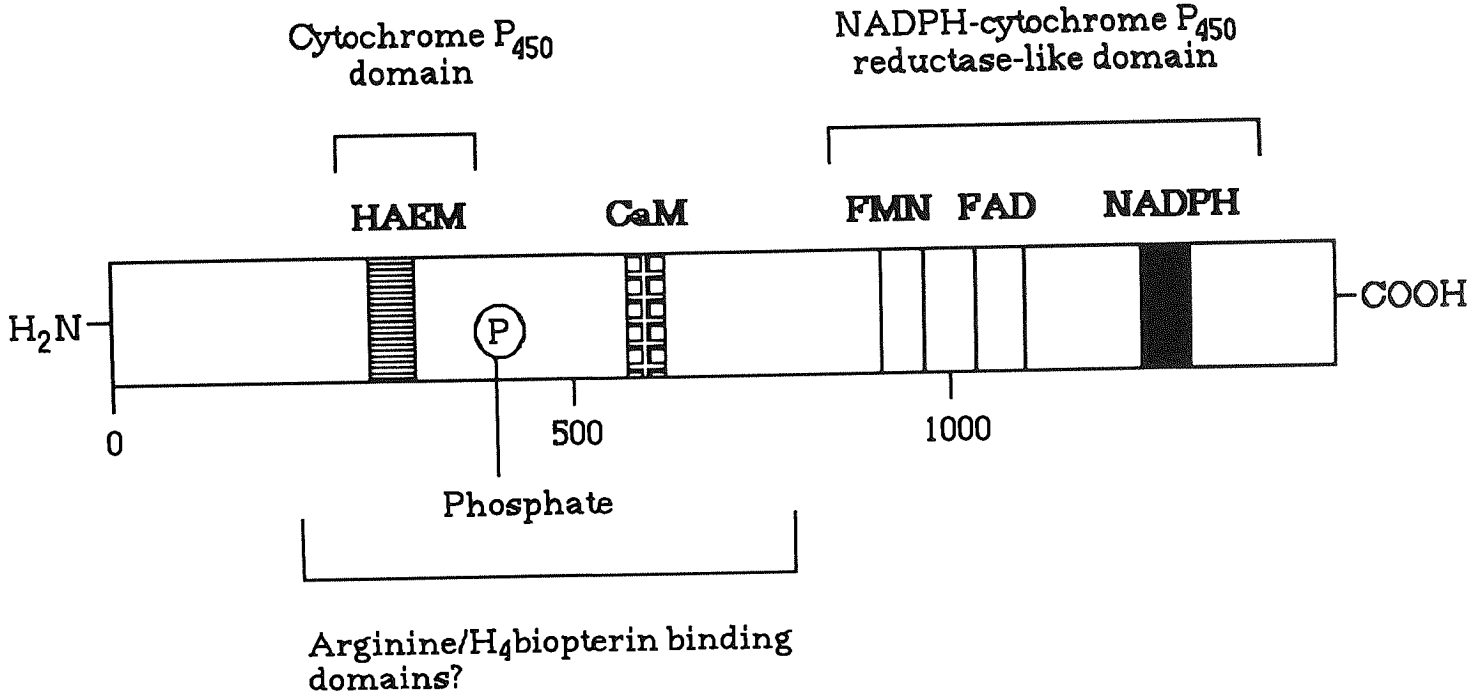


Fig. 5.0.2 Protein domains and binding sites of nitric oxide synthases.

The third gene for nitric oxide synthase codes for a form found in the endothelium. It is a constitutive, Ca^{2+}-dependent enzyme of molecular mass 133kDa. Endothelial nitric oxide synthase is primarily found in the particulate fraction and this is due to it being myristoylated on its N-terminus (Busconi and Michel, 1993). A cytosolic form of the endothelial enzyme is present only when it is phosphorylated on a serine residue and this may play a role in the regulation of enzyme action (Michel et al., 1993). The endothelial enzyme shows 50-60% homology in nucleotides with the macrophage and rat brain nitric oxide synthase (Lamas et al., 1992).

The presence of NADPH diaphorase activity is used as a histochemical marker for nitric oxide synthase. NADPH diaphorase is a partial activity of nitric oxide synthases. In the absence of arginine the enzyme reduces the dye nitroblue tetrazolium to a water-insoluble formazan product. All known forms of nitric oxide synthase, including the endotoxin-induced type, exhibit NADPH diaphorase activity, but not all diaphorase activity can be assigned to nitric oxide synthases (Tracey et al., 1993). NADPH diaphorase acitvity has been observed in the myenteric plexus of the rat small intestine (Belai et al., 1992).

5.0.4 Assay for nitric oxide synthase.

The assay for nitric oxide synthase activity used in this work relies on the ability of nitric oxide synthase to convert [^{14}C] L-arginine to [^{14}C] L-citrulline. L-citrulline has no net charge at neutral pH but L-arginine has a net charge of 1^+. Dowex AG 50W-8 resin, at neutral pH, is a negatively charged cation exchange resin and when added to a mixture of enzyme and radiolabelled substrate after incubation will attract the remaining L-arginine but will leave the L-citrulline that was produced by the reaction in solution. The amount of radioactivity remaining in

solution after the addition of the Dowex resin can be used to calculate the amount of L-citrulline formed. Valine is added to the reaction mixture to inhibit any arginase which would convert arginine to ornithine, and NADPH is added as a cofactor. The activity of nitric oxide synthase is calculated from the difference in radioactivity remaining between the assays performed with and without N^G-monomethyl-L-arginine (L-NMMA). L-NMMA is a specific and competitive inhibitor of all known forms of nitric oxide synthase (Palmer et al., 1988b). Ca^{2+}-independent, and therefore probably inducible nitric oxide synthase, is determined by the difference between the activity inhibitable by L-NMMA (total) and that by EGTA (Ca^{2+}-dependent). Blank values are obtained when cellular homogenate is replaced by homogenisation buffer.

5.0.5 The Roles of Nitric Oxide.

Nitric oxide produced by constitutive enzymes is manufactured in small amounts and acts as a messenger molecule. Endothelial nitric oxide is a vasodilator and also, in conjunction with that produced by platelets, inhibits the aggregation of platelets (Azuma et al. 1986) and their adhesion to endothelial monolayers, extracellular matrix and collagen fibrils (Radomski et al., 1987a, b).

Large amounts of nitric oxide synthase activity are found in the cerebellum. Significant amounts of the enzyme are present in the nerves of the posterior pituitary and the autonomic nerves of the retina (Bredt et al., 1990). Elsewhere in the body nerves containing nitric oxide synthase are present in the myenteric plexus of the stomach and intestine and in the adrenal medulla. In the brain nitric oxide is a neuro-modulator and possibly a neurotransmitter (Garthwaite, 1991). Nitric oxide is one of the non-adrenergic, non-cholinergic neurotransmitters involved in

peristalsis, relaxation of the fundus (Boeckxstaens et al., 1992) and relaxation of the sphincter muscles (Heath, 1991).

Nitric oxide plays an important role in the immune system as a primary defence against the invasion of intracellular micro-organisms. Nitric oxide produced by activated macrophages is made in large amounts. It can inactivate iron and iron-sulphur containing enzymes (Ignarro, 1991), cause nitrosylation of proteins and induce mutagenesis.

Endotoxic shock is a leading cause of death in intensive-care units (Culotta and Koshland, 1992). Excessive production of nitric oxide by the immune system in response to infection has been implicated in the hypotension and reduction in the response to vasoconstrictors seen in this condition (Rees et al., 1990). In a number of cases of septic shock nitric oxide synthase inhibitors such, as L-NMMA, have been used as treatment (Culotta and Koshland, 1992).

The presence of endotoxin can lead to acute intestinal damage from vasocongestion, plasma leakage and haemorrhage into the lumen. In the presence of the nitric oxide generator SNAP (S-nitroso-N-acetyl-penicillamine) this damage is decreased (Stark et al., 1992). It is possible that the endotoxin is causing the production of superoxide radicals which lead to injury. Nitric oxide interacts with these free radicals to form peroxynitrite, which breaks down to yield hydroxyl (Stark et al., 1992). Induction by intravenous lipopolysaccharide has been seen in tissues such as aortic smooth muscle, macrophages, vascular endothelium, liver, lung and chondrocytes (Mitchell et al., 1992).

5.0.5.1 Role of nitric oxide in the Gastrointestinal Tract.

Inhibition of the production of nitric oxide reduces gastric mucosal blood flow (Whittle et al., 1991). Blood flow depends on a delicate balance between endogenous vasodilators and vasoconstrictors. Removal of nitric oxide, sensory neuropeptides and prostanoids produces extensive damage to the mucosa in the absence of exogenous vasoconstrictors (Whittle et al., 1990). Nitric oxide synthase is also known to be present in the myenteric plexi, in nerves running through the gastrointestinal circular muscle and may be present in gastric muscle cells. The enzyme at these sites is probably involved in regulation of gastrointestinal motility (Grider et al., 1992).

Whittle et al. (1991) found a considerable activity of nitric oxide synthase in the gastric mucosa. However, further information about the precise location and forms of nitric oxide synthase in the mucosa of the gastrointestinal tract was lacking, as was a cell line with which to study the regulation of gastrointestinal nitric oxide synthase. Consequently in this work an investigation of the presence of nitric oxide synthase in gastrointestinal cell lines and preparations of rat gastrointestinal tract was initiated.

As can be seen nitric oxide has a dual role in the body confering both harmful and beneficial effects and these are summarised in table 5.0.2.

5.0.6 Nitric Oxide: Transduction Mechanisms.

Nitric oxide activates soluble guanylate cyclase (Rapoport et al., 1983) and so promotes the formation of cGMP inside the cell. Activation of guanylate cyclase by nitric oxide probably involves a conformational change in guanylate cyclase induced by the binding of nitric oxide to the

Table 5.0.2 Comparison of the Benefits and Harm conferred by Nitric Oxide (Schmidt et al., 1992).

Tissue	Benefit	Harm
Brain	Neurotransmitter Potentiation	Neurotoxic
Stomach	Cytoprotection Reflex dilation Motility	Cytotoxic Mutagenic ?
Pancreas	Insulin release	β-cell destruction
Blood vessels	Vasodilator Antithrombic	Reperfusion injury Endotoxic shock Anaphylactic shock
Leucocytes	Immune defence	Endotoxic shock

haem prosthetic group. cGMP can modulate cGMP-gated ion channels, cGMP-inhibited cAMP phosphodiesterase, cGMP-stimulated cAMP phosphodiesterase and the cGMP-dependent protein kinase which exists in forms Iα, Iβ or II. There is approximately 5s between stimulation of a cell by nitric oxide and an increase in cGMP and another 5s between that and relaxation in vascular smooth muscle (Ignarro, 1991).

Gastrointestinal smooth muscle contains cGMP-dependent protein kinases and it is likely that these mediate nitric oxide's effects (Stark et al., 1992). In the smooth muscle of the canine gastric antrum nitric oxide inhibits voltage dependent calcium-channels and it may also reduce the calcium sensitivity of contractile elements via elevation of cGMP (Stark et al., 1992).

5.1 METHODOLOGY.

5.1.1 Preparation of homogenates.

5.1.1.1 Cells.

Cells were cultured and detached from flasks as described in section 2.0.1 and washed with Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (section 4.1.1.1). Rat gastric mucosal, ileal and colonic cells were isolated and, in the first case enriched, as described in sections 2.1 and 2.2.

Cells were resuspended in homogenisation buffer (Table 5.1.1) at 10^8 cells/ml and freeze fractured by placing in liquid nitrogen and then thawing rapidly at 37°C three times. Homogenates were subjected to centrifugation at 12,000g for 20min at 4°C and the supernatant used in the assay.

5.1.1.2 Tissue.

The gastrointestinal tract and brain were removed from an adult male Wistar rat under anaesthesia with Sagatal™ (procedure carried out by Mr J. F. Brown) and washed with ice-cold 0.9% (w/v) saline. The gastrointestinal tract was opened along its length and the mucosa removed from each section by gently scraping with a microscope slide. The gastrointestinal muscle and brain were roughly chopped. Each tissue was added to 5 volumes of homogenisation buffer and homogenised for 1min at maximum speed on ice using an Ultra Turrax tissue homogeniser. Samples were then subjected to centrifugation as in section 5.1.1.1.

5.1.2 Extraction of putative particulate nitric oxide synthase (after Hiki et al., 1992).

Rat gastric mucosa and muscle were prepared as before (section 5.1.1.2). After homogenisation the suspensions were subjected to centrifugation at 100,000g at 4°C for 30min. The resulting supernatant was decanted and assayed as the soluble fraction. The pellet was resuspended by agitation on a whirlymixer in a volume of homogenisation buffer equivalent to that of the supernatant and containing 1M KCl and 10% glycerol and then was left for 5min on ice. The suspension then underwent centrifugation again at 100,000g at 4°C for 30min and the supernatant was discarded. The pellet was resuspended in an equivalent amount of homogenisation buffer to which: 10% glycerol, and 20mM CHAPS had been added and left for 20min at 4°C on a rolling incubator. The suspension was subjected to centrifugation at 100,000g at 4°C for 30min and the resulting supernatant assayed as the particulate fraction.

60nM calmodulin and 10μM FAD was added to the particulate fraction since it was thought that these cofactors might have been removed during fractionation.

The modified homogenisation buffer used in the solubilisation of the pellet containing the particulate fraction was monitored for any inhibitory action on nitric oxide synthase activity. The activity of soluble nitric oxide synthase in normal homogenisation buffer was compared with that seen after additions of glycerol and CHAPS to give final concentrations the same as those found in the modified buffer. A correction was made for dilution of enzyme activity by these additions.

5.1.3 Induction of Nitric Oxide Synthase Activity *in Vivo*.

Lipopolysaccharide (isolated from the bacterial cell wall of E. col. serotype 0111:B4) was dissolved in pyrogen-free 0.9% (w/v) saline to give a concentration of 3mg/ml and injected into the tail vein of adult male Wistar rats at 3mg/kg body weight (procedure performed by Mr J. F. Brown). The animals were then left for 4h. Control animals received an equivalent amount of pyrogen-free 0.9% (w/v) saline.

5.1.4 Nitric Oxide Synthase Assay.

5.1.4.1 Preparation of Dowex Resin.

200ml of 1M NaOH was added to 50g of Dowex AG 50W-8 cation exchange resin to activate it and the suspension left for 1h. The supernatant was decanted and another 200ml of NaOH added. This was again left for 1h and the supernatant decanted. Double distilled water was added in 300ml aliquots for 1h periods until the pH of the supernatant was approximately 6.5. The prepared Dowex resin was then suspended 1:1 with double distilled water.

5.1.4.2 Nitric Oxide Synthase Assay.

Nitric oxide synthase activity was determined by the use of a radiochemical assay converting [U-^{14}C] L-arginine to [U-^{14}C] L-citrulline (derived from the procedure of Knowles et al., 1990b). 50µl aliquots of assay buffer pH 7.2 were prewarmed to 37°C and 20µl of homogenate was added to each tube at 10s intervals. The final mixture contained: 35mM dipotassium hydrogen phosphate, 0.7mM magnesium chloride, 143µM calcium chloride, 680mM NADPH, 6.8mM L-valine and 15µM [^{14}C] L-

arginine [700,000 d.p.m./ml]. In some incubations 70μM N^G-monomethyl-L-arginine (final concentration) or 694μM EGTA (final concentration) was present. After 10min of incubation at 37°C 500μl of a 1:1 suspension of Dowex resin was added, again at 10s intervals. 1ml of double distilled water was then added at room temperature to each tube and the resin allowed to settle for 15min. 975μl of the supernatant was added to 5ml of Optiphase Hisafe II for scintillation counting on the Tri-carb liquid scintillation analyser, model 1900TR (Packard-Hewlett).

Blanks were prepared by the addition of 20μl of homogenisation buffer rather than homogenate to tubes. Total label added was determined by adding 20µl of homogenisation buffer instead of homogenate, 250µl of double distilled water in place of the Dowex resin, and counting 975μl of the final mixture.

5.1.4.3 Calculation of nitric oxide synthase activity.

The amount of [^{14}C] L-citrulline formed in the nitric oxide synthase assay is a direct measurement of enzyme activity. The calculation to convert the values obtained with the assay to nitric oxide synthase activity in units of nmol/min/ml homogenate is:

$$\frac{\text{d.p.m. above result with L-NMMA}}{\text{Specific activity of label (dpm/nmol)}} \times \frac{\text{total aqueous volume}}{\text{volume removed for counting}} \times \frac{1000}{20 \times \text{Time (min)}}$$

Conversions can then be made for the number of cells present, the amount of protein the sample contains or the wet weight of the sample in 1ml of homogenate.

5.1.4.4 Calcium dependency.

Tissues were homogenised as previously described (section 5.1.1.2) in homogenisation buffer (Table 5.1.1) without EDTA and with the pH 7.2. Calcium chloride was added to the assay buffer to give a range of free calcium concentrations from 0 - 0.2mM in the final assay system. Free calcium concentrations were calculated using an association constant for Ca^{2+}-EGTA of 12052123.7 l^{-1} moles.

5.1.5 Alkaline Phosphatase Assay.

CaCo-2 cells were grown as described in section 2.0 for 20 days. The monolayer was washed with Ca^{2+}-, Mg^{2+}-free phosphate buffered saline and detached with a solution of 0.02% EDTA (w/v) in phosphate buffered saline at 37°C for 3min. The cell suspension was subjected to centrifugation at 100g for 3min at 15°C and washed twice with phosphate buffered saline. The pellet was resuspended in homogenisation buffer (Table 5.1.1 at 10^8cells/ml and freeze fractured in liquid nitrogen as described in section 5.1.1.1. To 25µl of homogenate was added 1ml of a buffer containing: 0.5M Tris HCl (pH9.4), 0.3mM $ZnCl_2$, 10mM $MgCl_2$ and 0.23mM p-nitrophenylphosphate. The mixture was incubated at 37°C for 15min after which 0.5ml of 0.5M NaOH was added and the solution mixed. The absorbance at 420nm was read on a spectrophotometer. A standard curve was prepared using p-nitrophenol in homogenisation buffer in the range 0 - 100µg from which the alkaline phosphatase activity in nmol/min/mg protein was calculated.

5.1.6 Preparation of Tissue for Sectioning on the Cryostat.

The stomach was removed from a rat anaesthetised by intraperitoneal injection of Sagatal™. 0.9% (w/v) saline was injected with a syringe

between the mucosa and muscle layers to form a blister, the mucosa cut off and placed in liquid nitrogen. Alternatively paraformaldehyde was prepared by heating a 4% (w/v) solution in phosphate buffered saline to exactly 65°C and then adding a few drops of 1M NaOH to clear it. The mucosa was fixed in paraformaldehyde for 2h and then placed in a 15% (w/v) solution of sucrose overnight. The prepared tissue or frozen sections were embedded in OCT and frozen with freezing spray (Anglia). Sections were cut on a Bright cryostat at 5μm and placed on microscope slides coated with 0.5% (w/v) gelatine and 0.05% (w/v) chrome alum. Slides were dried at 37°C for, at least, 30min before being stained.

