DEVELOPMENT OF A RADIOLIGAND BINDING ASSAY FOR DETECTION OF GASTRIN/CCK$_B$ RECEPTORS IN THE HUMAN GASTROINTESTINAL TRACT

by

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SUMMARY

The initial strategy of the thesis (Chapter 3) examined the presence and characterisation of gastrin/CCK_B receptors in the rat pancreatic cell line, AR42J. This cell line was chosen due to its continuous expression of high affinity gastrin/CCK_B receptors even after repeated cell culture. Following optimisation of the radioligand binding assay, gastrin/CCK_B receptors were characterised using a panel of receptor agonists and antagonists. The AR42J whole cell assay demonstrated that AR42J cells express high affinity gastrin/CCK_B receptors with a dissociation constant of 0.3nM and maximal binding capacity of 24fmols/10^6 cells. These results were similar to those found in the literature by several different groups. Inhibitory dissociation constants (Ki) for the receptor agonists and antagonists used in displacement experiments were also found to correlate closely to literature values thereby confirming the validity of the gastrin/CCK_B receptor properties of AR42J cells as measured using the assay developed.

The second series of experiments (Chapter 4) examined the preparation of crude membranes from AR42J cells and also the effect of membrane storage. Crude membrane fractions were found to retain the receptor characteristics and properties of receptors on AR42J whole cells. Storage of crude membranes for a limited period at -70°C in the presence of glycerol did not significantly affect receptor affinity or number. Receptor agonists/antagonists were found to displace gastrin from gastrin/CCK_B receptor sites with similar potencies to those previously determined in the AR42J whole cells thereby confirming that receptor properties were unaltered by the process of membrane preparation.

The first clinical study (Chapter 5) assessed the ability of the assay developed to detect and measure high affinity gastrin/CCK_B receptors in membrane extracts derived from human colonic normal and neoplastic tissues. Various methods of membrane preparation were explored, including pulverisation of tissues under liquid nitrogen which had been previously successful in the detection of these receptors by other researchers. Using a similar radioligand binding assay to that optimised for AR42J membranes in the previous study (Chapter 4), human membranes were evaluated for gastrin/CCK_B receptor status. Membranes prepared from both normal and neoplastic tissues were found to show little or no specific gastrin binding. Membranes that had low specific binding were subsequently found not to show displacement, even with high concentrations of unlabeled gastrin. When a similar assay was applied to membranes from normal and tumour tissues, freshly prepared by homogenisation, again there was no convincing evidence of high affinity gastrin/CCK_B receptors.

The second study (Chapter 6) examined the possible presence of high affinity gastrin/CCK_B receptors in membrane preparations from human gastric cancer and normal gastric body/antral tissues. Nine patients' tissue samples were collected in total; four were prepared as crude membranes from fresh tissue and a further five were prepared as crude membranes from frozen tissues that had been stored at -70°C for various periods of time.

Four patients' tissue samples collected fresh from theatre were immediately immersed in a buffer containing protease inhibitors and glycerol in order to protect the membrane structure prior to membrane preparation. Three of the four patients' gastric

body membranes expressed high affinity gastrin/CCK_B receptors with K_D and Bmax values between 0.4-2nM and 28-76fmol/mg protein respectively. Specific gastrin binding was displaced by gastrin/CCK_B receptor agonists and antagonists with similar potencies to those found with AR42J membranes.

Crude membranes were also prepared from five gastric cancer patients' tissue which had been stored at -70°C. However these tissues had not been immersed in buffer containing glycerol and protease inhibitors. They had been stored for varying periods of time at -70°C and only one patient's gastric body membrane preparation was found to exhibit high affinity gastrin/CCK_B receptors with K_D and Bmax of 0.7nM and 21 fmols/mg protein respectively.

None of the gastric antral or tumour membranes analysed exhibited high affinity gastrin/CCK_B receptors although a low level of specific binding was found in one tumour membrane sample which may indicate binding to low affinity gastrin binding sites.

In conclusion, the experiments in this thesis demonstrate that high affinity gastrin/CCK_B receptors are measurable in AR42J whole cells and that membrane preparation does not alter the receptor properties even after freezing at -70°C for a limited period of time. The clinical studies demonstrate that the optimised radioligand binding assay was successful in detection and characterisation of high affinity gastrin/CCK_B receptors in human gastric body tissues. No convincing evidence to support the presence of high affinity gastrin/CCK_B receptors in colorectal or gastric tumour tissue was found in this study.

CONTENTS

LIST OF TABLES

Appendix IV

LIST OF FIGURES

Chapter 4

Chapter 5

Chapter 6

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DECLARATION

I declare that this thesis was composed by myself and has not previously been submitted for consideration of a higher degree.

This research was conducted in the Department of Medicine and Therapeutics, University of Glasgow, under the supervision of Professor K.E.L. McColl and Dr C.A. Dorrian.

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ABBREVIATIONS

B	bound
Bmax	maximal binding capacity
BSA	bovine serum albumin
CCK	cholecystokinin
Ci (mCi, uCi)	Curie (millicurie, microcurie)
cGMP	cyclic guanosine monophosphate
cpm	counts per minute
CRC	Cancer Research Campaign
C-terminal	carboxy terminal
°C	degree(s) centigrade
DAG	diacylglycerol
dpm	disintegrations per minute
DMEM	Dulbecco's modified Eagle's medium
DMFO	dimethlyfluorioxide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBDA	equilibrium binding data analysis
ECL cells	enterochromaffin-like cells
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
F	free ligand
FCS	fetal calf serum
g (kg,mg,ug,ng)	gram (kilogram, milligram,microgram, nanogram)
G-cells	gastrin cells
GDP	guanosine diphosphate
GI	gastrointestinal

Gly	glycine
G-proteins	GTP-dependent regulatory proteins
G-17, G-34	gastrin-17, gastrin-34
GTP	guanosine triphosphate
^{3}H	tritium
HPLC	high performance liquid chromatography
^{125}I	^{125}Iodine
IC_{50}	half maximal concentration
K_D	dissociation constant
kDa	kilodalton
K_{+1}	association rate constant
K_{-1}	dissociation rate constant
K_{obs}	observed association rate
L (ml, ul)	litre (millilitre, microlitre)
[L]*	radiolabeled ligand concentration
M (mM, uM, nM, pM, fM)	molar (moles per litre; millimolar micromolar, nanomolar, picomolar, femtomolar)
mRNA	messenger ribonucleic acid
NMR	nuclear magnetic reasonance
NSB	non specific binding
ODC	ornithine decarboxylase
PCR	polymerase chain reaction
PKC	protein kinase C
PMSF	phenylmethylsulphonyl fluoride
RNA	ribonucleic acid
[R]	receptor concentration
[RL]*	radiolabeled ligand-receptor complex concentration
RIA	radioimmunoassay
SA	specific acivity
SD	standard deviation
$t_{1/2}$	half-time
um	micrometre

v/v	volume/volume
WHO	World Health Organisation
w/v	weight/volume

This thesis is dedicated to my father, ' A man you don't meet everyday',
the late Donald Mackenzie esq.

CHAPTER 1 GENERAL INTRODUCTION

1 GASTRIN

1.1 Introduction

The gastrointestinal hormone, gastrin, was first isolated in 1905 from extracts of porcine antral mucosa (Edkins *et al* 1905). It was subsequently purified and structurally identified as sulphated and non-sulphated gastrin (G17) hecadecapeptide forms (Gregory and Tracy 1964). The structure of the peptide (Figure 1.1), its mRNA and genomic sequence have since been established (Wiborg *et al* 1984) and in recent years it has been demonstrated that G17 functions as a trophic factor for much of the gastrointestinal mucosa. Consequently the C-terminal of G17 (Trp-Met-Asp-Phe-NH_2) was found to be the main biologically active site, although other C-terminal fragments may also be active (Tracy and Gregory 1964; Lin 1972). However, it is the C-terminal fragment of gastrin that binds to the gastrin/CCK_B receptor and therefore any changes in amino acids of the C-terminal may affect the biological activity of the peptide (Tracy and Gregory 1964).

1.2 Biosynthesis and processing

Gastrins' complex biosynthetic pathway has become well established and follows a pattern similar to that of most other peptide hormones (Rehfeld and Hilsted 1992). The majority of circulating gastrin originates from specialised G-cells which are situated within the pyloric glands of the antrum and proximal duodenum. G-cells have a flask-like shape and narrow neck, which opens onto the mucosal surface. Storage granules containing gastrin can be found at the base of the gland.

Gastrin is initially synthesised as preprogastrin, 101 amino acids in length (Figure 1.2). The signal peptide is enzymatically cleaved to yield progastrin, which is subjected to a sequence of proteolytic cleavages. The resulting glycine-extended

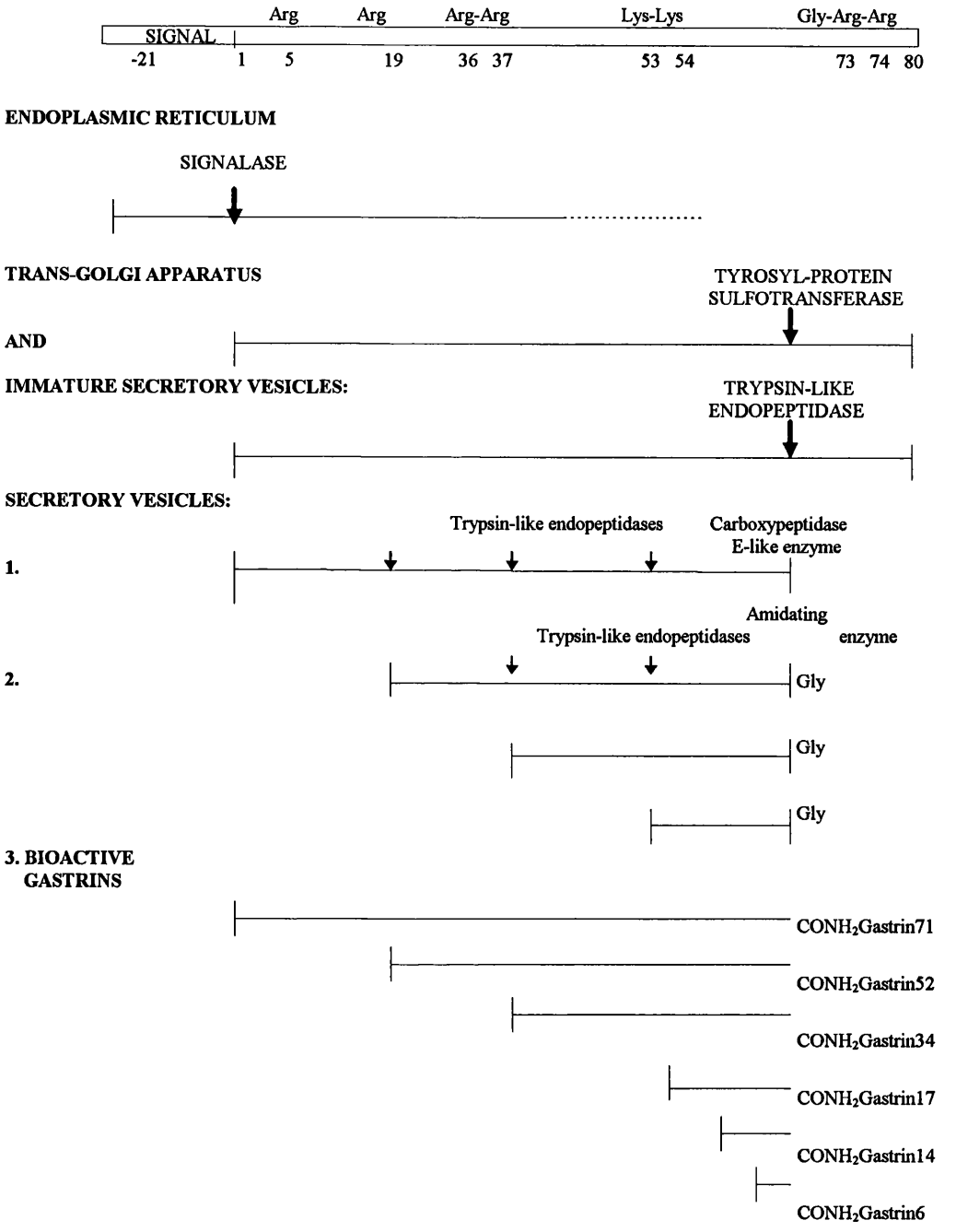

Figure 1.1 **Scheme of preprogastrin processing in antral G cells (Rehfeld *et al* 1993).**

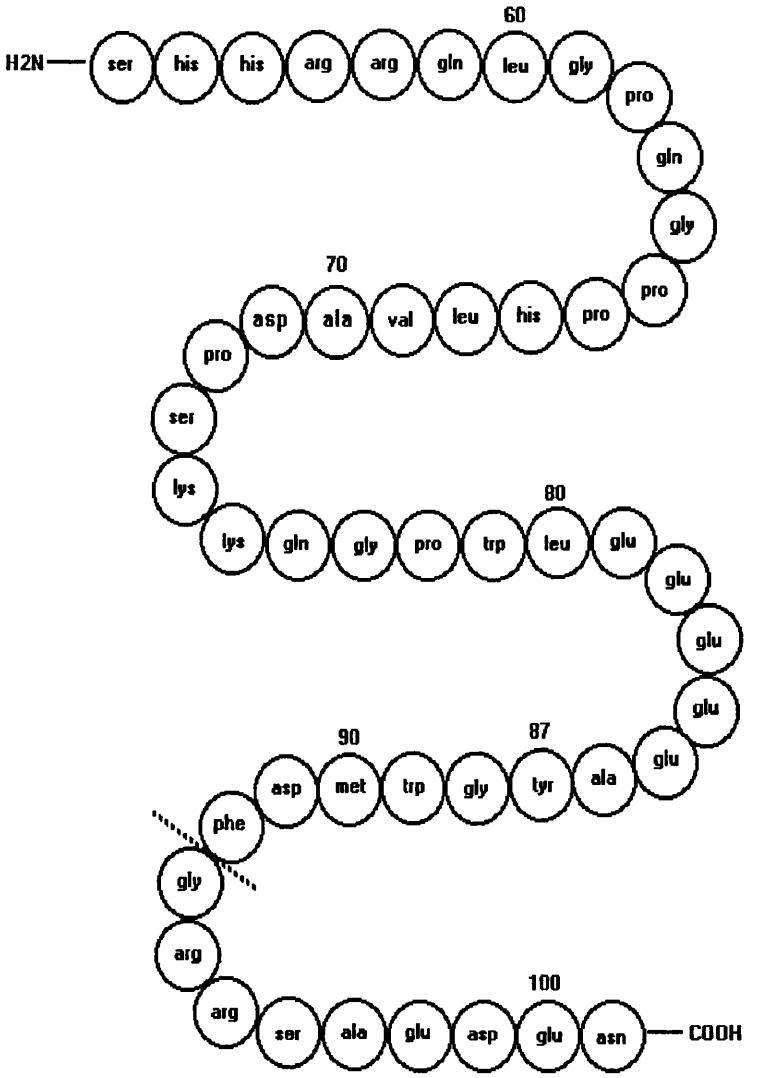

Figure 1.2 **Amino acid sequence of preprogastrin (Bardram and Rehfeld 1988)**

intermediate is carboxyamidated to produce the biologically active hormone (Rehfeld and Hilsted 1992; Walsh 1994). The tyrosyl residue in position six from the C-terminal distinguishes gastrin from the homologous peptide hormone, cholecystokinin.