5.1.7 NADPH Diaphorase Staining.

Pre-cut sections were stained with a solution containing: 50mM Tris HCl (pH8.0), 0.2% Triton-X 100, 0.5mM nitroblue tetrazolium and 1mM β-NADPH for 30min at 37°C. Control sections were stained with a solution without the 1mM β-NADPH. Sections were viewed by light microscopy and photographed using an Olympus OM4 camera using an automatic exposure setting and Kodak Gold 200ASA film.

Table 5.1.1 Composition of the Homogenisation Buffer used in the Nitric Oxide Synthase Assay.

10mM	HEPES pH7.4
0.32M	Sucrose
0.09mM	EDTA
1mM	Dithiothreitol
10μg/ml	Leupeptin
10μg/ml	Aprotinin
10μg/ml	Soybean trypsin inhibitor

5.2 RESULTS.

5.2.1 Cell Lines.

None of the gastrointestinal cell lines tested exhibited any nitric oxide synthase activity. Thus the d.p.m. remaining in the supernatant after the addition of Dowex was the same in uninhibited assays as when Ca^{2+} was removed by EGTA or when the nitric oxide synthase inhibitor L-NMMA was present. Examples of these negative results are shown in table 5.2.1.

CaCo-2 cells were shown to be differentiated by the presence of increased amounts of alkaline phosphatase activity (table 5.2.2).

5.2.2 Tissues of the Gastrointestinal Tract.

Gastric mucosa exhibited a significant amount of nitric oxide synthase activity above zero ($P<0.001$, t-test) (fig. 5.2.1). Gastric muscle, jejunal muscle, ileal muscle and colonic muscle all showed significant activity ($P<0.005$, paired t-test). Gastric mucosal activity was significantly greater than that of the jejunum, ileum and colon ($P<0.01$, Newman-Keuls test) which did not differ significantly from each other. Gastric and colonic muscle activities were significantly greater than those of jejunal and ileal muscle ($P<0.01$, Newman-Keuls test). Gastric and colonic muscle did not differ significantly from each other and neither did ileal and jejunal muscle (Newman-Keuls test). The activity inhibitable by EGTA in the gastric (1036±155pmol/min/g wet weight) and colonic muscles (1004±108pmol/min/g wet weight) was slightly but significantly greater than that inhibitable by L-NMMA ($P<0.007$ and 0.019 respectively, paired t test). Activity inhibitable by EGTA in all other tissues did not differ from that found in the presence of L-NMMA.

Table 5.2.1 The Absence of Nitric Oxide Synthase Activity in Gastrointestinal Cell Lines.

Cell line	pmol/min/10^8 cells. Uninhibited value	L-NMMA value	EGTA value
HGT-1	0.195±0.005	0.189±0.003	0.189±0.009
MKN-45	0.203±0.01	0.196±0.004	0.200±0.002
CaCo-2 (undifferentiated)	0.176±0.008	0.175±0.008	0.175±0.012
CaCo-2 (differentiated)	0.244±0.018	0.247±0.019	0.245±0.009

Each experiment is a mean ± SEM of triplicate determinations and is representative of: HGT-1 three experiments, MKN-45 one experiment, CaCo-2 undifferentiated two experiments and CaCo-2 differentiated ten experiments. No significant difference was found between uninhibited values and those in the presence of L-NMMA or EGTA in any of the cell lines (Analysis of variance plus Newman-Kuels test).

Table 5.2.2 Comparison of the Alkaline Phosphatase Content of CaCo-2 Cells Grown for Varying Lengths of Time.

Number of Days of growth	Alkaline Phosphatase Activity (nmol/min/mg protein*).
6	7.8±0.98
10	26.72±2.34
15	42.29±3.38
20	124.57±14.95

*Protein content was determined by the Folin-Lowry procedure (section 2.5).
Results are the mean of triplicate determinations from a single experiment.

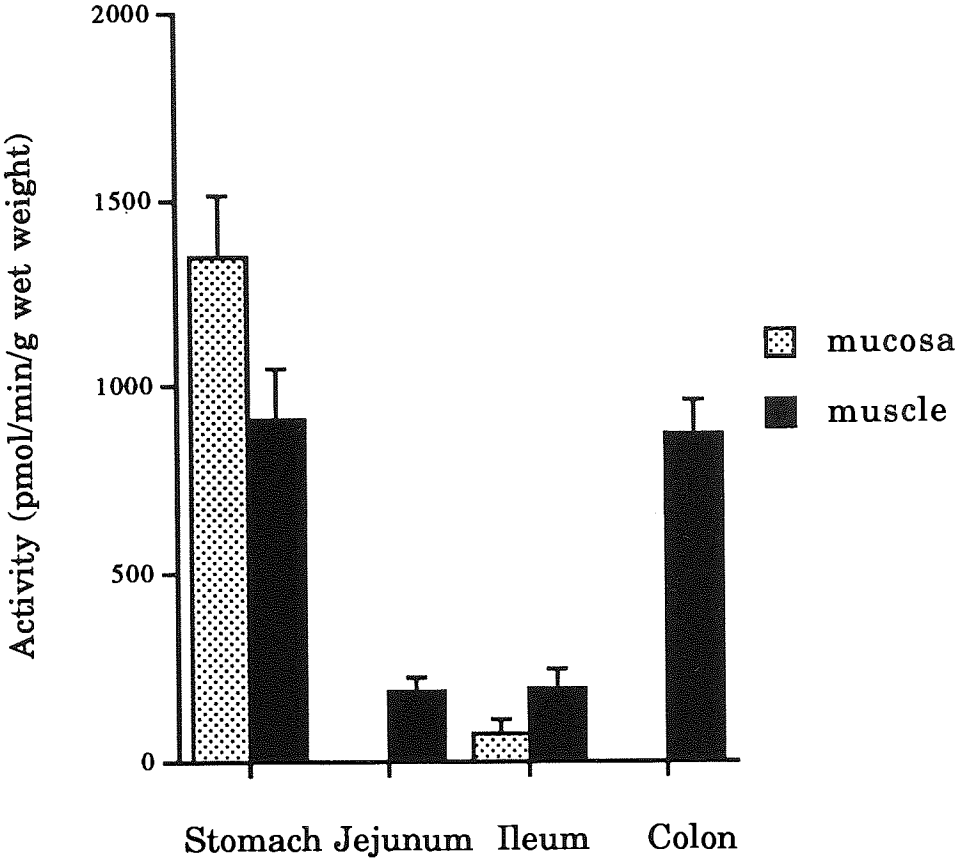


Fig 5.2.1 Distribution of Nitric Oxide Synthase in the mucosa and muscle of the gastrointestinal tract of the rat.

Results are mean ± SEM of stomach n=6, jejunum and ileum n=7 and colon n=4 rats respectively. Nitric oxide synthase activity measured as citrulline formation inhibitable by a final concentration of 70μM L-NMMA. Significant activity above zero was seen in the gastric mucosa ($P<0.001$) and all muscle fractions ($P<0.005$, paired t-test). Jejunal and colonic mucosa activities were 1.97±1.17 and 0±0.04pmol/min/g wet weight respectively and do not show up on the plot.

5.2.3 Subcellular Distribution of Nitric Oxide Synthase Activity in the Gastric Mucosa and Muscle.

In both the mucosa and the muscle of the stomach only the soluble fraction exhibited activity which was significantly greater than zero ($P<0.01$, paired t-test) (fig. 5.2.2). The solubilisation buffer had a significant effect on enzyme activity ($P=0.01$, t-test) but it did not completely destroy it (table 5.2.3).

5.2.4 The distribution of Nitric Oxide Synthase in Cells Isolated from the Mucosa of the Gastrointestinal Tract.

Nitric oxide synthase activity in crude and enriched gastric mucosal cells was 0.17 ± 0.046 and 1.412 ± 0.297 pmol/min/10^6 cells respectively. Ileal cells were difficult to count because they aggregated and activity in these cells was expressed per g dry weight. Gastric nitric oxide synthase activity obtained from isolated cells was re-expressed per g dry weight by using a factor (J. Hatt and P. J. Hanson, unpublished work) of 0.15mg dry weight / 10^6 cells to enable a comparison with ileal cells. Nitric oxide synthase activity in high density cells and in crude gastric mucosal cells was clearly significant ($P<0.01$, t-test) (fig. 5.2.3) as was also found in ileal cells ($P<0.025$, t-test). High density gastric cells differed significantly from unenriched gastric mucosal cells, ileal mucosal cells and colonic mucosal cells ($P<0.01$, Newman-Keuls test). There was no significant difference between activity inhibitable by L-NMMA and that by EGTA (paired t-test).

Nitric oxide synthase activity in scraped mucosa and in cells isolated from the mucosa of the gastrointestinal tract is compared in table 5.2.4. An hydration value of 3.5g water / g dry weight (Hanson and Parsons 1976) was used to convert results with tissue scrapings to a form

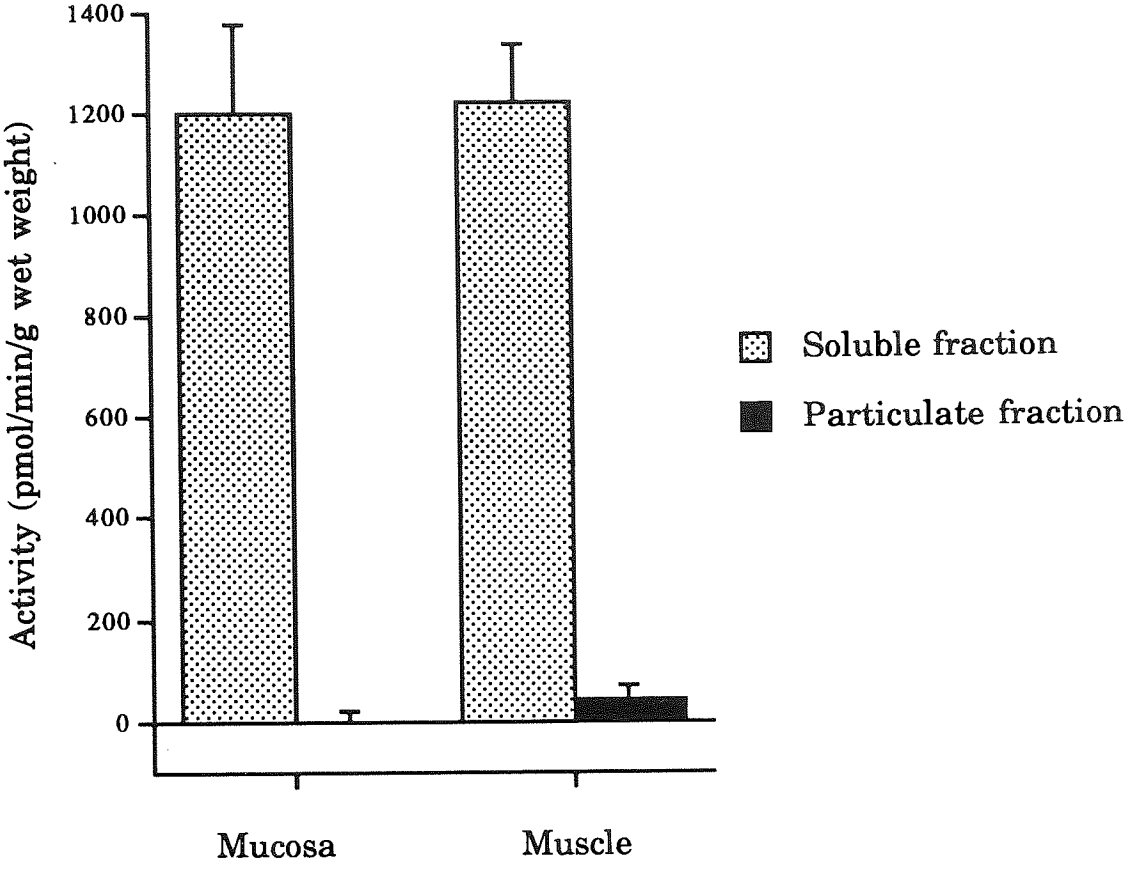


Fig. 5.2.2 Subcellular distribution of nitric oxide synthase in the gastric mucosa and muscle of the rat.

Results are mean ± SEM, n=4 rats. Nitric oxide synthase activity measured as citrulline formation inhibitable by a final concentration of 70μM L-NMMA. Significant activity was seen in the soluble fraction ($P<0.01$, t-test).

Table 5.2.3 The Effect of Modification of the Homogenisation Buffer on the Activity of Gastric Mucosal Nitric Oxide Synthase.

Nitric oxide synthase activity in normal homogenisation buffer.	0.954±0.018nmol/min/g wet weight
Nitric oxide synthase activity after modification of the homogenisation buffer.	0.684±0.031nmol/min/g wet weight

Results expressed as mean±SEM of triplicate determinations. One experiment typical of two. There was a significant difference between the two results (P=0.01, unpaired t-test).

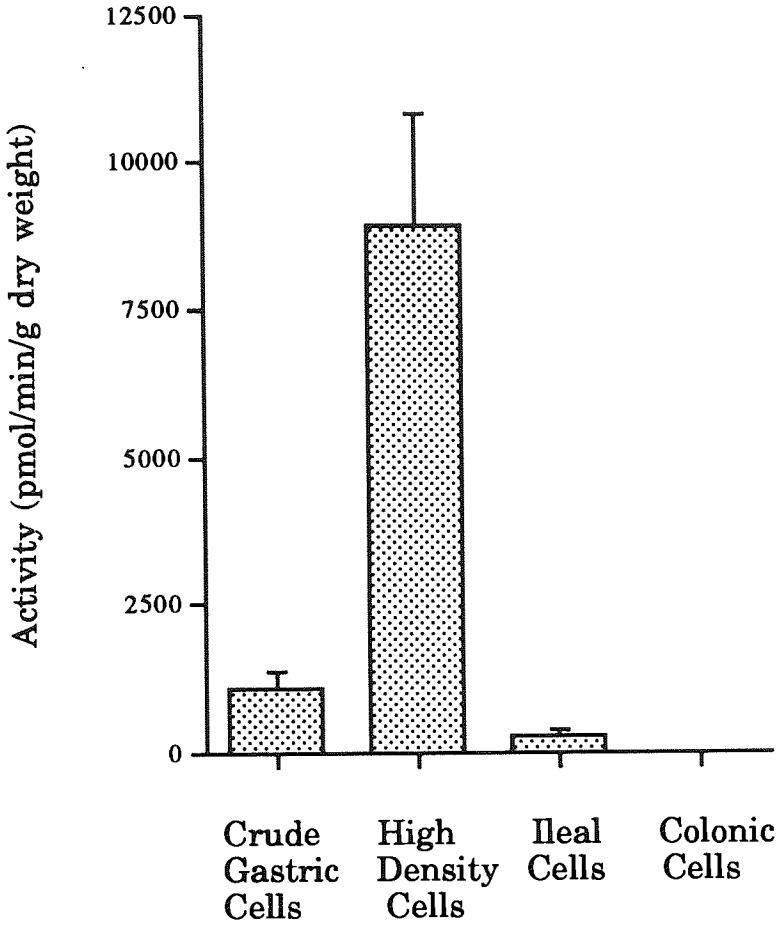


Fig. 5.2.3 Distribution of nitric oxide synthase in cells isolated from the mucosa of the gastrointestinal tract of the rat.

Results are mean ± SEM of crude cells n=8, high density cells n=9, ileal cells n=9 and colonocytes n=5 experiments respectively. Nitric oxide synthase activity measured as citrulline formation inhibitable by a final concentration of 70μM L-NMMA. Significant activity above zero was seen in crude gastric cells, high density cells ($P<0.01$) and ileal cells ($P<0.025$, t-test).

Table 5.2.4 Comparison of nitric oxide synthase activity in scraped mucosa and cells isolated from the mucosa of the gastro-intestinal tract.

(Activity per unit dry weight using a conversion of 3.5g water /g dry weight [Hanson and Parsons 1977]).

Region	pmol/min/g dry weight	
	Scraped	Isolated
Stomach (crude cells)	6066±752	1076±291
Ileum	340±171	275±93
Colon	0±0.18	0±7.5
Stomach (high density cells)		8937±1879

comparable with mucosal cell isolates. Due to the approximations involved in the manipulating of both sets of data, no statistical analysis were performed but some qualitative comments are possible. Scraped gastric mucosa appears to have more activity than unenriched isolated cells. Ileal values were approximately equal although only significant for isolated cells and colonic results were negligible for both preparations.

5.2.5 Localisation of NADPH Diaphorase Activity in the Rat Stomach.

The stain for NADPH diaphorase activity localised mainly in the gastric pits (plate 5.2.1) with a small amount of staining in the glands. No staining was observed in the absence of NADPH (plate 5.2.2).

5.2.6 Nitric Oxide Synthase Activity in the Mucosa and Muscle of the Gastrointestinal Tract of Rats Treated with Endotoxin.

Jejunal, ileal and colonic mucosae from rats pretreated with endotoxin all exhibited a significant increase above control in nitric oxide activity ($P<0.001$, unpaired t-test) (see fig. 5.2.4). Mucosa in the ileum and colon of endotoxin-pretreated rats showed a significant proportion of activity which was not inhibitable by EGTA ($P<0.02$, paired t-test) (fig. 5.2.5). This Ca^{2+}-independent enzyme activity was greatest in the ileum followed by the colon ($P<0.001$, Newman-Keuls test).

In the gastrointestinal muscle of rats pretreated with endotoxin there was a significant increase in the total activity of nitric oxide synthase in the jejunal, ileal ($P<0.01$) and colonic muscle ($P<0.05$) (unpaired t-test) (fig. 5.2.4). However, no significant proportion of this activity was found to be Ca^{2+}-independent (paired t test) (fig. 5.2.6).

Plate 5.2.1 Localisation of NADPH diaphorase activity in the rat stomach.

The plate shows a transverse section of the gastric wall with gastric pits (p) and glands (g). Magnification x156.25

Plate 5.2.2 Detection of NADPH diaphorase activity in the rat stomach in the absence of NADPH.

The plate shows a transverse section of the gastric wall. Magnification x156.25.