1.3 Homology and molecular forms

Gastrin and cholecystokinin (CCK) belong to the same mammalian peptide family, both being isolated from the GI tract and subsequently shown to be present in the brain. Different forms of these peptides are released into the circulation by differential proteolysis. Gastrin and CCK share the same carboxyl terminal pentapeptide sequence. The carboxy-terminus tetrapeptide represents the region responsible for biological activity of both hormones whereas the rest of the two molecules only modifies their selectivity and potency for different target cells. The main difference is in the tyrosine residue which is present in gastrin six amino acids from the carboxy-terminus whereas CCK has the tyrosine as the seventh amino acid. The tyrosine residues are sulphated in various peptides of CCK but only partially sulphated in gastrin. The presence of a sulphate residue increases the affinity of gastrin for its receptor and sulphated G17 has a 19 fold higher affinity for gastrin/CCK_B receptors compared to non-sulphated G17 (Huang *et al* 1989).

Processing of progastrin in the antral G-cell results in various N-terminal and C-terminal extended gastrins, glycine extended intermediates and mature bioactive carboxyamidated gastrins. Approximately ninety percent of gastrin released is G17 and about five percent is gastrin-34 (G34). Also, unknown amounts of sulphated and non-sulphated gastrin-14, gastrin-6 and an NH_2 terminal fragment 1-13 of G17 are found

in extracts of antral tissue. The biological activity of these peptides is uncertain. The differential clearance rate of G34 is approximately six to eight times slower than G17. Therefore G34 is the predominant form of gastrin in serum at any time (Jensen *et al* 1980). Conversion of G34 to G17 occurs in the antrum and consequently G17 is the predominant form in the antral G-cells. Gastrin-34 has a half-life which is approximately five times greater than G17 but it is also about five times less potent than G17 (Walsh 1974; Debas 1974).

1.4 Trophic effects

While gastrins' importance in the control and regulation of acid secretion has been well studied, the hormones trophic effects on the gastrointestinal tract have only become appreciated in the last two decades.

Gastrin was first described as a trophic factor after reports of increased synthesis of the enzyme histidine decarboxylase in the rat stomach (Kahlson *et al* 1964, 1973). Experimental evidence was reported which demonstrated that pentagastrin induced increased protein synthesis in the rat stomach (Crean *et al* 1969). At the same time, Johnson *et al* confirmed these results when pentagastrin was exogenously administered and stimulated protein synthesis (Johnson *et al* 1969). It was subsequently discovered that this trophic effect of gastrin was independent of acid secretion (Johnson *et al* 1977).

Gastrin may control growth of gastrointestinal mucosa and physiological concentrations of gastrin have been shown to stimulate DNA synthesis particularly in the acid producing oxyntic mucosa of the stomach (Majumdar and Johnson 1982). Since the

early studies, exogenously administered pentagastrin has been demonstrated to have trophic effects on the duodenum, colon and pancreas (Peitsch *et al* 1981). In addition, physiological concentrations of gastrin have also been shown to stimulate mRNA expression in isolated rat epithelial cells (Yassin *et al* 1991).

Further evidence for the trophic role of gastrin has been revealed by inhibition of endogenous gastrin via antrectomy which leads to atrophy of the GI mucosa and pancreas. This is reversable with exogenous gastrin (Seidel *et al* 1985). Endocrine cells within the oxyntic mucosa also respond trophically to gastrin. Stimulation of these enterochromaffin-like (ECL) cells by gastrin, enhances histamine release, enlarges the cells and increases proliferation (Håkanson and Sundler 1991).

1.5 Intracellular signalling

It has been shown in several species that following gastrin binding to its receptor in gastric parietal cells, there is a rapid turnover of inositol phosphates (Chew and Brown 1986; Chiba *et al* 1989). This is linked to an accumulation of intracellular calcium and activation of protein kinase C (PKC). Protein kinase C may also activate an autoregulatory mechanism which in turn may down regulate gastrin/CCK_B receptors (Yamada *et al* 1993). Gastrin also stimulates PKC activity in isolated normal rat colonic epithelial crypt cells (Yassin *et al* 1991); effects which may be reversed by the gastrin receptor antagonist proglumide (Yassin *et al* 1993). Furthermore gastrin and CCK8 both induce a rapid turnover of inositol phosphates in isolated non-parietal cells from rabbit gastric mucosa, an effect which is mediated through the gastrin/CCK_B receptor (Roche *et al* 1991). Nanomolar concentrations of gastrin also induce transcription of a wide range

of mRNA species in these cells and significantly increases protein synthesis (Yassin *et al* 1991).

Ornithine decarboxylase (ODC) catalyses the rate limiting step in the synthesis of polyamines, has a short half-life and is also under hormonal control. In normal tissues ODC activity is relatively low unless cells are actively dividing and it has been shown that in several parts of the normal gastrointestinal tract, including the colon, exogenous gastrin stimulates ODC activity and increases mucosal polyamine content in conjunction with gastrin mediated trophic effects (Seidel *et al* 1985; Majumdar 1990).

2 GASTRIN/CCK$_B$ RECEPTORS

1.1 Introduction

Whilst a great deal is known about the effects of gastrin and its possible role in gastrointestinal proliferation, comparatively little is known about the role of gastrin/CCK_B receptors. Since the trophic effects of gastrin are known to be mediated by binding of the hormone to gastrin/CCK_B receptors, their presence is of importance in mechanisms involved in growth of the GI tract.

Gastrin binding sites were first described using radioligand binding in isolated gastric tissue with an iodinated gastrin ligand. Specific gastrin binding sites were reported in the oxyntic gland mucosa of the rat (Brown and Gallagher 1978). Further studies using tritiated gastrin revealed gastrin binding to antral smooth muscle cells, gastric mucosal plasma membranes and fundic cells in the rat (Baur and Bacon 1976; Lewin *et al* 1976, 1977; Soumarmon *et al* 1977). Early reports of gastrin/CCK_B receptor binding sites in the stomach were not fully assessed and biological activity of the ligands used was not reported. Takeuchi and colleagues were the first to demonstrate physiological and specific binding of iodinated gastrin to gastrin/CCK_B receptors in rat oxyntic gland mucosal membrane preparations which satisfied receptor binding criteria (Takeuchi *et al* 1979).

1.2 Distribution in different tissues and species

Gastrin/CCK_B receptors have been identified in a variety of different animals and a diversity of tissues throughout the gastrointestinal tract, central nervous system and brain. Specific gastrin/CCK_B receptors were first isolated on parietal cells from the canine stomach by Soll *et al* who demonstrated specific G17 binding to canine fundic

mucosal cells (Soll *et al* 1984). Gastrin/CCK_B receptors on canine parietal cells were further characterised by affinity cross-linking (Matsumoto *et al* 1987). Gastrin/CCK_B receptors have also been identified on rabbit parietal cells (Magous and Bali 1982), histamine-containing cells from the fundic mucosa (Roche *et al* 1991) and guinea-pig fundic gastric glands (Ramani and Praissman 1989; Chang and Lotti 1986). In addition, gastrin/CCK_B receptors have been shown to be present on gastrointestinal smooth muscle both in the stomach and the gallbladder of rabbits (Bitak and Makhlouf 1982; Grider and Makhlouf 1990). Distinct receptors for both gastrin and CCK have also been found on the pancreatic acini of the guinea-pig (Yu *et al* 1987,1990), the canine parietal cells (Fourmy *et al* 1987) and chief cells from the guinea-pig stomach (Cherner *et al* 1988). Finally, Singh and colleagues demonstrated specific gastrin/CCK_B receptors in the rat stomach, distal duodenum and colorectal mucosa (Singh *et al* 1985).

Kumamoto and co-workers examined specific gastrin binding to human gastric, duodenal, colonic and pancreatic tissue (Kumamoto *et al* 1989). Specific binding was shown in both fundic and antral mucosa although the latter binding was lower. Specific binding to duodenal and pancreatic tissue was reported but specific binding to colonic mucosal tissue was found to be very low. It must be noted however that specific binding in the Kumamoto study was found after analysis of tissues from only one patient.

A more detailed study on human colonic mucosa by Upp *et al* reported high affinity gastrin binding sites which were present on 28 out of 59 of the normal colon mucosa examined (Upp *et al* 1989). The gastrin/CCK_B receptor content varied between 2 and 20 fmol/mg protein with the majority of high affinity gastrin/CCK_B receptors being

present in amounts less than 10 fmol/mg protein. Details of this study are discussed more fully in Chapter 5, 'Gastrin/CCK_B receptors in normal and neoplastic human colonic tissues.'

1.3 Methods of measurement of gastrin/CCK_B receptors

Three different techniques have been used to detect or measure gastrin/CCK_B receptors : radioligand binding, immunocytochemistry and molecular characterisation. Each method has its own advantages and disadvantages in the measurement of gastrin/CCK_B receptors, and these are discussed in more detail below.

1.3.1 Radioligand Binding

(i) Radioligand and methods of radiolabeling

Since gastrin has a high affinity for its receptor, only a small amount is required to saturate the binding sites. This necessitates a radioligand of high specific activity to permit accurate measurement of the bound fraction and therefore ^{125}Iodine is the label of choice. Takeuchi and co-workers utilised iodination of synthetic gastrin-17 for use in gastrin/CCK_B receptor assays and were the first to standardise the radioligand binding assay for measurement of gastrin/CCK_B receptors (Takeuchi *et al* 1979). Various methods of iodination of gastrin-17 have been studied to determine the retention of biological activity (Singh *et al* 1985). Iodination methods were compared using synthetic 15-leu-gastrin 17 and gastrin-17 containing methionine. Iodogen and enzymobead iodinations resulted in similar biological activities for both ligands. Another method, Chloramine T was found to completely abolish biological acivity with met-gastrin-17and

reduce it with leu-gastrin-17. As chloramine T is a strong oxidising agent it may cleave gastrin-17 and oxidise the sulphydrylgroups in the methionine residue.

To prevent oxidation of the methionine residue, gastrin analogues using leucine or norleucine substitutions for 15-methionine have been synthesised. These gastrin analogues have been reported to retain full biological activity and are more resistant to oxidation during iodination (Takeuchi *et al* 1979; Soll *et al* 1984; Magous *et al* 1982). In addition, 15-norleucine is chemically closer to 15-methionine and is theoretically better as a radioligand since similar receptor affinities and numbers were reported on isolated canine parietal cells compared to radiolabeled gastrin (Seet *et al* 1987).

(ii) Receptor preparations

Gastric mucosal membrane preparations

In 1979, Takeuchi *et al* described preparation of a crude membrane fraction from the rat fundic mucosa suitable for gastrin binding (Takeuchi *et al* 1979). Briefly, rat antral mucosa was scraped and homogenised followed by differential centrifugation at 270g, 30,000g and 60,000g. Specific ^{125}I-G17 binding to membranes was greatest in the 30,000g fraction. The gastrin/CCK_B receptor was later extensively studied and biochemically characterised (Johnson *et al* 1985). This method has never been reproduced by other researchers, although several other modifications have been described and detection of gastrin/CCK_B receptors has been subsequently reported (Singh *et al* 1985; Kleveland and Waldum 1986).

However, problems may be encountered with membrane preparation since homogenisation of tissues may expose the receptors to liberated proteolytic enzymes which may affect both the receptor and radiolabeled gastrin during assay incubation. In addition, there are problems with the heterogeneity of cells in tissues to be analysed. Since cells which express gastrin/CCK_B receptors may only constitute a small proportion of total fundic mucosal tissue or indeed tumour tissues, non-specific binding may be increased and in some cases it may be too high to permit detection of specific binding. This was evident from a study by Kumamoto *et al* where specific binding was almost non detectable and non-specific binding was high in membrane preparations from human gastric fundic mucosa (Kumamoto *et al* 1989)

There has been no evidence to confirm that gastrin/CCK_B receptors are stable stored as membrane preparations. However, a study by Upp and colleagues which reported gastrin/CCK_B receptors in human tissues stored at -80°C would support the theory that gastrin/CCK_B receptors are stable after freezing of human tissues (Upp *et al* 1989). In the same study, later findings by the same group confirmed high affinity gastrin/CCK_B receptors were detected on partially purified plasma membranes prepared from colonic tumour and normal tissues (Upp *et al* 1989; Chicone *et al* 1989). However, to date these results have never been confirmed by any other laboratory.