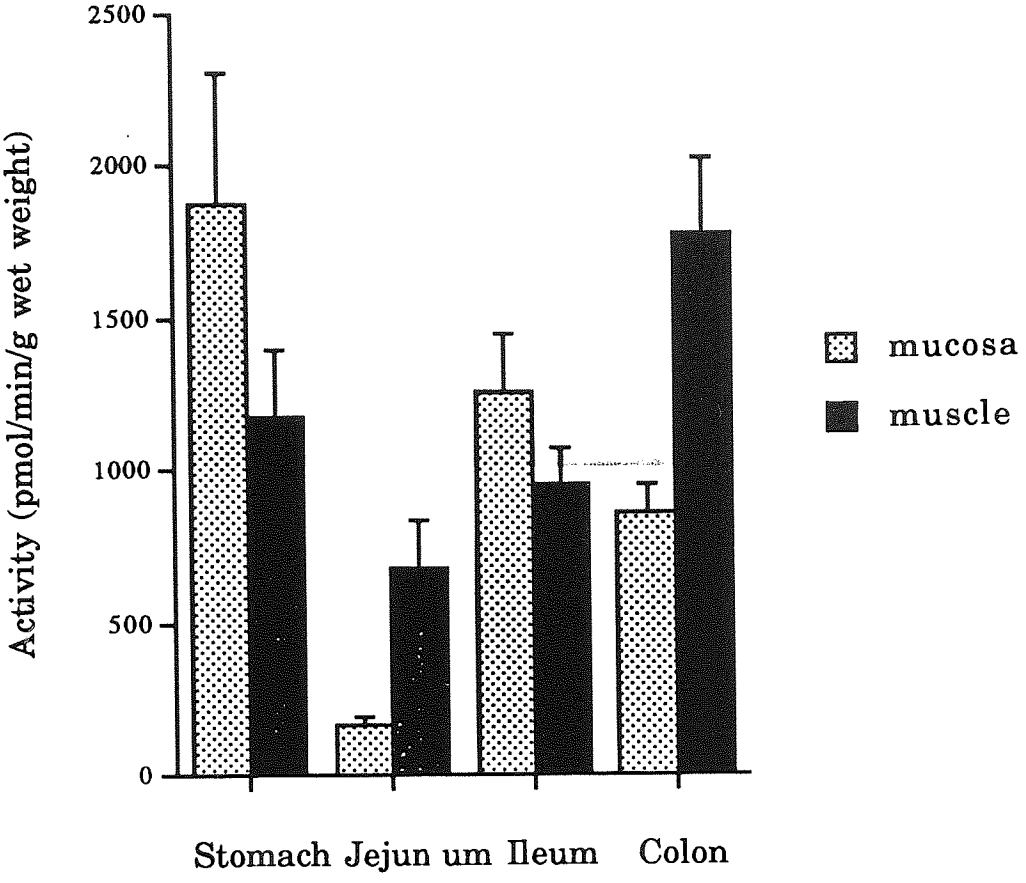


Fig. 5.2.4 Distribution of nitric oxide activity in the mucosa and muscle of the gastrointestinal tract of rats pretreated with intravenous endotoxin (3mg/kg body weight).

Results are mean ± SEM of stomach n=6, jejunum and ileum n=7 and colon n=4 rats respectively. Nitric oxide synthase activity was measured as citrulline formation inhibitable by a final concentration of 70μM L-NMMA. Gastric, jejunal, ileal and colonic mucosae all exhibited significant activity above control values ($P<0.01$, t-test) as did gastric, jejunal, ileal ($P<0.01$) and colonic muscle ($P<0.05$).

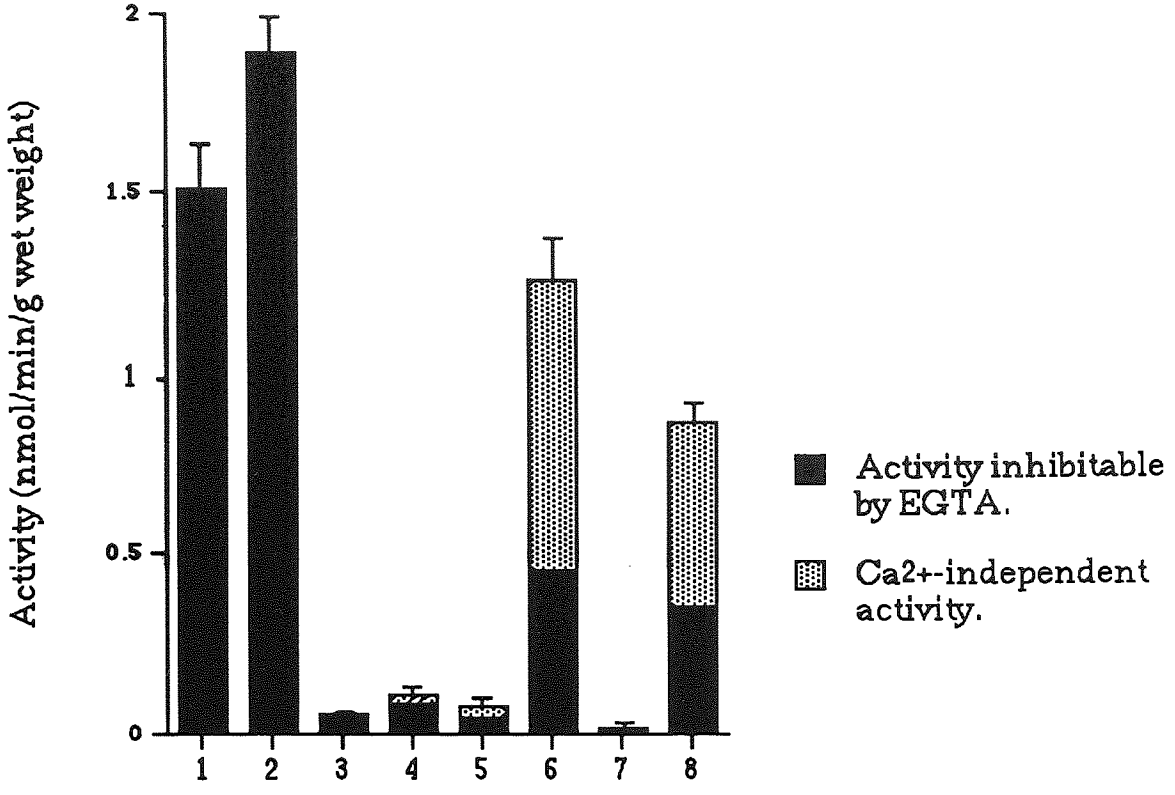


Fig. 5.2.5 Portion of nitric oxide synthase activity in the gastro-intestinal mucosa of control rats and those pretreated with endotoxin which required Ca2+ for activity.

Results are mean ± SEM from five rats pretreated with endotoxin and for the following numbers of control rats: stomach n=6, control jejunum and ileum n=7 and colon n=7. Key: 1 = control stomach, 2 = induced , stomach, 3 = control jejunum, 4 = induced jejunum, 5 = control ileum, 6 = induced ileum, 7 = control colon, 8 = induced colon. Nitric oxide synthase activity measured as citrulline formation inhibitable by a final concentration of 70μM L-NMMA. A significant amount of activity not inhibitable by EGTA was seen in the ileal and colonic mucosae ($P<0.02$, paired t-test).

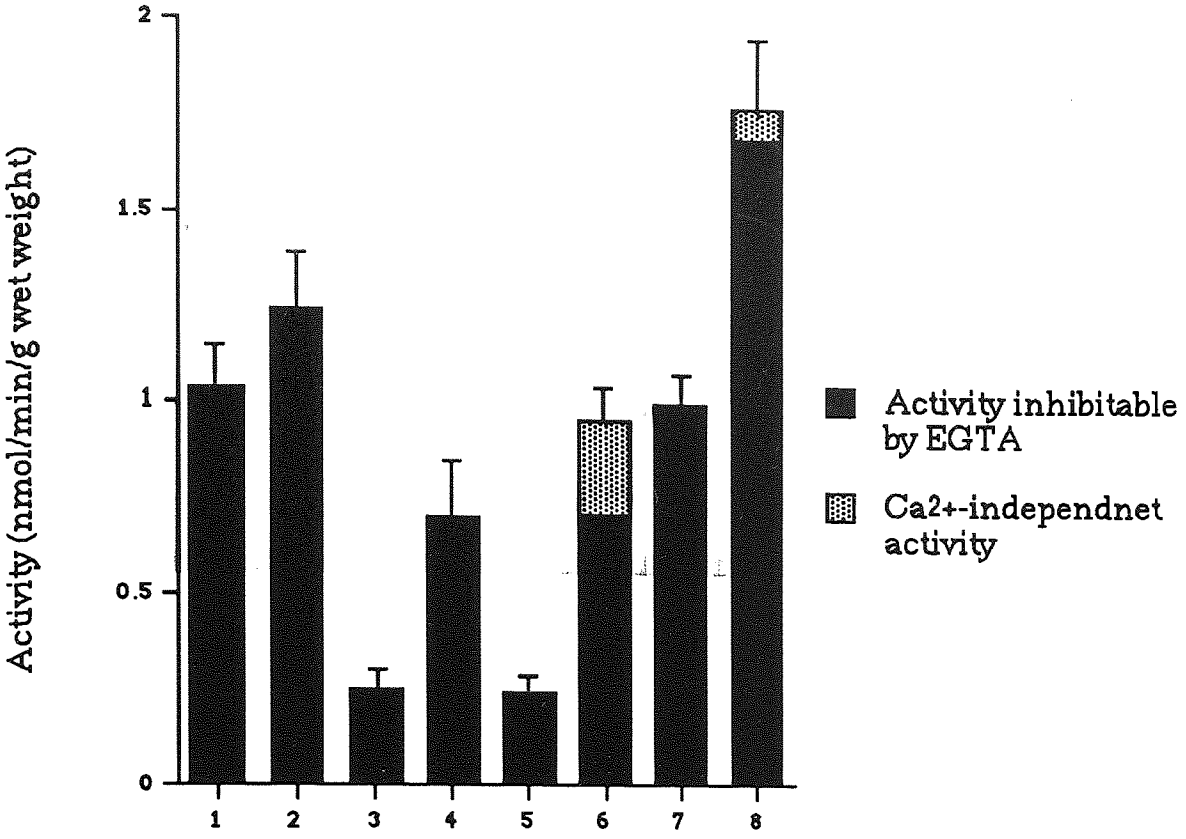


Fig 5.2.6 Portion of nitric oxide synthase activity in the gastro-intestinal muscle of control rats and those pretreated with endotoxin which required Ca2+ for activity.

Results are mean ± SEM from five rats pretreated with endotoxin and for the following numbers of control rats: stomach n=6, jejunum and ileum n=7 and colon n=7. Key: 1 = control stomach, 2 = inducedstomach, 3 = control jejunum, 4 = induced jejunum, 5 = control ileum, 6 = induced ileum, 7 = control colon and 8 = induced colon. Nitric oxide synthase measured as citrulline formation inhibitable by a final concentration of 70μM L-NMMA. No significant proportion of the activity was inhibitable by EGTA.

5.2.7 Ca^{2+}-independence of Nitric Oxide Synthase in Colonic Mucosa Pretreated with Endotoxin.

The activity induced by endotoxin in colonic mucosa appeared to be partially Ca^{2+}-dependent since no activity was present in this region of the gastrointestinal tract of control rats (fig 5.2.5). To investigate this further the effect of changing $[Ca^{2+}]$ on nitric oxide synthase activity was examined in gastric mucosal homogenates from normal rats and colonic mucosal homogenates of rats pretreated with endotoxin. The half-maximally effective Ca^{2+} concentration for the gastric enzyme was 400nM, but in this series of experiments the activity of the colonic enzyme from endotoxin-pretreated rats appeared totally independent of Ca^{2+}.

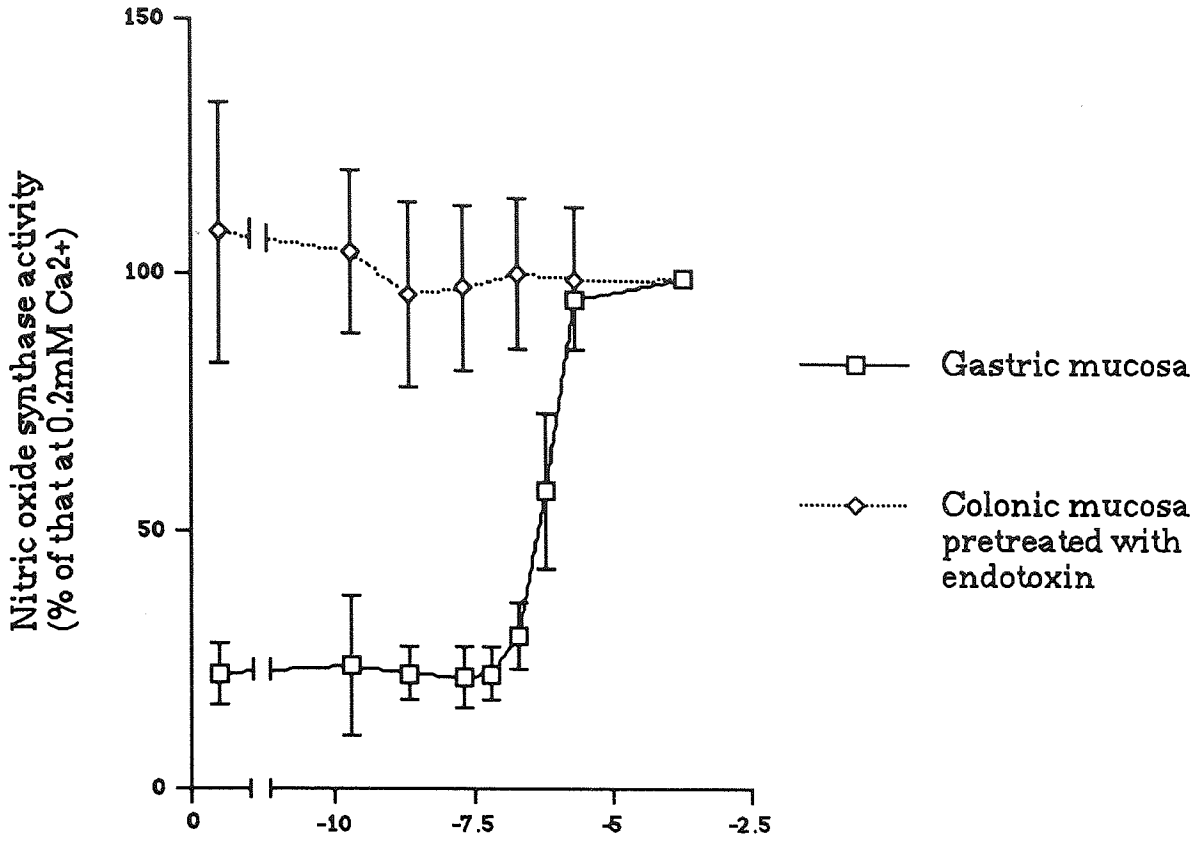


Fig 5.2.7 Comparison of the calcium-dependency of control gastric mucosa verses colonic mucosa pretreated with endotoxin.

Results are mean ± SEM of three experiments using data normalised so that the maximum control value is 100%. For the gastric enzyme control value at 0.2mM Ca^{2+} was 1.3nmol/min/g wet weight. Nitric oxide synthase activity measured as citrulline formation inhibitable by a final concentration of 70μM L-NMMA. Half-maximal activity was at 400nM Ca^{2+}.

5.3 DISCUSSION.

The initial aim of this section was to detect the presence of nitric oxide synthase activity in one or a number of gastrointestinal cell lines and to use cell lines to examine the cellular mechanisms involved in regulation of signalling via nitric oxide synthase. Unfortunately, none of the lines examined contained significant nitric oxide synthase activity (Table 5.2.1) and therefore it was decided to investigate the distribution and forms of the enzyme in the rat gastrointestinal tract as a whole.

Nitric oxide synthase activity was present in the muscle layer throughout the gastrointestinal tract but was only selectively expressed in the mucosa. Other workers have recently investigated the presence of nitric oxide synthase in the gastrointestinal tract or in mucosa and muscle but none have mapped the complete distribution (see table 5.3.1 for a comparison). Activities found by Salter et al. (1991) were somewhat higher, but this may reflect the saturating substrate concentration used by these workers, who detected nitric oxide formation by its reaction with oxyhaemoglobin. In the radiochemical method used in this work maintenance of a reasonable usage and specific activity of [^{14}C] L-arginine dictates the use of a total arginine concentration close to the Km for nitric oxide synthase (Knowles et al., 1990c). Tepperman et al. (1992) found significant nitric oxide synthase activity in rabbit jejunal and colonic mucosa which could be due to species variation and suggests that great care must be taken when comparing data between species. Smooth muscle of the gastrointestinal tract clearly contains nitric oxide synthase activity (table 5.2.1) but whether the activity is in the muscle cells themselves or solely in the nerves and myenteric plexus is still uncertain. Llewellyn-Smith et al. (1992), using the guinea-pig ileum stained immunochemically for nitric oxide synthase, showed the enzyme to be present in the myenteric neurones and to be less than 100nm from the

Table 5.3.1 Comparison of results of the present work with the distribution of nitric oxide synthase in the gastrointestinal tract found by others.

		Activity (nmol/min/g wet weight)		
Region		This thesis	Ref 1	Ref 2
Stomach	Mucosa	1.35		
	Muscle	0.9		
	Total		2.2	
Jejunum	Mucosa	0.002		0.21
	Muscle	0.183		0.75
	Total		0.96	
Ileum	Mucosa	0.076		
	Muscle	0.197		
	Total			
Colon	Mucosa	0		0.37
	Muscle	0.872		0.94
	Total		1.34	1.31

Ref 1 is Salter et al., 1991. Values are from the gastrointestinal tract of the rat.

Ref 2 is Tepperman, 1992. Values are from the gastrointestinal tract of the rabbit.

muscle cells. Forster et al. (1992) discovered nitric oxide synthase containing fibres innervating the gastric corpus in and around the myenteric and submucosal plexus of the circular muscle. Fibres were also seen in the mucosal layer. Stark et al. (1992) hypothesised that there is an ongoing release of nitric oxide in sphincters or smooth muscles that show a predominantly active tone or slow changes in tone, for example the gastric fundus, whereas smooth muscles with no ongoing tone but a predominantly phasic activity do not exhibit nitric oxide synthase, for example ileum and jejunum. Our results would agree with this to some extent as more activity was found in the gastric muscle than in the ileal or jejunal muscle but there was still some nitric oxide synthase activity present. Martinez-Cuesta et al. (1992) have shown that nitric oxide synthase is involved in peristalsis in the small intestine.

In the rat stomach all of the nitric oxide synthase activity was present in the soluble fraction of both the mucosa and the muscle. Table 5.0.1 illustrates the subcellular distribution of known isoforms of nitric oxide synthase. The present results clearly distinguish gastric mucosal nitric oxide synthase from the particulate enzyme found in endothelial cells Mitchell et al. (1992). In the myenteric neurones of the circular muscle of the ileum nitric oxide synthase was found not to be associated with any subcellular organelle or with the plasma membrane (Llewellyn-Smith et al., 1992), which is consistent with the present data on gastric muscle.