Dispersed cells

Isolated and dispersed cells prepared by enzyme disaggregation and enriched by elutriation have to some extent overcome the disadvantage of dealing with mixtures of different cell types from tissues. In a study by Soll *et al,* canine fundic mucosal cells were

dispersed by collagenase followed by enrichment of parietal cells by elutriation (Soll *et al* 1984). Specific ^{125}I-G17 binding was reported in cell fractions containing parietal cells and binding was saturable at 37°C with steady state attained after 30mins incubation. Comparable gastrin/CCK_B receptor binding properties were reported in isolated rat fundic plasma membranes (Singh *et al* 1985) and isolated rabbit gastric fundic cells (Magous *et al* 1982).

Tumour cell lines and tissues

Both animal and human tumour cell lines have been used extensively in the study of gastrin/CCK_B receptors using radioligand binding assays. One of the main cell lines used has been the rat pancreatic cell line AR42J. Whole cell gastrin/CCK_B receptor binding has been widely studied by different groups including Scemama and co-workers (Scemama *et al* 1987). Further whole cell binding assays in gastric cancer cell lines derived from primary human tumours used ^{125}I-G17 to bind to gastrin/CCK_B receptors (Weinstock and Baldwin 1988).

However, although these preparations allow detection and characterisation of gastrin/CCK_B receptors *in vitro*, they may not reflect *in vivo* the binding of gastrin to the receptor. More useful information may be obtained from the study of spontaneous human gastrointestinal tumours. To date however there has only been one published report of specific ^{125}I-G17 radioligand binding to human cancer tissues which has only ever been reproduced by the same researchers (Upp *et al* 1989; Chicone *et al* 1989). Specific binding was reported on partially purified membrane preparations from both normal and tumour tissues.

(iii) Assay conditions

In standardisation of assay conditions in the gastrin/CCK_B receptor assay, Takeuchi *et al* reported optimal binding of gastrin to rat fundic membranes at a temperature of 30°C after incubation for thirty minutes (Takeuchi *et al* 1979). However, this incubation temperature was found to give variable and mainly non-specific binding and significant peptide degradation was demonstrated at 30°C in plasma membranes from the rat fundic mucosa (Kleveland *et al* 1985). In addition iodinated gastrin was found to be degraded during incubation with enriched parietal cell preparations (Soll *et al* 1984; Janas *et al* 1984) and different enzyme inhibitors were found to be ineffective in preventing radiolabeled gastrin degradation. Assay incubation temperatures of 30-37°C cause added problems because of increased proteolytic activity which may damage the radiolabeled gastrin or receptors themselves. Substantial proteolytic damage was demonstrated in crude membranes from fundic rat mucosa (Kleveland and Waldum 1986) and lowering of incubation temperature inhibited both tracer degradation and destruction of the binding sites. Incubations at 15°C were performed and membranes from rat oxyntic glands bound ^{125}I-G17 with a K_D of 0.8nM (Kleveland and Waldum 1986).

Takeuchi *et al* used a standard Hepes buffer with added albumin in the first standardisation of the gastrin/CCK_B receptor assay which was shown to be pH dependent displaying optimum binding at pH 7.0 (Takeuchi *et al* 1979). Since its description there have been a variety of different buffers used in gastrin/CCK_B receptor radioligand binding assays and the buffer used would seem to depend on the receptor preparation analysed. Most isolated whole cell binding assays have used the standard Tris

or Hepes buffers (Seva *et al* 1990a; Scemama *et al* 1987; Frucht *et al* 1992). However, specific ^{125}I-G17 binding to AR42J whole cells attached to cell culture plates using Minimal Eagle's media and BSA (Watson *et al* 1991) demonstrated a higher receptor affinity for the gastrin/CCK_B receptor (K_D = 0.09nM) in comparison to results on AR42J cells (K_D =1.1nM) in a similar assay using a Krebs-Hepes based buffer (Seva *et al* 1990a). The discord between different researchers with the same cell line demonstrates the requirement for a standardisation of assay conditions in radioligand binding studies.

1.3.2 Immunocytochemistry

Histological analysis of gastrin/CCK_B receptors utilises specific anti-gastrin receptor antibodies and may provide an important screening technique for gastrointestinal tumours to determine the level and significance of gastrin/CCK_B receptors in these tumours.

Staining of specific gastrin/CCK_B receptors using murine monoclonal antibodies raised against the gastrin/CCK_B receptor have been used by several groups (Mu *et al* 1987; Nicolson *et al* 1992; Watson *et al* 1994). Specifically raised to the canine parietal cells, the IgM antibody, 2Cl, was found to dose-dependently inhibit ^{125}I-G17 binding to parietal cells with an IC_{50} of 10nM. The percentage of positively stained cells with 2Cl was found to correlate with previously determined gastrin receptor status by radioligand binding (Scemama *et al* 1987). Using human fundic mucosa as a positive control the 2Cl antibody was found to stain frozen unfixed sections of human colon carcinoma and revealed heterogeneous receptor expression. The antibody was also shown in this study to inhibit growth of a gastrin-sensitive carcinoma cell line (Nicolson *et al* 1992).

Anti-gastrin receptor antibodies may provide an important screening method for histological analysis of gastrin/CCK_B receptor expression in colorectal and gastric carcinoma patients. Large scale screening of tissues would also remove the problems associated with receptor preparations in radioligand binding techniques and may provide information on whether gastrin/CCK_B receptor positive cells also contain intracellular gastrin and therefore operate in an autocrine manner.

1.3.3 Molecular characterisation

Until recently, very little about the molecular background to gastrin/CCK_B receptors was known. Recently, the gastrin/CCK_B receptor was cloned, sequenced and characterised by different groups from a range of sources including the rat brain and pancreas (Wank *et al* 1992a), the human brain and stomach (Psiegna *et al* 1992), canine parietal cells (Kopin *et al* 1992) and ECL carcinoid tumours from *Mastomys natalensis* (Nakata *et al* 1992). A high degree of nucleic acid homology was found between *Mastomys natalensis* and canine parietal cells using polymerase chain reaction (PCR) to amplify transmembrane domain sequences. In addition Chiba *et al* demonstarted specific gastrin/CCK_B receptors on membranes from gastric carcinoid tissues of *Mastomys natalensis* (Chiba *et al* 1991). But whether gastrin/CCK_B receptors are identical in different tissues within the same species remains to be seen. Recent evidence by Kopins' group would suggest that they are the same (Lee *et al* 1993) although others have suggested on the basis of the molecular structure of the human gastrin/CCK_B receptor gene that alternate splicing pathways yielding receptor variants may exist (Song *et al* 1993).

3 TROPHIC EFFECTS OF GASTRIN SUPPORTING THE PRESENCE OF GASTRIN/CCK$_B$ RECEPTORS IN GASTROINTESTINAL CANCER

1.1 Introduction

Colorectal cancer poses a major problem worldwide as it is is the second commonest cancer after lung, with an estimated 755, 000 new cases occurring each year (CRC 1995). In addition, there are 19000 deaths each year in the UK from gastric cancer (CRC 1993). Survival rates of patients with advanced colorectal and gastric carcinoma are poor, with surgery offering the only hope of cure.

There is evidence that gastrin exerts trophic effects on various human gastrointestinal tumours and this has stimulated interest in the use of hormonal therapy which has already been successful in the management of some breast cancers. The factors involved in the mechanism of carcinogenesis of gastrointestinal cancer remain elusive, therefore much still needs to be learned about the control and regulation of normal and malignant gastrointestinal tissue by the gastric antral hormone, gastrin.

1.2 Trophic effects of gastrin in tumour cells

Growth of gastrointestinal mucosa is regulated by various hormones and growth factors, but the underlying mechanism of gastrointestinal tumour growth is still not completely understood. Several studies have shown that gastrin plays an important role in the regulation of the gastrointestinal tract and possibly in the proliferation of tumour cells arising in the GI tract (Townsend *et al* 1988; Morris *et al* 1989; Watson *et al* 1988, 1989a,1989b). In addition, gastrin/CCK_B receptors have been shown to be expressed in tumours arising from the gastrointestinal tract as well as normal gastrointestinal mucosa (Upp *et al* 1989; Chicone *et al* 1989). Both human and animal carcinoma cell lines have been widely reported to express high affinity gastrin/CCK_B receptors and have been

characterised using receptor agonists and antagonists (Singh *et al* 1985; Scemama *et al* 1987; Seva *et al* 1990a; Watson *et al* 1991; Frucht *et al* 1992).

1.3 Trophic effects of progastrin in tumour cells

Following the important discovery of gastrin mRNA in extracts from colonic cancer cells, interest in the measurement of progastrin and different molecular forms of gastrin in GI tumours has increased. Kochman *et al* found increased levels of progastrin in extracts of colorectal tumours and corresponding normal mucosa using a panel of specific antibodies (Kochman *et al* 1992). While tumours contain more immature glycine extended gastrins than normal mucosa, the latter contain greater amounts of mature amidated gastrin. In comparison to gastric antrum, the amounts detected were small and the ratio of amidated to glycine extended gastrin was different, suggesting altered post translational processing of gastrin in tumours. No correlation was found between gastrin content and either tumour site or stage. At the same time, Nemeth *et al* in 1993, reported similar findings. They examined forty tumours and found all to contain progastrin with only eleven containing mature gastrin. The mature gastrin was more abundant in normal mucosa, while the tumours contained more progastrin. Although gastrin mRNA was present in large amounts in the tumours, they suggested the processing to mature gastrin was impaired. This was confirmed by Van Solinge *et al,* who detected the same patterns with the more sensitive technique of PCR (Van Solinge 1993).

It was later found that the more abundant non amidated forms of gastrin may possess trophic effects (Dia *et al* 1992). Several groups have since shown that progastrin

derived glycine extended intermediates may possess trophic properties for AR42J cells (Seva *et al* 1994; Nègre *et al* 1994; Kaise *et al* 1994).

It has also recently been shown that glycine-extended forms of gastrin may play an important role in growth, a process which may act through a receptor other than the gastrin receptor (Seva *et al* 1995; Singh *et al* 1995; Kaise *et al* 1995). Over the years there has been growing recognition of the association between gastrin and carcinomas of the stomach and colon. Therefore the presence of gastrin/CCK_B receptors and/or precursor receptors may be of clinical and therapeutic importance with respect to receptor antagonists which may be used in treatment of these carcinomas.

The exact cellular location of gastrin precursors has not yet been unequivocally shown because the previous studies depend on the homogenisation of tissues to release the peptide of interest. Gene expression in the colon was examined immunohistochemically by Finley *et al* in 1993 and normal colonic mucosa was found to contain occasional crypt cells which stained for progastrin, gastrin and chromagranin A suggesting that these cells normally express gastrin. In contrast, in twenty-two out of twenty-three colon cancers studied, 50% of the cells stained for gastrin and progastrin. The majority of these cells were not neuroendocrine as assessed by the absence of chromagranin A staining. No gastrin was found in six benign polyps suggesting that gastrin synthesis is a late event in the carcinogenic process.

Gastrin mRNA detected in tumours by PCR was identical to the published sequence of human gastrin (Finley *et al* 1993). They reported expression of gastrin

mRNA in two human colorectal cancer cell lines and found that somatostatin inhibited both gastrin mRNA expression and cell growth, the latter being prevented when gastrin was supplied along with somatostatin (Lebovitz *et al* 1993). Therefore strong evidence exists that the normal and malignant colonic epithelium may synthesise gastrin .

Gastrin has also been suggested to have an autocrine/paracrine growth factor effect outside the colon in gastric carcinoma cell lines (Van Solinge and Rehfeld 1992; Reimy-Heintz *et al* 1993), a rat pancreatic cell line (Blackmore and Hirst 1992), bronchogenic carcinomas (Rehfeld *et al* 1989), ovarian cancers (Van solinge *et al* 1993) and a variety of uncommon neural and endocrine tumours (Rehfeld and Hilsted 1992). The ability to express and synthesise gastrin appears to be relatively common in many neoplastic cells of diverse origins and may lead to the disordered growth control in these tumours.

Recent studies demonstrated gastrin gene expression in some colonic cancer cell lines (Baldwin and Zhang 1992) and progastrin derived peptides have been found in human tumours (Kochman *et al* 1992; Nemeth 1993) therefore supporting a role for gastrin as an autocrine growth factor.

1.4 Autocrine trophic effects

Considerable evidence to support the trophic effects of gastrin in proliferation of human cancer cells has already been discussed. It is also now widely recognised that many hormones can act as autocrine growth factors in human carcinomas (Cuttitta 1990). An autocrine role for gastrin was first proposed by Hoosein *et al* who reported that

polyclonal anti-gastrin antibodies inhibited growth of two human colon cancer cell lines *in vitro* (Hoosein *et al* 1989). It was also noted that pre-incubation of the antiserum with gastrin reversed the inhibitory effect. In addition, the human gastric cancer cell line HGT-1 was reported to have high affinity gastrin/CCK_B receptors but when grown in serum supplemented with gastrin, growth was not enhanced (Remy-Heintz *et al* 1993). In contrast, Guo *et al* reported that anti-gastrin antibodies had no effect on cell growth of the murine colon cancer cell line MC26 *in vitro* (Guo *et al* 1990). Gastrin-like peptides were detected in both human colon cancer cells and gastric cancer cell supernatant by RIA analysis. These gastrin/CCK antibodies inhibited cell proliferation at very high dilutions. This suggests the gastrin-like peptide may be acting as an autocrine growth factor in human GI cancer cells.

In another report by Baldwin *et al*, gastrin mRNA was detected in two human cell lines only after using the more sensitive technique of PCR. (Baldwin *et al* 1990).With the use of quantitative PCR, Baldwin *et al* were able to detect gastrin mRNA in seven colonic carcinoma cell lines (Baldwin and Zhang 1992). Others have demonstrated gastrin in samples of colonic cancers but not in normal mucosa (Monges *et al* 1993) and demonstrated the capacity for gastrin gene transcript in some colon cancer cell lines (Tillotson *et al* 1993).

A significant growth promoting effect of gastrin was recently found on both colorectal and gastric cancer cells that were either freshly disaggregated from patient tumours or were primary tumour cell lines at an early passage (Watson *et al* 1988). Using a specific anti-gastrin antibody, with immunofluorescence and flow cytometry,

Watson *et al*, found that six out of twenty-eight freshly disaggregated human colorectal tumours possessed more than 20% gastrin positive cells (Watson *et al* 1991). Corresponding tumour-free mucosa contained less than 5% of gastrin positive cells in the majoritory of cases.