When mucosal cells were isolated from various regions of the gastrointestinal tract a similar pattern to that seen with mucosal scrapings was seen. Gastric mucosal cells exhibited, relatively, the highest activity and this was even more obvious in a fraction enriched with high density cells. Values obtained were similar to those seen by Brown et al. (1992) of 0.15pmol/min/10^6 cells for homogenates of unenriched cells (this thesis 0.17pmol/min/10^6 cells) and 0.7 pmol/min/10^6

cells for the high density fraction (this thesis 1.4pmol/min/10^6 cells). Brown et al. (1992) found that, by separating the cells on an elutriator rotor, the predominant activity was in a fraction enriched with mucous epithelial cells.

Although NADPH diaphorase activity is not a definite indication of nitric oxide synthase activity (Tracey et al., 1993) there probably cannot be any nitric oxide synthase activity without NADPH diaphorase activity. The gastric pits, where the NADPH diaphorase activity was predominant, contain surface mucous cells and no parietal cells. Brown et al., (1992) found that nitric oxide synthase activity was greatest in the fraction of the gastric mucosa containing mucous epithelial cells. There is also a small amount of staining in the gastric glands and this is possibly due to the mucous neck cells.

There is a clear change in the pattern of distribution of nitric oxide synthase activity in the gastrointestinal tract of rats pretreated with endotoxin with induction of activity occuring in regions previously low in nitric oxide synthase activity. Salter et al. (1991) showed no induction of nitric oxide synthase activity in the whole stomach and an increase in activity in the whole ileum but their results differ from the present findings in that they found no induction in the colon (see table 5.3.2 for comparison). Recent work implying an involvement of nitric oxide in ulcerative colitis suggests that the present findings of induced activity in the colon may be of importance. Boughton-Smith et al. (1992) found that normal human colonic mucosa had a nitric oxide synthase activity of 0.1 ± 0.03 nmol/min/g whereas the colonic mucosa from patients with ulcerative colitis had an activity of 0.72 ± 0.21nmol/min/g. There was no difference in colonic muscle.

Table 5.3.2 Comparison of the distribution of nitric oxide synthase in the gastrointestinal tract after treatment of rats with intravenous endotoxin.

Region		Activity (nmol/min/g wet weight) This thesis	Salter (1991)
Stomach	Ca^{2+}-dependent	3.127	2.31
	Ca^{2+}-independent	0	0
Jejunum	Ca^{2+}-dependent	0.786	
	Ca^{2+}-independent	0.082	
Ileum	Ca^{2+}-dependent	1.167	0.65
	Ca^{2+}-independent	1.053	0.62
Colon	Ca^{2+}-dependent	2.056	1.39
	Ca^{2+}-independent	0.586	0

Salter et al. (1991) did not make measurements on separated muscle and mucosa. From our work it is clear that induction of nitric oxide synthase by endotoxin occurs in the ileal and colonic mucosa and in the jejunal, ileal and colonic muscle layers.

The archetypal induced form of nitric oxide synthase, purified macrophage nitric oxide synthase, is a calcium-independent enzyme which contains tightly-bound calmodulin (Cho et al., 1992). Therefore it is not surprising that at least part of the increased activity in the ileum and colon of rats pretreated with endotoxin was Ca^{2+}-independent (not inhibitable by EGTA). However, since the colonic mucosa in control rats has no Ca^{2+}-dependent activity it is evident that endotoxin pretreatment caused the appearance of some Ca^{2+}-dependent, as well as some Ca^{2+}-independent, activity. There are two possible explanations. Firstly, the induced enzyme as in chondrocytes (Palmer et al., 1992) shows a low Ca^{2+}-dependency such that a partial activity might be present even with 1mM EGTA or, secondly, that a separate Ca^{2+}-dependent enzyme is also induced by endotoxin. This latter suggestion was made by Salter et al. (1991) to explain their results with whole ileum from endotoxin pretreated rats.

To clarify the situation the Ca^{2+}-dependency of nitric oxide synthase from control gastric mucosa and colonic mucosa from rats pretreated with endotoxin was compared using a series of Ca^{2+}/EGTA buffered assay media. As expected constitutive gastric mucosal nitric oxide synthase was highly sensitive to the amount of free Ca^{2+} present and, as was found by Forstermann (1991), the major increase in enzyme activity came between 100 - 500nM free Ca^{2+}. Contrary to expectation, the colonic mucosal enzyme activity was completely Ca^{2+}-independent. The only difference from earlier preparation of colonic mucosa was that EDTA was omitted from the homogenisation buffer. The possibility that the

conditions of homogenisation can affect subsequent enzyme activity requires further investigation.

A number of suppositions can be drawn from the data presented in this chapter. The prevalence of nitric oxide synthase activity in the gastric mucosa would point to a role for nitric oxide in regulation of epithelial cell integrity or secretion. The form of enzyme present in the gastric mucosa appears not to be the endothelial or the macrophage enzyme because it is soluble and Ca^{2+}-dependent and this will be investigated more fully in Chapter 6. The presence of enzyme activity in the muscle throughout the gastrointestinal tract is in line with findings that nitric oxide is probably responsible for non-aderenergic, non-cholinergic transmission in the gastrointestinal tract. The exact localisation of nitric oxide in the muscle layers is still to be elucidated. Induction of nitric oxide synthase activity in the gastrointestinal tract occurred mostly in areas of low constitutive activity such as the intestinal mucosa. This increased activity is possibly responsible for a major defensive response in the small intestine in which, unlike the stomach where there is a low pH, there is a propensity for bacterial colonisation. In the colon bacteria are, of course, always present but they may be involved in the triggering of an immune response in inflammatory bowel disease.

5.4 SUMMARY.

Nitric oxide synthase activity was not found in the cell lines HGT-1 MKN-45 or CaCo-2. Gastric mucosa exhibited a significant amount of Ca^{2+}-dependent activity as did all of the gastrointestinal muscle layers. Gastric mucosal enzyme activity appeared to be predominantly in the soluble fraction and showed half-maximal activation at a Ca^{2+} concentration of 400nM. Treatment of rats with endotoxin gave rise to an induction of activity in the jejunal, ileal and colonic mucosa. 63.7% and 58.7% of induced nitric oxide synthase activity in the ileum and colon respectively was Ca^{2+}-independent. Jejunal, ileal and colonic muscle all exhibited an increase in nitric oxide synthase activity on pretreatment of rats with endotoxin but this activity was substantially Ca^{2+}-dependent.

CHAPTER 6.

PROPERTIES OF NITRIC OXIDE SYNTHASE IN THE GASTRIC MUCOSA.

6.0 INTRODUCTION.

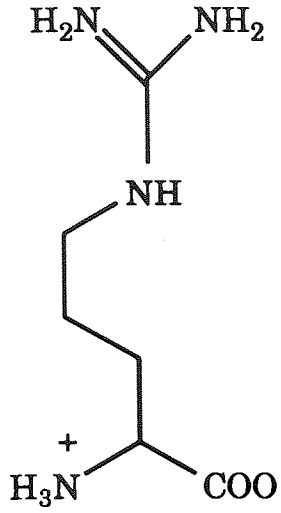
The main aims of this chapter were to further characterise gastric mucosal nitric oxide synthase by using inhibitors and to investigate mechanisms for intracellular control of nitric oxide synthase in the gastric mucosa.

Nitric oxide synthase activity is present in high activity in an high density fraction of gastric mucosal cells and all of the enzyme in the gastric mucosa appears to be soluble (Chapter 5). However, Bredt et al. (1991) were unable to detect mRNA for nitric oxide synthase in stomach extracts using Northern blotting with a brain cDNA probe. It is possible that the brain and gastric mucosal enzymes are therefore different isoforms.

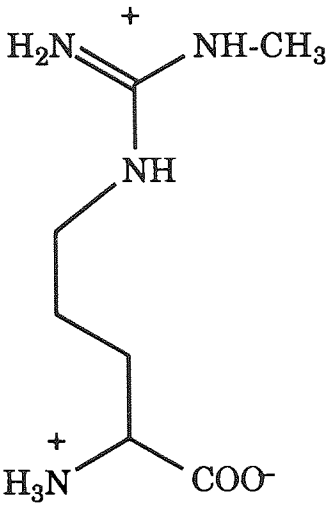
6.0.1 Inhibitors of Nitric Oxide Synthase.

N^G-Monomethyl-L-arginine (L-NMMA) (fig. 6.0.1) inhibits nitric oxide synthase competitively and does not affect other arginine-metabolising enzymes, such as arginase or arginine decarboxylase, (Granger et al., 1990). L-NMMA is an inhibitor for all known forms of nitric oxide synthase (Schmidt, 1993).

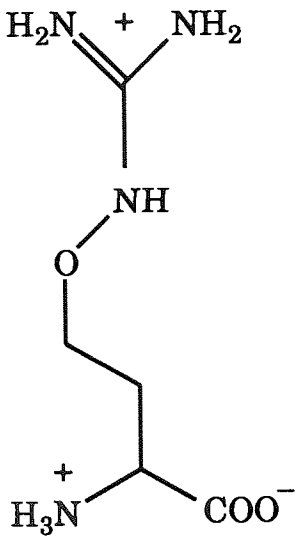
L-Canavanine (fig. 6.0.1) is a broad inhibitor of arginine utilising reactions. It is capable of inhibiting nitric oxide synthase in macrophages but apparently not in endothelial cells or the brain (Marletta, 1989).



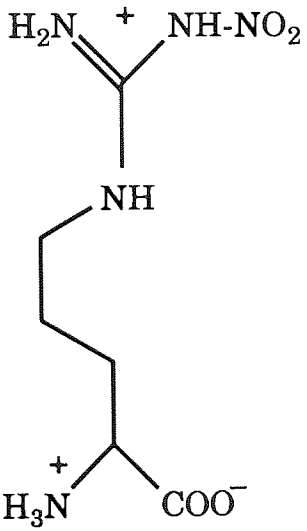
L-Arginine



N^G-Monomethyl-L-arginine



L-Canavanine



N^G-Nitro-L-arginine

Fig. 6.0.1 Inhibitors of nitric oxide synthase.

N^G-nitro-L-arginine (fig. 6.0.1) inhibits the endothelial form of nitric oxide synthase (Rees et al., 1990b) is a irreversible inhibitor of brain nitric oxide synthase and at higher concentrations a reversible inhibitor of macrophage nitric oxide synthase (Dwyer et al., 1991).

Trifluoperazine is a calmodulin antagonist. It is a tricyclic phenothiazine which binds to the active form of calmodulin thereby blocking the binding of calmodulin to proteins and activation of calmodulin-requiring enzymes (Brostrom et al., 1981). Calmodulin has four binding sites for Ca^{2+} and it binds two molecules of trifluoperazine reversibly with high affinity. If EGTA is added this will break the binding as it removes the Ca^{2+}. Trifluoperazine should therefore inhibit Ca^{2+}-dependent forms of nitric oxide synthase.

6.0.2 Regulation of Nitric Oxide Synthase by phosphorylation.

Nitric oxide synthase contains serine, threonine and tyrosine residues and this means that it is a possible candidate for phosphorylation by protein kinases. The amino acid sequence for brain nitric oxide synthase predicted from the cDNA contains a site where phosphorylation by cAMP-dependent protein kinases is highly probable. However, increases in cAMP or activation of cAMP-dependent protein kinase do not seem to regulate nitric oxide synthase (Schmidt et al., 1993).

Involvement of phosphorylation in the regulation of enzyme activity can be investigated by using inhibitors of protein kinases and phosphatases. A protein phosphatase inhibitor is okadaic acid which belongs to a family of diarrhetic shellfish toxins produced by dinoflagellates. It is a potent inhibitor of serine/threonine-specific protein phosphatases 1, 2A, (table 6.0.1) and at high concentrations inhibits phosphatase 2B. Protein tyrosine phosphatases, other phosphatases or kinases are apparently

Table 6.0.1 Specificity of Okadaic acid for different protein phosphatases.

Phosphatase	IC_{50} for okadaic acid (nM)
Rabbit skeletal muscle type PP-1	60 - 200
Rabbit skeletal muscle type PP-2A	0.5 - 1.0
Bovine brain PP-2B	10,000
Rabbit liver PP-2C	no effect at 10μM

unaffected. The effects seen in a cell with okadaic acid will therefore be due to a net increase in the level of phosphorylated proteins, which may be equivalent to the effects of activation of several protein kinases.

There are now some protein kinase inhibitors which have an high affinity for their particular kinase and a low affinity for other kinases and which therefore exhibit at least partial specificity. Chelerythrine chloride is a water-soluble specific inhibitor of protein kinase C (table 6.0.2). It is a tetracyclic aromatic alkaloid of the benzophenanthridine class which contains a methylated aromatic nitrogen and therefore has a positive charge (fig. 6.0.2). Chelerythrine acts competitively with the protein kinase C substrate histone IIIS but not competitively with ATP (Herbert et al., 1990). cAMP-dependent protein kinase inhibitory peptide is a specific cAMP-dependent protein kinase inhibitor (table 6.0.2). This peptide binds to the catalytic subunit of cAMP-dependent protein kinase and the phosphorylating ability of the kinase is inhibited. 1-[N,O-Bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) is a selective inhibitor (table 6.0.2) of rat brain Ca^{2+}/calmodulin kinase II. KN-62 also inhibits autophosphorylation but it cannot inhibit previously autophosphorylated Ca^{2+}/calmodulin kinase II. KN-62 is competitive with respect to calmodulin and binds to the calmodulin binding site (Tokumitsu et al., 1990).

Table 6.0.2 Comparison of the Effects of Specific Protein Kinase Inhibitors.

Inhibitor	IC_{50} (μM)		
	Protein kinase C	cAMP-dependent protein kinase	Ca^{2+}/ calmodulin dependent protein kinase
Chelerythrin	0.66	170	>100
cAMP-dependent protein kinase inhibitor	-	2.3	-
KN-62	>100	>100	0.9

6.1 METHODOLOGY.

6.1.1 G25 Sephadex Column Chromatography of Homogenates.

The Sephadex resin was allowed to swell overnight at 4°C in homogenisation buffer (Table 5.1.1) without sucrose or protease inhibitors. A column was set up with a 3.33ml (8.12cm height and 0.72cm diameter) bed volume which was able to accomodate a sample volume of 500μl (15% of the bed volume). To calibrate the column blue dextran (1mg/ml) was run through and the drops counted for the dextran to first appear in the eluent and to complete its passage. This calibration indicated the void volume of the column in which any nitric oxide synthase activity would be expected to appear. Normal homogenisation buffer was used to elute the sample and the void volume fraction collected. This procedure serves to remove endogenous arginine from nitric oxide synthase and was used in all of the nitric oxide synthase inhibitor experiments. The presence of arginine would effect the concentration dependence of inhibition of gastric mucosal nitric oxide synthase.

6.1.1 Inhibitors of nitric oxide synthase.

A range of inhibitors were added to the assay buffer to give the following final concentrations in the assay system. N^G-Monomethyl-L-arginine (L-NMMA) 0-71μM, N^G-Nitro-L-arginine 0-17.5μM, L-Canavanine 0-7.1mM, Trifluoperazine 0-0.4mM and Okadaic acid 0-4.43μM. All chemicals were diluted in nitric oxide synthase assay buffer (Chapter 5) before being added to the assay system.

Inhibitors, at their maximum concentration, were also added to the assay blanks to ensure that they were not interfering with the binding of [^{14}C] L-arginine to the Dowex resin.

6.1.2 Experiments Performed in Intact Cells.

Rat gastric cells were isolated and purified as previously described (section 2.1). A suspension of 10^7 cells/ml was incubated in pregassed medium B' (Table 2.1.2) containing 1μM okadaic acid in DMSO for 30min in a shaking water bath at 37°C. Control cells were incubated in medium B' containing DMSO (0.51% v/v). The cells were then subjected to centrifugation at 12,000g for 10s, the supernatant decanted and the pellet frozen in liquid nitrogen. Each pellet was resuspended at $6.7x10^7$ cells/ml in a buffer containing: 50mM Tris, 50mM NaF, 10mM sodium pyrophosphate, 1mM EDTA, 1mM orthovanadate, 1mg/100ml soybean trypsin inhibitor, 1mg/100ml leupeptin, 1mg/100ml aprotinin, 1mg/100ml PMSF, 1μM okadaic acid, pH7.4, freeze-thawed three times and subjected to centrifugation at 12,000g for 20min at 4°C. When the samples were assayed for nitric oxide synthase activity the concentration of calcium chloride in the assay buffer was increased to 0.56mM to counteract the increased EDTA concentration deriving from the homogenisation buffer.

6.1.3 The addition of Mg^{2+}, ATP, β,γ-Methylene ATP and GTP to the assay.

A range of chemicals were added separately to the homogenisation buffer (Table 5.1.1) to give the following final concentrations: magnesium chloride, 10mM; adenosine 5'- triphosphate, 5mM; β,γ-methyleneadenosine 5'-triphosphate, 5mM and guanosine 5'-triphosphate, 5mM. Homogenisation buffers containing these chemicals were also added to the assay blanks. The concentrations of ATP and Mg^{2+} were those selected by Bredt et al. (1992) for studies on phosphorylation of purified nitric oxide synthase.

In some cases the above chemicals were added directly to the assay buffer to give the same final concentration as would have occurred had they been present during the homogenisation. This procedure was used to detect if they were having a direct effect on the assay for nitric oxide synthase.

6.1.4 Protein kinase inhibitors.

Protein kinase inhibitors were added to the homogenisation buffer (Table 5.1.1) to give the following final concentrations: chelerythrine chloride, 7μM; cAMP-dependent protein kinase inhibitor, 2μM; and KN-62, 10μM. The first two inhibitors were dissolved in water but KN-62 was dissolved in DMSO and so an equivalent amount was added to control homogenisation buffer to give a 1% v/v final concentration. This procedure was carried out to verify that any effects were not caused by the solvent.

Homogenisation buffer containing protein kinase inhibitors was also added to the assay blanks and directly to the assay buffer to give the final concentration that would have been achieved had they been added with the homogenisation buffer.

6.2 RESULTS.

6.2.1 Effect of arginine analogues on inhibitors of nitric oxide synthase activity.

L-NMMA exhibited a dose-dependent inhibition of nitric oxide synthase activity. The IC_{50} for L-NMMA was approximately 18μM (from interpolation) (fig. 6.2.1).

Nitroarginine exhibited a dose-dependent inhibition of nitric oxide synthase activity from gastric mucosal cells (fig. 6.2.2). The Ki (a curve was fitted to the experimental data and the Ki calculated from this) was 0.8 ± 0.09μM. The curve obtained with whole brain homogenate was very similar.

L-Canavanine exhibited a dose-dependent inhibition of nitric oxide synthase activity in gastric mucosal cells (fig. 6.2.3). The Ki for the high density cell extract was 147 ± 22μM. The curve for a brain homogenate was again very similar to that for the gastric cells.

6.2.2 The effect of Trifluoperazine on the activity of nitric oxide synthase in high density gastric mucosal cells.

Trifluoperazine exhibited a dose-dependent inhibition of nitric oxide synthase activity in gastric mucosal cells (fig. 6.2.4). The Ki was 140 ± 11μM.

Neither 100μM L-NMMA, 21μM nitro-L-arginine nor 400μM trifluoperazine had any effect on blank values (two way analysis of variance) (table 6.2.1). 6.9mM L-canavanine had a small but signinficant effect on the assay blank value ($P<0.01$, two way analysis of variance). A

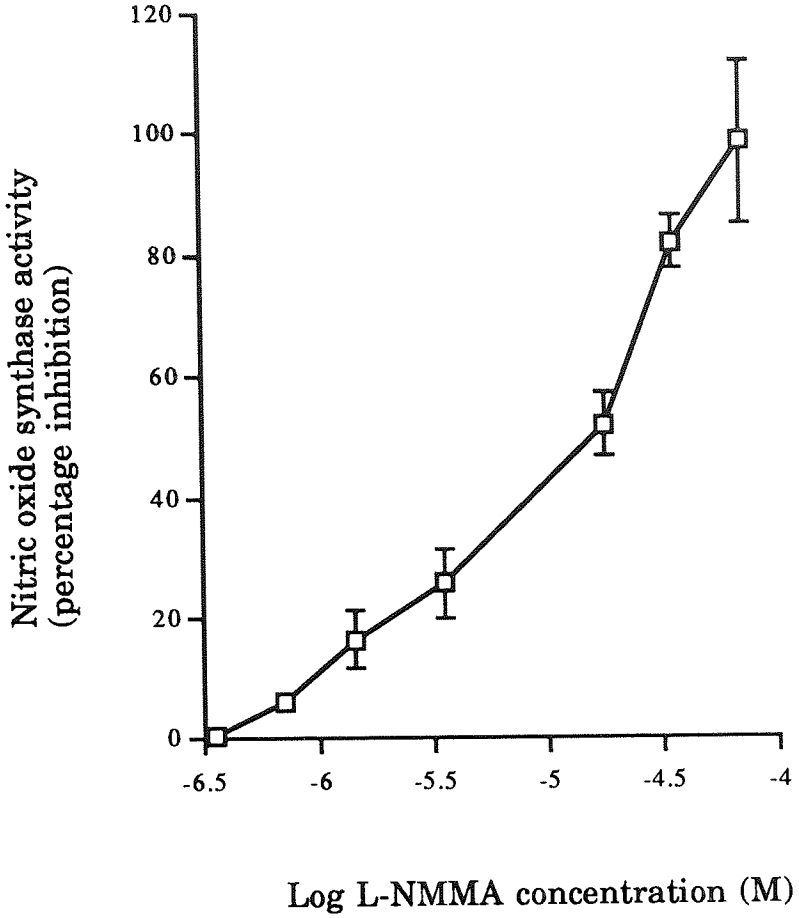


Fig. 6.2.1 The effect of L-NMMA on the activity of nitric oxide synthase in an homogenate of an high density fraction of gastric mucosal cells.

Results are mean ± SEM of three separate experiments.

Uninhibited activity 1.289 ± 0.51 pmol/min/106 cells.

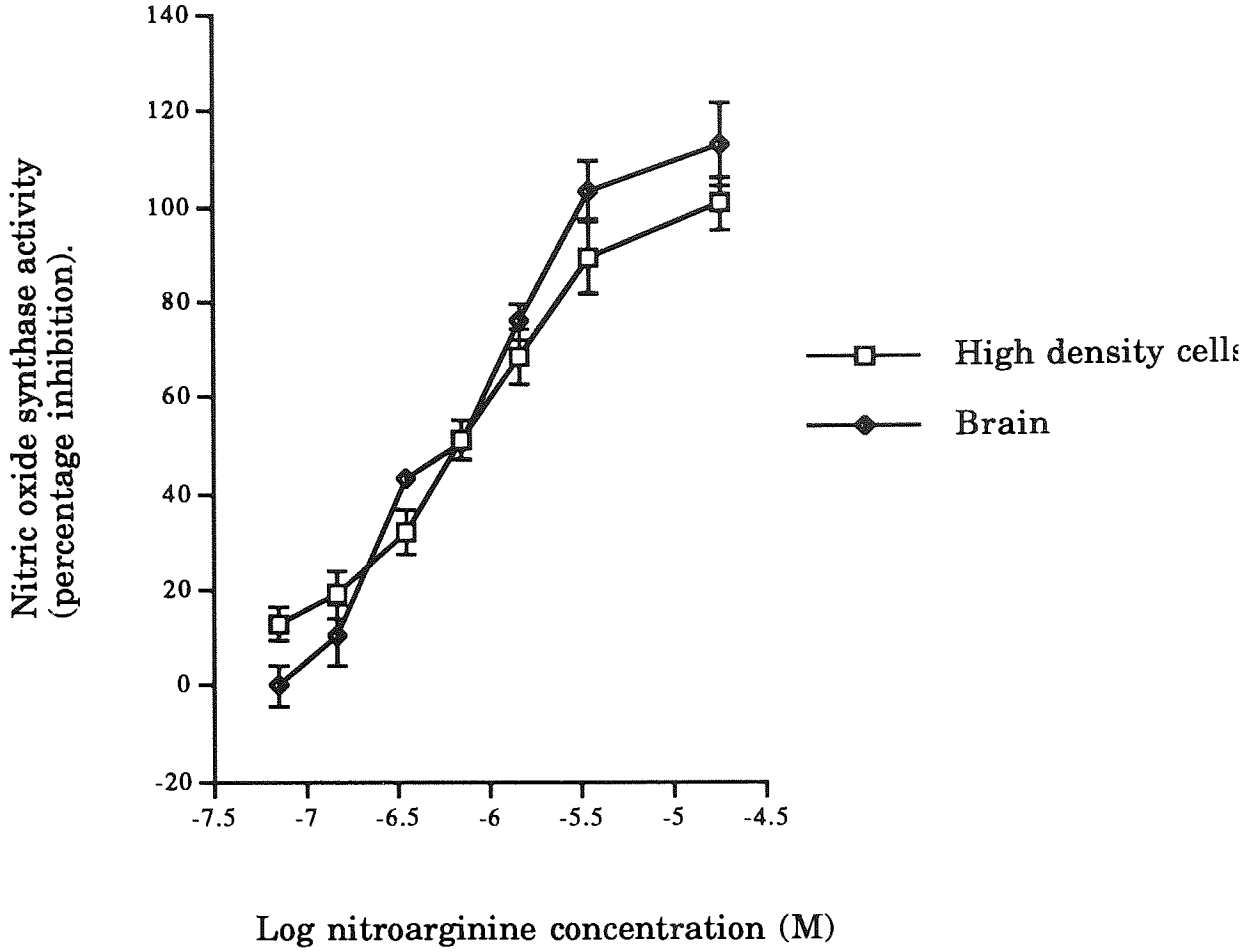


Fig. 6.2.2 The effect of nitroarginine on the activity of nitric oxide synthase in an homogenate of an high density fraction of gastric mucosal cells and rat brain homogenate.

Results are mean ± SEM of three separate experiments. Uninhibited activity in gastric mucosal cells was 1.665 ± 0.77 pmol/min/106 cells. Uninhibited activity in rat brain was 2.53nmol/min/g wet weight.

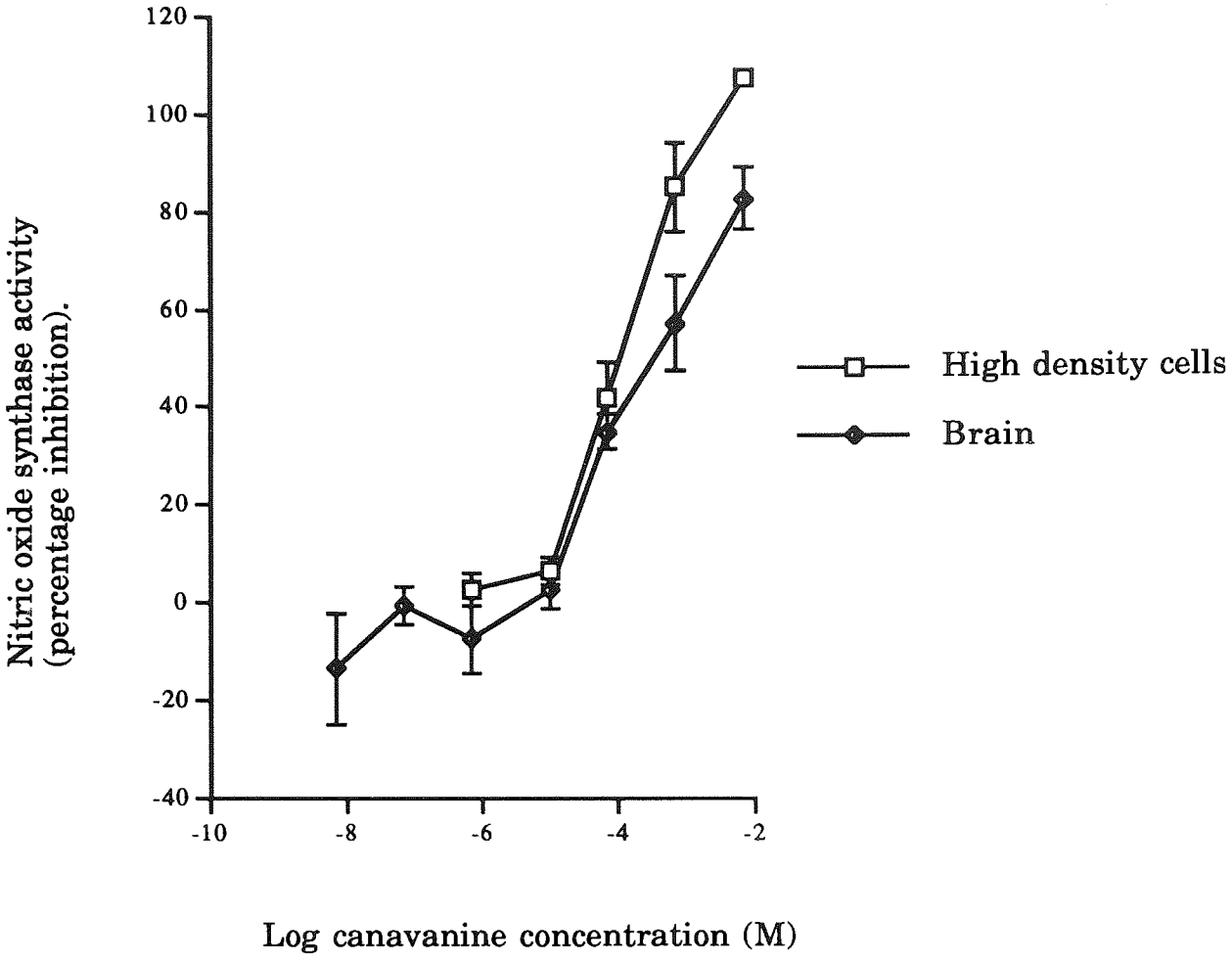


Fig 6.2.3 The effect of L-Canavanine on the activity of nitric oxide synthase in an homogenate of an high density fraction of gastric mucosal c and rat rain homogenate.

Results are mean ± SEM of three separate experiments. Uninhibited activity in gastric mucosal cells was 1.281 ± 0.4 pmol/min/106 cells. Uninhibited activity in rat brain was 1.95nmol/min/g wet weight.

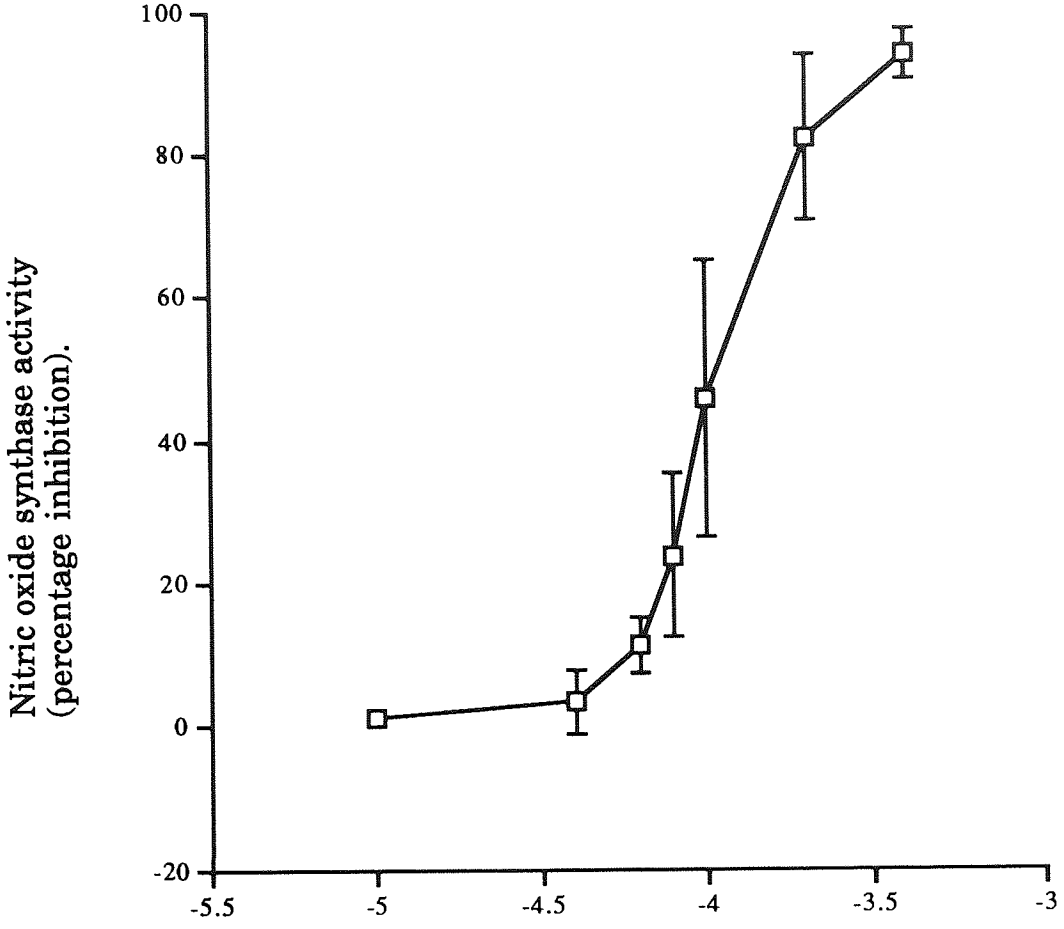


Fig 6.2.4 The effect of trifluoperazine on the activity of nitric oxide synthase in an homogenate of an high density fraction of gastric mucosal cells.

Results are mean ± SEM of five separate experiments.

Uninhibited activity was 1.866 ± 0.67 pmol/min/106 cells.

Table 6.2.1 Analysis of the effect of nitric oxide synthase inhibitors on assay blank values.

Inhibitor	dpm remaining in supernatant after the addition of Dowex resin.	
	No inhibitor	With Inhibitor
L-NMMA (100μM)	373±48	377±51
Nitro-L-arginine (21μM)	356±35	359±21
L-canavanine (6.9mM)	412±123	356±91*
Trifluoperazine (400μM)	280±21	256±12

Results are mean ± SEM of three experiments with triplicate determinations. Results were analysed by two way analysis of variance to remove the effect of variation between experiments. The result in the presence of L-canavanine was significantly different from control ($P<0.01$).

correction was made for this effect in calculating the percentage inhibition obtained at the highest canavanine concentration.

6.2.3 The effect of preincubation of high density gastric mucosal cells with okadaic acid.

Preincubation of intact high density cells with okadaic acid for 30min prior to homogenisation caused a significant reduction in nitric oxide synthase activity ($P<0.05$, Dunnett's test) (table 6.2.2). Direct addition of okadaic acid to the assay system produced essentially no inhibition of nitric oxide synthase activity until the concentration was greater than 0.5μM (fig. 6.2.5). When present in homogenisation buffer the final concentration of okadaic acid in the assay system was 0.28μM. Okadaic acid (1μM) in the assay buffer had no significant effect on the blank values, or when it was added at a concentration of 1μM to the homogenisation buffer used to prepare blanks (paired t-test) (table 6.2.3).

6.2.4 The effect of homogenisation with Mg^{2+}, ATP, β,γ-methylene ATP and GTP on the activity of nitric oxide synthase extracted from gastric mucosal cells.

Mg^{2+}, Mg^{2+}/β,γ-methylene ATP and Mg^{2+}/GTP produced no significant effect, however, activity after freeze-thawing in the presence of Mg^{2+}/ATP was significantly lower than control ($P<0.01$, Dunnett's test) (fig. 6.2.6). Blank values for the uninhibited enzyme assay system and assay plus L-NMMA were not affected by the addition of Mg^{2+}, ATP, Methylene ATP or GTP (Dunnett's test) (table 6.2.4).

Table 6.2.2 The effect of preincubation of intact high density cells with okadaic acid prior to homogenisation and assay.

	Nitric oxide activity pmol/min/10^6 cells
Control	0.44 ± 0.062
Okadaic acid (1μM)	0.24 ± 0.68

Results are mean ± SEM of five experiments each containing triplicate determinations. A significant effect was seen in the presence of okadaic acid ($P<0.02$, paired t-test).