1.5 Intracellular signalling

Although much is known about the post-receptor second messengers in the gastric parietal cell involved in acid secretion (Yamada *et al* 1993), this may not be applicable to GI tumour cells. Indeed, the gastrin/CCK_B receptor on gastric parietal cells may not be the same as the receptor mediating trophic responses in the colon or tumour cells. Therefore, it is important to understand the mechanisms involved in cellular signalling, for even if they are the same receptor, their regulation may be different due to binding to different G-proteins.

Physiological concentrations of gastrin stimulate growth of several human colorectal cancer cell lines with a parallel stimulation of phosphoinositol hydrolysis (PI) and intracellular calcium mobilisation (Ishizuka *et al* 1994). Effects are blocked by the selective gastrin/CCK_B receptor antagonist JMV320. Other cell lines respond to gastrin with an increase in cAMP turnover but without any change in PI or intracellular calcium. A study by Bold and colleagues showed that gastrin stimulates growth of the human colon cancer cell line LoVo, an effect irreversible by either CCK_A or gastrin/CCK_B receptor antagonists. LoVo cells were devoid of mRNA transcripts for CCK_A and CCK_B receptors as assessed by northern hybridisation, yet gastrin stimulated the production of cAMP but not phospholipase C. It was suggested that growth of these cells occurred via

a receptor other than CCK_A or CCK_B and perhaps one which has not yet been isolated (Bold *et al* 1994). Therefore gastrin signalling in colonic cancers cells appears to occur through two differing routes or mechanisms.

Gastrin also stimulates mobilisation of intracellular calcium independently of PI hydrolysis in a gastric carcinoma cell line (AGS) which possesses gastrin/CCK_B receptors and responds trophically to gastrin (Ishizuka *et al* 1992). The hormone had no effect on intracellular calcium in a subclone lacking gastrin/CCK_B receptors. Similar effects with addition of gastrin to SCLC cells causes a rapid and transient rise in intracellular calcium concentrations (Sethi *et al* 1993) and in NIH3T3 fibroblasts expressing the cloned human gastrin/CCK_B receptor (Taniguchi *et al* 1994). Gastrin may act as a direct growth factor through gastrin/CCK_B receptors on some SCLC (Herget *et al* 1992). However, CCK_A receptors are preferentially expressed in SCLC and therefore increased intracellular calcium mobilisation and growth in SCLC cells may be mediated through either CCK_A or CCK_B receptor subtypes (Sethi *et al* 1993). These effects were also observed and blocked by gastrin/CCK_B and CCK receptor antagonists in *Mastomys natalensis* ECL gastric carcinoid tumour cells (Inomoto *et al* 1992).

In contrast, proliferation of pentagastrin stimulated growth of a xenotransplantable human gastric tumour in nude mice was found to be linked to cAMP metabolism (Sumiyoshi *et al* 1984) whereas CCK8 inhibited both increases in cAMP and activation of the cAMP dependent protein kinase C (Yasui *et al* 1986).

Polyamines have been implicated in the regulation of cellular proliferation and have important roles in cell growth and differentiation that are not completely understood. An increase in activity of the enzyme ornithine decarboxylase (ODC) is indicative of cellular proliferation (Johnson *et al* 1993). High levels of polyamines and increased ODC activity occur in colorectal tumours (Kingsnorth *et al* 1984; Lamuraglia *et al* 1986). Gastrin also stimulates polyamine turnover in both colonic cancer cell lines and xenografts (Eggstein *et al* 1991; Smith *et al* 1993). Freshly resected colorectal carcinomas were found to have higher levels of polyamines in gastrin receptor positive cancers than compared with gastrin/CCK_B receptor negative tumours (Upp *et al* 1988). Levels of polyamines were higher in normal colon mucosa from patients with cancer than from those without cancer (Upp *et al* 1987). The presence of polyamines in colon cancers with gastrin receptors provides evidence that gastrin may play a trophic role in human colon cancers and it was thought that some tumours may have been treated with polyamine biosynthesis inhibitors. Polyamine levels were also found to be elevated in oestrogen receptor positive gastric tumours (Linsalata *et al* 1994).

Several studies have found that DFMO (α-difluoromethylornithine) an irreversible inhibitor of ODC inhibits proliferative effects of gastrin on the colon *in vivo* and *in vitro* (Seidel *et al* 1985; Majumdar *et al* 1990; Eggstein *et al* 1991; Smith *et al* 1993). Cell proliferation in primary colonic tumours was substantially reduced by DFMO (Tutton and Barkla 1986). AR42J cells differ from normal acinar cells since they proliferate rapidly and express gastrin/CCK_B receptors (Christophe 1994). DFMO was found to inhibit AR42J cell growth since growth is dependent on adequate intracellular polyamine concentrations (Scemama *et al* 1987; Logsdon *et al* 1992). Results obtained

with gastrin/CCK_B receptor antagonists demonstrate that ODC stimulation in AR42J cells is mediated via the gastrin/CCK_B receptor (Scemama *et al* 1989). Coupling of gastrin/CCK_B receptors to PKC activation also occurs in AR42J cells (Seva *et al* 1990b). Polyamines are therefore likely to be involved in the trophic response to gastrin in carcinoma cells.

4 GASTRIN/CCK$_B$ RECEPTORS IN GASTROINTESTINAL CANCER

1.1 Introduction

Gastrin exerts its biological actions by interacting with specific cell surface receptors. If gastrin is of relevance to human gastrointestinal cancer, then detection and characterisation of gastrin/CCK_B receptors on tumour cells is of importance before gastrin/CCK_B receptor antagonists can be considered in the therapy of gastrointestinal cancer.

1.2 Animal tumour cells

One of the most widely studied animal tumour cell lines has been the rat pancreatic cell line AR42J. Receptor affinities for gastrin were found to differ from those found on normal rat pancreatic acinar cells (Blackmore and Hirst 1992) and have been characterised with gastrin receptor antagonists (Scemama *et al* 1987; Seva *et al* 1990a; Watson *et al* 1991). The presence of the gastrin/CCK_B receptor on these cells is discussed in more detail in Chapter 3, 'Gastrin/CCK_B receptors in AR42J cells.'

High affinity gastrin/CCK_B receptors were detected on the murine colon cancer cell line, MC26 (Singh *et al* 1985) and subsequent studies confirmed the presence of gastrin/CCK_B receptors demonstrating a requirement for gastrin to maintain both receptor affinity and number as the tumours increased in size (Singh *et al* 1986, 1987, 1993; Chicone *et al* 1989; Guo *et al* 1990).

Tumours in rats which were not treated with pentagastrin showed a significant reduction in gastrin/CCK_B receptor affinity indicating that the concentration of endogenous gastrin was too low to maintain receptor affinity (Singh *et al* 1987). It was

suggested that *in vivo*, tumour cells may de-differentiate resulting in possible conformational changes in expression of the gastrin/CCK_B receptor in the absence of high levels of circulating gastrin (Singh *et al* 1986). This study also supports previous work which suggested that gastrin up regulates its own receptor (Takeuchi *et al* 1980).

1.3 Human tumour cells

1.3.1 Gastric

Many groups have studied human gastrointestinal tumour cell lines which respond trophically to gastrin (Ishizuka *et al* 1992; Watson *et al* 1989a, 1989b; Singh *et al* 1985; Ochiai *et al* 1985). Gastrin/CCK_B receptors have also been reported on cancer cell lines from a variety of species including several human gastric cancer cell lines. The human gastric cell line TMK-1, has been reported to respond trophically to gastrin, an effect which appears to be mediated by a high affinity gastrin/CCK_B receptor (Ochiai *et al* 1985).

Human gastric cancer cell lines have been screened using a single saturating dose of ^{125}I-G17. The gastric cell line AGS was found to be strongly positive for specific gastrin binding sites, with an affinity (K_D < 1nM) similar to the normal rat fundus (Singh *et al* 1985). Seven AGS clones were established and four were positive for gastrin binding sites (>12 fmols/mg protein). Of the others, one was found to be negative and two exhibited gastrin binding sites of less than 3.3 fmols/mg protein. Although details of how the AGS cells were grown was not given in the report, it is known from other studies that gastrin-responsiveness may be lost in established cell lines after repeated subculture (Watson *et al* 1988). Loss of responsiveness to gastrin has been shown in

established cell lines on repeated subculture *in vitro* (Watson *et al* 1988). Gastrin responsiveness could be retained by transplanting cells into nude mice and growing *in vivo* before re-establishment *in vitro*. The same group also reported that several human gastric cell lines lacked any mitogenic response to gastrin at passage >250 *in vitro* when compared to freshly derived primary gastric tumours, where ~50% were gastrin responsive (Watson *et al* 1989b). This variation in ability of gastrin to induce mitogenic effects may be due to up and down regulation of receptors. This must be taken into account when examining gastrin receptor status of gastrointestinal tumour cells and caution in interpretation of negative gastrin/CCK_B receptor cell lines is required.

Weinstock and Baldwin examined five human gastric cancer cell lines using whole cells in radioligand binding studies (Weinstock and Baldwin 1988). Isolated canine parietal cells were used as positive controls with K_D's of 1.7 and 0.2nM for ^{125}I-met G17 and ^{125}I-leu G17 respectively. However, it was noted that affinity constants for the human gastric tumour cell lines varied between 0.2 and 1.3uM exhibiting receptor affinities around 1000 times less than found in parietal cells. This variation in affinities for the gastrin/CCK_B receptor did not affect displacements with unlabeled gastrin-17 and CCK8 which were shown to have comparable affinities for the receptor. The authors postulated that the gastrin/CCK_B receptor can exist in different conformational states thereby altering receptor affinity and go on to describe experiments with isolated canine parietal cells where membranes are extracted using detergents resulting in a low affinity gastrin receptor. In addition, there was no increase in *in vitro* cell proliferation of the gastric cell lines in the presence of gastrin-17, indicating that the low affinity gastrin/CCK_B receptor is not involved in any trophic response. The relevance of these

low affinity gastrin/CCK_B receptors in a possible autocrine role for gastrin has been discussed elsewhere.

Radioligand binding studies were performed on human scirrhous gastric carcinomas by Kumamoto *et al* (1988). Using membrane preparations and ^{125}I-G17, four out of five carcinomas showed a high degree of specific binding. Although no affinities were reported, specific binding ranged from 1.1-18.2fmols/mg protein. The presence of gastrin/CCK_B receptors was more frequent in the poorly differentiated scirrhous carcinomas (Borrman type IV) than in other gastric adenocarcinomas examined (Borrman type II or III). A study by the same group in 1989 demonstrated specific ^{125}I-G17 binding to human gastric fundic mucosa with K_D of 1.6nM and receptor capacity of 15fmol/mg protein. Antral mucosa was shown to exhibit little specific binding.

1.3.2 Colorectal

In a report by Singh and colleagues, high affinity gastrin/CCK_B receptors (K_D of 0.25-0.6nM) were found to be present on membranes from a human colonic cancer cell line (LoVo). Another human colonic cell line, HT29 showed little specific binding (Singh *et al* 1985) in this study but was later reported by another group to exhibit specific gastrin binding when cells were grown in serum free medium (McRae *et al* 1986). Pentagastrin was also shown to stimulate growth of the HT29 cells.

A recent study by Frucht *et al* examined functional receptors for a wide range of GI hormones on human colon cancer cell lines (Frucht *et al* 1992). Only one out of the

ten cell lines examined expressed gastrin/CCK_B receptors. This low gastrin/CCK_B receptor expression in human tumour cell lines may be due to methodological problems, in particular the length of time in culture as has been suggested by Watson *et al* as human colorectal cell lines lost their gastrin responsiveness when passaged more than 250 times (Watson *et al* 1989b).

Eggstein and co-workers examined the mechanisms involved in the mitogenic action of gastrin mediated by gastrin/CCK_B receptors on human colonic carcinoma cells (Eggstein *et al* 1991). Growth of the colonic cell line SW403 was increased by gastrin *in vitro* and this was shown to be specifically mediated by gastrin receptors since the gastrin receptor antagonist benzotript reversed this stimulation.

Several reports have demonstrated the *in vitro* gastrin responsiveness of fresh human tumour tissue (Watson *et al* 1989b) and used this as a marker of gastrin/CCK_B receptor status, but few have directly shown the presence of specific high affinity gastrin/CCK_B receptors on human tumour tissues. The first study to demonstrate specific gastrin/CCK_B receptors on primary colon cancer tissue was by Rae-Venter *et al* in 1980. Seven out of eight (87.5%) colon tumours expressed gastrin/CCK_B receptors with a K_D of 0.4-0.6nM and receptor density of 0.5-1.3fmols/mg protein. Further publications by the same group examined gastrin/CCK_B receptor status in freshly resected colorectal tumours and healthy normal mucosa (Upp *et al* 1989). Thirty-eight of the sixty-seven (56.7%) cancers had high affinity receptors (K_D < 1nM) and seven had low affinity receptors (K_D >1nM). Twenty of the thirty-eight people with gastrin/CCK_B receptor positive tumours had receptor densities above 10fmol/mg of protein but no correlation

between gastrin/CCK_B receptor content and patient age, sex, serum CEA concentration or degree of differentiation was found. The mean receptor density of Dukes' A or B tumours was twice that of Dukes' stage C or D lesions. Twenty-two out of fifty-nine (37%) of samples had no detectable gastrin/CCK_B receptors. There was a highly significant correlation between the presence of gastrin/CCK_B receptors on normal mucosa and corresponding tumours.

5 GASTRIN/CCK$_B$ RECEPTOR ANTAGONISTS

1.1 Introduction

In recent years, there has been increasing interest in the ability to inhibit gastrin/CCK_B receptor mediated growth in tumour cells. This is due to the accumulating evidence of the presence of gastrin/CCK_B receptors in tumour cell lines and tissues. Subsequently, over the past decade there has been a vast production of receptor antagonists for gastrin/CCK_B peptides. In addition, human cancer cell lines and tissues have been found to possess gastrin/CCK_B receptors leading to therapeutic implications for gastrin/CCK_B receptor antagonist therapy in some gastrointestinal tumours.

Competitive receptor antagonists should be effective at physiological concentrations and therefore require to be selective and of high affinity. They are established as the main tool for study and characterisation of receptors and have been used extensively in the classification of receptor subtypes and second messenger systems within the gastrointestinal tract.

Since the gastrin/CCK_B receptor is known to have equal affinities for gastrin and cholecystokinin (Jensen *et al* 1989; Roques *et al* 1989; Freidinger *et al* 1989) it follows that gastrin/CCK_B receptor antagonists also antagonise the effects of CCK. Consequently the development of gastrin/CCK_B receptor antagonists was simultaneous with the development of CCK receptor antagonists.

At least eight classes of gastrin/CCK_B receptor antagonists have been presented in the literature (Jensen *et al* 1990; Presti and Gardner 1993) which are highly selective with the capacity to distinguish between CCK_A and gastrin/CCK_B receptors. The

majority of studies have concentrated on the inhibitory effects of antagonists on growth of tumour cell lines and tumour cells and not on normal epithelial cells (Watson *et al* 1991, 1992a, 1992b).

1.2 Gastrin/CCK_B and CCK_A receptor antagonists

1.2.1 Glutaramic acid derivatives

During the 1960s, Rovati developed the gastrin receptor antagonist proglumide ((+/-)-4-(benzolyamino)-5-(dipropylamino)-5-oxopentanoic acid), a glutaramic acid derivative (Rovati 1968). Proglumide was used for several years in the treatment of peptic ulcer disease because of its ability to competitively inhibit gastrin-stimulated gastric acid secretion (Rovati 1979). The compound was later found to competitively inhibit pentagastrin-stimulated increases in DNA, RNA and protein content in the rat oxyntic mucosa as well as the rate of DNA synthesis (Johnson and Guthrie 1984).

Following an earlier study which found that pentagastrin enhanced growth of MC26 tumours and reduced survival in mice (Winsett *et al* 1985), the effects of proglumide were found to reduce growth, DNA and RNA content in both tumours and

normal colonic mucosa (Beauchamp *et al* 1985). Others have also found that proglumide inhibits gastrin or pentagastrin-stimulated growth in colon cancer cells *in vivo* (Singh *et al* 1986, 1987) but not basal growth of tumour cells. However, not all reports have been able to show an inhibitory effect of proglumide on colorectal cancer cells (Romani *et al* 1994) and inhibitory effects that have been found were not in the physiological range. In addition, proglumide has only been used in one small study of patients with advanced colorectal cancer, in which no effect on survival or tumour growth was demonstrated (Morris *et al* 1990).

Subsequent developments produced an amino acid tryptophan derivative, benzotript (Rovati 1976). However both proglumide and benzotript were shown to competitively antagonise effects of cholecystokinin on the CCK_A receptor (Hahne *et al* 1981). But proglumide and benzotript are relatively weak antagonists of the gastrin/CCK_B receptor, with relatively weak inhibitory effects in both human and animal colorectal cell lines within the millimolar range (Hoosein *et al* 1989; Guo *et al* 1990; Eggstein *et al* 1991).

A requirement for new gastrin/CCK_B receptor antagonists with greater potency in the physiological range led to the development of non-peptide derivatives of proglumide, lorglumide (Makovec *et al* 1985) and loxiglumide (Setnikar *et al* 1987) and recently the (R)-4-benzodiamdo-5-oxopentanoic acid non-peptidic derivatives CR1795, CR2093 and CR2194, which discriminate between different CCK receptor subtypes (Makovec *et al* 1992) but are still weak CCK receptor antagonists. CR2093 inhibited *in vitro* growth of AR42J cells and the human gastric cell line MKN45 (Watson *et al*

1992b). However when human colonic cancer cells C523, which have been reported to have high affinity gastrin/CCK_B receptors (K_D = 0.22nM), were grown as xenografts in nude mice, neither basal nor gastrin stimulated growth was inhibited by continuous intravenous infusion of CR2093 (Watson *et al* 1992b).

1.2.2 Benzodiazepine derivatives

The benzodiazepine derivative gastrin/CCK_B and CCK_A receptor antagonists originated from asperlicin (Chang *et al* 1985). Asperlicin had little potency, but the 1,4-benzodiazepine ring system led to derivatives with increased potency.

Devazepide (MK-329 or L364718), a selective and competitive receptor antagonist of CCK at CCK_A receptors with an affinity comparable to that of CCK8 (IC_{50} = 0.08nM) has greater than 1000 fold selectivity over the gastrin/CCK_B receptor (Evans *et al* 1986). This was confirmed by Chang and Lotti by *in vitro* studies using CCK induced contractions of the guinea-pig ileum and colon where L364718 acted as a CCK antagonist without any agonist action (Chang and Lotti 1986).

Several structural modifications of devazapide led to the discovery of the gastrin/CCK_B receptor antagonist L365260 which exhibited a 100 fold greater affinity for gastrin/CCK_B receptors (IC_{50} = 1nM) than CCK_A receptors (Bock *et al* 1989; Lotti and Chang 1989). Following growth of AR42J cells as xenograft tumours in nude mice, the gastrin/CCK_B receptor antagonist L365260 was administered via an osmotic mini pump to mice that had previously received either PBS control or G17. Gastrin increased growth of AR42J xenografts and L365260 was found to suppress only gastrin stimulated growth (Watson *et al* 1991). This effect was also found in MC26 xenografts with

proglumide (Singh *et al* 1987). The gastrin/CCK_B receptor antagonist, L365260 was one of the most potent (Presti and Gardner 1993; Hughes *et al* 1993) which also inhibited basal growth rates of human colon cancer cell lines *in vitro* (Watson *et al* 1991). Further studies confirmed L365260 as a selective gastrin/CCK_B receptor antagonist (Roche *et al* 1991; Durieux *et al* 1991) although a further report did not show any effect on growth of two human colorectal cancer cell lines *in vitro* (Thumwood *et al* 1991).

Attempts to produce receptor antagonists with increased affinity for the gastrin/CCK_B receptor resulted in a range of acidic derivatives of L365260. Biological activity and CNS penetration were examined and increased in a number of tetrazole derivatives. The compound L368730, the tetrazole analogue of L365260 showed an eight fold increase in affinity for the gastrin/CCK_B receptor which increased CCK_B/CCK_A selectivity by more than seven percent. The isobutyl derivative L368935, (K_D = 0.1nM) is more potent than other recently described gastrin/CCK_B receptor antagonists C1988 (Hughes *et al* 1990) and LY262691 (Howbert *et al* 1992). The latter has weak affinity for gastrin/CCK_B receptors whereas C1988 has limitations because of poor oral bioavailability and CNS penetration (Hinton *et al* 1991). The CCK_A and gastrin/CCK_B receptor antagonist CAM1481, reduced growth of LoVo cells grown as xenografts by 53% after oral administration in nude mice (Romani *et al* 1994).

The most potent gastrin/CCK_B receptor antagonists have originated from benzodiazepine derivatives and one in particular with high affinity (IC_{50} = 0.04nM) for the gastrin/CCK_B receptor, L740093 [N-[(3R)-5-(3-azabicyclo[3.2.2]nonan-3-yl)-2,3-dihydro-1-methyl-2-oxo-1H-1,4-benzodiazepin-3yl]-N'(3-methylphenyl)urea] is the most

potent and selective gastrin/CCK_B receptor yet known (Patel *et al* 1994). This receptor antagonist was found to have 210 fold higher affinity than L365260 for gastrin/CCK_B receptors and 3 fold higher for CCK_A receptor sites. The antagonist properties of L740093 were also confirmed in *in vivo* binding studies. In addition, L740093 demonstrated increased solubility and CNS penetration therefore offering an attractive profile to characterise the role of gastrin/CCK_B receptors in physiology and disease.

1.2.3 Other gastrin/CCK_B receptor antagonists

Analogues and fragments of CCK8 have been evaluated for their potential use as gastrin/CCK_B receptor antagonists. The CCK_A receptor binds CCK8 with high affinity but CCK4 only displays low affinity for the receptor subtype. In contrast, the gastrin/CCK_B receptor binds both CCK8 and CCK4 with high affinity and exploitation of this feature was deemed likely to produce a highly selective gastrin/CCK_B receptor antagonist (Lin *et al* 1989). This led to the development of derivatives of the c-terminal tetrapeptide of CCK8. Derivatives incorporating either BOC or succinyl groups at the N-terminal end were reported to exhibit high affinity for guinea-pig cortical gastrin/CCK_B receptors (Harbammer *et al* 1991).

Other potent and selective gastrin/CCK_B receptor antagonists have been produced and a series of phenethyl ester derivative analogues of the C-terminal tetrapeptide of gastrin have been described (Martinez *et al* 1986). Modification of amino acid side chains by replacing the phenylalanyl residue with a phenethyl group to produce these derivatives was found to affect receptor affinity (Harbammer *et al* 1991).

Alternatively, cyclization of the N-terminal portion of CCK8 was also found to produce gastrin/CCK_B receptor antagonists with high potency (Charpentier *et al* 1988).

Novel gastrin/CCK_B receptor antagonists have been isolated by screening of the antibiotic virginiamycin M_1 from guinea-pig mucosal glands. Three analogues, L-156,586, L-156,587 and L-156,588 were shown to selectively bind to gastrin/CCK_B receptors in the nanomolar range (Lam *et al* 1991).

1.3 Anti-gastrin antibodies

Alternatives to gastrin/CCK_B receptor antagonists in the form of antibodies directed against gastrin may have the potential to neutralise gastrin in serum without interaction at the receptor level and may also neutralise autocrine growth mediated via gastrin.

Recently specific neutralising anti-G17 antibodies were raised using a novel immunogen, Gastrimmune, in which the N-terminal of human G17 was conjugated to diptheria-toxoid (Makishima *et al* 1994). The resulting antiserum did not cross react with either G34 or CCK (Watson *et al* 1993, 1994) and prevented ^{125}I-G17 binding to gastrin receptors on AR42J cells (Watson *et al* 1995a).

The *in vitro* growth of the human gastric cell line MGLVA1 was significantly reduced in the presence of antiserum and mice which were injected with the cells and treated with the antiserum also showed a significant increase in survival rates (Watson *et al* 1995b). In experimental animal models, Gastrimmune treatment significantly inhibited basal and gastrin-stimulated growth of a human primary colorectal tumour AP5. When

AP5 cells were grown as xenografts in nude mice, the trophic effects of gastrin were maintained and both basal and gastrin-stimulated growth was significantly inhibited by *i.v.* infusion of the antiserum when compared to controls treated with anti-DT antisera alone (Watson *et al* 1995a). Results from clinical trials in patients with colorectal and gastric cancer would provide valuable information about the possible therapeutic role of anti-gastrin antibodies.

1.4 Therapeutic applications

Although there is a growing array of gastrin/CCK_B receptor antagonists, their potential clinical application in the possible treatment of gastrointestinal malignancy remains speculative. Much work has been done in the area of inhibition of basal and gastrin stimulated growth. In cultured cells derived from human colonic, gastric and pancreactic cancers shown to possess gastrin/CCK_B receptors this provides evidence of a possible therapeutic application based on gastrin/CCK_B receptor antagonist inhibition. In particular, new benzodiazepine derivatives such as L740093 which has a higher affinity for the gastrin/CCK_B receptor than the natural agonist G17, may have potential therapeutic value.

Many researchers have demonstrated *in vitro* and *in vivo* inhibitory effects of specific gastrin/CCK_B receptor antagonists on human colonic and gastric cell lines. But to date only one substantial report has demonstrated gastrin/CCK_B receptors directly on human colonic carcinomas (Upp *et al* 1989). This paper has been widely quoted yet there has been no subsequent report from any other group despite numerous reports of gastrin/CCK_B receptors on colonic carcinoma cell lines. The therapeutic value of the

growing number of gastrin/CCK_B receptor antagonists must follow the unequivocal demonstration and examination of the level and distribution of gastrin/CCK_B receptors in human gastrointestinal tumours.

6 RADIOLIGAND BINDING METHODOLOGY

1.1 Introduction

Radioligand binding assays are a relatively simple but extremely powerful tool for studying receptors and as a result, radioligand binding is important in many biological sciences.

The principle of radioligand binding is incubation of a biologically active radioligand with a receptor preparation until steady state has been reached. Bound radiolabel is then separated from free, either by centrifugation or filtration, and the bound fraction counted. Reliable measurement of any receptor requires fully optimised radioligand binding assay methodology for the receptor of interest. This chapter will discuss the main factors involved in the optimisation of radioligand binding methodology. The mathematical basis of radioligand binding theory is presented in Chapter 1, section 7.

1.2 Choice of radioligand and method of radiolabeling

When studying hormone receptors, the radioligand of choice would be the endogenous peptide expected to bind to the receptor of interest. It is important that the radioligand should retain its biological activity yet still be indistinguishable from the unlabeled peptide after labeling. While tritiated labels (^{3}[H]) have the advantage of leaving the structure of the peptide intact, the labels prepared are of inherently low specific acivity and therefore are of limited use in the detection of high affinity binding sites (Bylund and Yamamura 1990). Use of iodinated radioligands permits the preparation of high specific activity labels, necessary for detection of high affinity binding sites. But incorporation of an iodine atom and/or the iodination procedure itself may diminish the biological activity

of a peptide. Therefore the choice of iodination protocol is important and will be individually applicable to each peptide/receptor system.

1.3 Receptor preparations

Measurement of receptor expression on both normal and tumour tissues is most widely studied in crude membrane preparations of either cultured cells or tissues of interest although whole cells and isolated cells from tissues also provide valuable information about binding kinetics.

1.3.1 Cell lines and crude membrane preparation

Cell lines have been used extensively as a source of receptors for radioligand binding assays as they provide the advantage of a plentiful supply of homogenous receptor preparation for extended periods of time. Isolated cells from tissues may also be prepared by enzyme disaggregation and enriched by elutriation. To some extent, this has overcome the disadvantage of dealing with a mixture of different cell types from a single tissue. However, there still remains variablity between individual animals of the same species.