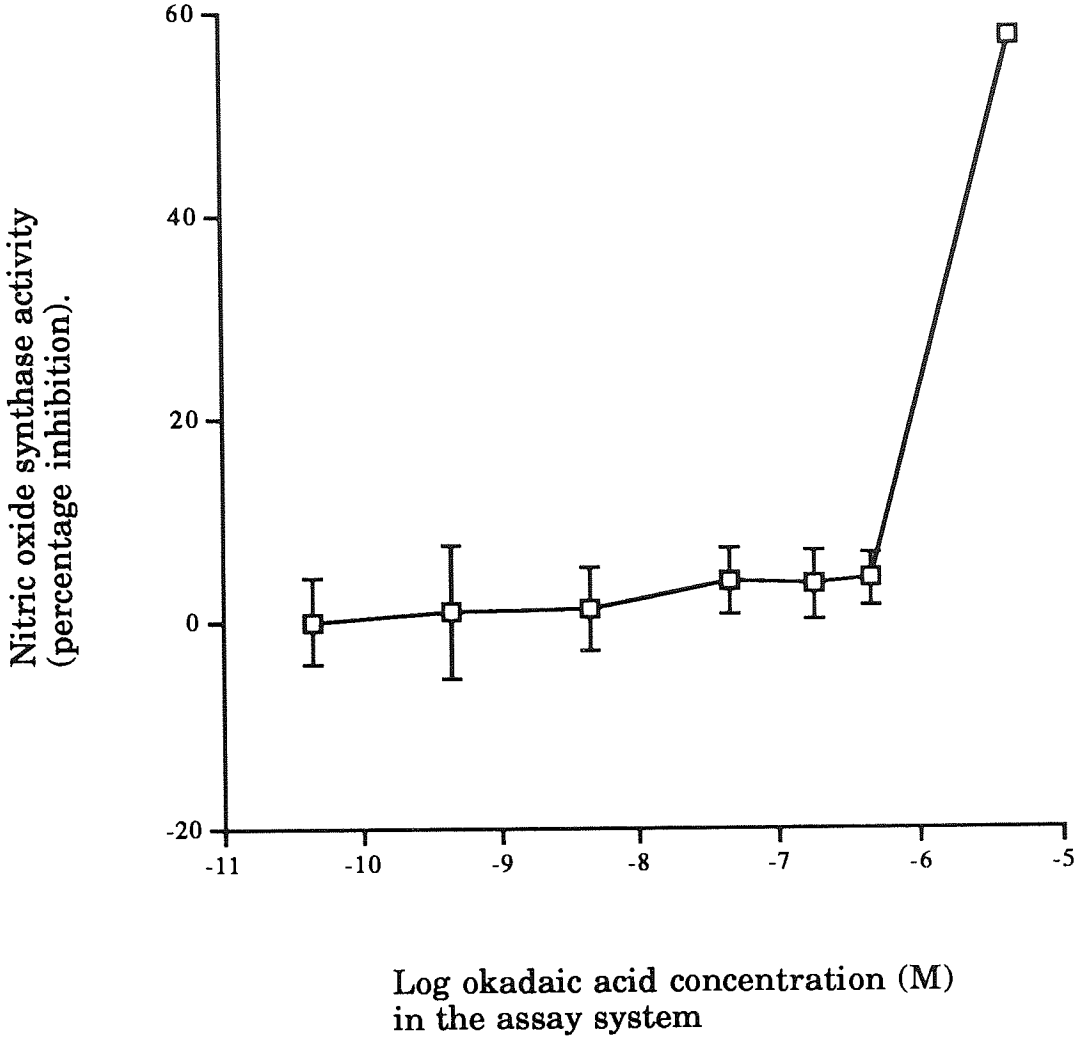


Fig 6.2.5 The effect of direct addition of Okadaic acid to the assay system on the activity of nitric oxide synthase in an homogenate of a high density fraction of gastric mucosal cells.

Results mean ± SEM of triplicate determinations from a single experiment. The uninhibited enzyme activity was 1.22pmol/min/10^6cells.

Table 6.2.3 Analysis of the effect of okadaic acid on assay blank values.

	dpm remaining in the supernatant after the addition of Dowex resin.	
	Control	Okadaic acid
1μM Okadaic acid added to the assay buffer	265.4±11.7	283.1±15.2
1μM Okadaic acid added to the homogenisation buffer	380.8±13.3	347.4±20.5

Results are mean ± SEM from one experiment with triplicate determinations. There was no significant effect in either case (unpaired t-test).

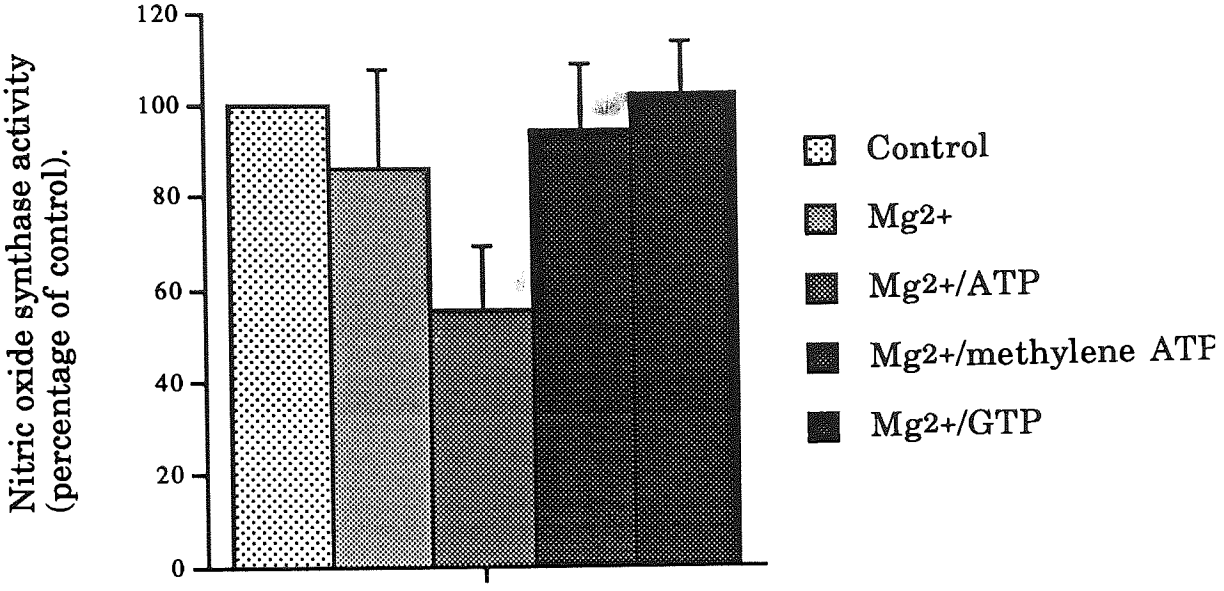


Fig. 6.2.6 The effect of the presence of Mg2+, ATP, β,γ-methylene ATP and GTP in the homogenisation buffer on the activity of nitric oxide synthase extracted from high density gastric mucosal cells.

Results are mean ± SEM of three separate experiments.

Uninhibited activity was 0.992pmol/min/106 cells.

Significant difference from control values was seen in the presence of ATP ($P<0.01$, Dunnett's test).

Table 6.2.4 Analysis of the effect of Mg^{2+}, ATP, β,γ-Methylene ATP and GTP on assay blank values.

	Percentage of control dpm.	
	Uninhibited	L-NMMA
Control	100	100
Mg^{2+}	103 ± 3.5	103 ± 5
Mg^{2+}/ATP	114 ± 5	107 ± 2
Mg^{2+}/β,γ-methylene ATP	112 ± 8	109 ± 6
Mg^{2+}/GTP	112 ± 6	97 ± 3

Results are mean ± SEM of triplicate determinations from one experiment. There was no significant effect on either the uninhibited assay blanks or those containing L-NMMA (Dunnett's test) by any of the compounds.

6.2.5 The effect of protein kinase inhibitors on the activity of nitric oxide synthase in high density gastric mucosal cells freeze-thawed in the presence of Mg^{2+}/ATP.

The inhibition in the presence of 7μM chelerythrine differed significantly from the value obtained with Mg^{2+}/ATP alone ($P<0.02$, Dunnett's test) (fig. 6.2.7). There was no significant difference between blanks in the presence and absence of protein kinase inhibitors (Dunnett's test). Also there was no difference in enzyme activity when protein kinase inhibitors were added to the assay buffer (Dunnett's test).

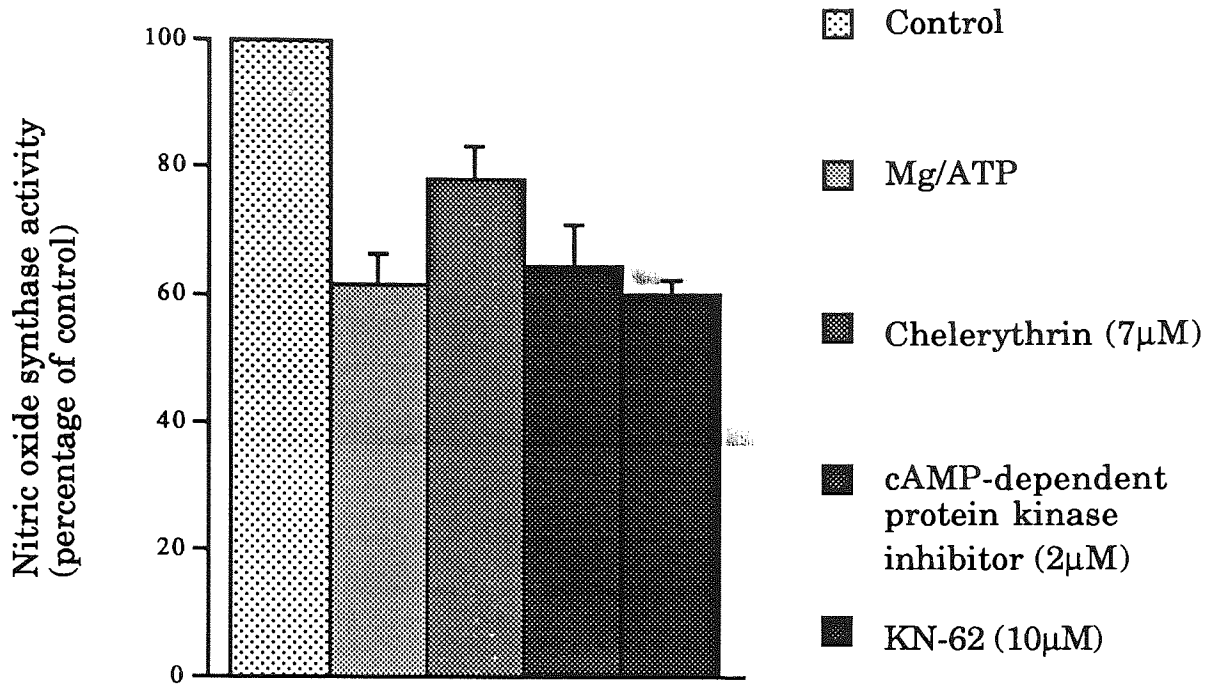


fig. 6.2.7 The effect of protein kinase inhibitors on the activity of nitric oxide synthase extracted from high density gastric mucosal cells. Results are mean ± SEM of six experiments each with triplicate determinations. The inhibition seen with chelerythrin was significantly different from that with Mg^{2+}/ATP ($P<0.05$, paired t-test). Control value was 1.22pmol/min/10^6 cell.

6.3 DISCUSSION.

From the results obtained in this chapter an order of inhibitor potency can be suggested for the gastric mucosal enzyme as follows; N^G-nitro-L-arginine > L-NMMA > trifluoperazine > L-canavanine.

The inhibition of nitric oxide synthase activity by L-NMMA has been established in a number of tissues. Cerebellar nitric oxide synthase has an IC_{50} for L-NMMA of 11μM (Aldir et al., 1990) whereas for neutrophils it is 30μM and for the macrophage cell line J774 7.5μM (McCall et al., 1991). In macrophages L-NMMA can inhibit the formation of NO_2^- by 50% at 130μM and this is similar in endothelial cells (Marletta, 1989). Gastric mucosal nitric oxide synthase has an IC_{50} for L-NMMA similar to that of the cerebellar enzyme and L-NMMA has been found to induce a dose-dependent fall in resting gastric blood flow by the inhibition of nitric oxide production indicating a role for nitric oxide in the regulation of gastric mucosa blood flow (Whittle et al., 1990).

Nitroarginine is the most potent inhibitor of brain nitric oxide synthase described to date (East and Garthwaite, 1990). East and Garthwaite (1990) described a biphasic response with the cerebellar enzyme with peaks of inhibition at 6nM and 600nM which indicates the possibility of two enzymes being present. Knowles et al. (1990c) found forebrain nitric oxide synthase to have a Ki of 0.4μM. The inhibitory constant for gastric mucosal nitric oxide synthase is very close to those reported for the brain enzyme and this is further confirmed by the similarities in the inhibition curves found in this work (fig. 6.2.2).

Rat forebrain nitric oxide synthase has previously been suggested to be unaffected by L-canavanine (Knowles et al., 1990b). The findings of this work did not agree with this and this could be due to the fact that whole

brains were used as opposed to crude forebrain synaptosomal enzyme. This would again indicate the possibility of two forms of nitric oxide synthase in the brain. The inhibition curves for L-canavanine with the brain and gastric mucosal enzyme were, again, very similar (fig. 6.2.3) but the compound was much less potent than nitroarginine or L-NMMA.

Calcium-dependent nitric oxide synthases which are not inhibited by the calmodulin binding protein trifluoperazine have been isolated in chondrocytes and in neutrophils (Palmer et al., 1992; Yui et al., 1991). Macrophage nitric oxide synthase has also been found to be resistant to the effects of trifluoperazine (Lyons et al., 1992) and this is, no doubt, due to the fact the calmodulin is extremely tightly bound in this form. Cerebellar nitric oxide synthase, however, is inhibited by trifluoperazine and has an IC_{50} of approximatley 10μM (Bredt and Snyder, 1990). Trifluoperazine is a weaker inhibitor of the endothelial form of nitric oxide synthase than it is in the brain, but 30μM has been found to work (Förstermann et al., 1991). The form of nitric oxide synthase which is found in high density gastric mucosal cells is inhibited by trifluoperazine. Trifluoperazine has been shown to inhibit the relaxation of smooth muscle and inhibit the generation of nitric oxide in bovine artery (Ignarro, 1991) so it probably inhibits the endothelial cell enzyme as well.

From the information ascertained from the four inhibitors there is an indication of the form of nitric oxide synthase present in the gastric mucosa. The IC50 for L-NMMA was near to that for the brain form. The Ki for nitroarginine was almost identical to that seen with the brain enzyme. The inhibition curves for gastric mucosal enzyme and brain enzyme were very close. Lastly gastric mucosal nitric oxide synthase, like brain nitric oxide synthase was inhibited by trifluoperazine. One other factor that points to the gastric mucosal enzyme being similar to that in the brain is the order of potency. Nitroarginine in cerebellar nitric oxide

synthase is a much more potent inhibitor than L-NMMA (the reverse is true in macrophages) (Dwyer et al., 1991) and this also true of the gastric mucosal enzyme.

Bredt (1991) found that when the stomach was probed for brain mRNA using Northern blotting techniques none was found. However, a moderate amount of brain mRNA was found by Imai (1992) using a similar technique. Our data would strongly agree with the latter findings.

The significant decrease in nitric oxide activity activity after preincubation with okadaic acid is possibly a consequence of this chemical inhibiting protein phosphatase activity and thereby increasing the level of phosphorylated nitric oxide synthase with phosphorylation of nitric oxide synthase causing a decrease in activity. Alternative explanations are that okadaic acid might cause the phosphorylation and activation of a kinase which, in turn, inactivates nitric oxide synthase, or the phosphorylation and inactivation of a kinase essential for nitric oxide synthase activity. No direct effect of okadaic acid could be detected in the assay system at concentrations that would have occurred from carry-over from the homogenisation buffer. Indeed homogenisation buffer contained okadaic acid irrespective of whether cells had been preincubated with this agent to prevent further changes in phosphorylation during homogenisation. As only 1μM okadaic acid was used it is probable that PP-2A was being inhibited in the intact cells and possibly PP-1.

The decrease in nitric oxide synthase activity seen in the presence of Mg^{2+}/ATP would further indicate a possible role for phosphorylation in enzyme regulation. The increased amount of ATP would increase enzyme phosphorylation of either nitric oxide itself or in addition a kinase in the cascade thereby causing inhibition. The Mg^{2+} was present in the

mixture as an enzyme cofactor for the conversion of ATP to ADP plus phosphate. GTP was tested for any inhibitory activity as it was possible that the ATP used might have been contaminated with minute amounts of it and that GTP was causing the effect seen via a G-protein. This proved not to be the case. The lack of effect of the non-hydrolysable analogue. β,γ-methylene ATP confirms that phosphate transfer was required for the action of ATP.

The effects of ATP were demonstrated at a concentration of 5mM so kinase inhibitors which exerted their effect other than competitively at the ATP binding site were required. Of those used only the presence of 7μM chelerythrine reduced the inhibition of nitric oxide synthase activity seen on addition ATP to the homogenisation buffer. Chelerythrin is a protein kinase C inhibitor and therefore inhibition of this enzyme, by some mechanism, may prevent inhibition of nitric oxide synthase activity. The simplest explanation is that protein kinase C phosphorylates nitric oxide synthase and thereby reduces its activity. The inhibition by chelerythrin was incomplete but higher concentrations of this agent could not be used because they interfered directly with the nitric oxide synthase assay. Other kinases could therefore also be involved in the inhibition of gastric mucosal nitric oxide synthase. Since protein kinase C is not usually regulated by phosphorylation okadaic acid may be acting by enhancing the effect of a basal protein kinase C activity by inhibiting the removal of phosphate from nitric oxide synthase. There have been conflicting reports on the effect of protein kinase C on brain nitric oxide activity. Brune and Lapetina (1991) found that prctein kinase C had no effect on purified enzyme activity . Bredt et al. (1992) found that protein kinase C phosphorylated nitric oxide stoichimetrically on a serine residue and this inhibited activity rapidly by 50%. Nakane et al. (1991) also found that nitric oxide synthase was phosphorylated by protein kinase C but on nine

different sites and this increased enzyme activity to 140%. Our data would appear to support with Bredt's findings.

The lack of effect with the other protein kinase inhibitors suggests cAMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent kinase II may not be responsible for the inhibitory effect of ATP on nitric oxide synthase activity in homogenates. cAMP-dependent protein kinase has been shown to phosphorylate brain nitric oxide synthase stoichimetrically on a serine residue and this produces either no change in enzyme activity (Brune and Lapetina, 1991) or an inhibition in activity (Bredt et al., 1992). Ca^{2+}/calmodulin-dependent protein kinase II is also known to phosphorylate brain nitric oxide synthase. Brune and Lapetina (1991) reported that this to have no affect on enzyme activity but both Bredt et al. (1992) and Nakane et al. (1991) found to be inhibitory. Bredt again found the phosphorylation to be stoichiometric but Nakane found nine phosphorylations per nitric oxide synthase molecule.

Further experiments should be performed to assess the concentration-dependence of the effect of ATP/Mg^{2+}. If lower concentrations of ATP could be used then it might prove possible to use other inhibitors which compete with ATP, such as H7, and thereby rule out the involvement of kinases such as cGMP-dependent protein kinase.

6.4 SUMMARY.

Nitroarginine, L-NMMA, Trifluoperazine and L-Canavanine all inhibit nitric oxide synthase activity in high density gastric mucosal cells (in descending order of potency). Gastric mucosal nitric oxide synthase appeared to be very similar to the brain form of the enzyme in its inhibition by the aforementioned compounds. Okadaic acid inhibited nitric oxide synthase activity when preincubated with gastric mucosal cells for 30min. ATP/Mg^{2+}, but not Mg^{2+}, β,γ-methylene ATP or GTP, inhibited nitric oxide activity when present in the homogenisation buffer during freeze-thawing. This action was partially antagonised by the protein kinase C inhibitor chelerythrin. Neither cAMP-dependent protein kinase inhibitor nor Ca^{2+}/calmodulin -dependent kinase II inhibitor had any effect on nitric oxide synthase activity.