Membranes from cultured cells or tissues are easy to prepare and can be stored and used for screening of receptors from most cell types. Crude membrane preparation involves homogenisation of the tissue or cells in a hypotonic buffer using a mechanical homogeniser such as a polytron. Large debris can be removed by filtration through a nylon mesh (~50um) and/or slow speed centrifugation.A crude membrane fraction is sedimented after centrifugation at 30000g.

1.4 Assay conditions

The theoretical model for saturation requires the reaction to be at equilibrium (or at least steady state). The time for a reaction to reach steady state is dependent on both the radioligand concentration and temperature used. While it is convenient to perform experiments at room temperature, the use of 37°C may be more physiologically relevant. However, problems can occur with radiolabel degradation at 37°C and also reaction kinetics may be so fast that precision is difficult to achieve. For these reasons a temperature of 4°C may be advantagous.

Generally, the pH should be in the physiological range between pH7 and 8 (Bylund and Yamamura 1990). The type of buffer may depend on the receptor preparation and although Tris(hydroxymethyl) aminomethane (Tris) buffer is often used, it is not necessarily the best. It is therefore important to try other buffers in order to obtain optimum binding. Buffers are often supplemented with ions such as Mg^{2+} which may enhance binding of the radioligand. Addition of monovalent or divalent cations may either increase or decrease the affinity of competing ligands for the receptor site. For example addition of Mg^{2+} ions at concentrations of 0.1-10mM promote agonist binding to many G-protein-coupled receptors in membranes by favouring the formation of the high affinity agonist-receptor-G-protein complex (Hulme and Birdsall 1992). Other additions often included in radioligand binding buffers are protease inhibitors but this depends mainly on the receptor preparation being analysed.

Finally the choice of separation method for bound and free radioligand is important in preventing significant dissociation of the receptor radioligand complex since

this is the measured parameter. Membrane assays are generally separated using filtration methods where membrane fragments that contain the radioligand receptor complex are retained on the filter and the free radioligand passes though it. However there are drawbacks since non-specific binding to the filter may be high. This problem may be overcome by pre-soaking filters with 1% (v/v) aqueous polyethylenimine in order to reduce non-specific binding to the filter. Alternative methods involve centrifugation of assay reactants forming a pellet containing the radioligand bound to the membrane fragments. Centrifugation is most often used in assays where the affinity of the radioligand is in the 10nM to 1uM range or non-specific binding to the filter is prohibitively high. An additional advantage of the centrifugation technique is that the extent of dissociation of the radioligand-receptor complex is minimal.

7 RECEPTOR CHARACTERISATION

1.1 History

The origins of receptor theory came from Langley in 1878, who studied the effects of pilocarpine and atropine on salivary action. From these studies he declared that "there is some 'receptor' substance with which both atropine and pilocarpine are capable of forming complexes according to some law in which their relative mass and chemical affinity for the substance are factors" (Langley 1878). Later he noticed that nicotine and curare gave an effect and blockage respectively on small areas of muscle surface. He concluded in 1905 that "there is a chemical combination between the drug and a constituent of the cell - the receptive substance" (Langley 1905).

In 1885, Ehrlich independently postulated that specific activities of cellular protoplasm could be moderated by side chains or receptors. His work on the binding of drugs to these receptors is summed up by saying "substances that bind have an effect". Ehrlich also suggested from Langley's work that drug action on receptors is loose, reversible and involves weak chemical bonds (Ehrlich 1900), which led to the speculation of cell surface recognition sites.

Further work by Ehrlich and Hill provided evidence for the chemical nature of the receptor-ligand reaction (Hill 1909), but it was not until twenty-four years later that Clark provided the evidence for receptor occupancy (Clark 1933). These studies led Clark to assume that the biological or functional response to receptor activation was directly proportional to the number of receptors occupied by the ligand at equilibrium.

Gaddum developed the extension of receptor theory with competitive antagonism, where an antagonist interacts reversibly with receptors to form a complex but does not elicit a response. He proposed that an antagonist might bind to the same receptor as the agonist and compete for its occupancy (Gaddum 1936). Gaddum observed that the agonist dose response curves were rightwardly shifted in parallel in the presence of an antagonist. Consequently higher agonist concentrations than previous were required to displace the antagonist and adhere agonist occupation of all receptors.

1.2 Mathematical basis of radioligand binding

Familiarity with the theoretical background to receptor characterisation is necessary for correct interpretation of the results of binding studies. What follows is a brief summary of the binding kinetics, parameters and factors which may affect binding. Only the simple binding reactions are described due to the complex mathematical nature of multiple binding sites.

1.2.1 Kinetic analysis

(i) Association

In the simplest case, the binding of a labelled ligand [L]* to a receptor [R] is a simple bimolecular association reaction. Association of a radioligand is a second order process where the rate of formation of radioligand complex is dependent on both ligand [L]* and binding site [R]. The amounts of receptor and radioligand are constant and the concentration of radioligand bound to the receptor is determined as a function of time. The rate of association or rate of formation of $[RL]^* = K_{+1}.[L]^*.[R]$ where K_{+1} is the

forward rate constant or association rate, [L]* is the free radioligand concentration and [R] is the free receptor concentration.

$$[R] + [L]^* \xrightarrow{K_{+1}} [RL]^* \qquad (1)$$

Association experiments determine when steady-state is reached. This information is critical as inhibition experiments are usually performed at steady-state. The time to reach equilibrium is dependent not only on the rate constant but also the radioligand and receptor concentrations. Technically it is difficult to calculate K_{+1} as it is not possible to prevent concurrent dissociation of the complex. However by holding the ligand concentration [L]* constant in an experiment i.e. less than 10% bound at equilibrium, the above equation is reduced to a pseudo first order process. By doing this, the equation for association of a radioligand to a binding site describes an increasing curve as a function of time.

$$[Rbt] = \sum_{i=1}^{i=n} [Rei].\,(1-e^{-Kobs.t}) \qquad (2)$$

Where [Rbt] is the amount of radioligand bound at time t, [Rei] is the amount of radioligand bound at equilibrium to site i out of a possible n sites and K_{obsi} is the

observed association rate constant. The data is fitted again by non-linear regression or the natural logarithm of the ratio of the amount of [R] bound at steady-state (Be), which is divided by the difference between the [R] bound at steady state and the amount bound (B) at time t is plotted against time.

Pseudo first order association plot:

$$\text{Ln (Be/Be-B)} = (K_{+1} \cdot F + K_{-1})\, t + K_{obs} \cdot t \qquad (3)$$

K_{obs} is the slope of the plotted line and so if K_{-1} (dissociation constant) is known from separate experiments, then K_{+1} can be calculated:

$$K_{+1} = (K_{obs} - K_{-1}) \,/\, \text{Free ligand } (\eta M) \qquad (4)$$

$$K_D = K_{-1} / K_{+1} \qquad (5)$$

If accurate the K_D from association should be in agreement with the dissociation constant from saturation studies and this is a good check on the internal consistency of the binding data at steady-state.

(ii) Dissociation

The [RL]* complex is the initial radioligand concentration bound to the receptor, [R] is the free receptor and [L]* is the free ligand. The rate of breakdown of [RL]* to [R] and [L]* can be expressed as the dissociation (K_{-1}) rate constant.

$$[RL]^* \xrightarrow{K_{-1}} [R] + [L]^* \qquad (6)$$

Dissociation is a first order reaction since it depends only on the initial concentration of the complex. It can be described with the first order rate equation :

$$[Rbt] = \sum_{i=1}^{i=n} [Roi] \cdot e^{-K_{-1i}.t} \qquad (7)$$

Where [Rbt] is the amount of radioligand bound at time t, [Ro] is the amount bound at time t=0 and K_{-1} is the dissociation rate constant for each site i out of a possible n sites.

The radioligand [L]* is incubated with receptor [R] until steady-state is achieved. Further binding of [L]* to [R] is prevented by either a 50 fold or greater dilution of the incubation mixture or addition of excess unlabeled drug in order to occupy all the free receptors. An excess of unlabelled drug is defined as 100 times the IC_{50} value. In either case association is effectively prevented so only the dissociation reaction is measured.

The natural logarithm (Ln) of radioligand bound divided by bound at time zero i.e. at steady-state, can be plotted as a function of time. The semilogarithmic plot of dissociation data produces a straight line, the slope of which is $-K_{-1}$. Therefore the dissociation rate constant, K_D which is equal to K_{-1}/K_{+1} can be calculated from a combination of association and dissociation reactions.

1.2.2 Saturation analysis

As has been previously described, when a receptor [R] is incubated with a radioligand [L]* for a period of time t , [RL]* will form according to the simple biomolecular reaction :

$$[L]^* + [R] \rightleftharpoons [RL]^* \qquad (8)$$

In a saturation experiment, the amount of [RL]* complex formed is measured as a function of the free radioligand concentration. At equilibrium (steady state) where the forward and reverse reactions are progressing at the same rate, the law of mass action states that:

$$K_D = \frac{[L]^* \cdot [R]}{[RL]^*} \qquad (9)$$

where K_D (the dissociation constant) = K_{-1}/K_{+1}. If the total receptor concentration is equal to Bmax then the mathematical equation that relates the concentration of [RL]* (also termed B for Bound) and free ligand [L]* (termed F for free) is as follows:

$$B = \frac{Bmax \,.\, F}{(K_D + F)} \qquad (10)$$

This equation describes a rectangular hyperbola but due to the non-linearity of radioreceptor saturation curves, both the apparent Bmax and K_D values can only be approximated from the plot. Therefore transformation of the data into a linear form as with a scatchard plot or Rosenthal plot is generally required (Scatchard 1949; Rosenthal 1967).

Expanding and re-arranging:

$$\frac{B}{F} = \frac{-1}{K_D} \,.\, B + \frac{Bmax}{K_D} \qquad (11)$$

Although the terminology of a Rosenthal plot is more accurate since it belies a derivation of the scatchard, the name scatchard is more commonly recognised to mean the same as the latter. The scatchard plot involves bound/free ligand plotted as function of the bound. Equation eleven is in the form $y = mx + C$ and so it follows that y is equal to B/F, m is equal to $-1/K_D$, x is equal to B and C is equal to $Bmax/K_D$. The receptor density or Bmax as it is commonly known is the value obtained from the intercept on the abscissa and the K_D is the negative reciprocal of the slope of the line (Figure 1.3). To represent receptor binding, the calculated K_D should be similar to the concentration of the unlabeled hormone displacing 50% of the labeled hormone (IC_{50}). Additionally the K_D and IC_{50} should be compatible with the physiological plasma concentration for the

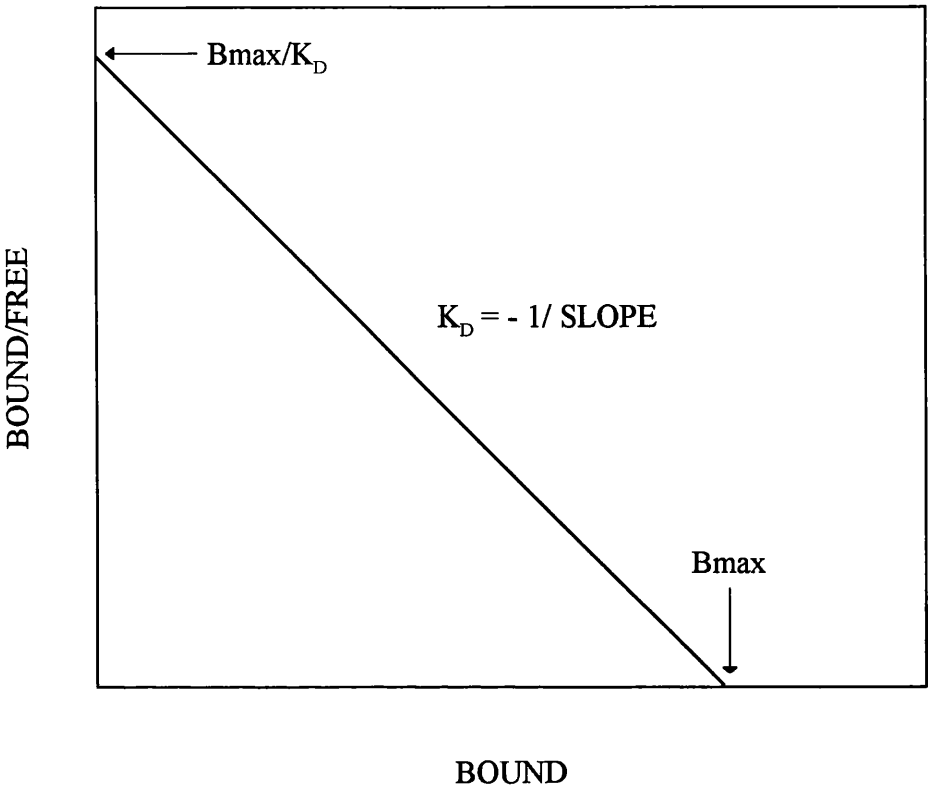

Figure 1.3 **Example of a scatchard plot**

hormone of interest. The binding should be saturable, thereby indicating a finite number of receptors. Specificity for the hormone and binding affinities of analogues should reflect their biological activity and should be linked to a biological response.

Where the radioligand binds to only one site, a linear scatchard (Figure 1.3) is observed and reasonable estimates of Bmax and the K_D can be generated. If a non-linear scatchard is observed this could mean that the ligand is binding to more than one site or receptor state and therefore computer generation of these values is compulsory. Interpretation of these plots is essential since linear scatchards can also be obtained when a non-selective radioligand binds to more than one site with the same affinity.

A further derivation of the scatchard plot is the Eadie-Hofstee plot (Hoftsee 1959). This is identical in form to the scatchard plot previously described (Molinoff *et al* 1981). Linear regression may be performed with the Hoftsee plot to obtain initial estimates of K_D and Bmax. One problem with this plot is that it is subject to influence by points close to either 0 or 100% specifically bound. To avoid this the Hoftsee plot only contains data which are in the region 5% to 95% of specific binding and therefore it is possible to loose some data.

1.2.3 Competition analysis

For a competition experiment, the receptor concentration, the radioligand concentration and the time are all constant with the variable being the concentration of unlabeled competing drug. When the drug concentration is zero only a small fraction of the receptors are bound as radioligand-receptor complex but as the concentration of drug

increases, it competes with the radioligand for the receptor binding site. This decreases the concentration of free receptors and therefore the concentration of radioligand-receptor complex is also reduced. The equation relating the concentration of bound radioligand to the drug concentration is :

$$B = \frac{Bmax.\ F}{F + K_D\ (1 + 1/Ki)} \qquad (12)$$

One useful method of linearizing inhibition data is to use a logit-log plot. Data are calculated in terms of percent bound (P) where 100% is the the amount specifically bound in the absence of inhibitor i.e Bo. The percent specifically bound at each concentration of inhibitor is calculated and the data is manipulated by logit transformation :

$$\text{logit} = \text{Ln}\ (P/(100-P)) \qquad (13)$$

The IC_{50} is 50% of specific binding, and the logit of 50% [Ln(1)] is 0, therefore the IC_{50} value can be obtained either by linear correlation or by plotting the logit data against log concentration of inhibitor and graphically determining the IC_{50}.

The Cheng-Prusoff relationship makes the assumption that the receptor concentration is much less than the ligand concentration and is only valid when [R] < 0.1 [F] (Cheng and Prusoff 1973). So by re-arranging equation 11 :

$$Ki = \frac{IC_{50}}{1 + [F]/K_D} \qquad (14)$$

Where Ki is the inhibition constant and is the affinity of the inhibitor for the receptor. Inhibition data are visualised by plotting the amount of bound radioligand on the ordinate against the log concentration of inhibitor on the abscissa. The concentration of competing drug required to inhibit 50% of the specific binding, IC_{50} is always greater than the Ki value.

1.3 Computation

Studies of radioligand binding to receptors have increased in importance for quantification and characterisation of a wide variety of receptors in different scientific fields. Before the advent of personal computers, most binding studies were analysed by simple manual graphical and very subjective methods which were often based on approximations. Today however, computers provide a more exact analytical tool which removes the tedious manipulation of data into a meaningful form and also provides an exact mathematical model with weighted least squares curve fitting.

Most, if not all, of the current computerised packages available for analysis of binding data are based on the method of Feldman (1980). Using non-linear regression analysis, the best fit of the mathematical model is determined through successive iterations. Initially the errors between the theoretical data points of the selected model and the actual data points are squared and summed, resulting in the sum of the squares of the residuals.

The LIGAND program is a versatile computerised approach for characterisation of ligand binding systems and can be used in the analysis of binding data from kinetic, saturation and displacement studies. Various versions of the program LIGAND exist (McPherson 1985; Munson and Rodbard 1980) and all will provide optimal initial and final estimates of binding parameters for ligands interacting with receptors.

8 OBJECTIVES

Since Takeuchi and co-workers first demonstrated the specific and physiologically relevant binding of gastrin to gastrin/CCK_B receptors in rat oxyntic gland mucosal membranes (Takeuchi *et al* 1979), many groups have shown binding of gastrin to a variety of tissues from different species (Magous and Bali 1982; Roche *et al* 1991; Chang *et al* 1986; Soll *et al* 1984; Ramani and Praismann 1989; Bitak and Makhlouf 1982; Grider and Makhlouf 1990; Kuamamoto *et al* 1989; Upp *et al* 1989; Singh *et al* 1985). The initial aim of this thesis was to optimise and standardise a gastrin/CCK_B receptor binding assay using the rat pancreatic acinar cell line AR42J, followed by characterisation of the gastrin/CCK_B receptor with established and novel receptor agonists and antagonists.

Following optimisation of the assay and characterisation of the gastrin/CCK_B receptor in whole cells, the next objective was to ensure that preparation of crude membranes from the cells did not alter the previously determined receptor properties. In addition, the crude membranes were to be used in stability studies to determine the most suitable and appropriate conditions for maintenance of gastrin/CCK_B receptor activity. This was considered important as it was hoped that human tissues could be collected and stored prior to assay.

To date the only study that has directly demonstrated specific gastrin/CCK_B receptors on human colonic normal and tumour tissue is by Upp *et al* in 1989. Their report provides little information about the kinetics and standardisation of the gastrin/CCK_B receptor assay used. No other group has published reports confirming or disproving these results. Therefore the aim of the first clinical study was to use the

optimised radioligand binding assay to determine if gastrin/CCK_B receptors were detectable on human colorectal tumour and normal tissues.

Finally, gastrin/CCK_B receptors may be present on parietal cells and possibly ECL cells located in the gastric body of humans (Praismann and Brand 1991). Although high affinity gastrin/CCK_B receptors have been demonstrated using radioligand binding (Kuamamoto *et al* 1989), they have not been characterised using gastrin/CCK_B receptor agonists and antagonists. Therefore characterisation of gastrin/CCK_B receptors in the human stomach would provide useful information especially in comparison to data gathered using AR42J cells and membranes.

CHAPTER 2 MATERIALS AND GENERAL METHODS

1 MATERIALS

1.1 Cell culture

DMEM amd RPMI 1640 media, glutamine and sodium bicarbonate were all obtained from Life Technologies Ltd, Paisley, Scotland. F10 HAMS medium and trypan blue were from ICN Biomedicals Inc, Thame, Oxfordshire, UK. Foetal calf serum (FCS) was obtained from Globe Pharmaceuticals Ltd, Surrey, UK. Dimethylsulphoxide (DMSO) was from Fisons Scientific Equipment, Loughborough, UK. Sodium hydroxide, Ethylenediaminetetra-acetic acid (EDTA) and sodium chloride were all purchased from BDH Chemicals Ltd, Poole, Dorset, UK. Tissue culture flasks and cryovials were from Nunclon, U.K. Disposable cell scrapers were purchased from Costar, UK. Centrifuge tubes and 19G microlance needles were from Becton Dickinson Labware, UK. All other plastic consumables were from Bibby Sterilin Ltd, Staffs, UK.

1.2 Cell lines

The rat pancreatic cell line AR42J was kindly donated by Dr.S.A. Watson, CRC Nottingham as were the human colonic cell lines LoVo and HT29. The human colonic cell line DLD1 was a gift from Dr. P.Scott, CRC Beatson, Glasgow.

1.3 Radioligand binding

Aprotinin, bacitracin, bestatin, bovine serum albumin (BSA), calcium chloride ($CaCl_2$), cholecystokinin-8 (CCK8), cholecystokinin-8-sulphated (CCK8S), dimethylsulphoxide (DMSO), DL-dithiothreitol (DTT), human gastrin-17-I (G17), human gastrin-34 (G34), N-[2-Hydroxyethyl]-piperazine-N'-[2-ethanesulphonic acid] (HEPES), magnesium

chloride ($MgCl_2.6H_2O$), sodium hydroxide, soya bean trypsin inhibitor type 1-S (SBTI), glycerol, pepstatin A, phenylmethylsulphonyl fluoride (PMSF) and phosphoramidon were all purchased from Sigma Chemical Co, Poole, Dorset, UK. Ringer lactate solution was from Baxter Healthcare Ltd, Norfolk, UK. BCA protein reagent assay kit was purchased from Pierce Chemical Co, UK. ^{125}I-tyr-human gastrin 17 (^{125}I-G17) was purchased from NEN Dupont, Stevenage,UK.

1.4 Gastrin/CCK_B and CCK_A receptor antagonists

L364718 (Devazepide) and L365260, highly selective and potent non-peptide antagonists capable of distinguishing CCK_A and gastrin/CCK_B receptors respectively, were kindly provided by Dr. B. Evans of Merck Sharp and Dohme, West Point, Pennsylvania, USA. L740093, a highly selective gastrin/CCK_B receptor antagonist, was donated by Dr. S. Patel of Merck Sharp and Dohme, Neuroscience Research Centre, Harlow, UK. CAM1028 (Meglumide), a gastrin/CCK_B antagonist, was a gift from Professor J. Hughes, Parke Davis, Neuroscience Research Centre, Addenbrookes Hospital Site, Cambridge. Loxiglumide-Na (CR1409) and Lorglumide-Na (CR1505) were gifted by Professor L. Rovati, Rotta Laboratories, Milan, Italy. All antagonists were dissolved in assay buffer 1 (section 2.3), except for L364718, L365260 and L740093 which were dissolved in DMSO and stored at a concentration of 1mM at -20°C.

2 BUFFERS

2.1 Cell culture media 1 : RPMI 1640

400mls distilled H_2O

45mls RPMI 1640 (10X concentrated)

13mls sodium bicarbonate

50mls FCS

5mls 200mM glutamine

pH to 7.4 with 2-2.5mls 0.1M sodium hydroxide

2.2 Cell culture media 2 : F10 HAMS/DMEM

400mls distilled H_2O

22.5mls F10 HAMS (10X concentrated)

22.5mls DMEM (10X concentrated)

13mls sodium bicarbonate

50mls FCS

5mls 200mM glutamine

pH to 7.4 with 2-2.5mls 0.1M sodium hydroxide

2.3 Assay buffer 1 : whole cell assay

50mM HEPES

10mM magnesium chloride

0.1% BSA (Fraction V, protease free)

pH 7.0

2.4 Assay buffer 2 : cell membrane assay

50mM HEPES

10mM magnesium chloride

1uM soya bean trypsin inhibitor

0.1% BSA

pH7.0

2.5 Assay buffer 3 : human tissue membrane assay

50mM HEPES

10mM magnesium chloride

1uM soya bean trypsin inhibitor

1uM bestatin

1uM bacitracin

1mM aprotinin

1.5mM DL-dithiothreitol

1uM PMSF

10%glycerol

0.1% BSA

pH 7.0

Filter buffer through a 0.2um sterile acrodisc filter prior to use.

3 GENERAL METHODS

3.1 Cell culture

All cell culture was carried out under sterile conditions in a laminar flow hood. AR42J cells and DLD1 cells were grown in RPMI 1640 medium supplemented with 10% v/v FCS and gassed with 5% CO_2. The cells were passaged twice weekly as described below. The human cell lines LoVo and HT29 were grown in F10 HAMS/DMEM medium supplemented with 10% v/v FCS and gassed with 5% CO_2. The cells were passaged at least three times weekly as described below.

Cells were grown consecutively in $25cm^2$, $75cm^2$ and $150cm^2$ tissue flasks and incubated after gassing with 5% CO_2 at 37°C. Once cells were confluent, the culture medium was removed and 1mM EDTA was added to the flask. The flask was incubated for five minutes at room temperature. The cells were detached from the flask surface by gentle pipetting. The suspension was removed into plastic universals and centrifuged at 1200g for 5mins. The supernatant was poured off and the cell pellet resuspended in 10mls of RPMI medium and 10% FCS. A single cell suspension was prepared by gentle resuspension approximately five times through a 19G needle.

All cell lines were counted in an electronic coulter counter using the same settings. The coulter counter was preset with an amplitude of 8, aperture current 1/2, lower and upper thresholds at forty and affinity respectively. The mean of three separate readings was taken. Cell viability was assessed by phase contrast microscopy using trypan blue exclusion.

Cell lines were frozen at a concentration of 3 x 10^6 cells per ml in 10% v/v DMSO in RPMI. Cells were initially frozen at -70°C overnight at a rate of 1°C per minute and transferred to liquid nitrogen for long term storage.

Frozen cells were thawed quickly at 37°C. 10mls of RPMI was added slowly to 3 x 10^6 cells per ml and the cells were centrifuged immediately at 1200g for 5mins to remove the DMSO. The cell pellet was resuspended in a further 10mls of RPMI by gentle agitation with a pipette. The cells were seeded at approximately 3 x 10^5 cells per ml into 25cm^2 flasks and incubated in a humidified atmosphere at 37°C with 5% CO_2.

3.3 Collection and storage of human tissue

Samples of normal and tumour tissue were obtained at surgical resection. The specimen was cut to expose the tumour and washed with ice cold ringer lactate solution, paying particular attention to the tumour surface. Macroscopically normal tissue was treated in the same way. The tissue was immersed in ice cold assay buffer 3 (section 2.5) without BSA and stored in ice for transport to pathology. Paired samples of human colorectal or gastric tumours and macroscopically normal mucosa were obtained and washed free of any endogenous blood. Membranes were either prepared fresh or tissues immersed in assay buffer 3 and snap frozen in liquid nitrogen and stored in a -70°C freezer until required.

3.4 Preparation of cell plasma membranes

AR42J cells were harvested with 1mM EDTA and a single cell suspension was prepared by dispersal through a 19G needle. Cells were diluted in RPMI containing 10% FCS and

counted using a coulter counter. Following centrifugation (1000g, 10mins, 4°C) the supernatant (fraction I) was retained on ice. The pellet was resuspended in ice cold assay buffer 2 (without BSA) and sonicated on ice in three 15 second bursts using an ultrasonic probe. Following centrifugation (1000g, 10mins, 4°C) the supernatant (fraction II) was retained on ice while the pellet was resuspended, resonicated and recentrifuged. This supernatant was pooled with fraction II above. Fractions I and II were centrifuged at 30,000 g, 4°C for thirty minutes. The resulting membrane pellets were resuspended in ice cold assay buffer 2 without BSA to give membranes I and II respectively. These were then processed immediately for protein estimation using a Pierce protein assay kit. Aliquots which were not used immediately for ligand binding were stored frozen at -70°C in assay buffer 2 containing 15% glycerol.

3.5 Processing of frozen human tissue membranes

To assess whether the method of tissue preparation affected gastrin binding, frozen tissues were initially processed in one of two ways :

3.5.1 Cryostat method

Paired tumour and normal tissues were collected without immersion in assay buffer 3 and mounted on ice in a cryostat (-30°C) from which multiple sections were cut and placed in tubes precooled in dry ice. All tissue was stored at -70°C until membrane preparation (section 3.6).

3.5.2 Pulverisation method

Paired tumour and normal tissues were collected and stored with and without immersion in assay buffer 3. Tissues were pulverised under liquid nitrogen either in a mechanical

dismembranator or by hand using a stainless steel percusson mortar followed by a pestle and mortar. Tissues were weighed before and after pulverisation and stored at -70°C in 0.2g aliquots until membrane preparation (section 3.6).