CHAPTER 7.

GENERAL CONCLUSIONS.

This study was commenced with the aim of investigating signal transduction systems in the gastrointestinal tract. A promising way to proceed with this project appeared to be by the use of cell lines as their use abolished the problem of contamination of isolated cell preparations with unwanted cell types and they are easily grown. Work began on the HGT-1 cell line. The parameters governing cell multiplication were examined and the cells showed a density dependent growth. The possibility of autocrine regulation of growth was proved to be correct since a conditioned medium from HGT-1 cells stimulated replication in cells at low density. The precise factor involved was unknown as there are a great many known growth factors and possibly a number of undiscovered ones too. It was also possible that there was more than one substance involved in this stimulation. EGF and insulin and the peptide gastrin-17 were found to be a mitogens for HGT-1 cells grown at low density. As HGT-1 cells are from the gastric mucosa and gastrin is a gastrointestinal mitogen it is feasible that this could be a reflection of a physiological growth stimulus in this cell line.

The HGT-1 cell line appeared an interesting model for the investigation of the biochemical mechanisms regulating gastrin-dependent growth of the gastric mucosa but unfortunately there were some practical difficulties in developing the experimental procedures. The main problem was that effects of gastrin were only seen in cells at low density which meant that enormous numbers of plates would be necessary to obtain enough cells for biochemical analysis.

A large amount of work had previously been done on the histamine H_2-receptor found on the HGT-1 cell line. As the receptor is a model for the one found in the human gastric mucosa it was of extreme interest to determine its regulation by intracellular signalling pathways. Past investigations had already established that the receptor functioned via the generation of cAMP and underwent modulation by phorbol esters. This latter fact indicated a role for protein kinase C in intracellular signalling, with this enzyme probably providing a "cross-talk" pathway with its site of action very near to the receptor itself. There are various isoforms of protein kinase C and investigations into which forms were present in HGT-1 cells was initiated. HGT-1 cells were found to have both α and ε isoforms but not β or γ which was very similar to the pattern seen in stomach mucosa. Of the two isoenzymes present only α was down-regulated by phorbol esters. TPA had previously been shown to inhibit the histamine-stimulation of cAMP and this thesis assembled evidence that it was protein kinase C α which was responsible for the effects of TPA.

Over the past few years a new intracellular messenger, nitric oxide, has assumed increasing importance. A gastrointestinal cell line containing nitric oxide synthase would have been an extremely useful tool but unfortunately all of the gastrointestinal cell lines tested were negative for nitric oxide synthase activity. The next step was to investigate the presence of activity in gastrointestinal tissue and to try and locate major sites of production. Of the mucosal regions the gastric mucosa was found to have, by far, the most activity. High amounts of activity in this region correlate with the proposed role of nitric oxide as a protector against mucosal damage. Induction by endotoxin led to a rise in nitric oxide synthase activity in the mucosa and muscle of the small and large intestines. This activity was attributable in the mucosa to the production of the calcium-independent form of nitric oxide synthase.

The role for nitric oxide as a protector of gastric mucosal integrity suggests that it could represent a target for anti-ulcer therapy and therefore the properties of the enzyme were examined in more detail. A number of inhibitors of nitric oxide synthase were tested for their potency which was found to be: nitroarginine > L-NMMA > trifluoperazine > canavanine. From inhibition curves the gastric mucosal enzyme appeared to be very similar to the brain form. This was also supported by its Ca^{2+}-dependency and presence in the cytosolic fraction of the cell.

The inhibition of nitric oxide synthase by preincubation with okadaic acid indicated that phosphorylation was a negative modulator of activity. This was further suggested by the finding that the addition of ATP/Mg^{2+} to the homogenisation buffer also caused a fall in activity. Phosphorylation occurs by the action of kinases and so inhibitors of some of the likely kinases in the cell were used. Only the inhibition of protein kinase C antagonised the decrease in activity seen with ATP/Mg^{2+}. The deduction from this would be that protein kinase C, either directly or indirectly, is involved in the regulation of gastric mucosal nitric oxide synthase.

It was unfortunate that the initial hope that gastrointestinal cell lines would be useful to investigate cellular signalling in the gastrointestinal tract was not completely fulfilled. However, in this thesis, important and novel information was provided on the isoform of protein kinase C involved in regulation of histamine H_2 receptor-stimulated adenylate cyclase, on the distribution of nitric oxide synthases in the gastrointestinal tract and on the characterisation and regulation of gastric mucosal nitric oxide synthase.

PUBLICATIONS.

Protein kinase C inhibits histamine H2 receptor-mediated stimulation of cyclic AMP content in the human gastric cancer cell line HGT-1.
McKenna, P. J., J. M. Williams, C. Gespach, P. J. Hanson.
Biochemical Society Transactions (1992) 20 128S.

Protein kinase C α is the isoform responsible for inhibition of histamine H2 receptor mediated stimulation of adenylate cyclase in the human gastric cancer cell line HGT-1.
McKenna, P. J., J. M. Williams, P. J. Hanson.
Biochemical Society Transactions (1993) 21 192S.

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

REFERENCES.

Adams, J. C. and W. J. Gullick (1989). Differences in phorbol-ester induced down-regulation of protein kinase C between cell lines. Biochem. J. 257 905-911.

Aldir, H. C., D. Lang and M. J. Lewis (1990). A comparison of three nitric oxide synthase inhibitors on endothelium-dependent relaxation in rabbit aorta and on NO synthase activity in rabbit cerebellum. Gastroenterology 202P.

Ase, K., N. Berry, U. Kikkawa, A. Kishimoto and Y. Nishizuka (1988). Differential down-regulation of protein kinase C subspecies in KM3 cells. FEBS 236 396-400.

Azuma, H (1986). Endothelium-dependent inhibition of platelet aggregation. Br. J. Pharmacol. 88 411-415.

Azzi, A., D. Boscoboinik and C. Hensey (1992). The protein kinase C family. Eur. J. Biochem. 208 547-557.

Belai, A., H. H. H. W. Schmidt, C. H. V. Hoyle, C. J. S. Hassall, M. J. Saffrey, J. Moss, U. Förstermann, F. Murad and G. Burnstock (1992). Colocalisation of nitric oxide synthase and NADPH diaphorase in the myenteric plexus of the rat gut. Neuroscience Letters 143 60-64.

Boeckxstaens, G. E., P. A. Pelckmans, J. G. De Man, H. Bult, A. G. Herman and Y. M. Van Maercke (1992). Evidence for a differential release of nitric oxide and vasoactive intestinal peptide by nonadrenergic noncholinergic nerves in the rat gastric fundus. Arch. Int. Pharmacodyn. 318 107-115.

Boughton-Smith, N. K., S. M. Evans, A. T. Cole, B. J. R. Whittle and C. J. Hawkey (1992). Increased nitric oxide synthase activity in inflamed colon from ulcerative colitis patients. Gut 33 (S2) W44.

Bredt, D. S. and S. H. Snyder (1990). Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. P.N.A.S. 87 682-685.

Bredt, D. S. (1990). Localisation of nitric oxide synthase indicating a neural role for nitric oxide. Nature 347 768-770.

Bredt, D. S., P. M. Hwang, C. E. Glat, C. Lowenstein, R. R. Reed and S. H. Snyder (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 351 714-718.

Bredt, D. S., C. D. Ferris and S. H. Snyder (1992). Nitric oxide synthase regulatory sites. J. Biol. Chem. 267 10976-10981.

Brown, J. F., B. L. Tepperman, P. J. Hanson, B. J. R. Whittle and S. Moncada (1992). Differential distribution of nitric oxide synthase between cell fractions isolated from the rat gastric mucosa. B.B.R.C. 184 680-685.

Brostrom, C. O. and D. J. Wolff (1981). Properties and functions of calmodulin. Biochem. Pharmacol. 30 1395-1405.

Brüne, B. and E. G. Lapetina (1991). Phosphorylation of nitric oxide synthase by protein kinase A. B.B.R.C. 181 921-926.

Burgess, A. W. (1989). Epidermal growth factor and transforming growth factor α. Br. Med. Bull. 45 (2) 401-424.

Busconi, L. and T. Michel (1993). Endothelial nitric oxide synthase. J. Biol. Chem. 268 8410-8413.

Cabero, J. L., J. F. Rehfeld and S. Mårdh (1989). The effects of various gastrins on intracellular free Ca^{2+} in isolated pig parietal cells. Acta Physiol. Scand. 136 301-307.

Carpenter, G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. Ann. Rev. Biochem. 56 881-914.

Cezard, J., M. Forgue-Lafitte, M. Chamblier and G. E. Rosselin (1981). Growth-promoting effect, biological activity, and binding of insulin in human intestinal cancer cells in culture. Can. Res. 41 1148-1153.

Cheng, H. C., B. E. Kemp, R. B. Pearson, A. J. Smith, L. Misconi, S. M. Van Patten and D. A. Walsh (1986). A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. J. Biol. Chem. 261 989-992.

Cho, H. J., Q. Xie, J. Calaycay, R. A. Mumford, K. M. Swiderek, T. D. Lee and C. Nathan (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. J. Exp. Med. 176 599-604.

Choi, P. M., K. M. Tchou-Wong and I. B. Weinstein (1990). Overexpression of protein kinase C in HT29 colon cancer cells causes growth inhibition and tumour suppression. Mol. Cell Biol. 10 4650-4657.

Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new born animal. J. Biol. Chem. 237 1555-1562.

Culotta, E. and D. E. Koshland (1992). NO news is good news. Science 258 1862-1865.

Dwyer, M. A., D. S. Bredt and S. H. Snyder (1991). Nitric oxide synthase: irreversible inhibition by L-N^G-nitroarginine in brain *in vitro* and *in vivo*. B.B.R.C. 176 1136-1141.

East, S. J. and J. Garthwaite (1990). Nanomolar N^G-nitroarginine inhibits NMDA-induced cyclic GMP formation in rat cerebellum. Eur. J. Pharmacol. 184 311-313.

Edkins, J. S. (1905). On the chemical mechanism of gastrin secretion. Proc. R. Soc. (London) [Biol] 76 376-379.

Emami, S., C. Gespach, M, Forgue-Lafitte, Y. Broer and G. Rosselin (1983). Histamine and VIP interactions with receptor-cyclic AMP systems in the human gastric cancer cell line HGT-1. Life Sci. 33 415-423.

Emami, S., E. Chastre, H. Bodéré, C. Gespach, D. Bataille and G. Rosselin (1986). Functional receptors for VIP, GIP, glucagon-29 and -37 in the HGT-1 human gastric cancer cell line. Peptides 7 (S1) 121-7.

Emami, S. and C. Gespach (1986b). Desensitization by histamine of H_2 receptor activity in HGT-1 human cancerous gastric cells. Agents and Actions 18 129-133.

Forster, E. R. and E. Southam (1992). Location of nitric oxide synthase in rat gastric corpus. Reg. Peptides 40 146.

Förstermann, U., H. H. H. W. Schmidt, J. S. Pollock, H. Sheng, J. A. Mitchell, T. D. Warner, M. Nakane and F. Murad (1991b). Isoforms of nitric oxide synthase. Biochem. Pharmacol. 42 1842-1857.

Förstermann, U., J. S. Pollock, H. H. H. W. Schmidt, M. Heller and F. Murad (1991). Calmodulin-dependent endothelium-derived relaxing factor / nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. P.N.A.S. 88 1788-1792.

Fraser, G. and G. I. Sandle (1990). High conductance K^+ channels in the plasma membrane of cultured human gastric cells (HGT-1).

Freshney, R. I. (1987). Culture of animal cells: A manual of basic technique, 2nd edition. Alan R. Liss inc., New York.

Furchgott, R. F. and J. V. Zawadzki (1980).The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature (London) 288 373-376.

Furchgott, R. F. (1988). Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In vasodilation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium ed. by P. M. Vanhoutte, pp401-414, Raven Press, New York.

Garen, A. and C. Levinthal (1960). A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of E. coli. Biochim. Biophys. Acta 38 470-83.

Garthwaite, J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervous system. TINS 14 60-67.

Gespach, C., S. Emami and G. Rosselin (1984). Gastric inhibitory peptide (GIP), pancreatic glucagon and vasoactive intestinal peptide (VIP) are cAMP-inducing hormones in the human gastric cancer cell line HGT-1. Homologous desensitization of VIP receptor activity. B.B.R.C. 120 641-649.

Gespach, C., S. Emami and E. Chastre (1988). Membrane receptors in the gastrointestinal tract. Bioscience Reports 8 199-232.

Granger, D. L., J. B. Hibbs, J. R. Perfect and D. T. Durack (1990). Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. J. Clin. Invest. 85 264-273.

Gschwendt, M., W. Kittstein and F. Marks (1991). Protein kinase C activation by phorbol esters: do cysteine-rich regions and pseudosubstrate motifs play a role? TIBS 16 167-169.

Gregory, R. A. and H. J. Tracy (1964). The constitution and properties of two gastrins extracted from hog antral mucosa. Gut 5 103-117.

Griffith, T. M. and D. H. Edwards (1984). The nature of endothelium-derived vascular relaxant factor. Nature (London) 308 645-647.

Guo, Y., M. Baijal, G. Jin, J. C. Thompson, C. M. Townsend and P. Singh (1990). Growth promoting effects of gastrin on mouse colon cancer cells in vitro: absence of autocrine effects. In Vitro Cell. Dev. Biol. 26 871-877.

Hansen, A. B., C. P. Gespach, G. E. Rosselin and J. J. Holst (1988). Effect of truncated glucagon-like peptide 1 on cAMP in rat gastric glands and HGT-1 human gastric cancer cells. FEB 236 119-122.

Hanson, P. J. and D. S. Parsons (1976). The utilization of glucose and production of lactate by *in vitro* preparations of rat small intestine: Effects of vascular perfusion. J. Physiol. 255 775-795.

Hanson, P. J. and J. F. Hatt (1989). Intracellular signalling and regulation of gastric acid secretion. Quarterly J. Experimental Physiology 74 607-634.

Hardie, D. G. (1991). Biochemical Messengers, first edition. Chapman and Hall.

Hassell, J. and D. L. Engelhardt (1977). Factors regulating the multiplication of animal cells in culture. Exptl. Cell Res. 107 159-167.

Hashimoto, Y., K. Chida, M. Huang, M. Katayama, T. Nishihira and T. Kuroki (1989). Levels of protein kinase C activity in human gastrointestinal cancers. B. B. R. C. 163 (1) 406-411.

Hatt, J. F. and P. J. Hanson (1989). Sites of action of protein kinase C on secretory activity in rat parietal cells. Am. J. Physiol. 256 G129-138.

Haystead, T. A. J., A. T. R. Sim, D. Carling, R. C. Honor, Y. Tsukitani, P. Cohen and D. G. Hardie. (1989). Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature 337 78-81.

Heath, R. (1991). The L-arginine:nitric oxide pathway. The second international meeting on the biology of nitric oxide.

Herbert, J. M., et. al. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. B.B.R.C. 172 993-999.

Hiki, K., R. Hattori, C. Kawai and Y. Yui (1992). Purification of insoluble nitric oxide snythase from rat cerebellum. J. Biochem. 111 556-558.

Hirai, M., S. Gamou, M. Kobayashi and N. Shimizu (1989). Lung cancer cells often express high levels of protein kinase C activity. Jpn. J. Can. Res. 80 204-208.

Holley, R. W., R. Armour, J. H. Baldwin, K. D. Brown and Y. C. Yeh (1977). Density-dependent regulation of growth of BSC-1 cells in cell culture: Control of growth by serum factors. PNAS (USA) 74 5046-5050.

Homan,E. C., D. E. Jensen and J. J. Sando (1991). Protein kinase C isozyme expression in phorbol ester-sensitive and -resistant EL4 thymoma cells. J. Biol. Chem. 266 5676-5681.

Hortelano, S., A. Genaro and L. Bosca (1992). Phorbol esters induce nitric oxide synthase activity in rat hepatocytes. J. Biol. Chem. 267 24937-24940.

Houslay, M. D. (1990). 'Cross-talk': a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. J. Biochem. 195 9-27.

Hug, H. (1993). Protein kinase C isoenzymes: divergence in signal transduction? Biochem. J. 291 329-343.

Ignarro, L. J., R. E. Byrns and K. S. Wood (1988). Biochemical and pharmacological properties of endothelium-derived relaxing factor and its similarity to nitric oxide radical. In Vasodilation: Vascular Smooth Muscle. Peptides Autonomic Nerves and Endothelium. ed. by P. M. Vanhoutte, pp427-436, Raven Press,New York.

Ignarro, L. J. (1991). Signal transduction mechanisms involving nitric oxide. Biochem. Pharmacol. 41 485-490.

Imai, T., Y. Hirata and F. Marumo (1992). Expression of brain nitric oxide synthase mRNA in various tissues and cultured cells of rat. Biomed. Res. 13 371-374.

Ishizuka, J., J. Martinez, C. M. Townsend and J. C. Thompson (1992). The effect of gastrin on growth of human stomach cancer cells. Ann. Surg. 215 (5) 528-534.

Ito, S. (1987). Functional gastric morpholgy in Physiology of the gastrointestinal tract. Ed. L. R. Johnson. Raven Press, New York.

James, R. (1984). Polypeptide growth factors. Ann. Rev. Biochem. 53 259-292.

Johnson, L. R. (1987). Regulation of gastrointestinal growth. Physiology of the gastrointestinal tract, second edition, pp301-333. Raven Press, New York.

Karaki, H., et. al. (1989). Inhibitory effect of a toxin okadaic acid, isolated from the black sponge on smooth muscle and platelets. Br. J. Pharmacol. 98 590-596.

Knowles, R. G., M. Palacios, R. M. J. Palmer and S. Moncada (1990c). Formation of nitric oxide from L-arginine in the central nervous system:

A transduction mechanism for stimulation of the soluble guanylate cyclase. P.N.A.S 86 5159-5162.

Knowles, R. G., M. Salter, S. L. Brookes and S. Moncada (1990a). Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. B.B.R.C. 172 1042-1048.

Knowles, R. G., M. Merrett, M. Salter and S. Moncada (1990b). Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. Biochem. J. 270 833-836.

Knowles, R. G., M. Palacios, R. M. J. and S. Moncada (1990). Kinetic characteristics of nitric oxide synthase from rat brain. Biochem. J. 269 207-210.

Kobori, O., M. Vuillot and F. Martin (1982). Growth responses of rat stomach cancer cells to gastro-enteropathic hormones. Int. J. Cancer 30 65-67.

Kraft, A. S. and W. B. Anderson (1983). Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. Nature (London) 301 621-623.

Kusyk, C. J., N. O. McNeil and L. R. Johnson (1986). Stimulation of growth of a colon cancer cell line by gastrin. Am. J. Physiol. 251 G597-G601.

Laboisse, C. L., C. Augeron, M. Couturier-Turpin, C. Gespach, A. Cheret and F. Potet (1982). Characterisation of a newly established human gastric cancer cell line HGT-1 bearing histamine H_2-receptors. Can. Res. 42 1541-1548.

Lamas, S., P. A. Marsden, G. K. Li, P. Tempst and T. Michel (1992). Endothelial nitric oxide synthase: Molecular cloning and characterisation of a distinct constitutive enzyme isoform. PNAS. 89 6348-6352.

Lemoine, N. R., H. Y. Leung, W. J. Gullick (1992). Growth factors in the gastrointestinal tract. Gut 33 1297-1300.

Lim, I. K., D. J. Lee, K. H. Lee and T. K. Yun (1987). High activities of protein kinases C and M in fresh human stomach and breast tumors. Yonsei Med J. 28 255-260.

Liyanage, M., D. Frith, E. Livneh and S. Stabel (1992). Protein kinase C group B members PKC-δ, -ε, -ζ and PKC-L(η). Biochem. J. 283 781-787.

Llewellyn-Smith, I. J., Z. Song, M. Costa, D. S. Bredt and S. H. Snyder (1992). Ultrastructural localisation of nitric oxide synthase immunoreactivity in guinea-pig enteric neurons. Brain Res. 577 337-342.

Lyons, C. R., G. J. Orloff and J. M. Cunningham (1992). Molecular cloning and functional expression of an inducible nitirc oxide synthase from a murine macrophage cell line. J. Biol. Chem. 267 6370-6374.

Madara, J. L. and Trier J. S. (1987). Functioal morphology of the mucosa of the small intestine in Physiology of the gastrointestinal tract. Ed. L.R. Johnson. Raven Press, New York.

Makowske, M., R. Ballester, Y. Cayre and O. Rosen (1988). Immunochemical evidence that three protein kinase C isozymes increase in abundance during HL-60 differentiation induced by dimethyl sulfoxide and retinoic acid. J. Biol. Chem. 263 3402-3410.

Marletta, M. A. (1989). Nitric oxide: biosynthesis and biological significance. TIBS 14 488-491.

Martinez-Cuesta, M. A., B. J. R. Whittle and S. Moncada (1992). Endogenous nitric oxide regulates motility of the rat isolated duodenum. Br. J. Parm. 107(S) 400.

Matsumoto, H., R. H. Erickson, J. R. Gum, M. Yoshioka, E. Gum and Y. S. Kim (1990). Biosynthesis of alkaline phosphatase during differentiation of the human colon cancer cell line CaCo-2. Gastroenterology 98 1199-1207.

Mayer, B., M. John and E. Böheme (1990). Purification of a Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum. FEBS Lett. 277 215-219.

McCall, T. B., M. Feelisch, R. M. J. Palmer and S. Moncada (1991). Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. Br. J. Pharmacol. 102 234-238.

McKenna, P. J., J. M. Williams, C. P. Gespach and P. J. Hanson (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. J., J. M. Williams and P. J. Hanson (1993b). Protein kinase $C\alpha$ is the isoform responsible for inhibition of histamine H_2 receptor-mediated stimulation of adenylate cyclase in the human gastric cancer cell line HGT-1. Biochemical Society Transactions 21 192S.

Michel, T., G. K. Li and L. Busconi (1993). Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. PNAS. 90 6252-6256.

Mitchell, J. A., K. L. Kohlhaas, T. Matsumoto, J. S. Pollock, U. Förstermann, T. D. Warner, H. H. H. W. Schmidt and F. Murad (1992). Induction of NADPH-dependent diaphorase and nitric oxide synthase activity in aortic smooth muscle and cultured macrophages. Mol. Pharmacol. 41 1163-1168.

Nakane, M., J. Mitchell, U. Förstermann and F. Murad (1991). Phosphorylation by calcium calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase. B.B.R.C. 180 1396-1402.

Nakanishi, Y., J. Mulshine, P. G. Kasprzyk, R. B. Natale, R. Maneckjee, I. Avis, et. al. (1988). Insulin-like growth factor-I can mediate autocrine

proliferation of human small cell lung cancer lines *in vitro*. J. Clin. Invest. 82 354-359.

Nishida, E., and Y. Gotoh (1993). The MAP kinase cascade is essential for diverse signal transduction pathways. TIBS 18 128-131.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. Science 233 305-312.

Nishizuka, Y. (1992). Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. Science 258 607-614.

Ochiai, A., W. Yasui and E. Tahara (1985). Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. Jpn. J. Can. Res. (Gann) 76 1064-1071.

Ohmura, E., M. Okada, N. Onoda, Y. Kamiya, H. Murakami, T. Tsushima and K. Shizume (1990). Insulin-like growth factor I and transforming growth factor α as autocrine growth factors in human pancreatic cancer cell growth. Can. Res. 50 103-107.

Osada, S., K. Mizuno, T. Saido, Y. Akita, K. Suzuki, T. Kuroki and S. Ohno (1990). A phorbol ester receptor/protein kinase, nPKCη, a new member of the protein kinase C family predominantly expressed in lung and skin. J. Biol. Chem. 265 22434-22440.

Palmer, R. M. J., A. G. Ferrige and S. Moncada (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327 524-526.

Palmer, R. M. J., D. S. Ashton and S. Moncada (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 333 664-666.

Palmer, R. M. J., D. D. Rees, D. S. Ashton and S. Moncada (1988b). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. B. B. R. C. 153 1251-1256.

Palmer, R. M. J., T. Andrews, N. A. Foxwell and S. Moncada (1992). Glucocorticoids do not affect the induction of a novel calcium-dependent nitric oxide synthase in rabbit chondrocytes. B.B.R.C. 188 209-215.

Pinto, M., S. Robine-Leon, M. D. Appay, M. Kerdinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum (1983). Enterocyte-like differentiation and polarisation of the human coloncarcinoma cell line CaCo-2 in culture. Biol. Cell 47 323-330.

Primrose, S. B. (1987). Modern Biotechnology. Blackwell Scientific Publications, Oxford.

Prost, A., S. Emami and C. Gespach (1984). Desensitization by histamine of H_2 receptor-mediated adenylate cyclase activation in the human gastric cancer cell line HGT-1. FEBS 177 227-230.

Radomski, M. W., R. M. J. Palmer amd S. Moncada (1987a). The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. Biochem. Biophys. Res. Commun. 148 1482-1489.

Radomski, M. W., R. M. J. Palmer and S. Moncada (1987b). Endogenous nitric oxide inhibits human platlet adhesion to vascular endothelium. Lancet 2 1057-1058.

Radomski, M. W., D. C. Jenkins, L. Holmes, and S. Moncada (1991). Human colorectal adenocarcinoma cells: Differential nitric oxide synthesis determines their ability to aggregate platelets. Can. Res. 5 6073-6078.

Rapoport, R. M. and F. Murad (1983). Agonist induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cyclic GMP. Circ. Res. 52 352-357.

Rees, D. D., R. M. J. Palmer, H. F. Hodson and S. Moncada (1989). A specific inhibitor of nitric oxide formation L-arginine attenuates endothelium-dependent relaxation. Br. J. Pharmacol. 96 418-424.

Rees, D. D., S. Cellek, R. M. J. Palmer and S. Moncada (1990). Dexamethosone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxic shock. B.B.R.C. 173 541-547.

Rees, D. D., R. M. J. Palmer, R. Schultz, H. F. Hodson and S. Moncada (1990). Charaterisation of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. Br. J. Pharmacol. 101 746-752.

Rehfeld, J. F. and L. Hilsted (1992). Gastrin and cancer. Adv. Clin. Chem. 29 239-262.

Rein A. and H. Rubin (1971). On the survival of chick embryo cells at low concentrations in culture. Exptl. Cell Res. 65 209-214.

Remy-Heintz, N., S. Perrier-Meissonnier, I. Nonotte, M. Laliberté, C. Chevillard, C. Laboisse and J. Bali (1993). Evidence for autocrine growth stimulation by a gastrin/ CCK-like peptide of the gastric cancer HGT-1 cell line. Mol. and Cell. Endocrinol. 93 23-29.

Reyl-Desmars, F., C. Laboisse and M. J. M. Lewin (1986). A somatostatin receptor negatively coupled to adenylate cyclase in the human gastric cell line HGT-1. Reg. Peptides 16 207-215.

Roche, S. and R. Magous (1989). Gastrin and CCK-8 induce inositol 1,4,5-trisphosphate formation in rabbbit gastric parietal cells. Biochim. et Biophys Acta 1014 313-318.

Rousset, M., M. Laburthe, M. Pinto, G. Chevalier, C. Rouyer-Fessard. E. Dussaulx, G. Trugnan, N. Boige, J. Brun and A. Zweibaum (1985).Enterocytic differentiation and glucose utilisation in the human colon tumor cell line CaCo-2: modulation by forskolin. J. Cell. Physiol. 123 377-385.

Sakai, K., S. Mori, T. Kawamoto, S. Taniguchi, O. Kobori, Y. Morioka, T. Kuroki and K. Kano (1986). Expression of epidermal growth factor receptors on normal human gastric epithelia and gastric carcinomas. J. N. C. I. 77 (5) 1047-1052.

Salter, M., R. G. Knowles and S. Moncada (1991). Widespread tissue distribution, species distribution and changes in the activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. FEBS 291 145-149.

Sandle, G. I., G. Fraser, S. Long and G. Warhurst (1990). A cAMP-activated chloride channel in the plasma membrane of cultured human gastric cells. Pflügers Arch 417 259-263.

Savage, C. R., T. Inagami and S. Cohen (1972). The primary structure of epidermal growth factor. J. Biol. Chem. 247 7612-7621.

Schlessinger, J. (1993). How receptor tyrosine kinases activate Ras. TIBS 18 273-275.

Schmidt, H. H. H. W., T. D. Warner and F. Murad (1992). Double-edged role of endogenous nitric oxide. Lancet 339 986.

Schmidt, H. H. H. W., S. M. Lohmann and U. Walter (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochem et Biophys Acta 1178 153-175.

Schwartz, G. K., J. Jiang, D. Kelsen and A. Albino (1993). Protein kinase C: A novel target for inhibiting gastric cancer cell invasion. J. Natl. Can. Inst. 85 402-407.

Severn, A., M. J. O. Wakelam and F. Y. Liew (1992). The role of protein kinase C in the induction of nitric oxide synthase by murine macrophages. B.B.R.C. 188 997-1002.

Sorrentino, V. (1989). Growth factors, growth inhibitors and cell cycle control. Antican. Res. 9 1925-1936.

Springall, D. R., V. Riveros-Moreno, L. Buttery, A. Suburo, A. E. Bishop, M. Merrett, S. Moncada and J. M. Polak (1992). Immunological detection of nitric oxide synthase(s) in human tissues using heterologous antibodies suggesting different isoforms. Histochemistry 98 259-266.

Stoscheck, C. M. and L. E. King (1986). Role of epidermal growth factor in carcinogenesis. Can. Res. 46 1030-1037.

Strulovici, B., S. Daniel-Issakani, G. Baxter, J. Knopf, L. Sultzman, H. Cherwinski, J. Nestor, D. Webb and J. Ransom (1991). Distinct mechanisms of regulation of protein kinase C ε by hormones and phorbol diesters. J. Biol. Chem. 266 168-173.

Stark, M. E. and J. H. Szurszewski (1992). Role of nitric oxide in gastrointestinal and hepatic function and disease. Gastroenterology 103 1928-1949.

Takai, Y., A. Kishimoto, M. Inoue and Y. Nishizuka (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. J. Biol. Chem. 252 7603

Tepperman, B. L., B. J. R. Whittle and S. Moncada (1992). Biosynthesis of nitric oxide by preparations of rabbit intestinal mucosa and smooth muscle. Motility and Nerve-Gut Interactions A525.

Thompson, M. A., A. J. Cox, R. H. Whitehead and H. A. Jonas (1990). Autocrine regulation of human tumor cell proliferation by insulin-like growth factor II: an *in-vitro* model. Endocrinology 126 (6) 3033-3042.

Tokumitso, H., T. Chijiwa, M. Hagiwara, A. Mizutani, M. Terasawa and H. Hidaka (1990). KN-62, 1-[N,O-Bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase. J. Biol. Chem. 265 4315-4320.

Todaro, G. J. and H. J. Green (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol. 17 299-310.

Tracey, W. R., M. Nakane, J. S. Pollock and U. Förstermann (1993). Nitric oxide synthases in neuronal cells, macrophages and endothelium are NADPH diaphorases, but represent only a fraction of total cellular NADPH diaphorase activity. B.B.R.C. 195 1035-1040.

Vandenbark, G. R. and J. E. Niedel (1984). Phorbol esters and cellular differentiation. J. Natl. Can. Res. 73 1013-1017.

Van Wyk, J. J., L. E. Underwood, J. B. Baseman, R. L. Hintz, D. R. Clemmons and R. N. Marshall (1975). Exploration of the insulin-like and growth promoting properties of somatomedin by membrane receptor assays. Adv. Metab. Disord. 8 127-150.

Walsh J. H. and M. I. Grossman (1975). Gastrin. N. Eng. J. Med. 292 (25) 1324-1334.

Watson, S. A., L. G. Durrant, J. D. Crosbie and D. L. Morris (1989). The in vitro growth response of primary human colorectal and gastric cancer cells to gastrin. Int. J. Cancer 43 692-696.

Watson, S., L. Durrant and D. Morris (1989). Gastrin: growth enhancing effects on human gastric and colonic tumour cells. Br. J. Cancer 59 554-558.

Watson, S. A., L. G. Durrant, P. M. Wencyk, A. L. Watson and D. L. Morris (1991). Intracellular gastrin in human gastrointestinal tumor cells. J. Natl. Can. Inst. 83 (12) 866-871.

Whittle, B. J. R., J. Lopez-Belmonte and S. Moncada (1990). Regulation of gastric mucosal integrity with prostanoids and sensory neuropeptides in the rat. Br. J. Pharmacol. 99 607-611.

Whittle, B. J. R., S. Berry, J. Lopez-Belmonte, N. K. Boughton-Smith and S. Moncada (1991). Detection of the synthase enzyme that forms the

endogenous vasodilator, nitric oxide in the rat gastric mucosa. Gastroenterology 100 A184.

Yasui, W., H. Sumiyoshi, A. Ochiai and E. Tahara (1985). Calcium-activated, phospholipid-dependent protein kinase in human gastric mucosa and carcinoma. Jpn. J. Can. Res. 76 1168-1173.

Yoshida, K., E. Kyo, T. Tsujino, T. Sano, M. Niimoto and E. Tahara (1990). Expression of epidermal growth factor, transforming growth factor-α and their receptor genes in human gastric carcinomas; implication for autocrine growth. Jpn. J. Cancer Res. 81 43-51.

Yiu, Y., R. Hattori, K.Kosuga, H. Eizawa, K. Hiki, S. Ohkawa, K. Ohnishi, S. Terao and C. Kawai (1991). Calmodulin-independent nitric oxide synthase from rat polymorphonuclear neutrophils. J. Biol. Chem. 266 3369-3371.

APPENDIX A.

MATERIALS.

Chemical	Source
Adenosine 5'-triphosphate (Na^+ salt)	Sigma
Anti-protein kinase C antibodies (isoform specific)	Gibco BRL
Aprotinin	Sigma
L-arginine	Sigma
U-[^{14}C]-Arginine	Amersham
Biotinylated anti-rabbit IgG antibody	Amersham
Bovine serum albumin, fraction V	ICN
Calmodulin	Sigma
cAMP-dependent protein kinase inhibitor	Sigma
L-canavanine	Sigma
CHAPS	Sigma
Chelerythrine	Calbiochem
DE-52 ion exchanger	Whatman
Dextran (molecular mass 40,000da)	Sigma
Diethanolamine	Amersham
Dimethylsulphoxide	Sigma
Dithiothreitol	Sigma
Dowex AG 50W-8 ion exchanger	Sigma
Eagle's minimum essential medium	Sigma
EDTA	BDH
EGTA	BDH
Epidermal growth factor (mouse)	Sigma
FAD	Sigma
Foetal calf serum	Gibco BRL
Folin-Ciocalteu's reagent	BDH
G25 Sephadex (gel filtration)	Sigma
[Leu^{15}]-Gastrin I (human)	Sigma
Gentamicin	Sigma
Guanine 5'-triphosphate	Sigma
HEPES	Sigma
Hybond C-Super nitrocellulose	Amersham
Insulin	Sigma
KN-62	Calbiochem
Leupeptin	Sigma
Lipopolysaccharide (E. col. serotype 0111:B4)	Sigma
β,γ-Methyleneadenosine 5'-triphosphate	Sigma
N^G-monomethyl-L-arginine	Wellcome
NADPH	Sigma
L-Nitroarginine	Sigma
Okadaic acid	Calbiochem
Optiphase Hisafe II	LKB, Wallace
Pepstatin	Sigma
Percoll	Sigma
Phenylmethylsulphonyl fluoride	Sigma
Phorbol-12,13-dibutyrate	Sigma
[3H] Phorbol-12,13-dibutyrate	Amersham
Phosphate buffered saline tablets	Oxiod
Pyrogen-free 0.9% saline	Sigma
Pronase (70,000 PUK/g)	BDH
Rainbow markers for electrophoresis	Amersham

Sagatal	Rhone Poulenc
Sodium dodecylsulphate	Sigma
Soybean trypsin inhibitor	Sigma
Streptavidin-alkaline phosphate conjugate	Amersham
Trifluoperazine	Sigma
Triton-X 100	Sigma
Trypan Blue	BDH
L-valine	Sigma

Any unlisted organic chemicals were from Sigma and inorganic chemicals from BDH.

APPENDIX B.

ANIMALS.

Male Wistar rats were obtained from Bantin and Kingman, Hull, U.K. They were fed ad libitum on Heygates' breeding diet from Pilsbury, Edgbaston, Birmingham, U.K.

APPENDIX C.

STATISTICAL ANALYSIS.

Analysis of Variance (AnoVar).

This method uses a two-way, random block design. The mean squares are calculated by dividing the sums of squares by the degrees of freedom for each factor. The mean squares for each factor is then divided by the residual mean square to give a F-ratio. The F-ratio is then compared with tabulated F-ratios with the degrees of freedom associated with the residuals and the factor.

If the F-ratio for the effect of treatments was significant the data could be analysed using a Dunnett's test.

Dunnett's Test.

This compares a control mean to all of the other treatments. The diference between the group means is calculated and divided by the standard error ($\sqrt{}$ 2 x residual mean sq all divided by n). If the resulting q' value exceeds the appropriate tabulated value then there is a significant difference between those two means.

Newman-Keuls Test.

This is again applied to data which has a significant F-ratio in the AnoVar and compares all means to the others in the group.