3.6 Preparation of human tissue membranes

Paired patient tumour and normal tissues were weighed. Fresh tissue was cut into small sections. Both fresh tissue and powdered frozen tissue (0.2g) were homogenised in a precooled polytron in 10mls of ice cold assay buffer 3 on ice for three 15 second bursts at setting two. The homogenate was filtered through a guaze mesh (100um) and centrifuged at 400g, 4°C for 5mins. The supernatant was centrifuged at 30,000g, 4°C for 60mins and the resulting pellet resuspended in 2mls of ice cold assay buffer 3 without BSA. The suspension was homogenised by hand in a precooled 5ml teflon-in-glass homgeniser on ice. The protein content was estimated using the Pierce BCA protein kit and membranes adjusted to 2mg/ml prior to radioligand binding.

3.7 Radioligand Binding

3.7.1 Cells and cell membranes

Reagents for the whole cell and membrane assays were prepared in assay buffers 1 and 2 respectively. AR42J cells (1 x 10^6 cells/tube, 200ul) and membranes (100ug/tube, 200ul) were incubated in duplicate with 0.114nM ^{125}I-G17 with a final concentration in the tube of 0.029nM (NEN Dupont, Stevenage, UK, 2200Ci/mmol). The reaction mixture was incubated in a final volume of 400ul for 180mins at 22°C for the measurement of total binding. Non-specific binding was determined in the presence of 0.119uM unlabeled G17 (0.029uM final concentration). The reaction was terminated by the addition of 0.8mls of

ice cold assay buffer. Whole cell and membrane bound ^{125}I-G17 was separated immediately by centrifugation (13,000g, 3min, RT) followed by aspiration of the supernatant. The pellet was washed twice with 0.8mls of ice cold assay buffer and the resultant pellet counted in a gamma counter for 1min. Specific binding was calculated by subtracting non-specific from total binding and expressed as G17 bound /10^6 cells for whole cell assays or fmol G17 bound /mg protein for membrane assays.

3.7.2 Human tissue membranes

Human tissue membranes (100ug/tube, 50ul) were incubated in duplicate with 0.25nM ^{125}I-G17 (final concentration of 0.125nM) in assay buffer 3 containing 0.1% BSA. The reaction mixture was incubated in a final volume of 200ul for 15mins at 22°C for the measurement of total binding. Non-specific binding was determined in the presence of 0.25uM (final concentration of 0.125uM) unlabeled G17. The reaction was terminated by the addition of 0.8mls of ice cold assay buffer 3. Membrane bound ^{125}I-G17 was separated immediately by centrifugation (13,000g, 3min, RT) followed by aspiration of the supernatant. The pellet was washed twice with 0.8mls of ice cold assay buffer and the resultant pellet counted in a gamma counter for 1min. Specific binding was calculated by subtracting non-specific from total binding and expressed as fmol G17 bound /mg protein.

4 DATA ANALYSIS

4.1 Kinetic analysis

The program KINETIC was used to analyse binding data from both association and dissociation experiments (McPherson 1985). Analysis involved using a weighted non

linear curve fitting technique (Rodbard 1984) coupled with statistical testing to determine the most appropriate model (section 4.4.1). Data was entered manually followed by initial estimates of the amount of radioligand specifically bound at time zero and dissociation rate constant (K_D calculated from cold saturation experiments). KINETIC produced a plot of bound versus either time of association or time of dissociation and calculated the observed association (K_{obs}) or dissociation rate constants respectively. The kinetic dissociation constant (K_D) was calculated manually using equations (4) and (5) in Chapter 1, section 7.

4.2 Equilibrium binding data

Binding data was processed by the equilibrium binding data analysis (EBDA) program to provide initial parameter estimates and produce raw data in a form for use by LIGAND. Data was entered manually and processed by EBDA from the following types of equilibrium binding experiments:

4.2.1 Saturation with homologous unlabeled ligand

A homologous displacement study and saturation binding study are similar and in both, increasing concentrations of an unlabeled ligand are incubated with the receptor. The essential difference is the estimate of the proportion of ligand bound to the receptor. Data was entered manually and transformed by EBDA. Initial estimates obtained from EBDA were entered into the LIGAND program. The number of binding sites was selected and the LIGAND automatically determined the final parameters of dissociation constant (K_D) and receptor capacity (Bmax) after fitting was completed and statistically acceptable.

4.2.2 Competition with unlabeled drug

A sigmoidal curve fitting program was used by EBDA to fit displacement data in competition experiments. Log-logit transformation of the data was performed to obtain initial estimates of IC_{50} values (Chapter 1, section 7 equation (13)). Calculation of inhibition affinity constant (Ki) used the Cheng-Prusoff correction method (Cheng and Prusoff 1973). This required input of the dissociation constant (K_D) which was previously determined from unlabeled G17 saturation experiments. LIGAND was not used to calculate final estimates.

4.3 Statistical analysis

4.3.1 Binding data

Does the data provide evidence of a second class of receptors or does a single class explain the data sufficiently well, given the experimental noise? The binding data was fitted to the simplest one site model with consequent fitting to a two site model and assessed using an F-test criterion on the residual variances. For the second more complex model to provide a significantly better fit to data, the F-test statistical criterion was set with degrees of freedom at $p = 0.05$.

The binding data was also tested for 'goodness of fit' by the RUNS test which assumed that the order of the signs of the residuals (difference between the data and the fitted curve) was random. Non random residuals were a sign of poorly fit data to model and the level of significance was computed by LIGAND.

4.3.2 Optimisation data

Optimisation data in Chapters three and four were analysed using one way analysis of variance (ANOVA) using GraphPAD InStat. A Bonferroni t-test was used to determine which groups differed and statistical significance was set at $p<0.01$.

CHAPTER 3 GASTRIN/CCK$_B$ RECEPTORS IN AR42J WHOLE CELLS

1 INTRODUCTION

The rat pancreatic cell line AR42J (Jessop *et al* 1980) has been widely studied and characterised because of its expression of abundant high affinity gastrin/CCK_B receptors (Scemama *et al* 1987; Seva *et al* 1990a; Watson *et al* 1991). It therefore provides a suitable model with which to develop and optimise a gastrin/CCK_B receptor assay.

The presence of gastrin/CCK_B receptors on AR42J whole cells was first described using ^{125}I-G17 as the radioligand (Scemama *et al* 1987). The receptors found on these cells resembled those found on canine and guinea-pig pancreatic acini (Fourmy *et al* 1987; Yu *et al* 1990) as G17 and CCK8S competed with equal affinity thus suggesting the presence of gastrin/CCK_B rather than CCK_A receptor sites. Gastrin/CCK_B receptors found on AR42J cells mediated trophic effects (Logdson1986; Logdson *et al* 1992) and the co-existence of CCK_A and gastrin/CCK_B receptor subtypes on AR42J cells was subsequently shown by radioligand binding (Scemama *et al* 1987).

Both gastrin and CCK-induced stimulation of AR42J cell growth involves the ornithine decarboxylase (ODC) pathway, an early event associated with cell proliferation (DeVries *et al* 1987a; Scemama *et al* 1989). Affinities of gastrin and CCK peptides for the gastrin/CCK_B receptor and their order of potency in stimulation of ODC activity are similar (De Vries *et al* 1987b; Seva *et al* 1990b). Although both CCK and gastrin stimulate ODC activity, only gastrin and pentagastrin are efficient at stimulating ^{3}H-thymidine uptake, indicating that it is only the gastrin/CCK_B receptor that is involved in AR42J cell proliferation (Seva *et al* 1990b). Additionally, specific gastrin/CCK_B

receptor antagonists inhibited gastrin stimulated thymidine uptake whereas CCK_A receptor antagonists had no effect (Seva *et al* 1990b).

1.1 Objectives

The aim of this study was to develop a reliable and sensitive assay capable of detecting and characterising gastrin/CCK_B receptors on AR42J cells. This would provide control data to permit development of an assay for use with cell membrane preparations

2 METHODS

Materials and general methods can be found in Chapter 2

2.1 Kinetic assays

Radioligand binding was performed as described in Chapter 2, section 3.7.1. Association kinetics were examined at increasing time points until a point after steady state had been reached. Dissociation kinetics were studied by incubating cells with radioligand until equilibrium was reached, prior to a saturable concentration of unlabeled G17 being added and binding again measured at increasing time points. Specific binding was defined as total binding minus non-specific binding with excess unlabeled G17.

2.2 Optimisation assays

The gastrin receptor assay was optimised with respect to cell number, ^{125}I-G17 concentration, incubation buffer, pH, temperature and method of separation. The basic protocol was as described in Chapter 2, section 3.7.1, with each of the conditions under

study being varied in separate experiments. Non-specific binding was defined as binding in the presence of excess unlabeled G17.

2.3 Competition assays

Gastrin receptors on AR42J cells were characterised using the agonists G17, G34, CCK8 and CCK8S; the gastrin/CCK_B receptor antagonists L365260, L740093, and CAM1028; and the CCK_A receptor antagonists L364718, CR1505 and CR1409. Cells were incubated for 180mins at 22°C in assay buffer. All competition assays were performed with 0.029nM ^{125}I-G17 (final concentration) and increasing concentrations (10^{-16}-10^{-5}M) of the previously stated agonists and antagonists.

3 RESULTS

3.1 Assay Optimisation

3.1.1 Effect of cell number on binding of ^{125}I-G17 to AR42J cells

Increasing numbers of AR42J cells (0.2-2.4 x 10^6 cells/tube), were incubated with 0.029nM ^{125}I-G17 for 180mins at 22°C (Figure 3.1). Specific binding was found to be linear within the range 0.2-1 x 10^6 cells/tube and almost reached receptor saturation at 2.4 x 10^6cells/tube. A concentration of 1 x 10^6 cells/tube gave specific binding of less than 17% of total added and the highest ratio of specific to non specific binding. This was also the minimum concentration required to provide a reasonable size pellet, thus permitting acceptable precision in the assay.

3.1.2 Effect of incubation time on binding of ^{125}I-G17 to AR42J cells

AR42J cells (1 x 10^6cells/tube) were incubated with 0.029nM ^{125}I-G17 for increasing time intervals at 22°C. Binding reached a plateau after 180mins, confirming that the reaction had reached steady state (Figure 3.2). The *observed* association rate (K_{obs}) was calculated using a non linear regression program (LIGAND) and is represented graphically by Figure 3.2 inset, where K_{obs} is the slope of the line. Non-specific binding changed little throughout the duration of the assay and was less than 13% of total binding at 270 minutes of incubation. Addition of 10uM unlabeled G17 into the incubation medium at 180 minutes (i.e. when the reaction was at steady state) caused dissociation of bound radioactivity. The dissociation rate (K_{-1}) was calculated using LIGAND and the half-life for dissociation of G17 from the gastrin/CCK_B receptor was estimated to be 55mins. From equation 4 (Chapter 1, section 7), the *actual* association rate (K_{+1}) was also calculated. The dissociation rate constant (K_D) for AR42J cells was calculated from equation 5 (Chapter 1, section 7) as 0.02nM.

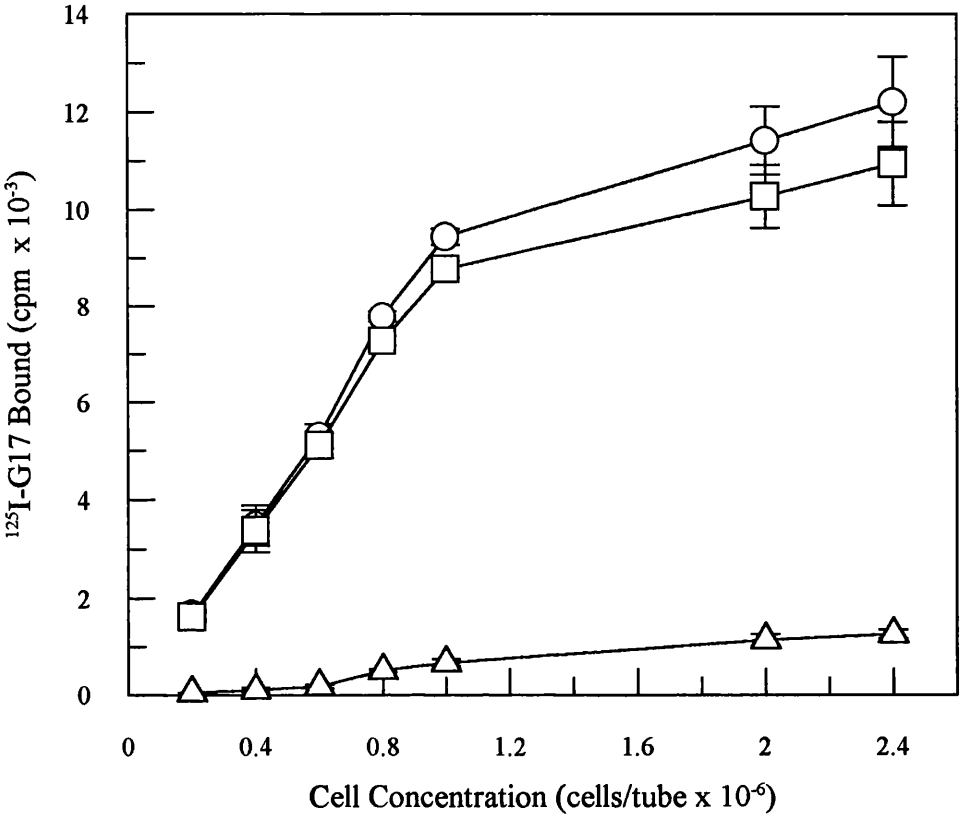

Figure 3.1 **Effect of increasing cell concentrations on ^{125}I-G17 binding to AR42J cells**

Binding of ^{125}I-G17 to increasing numbers of AR42J cells at 22°C for 180mins. Total binding (-○-); Non-specific binding (-△-) in the presence of 1000 fold excess unlabeled G17; Specifc binding (-□-). Each point is the mean (+/-SD) of six experiments performed in duplicate.

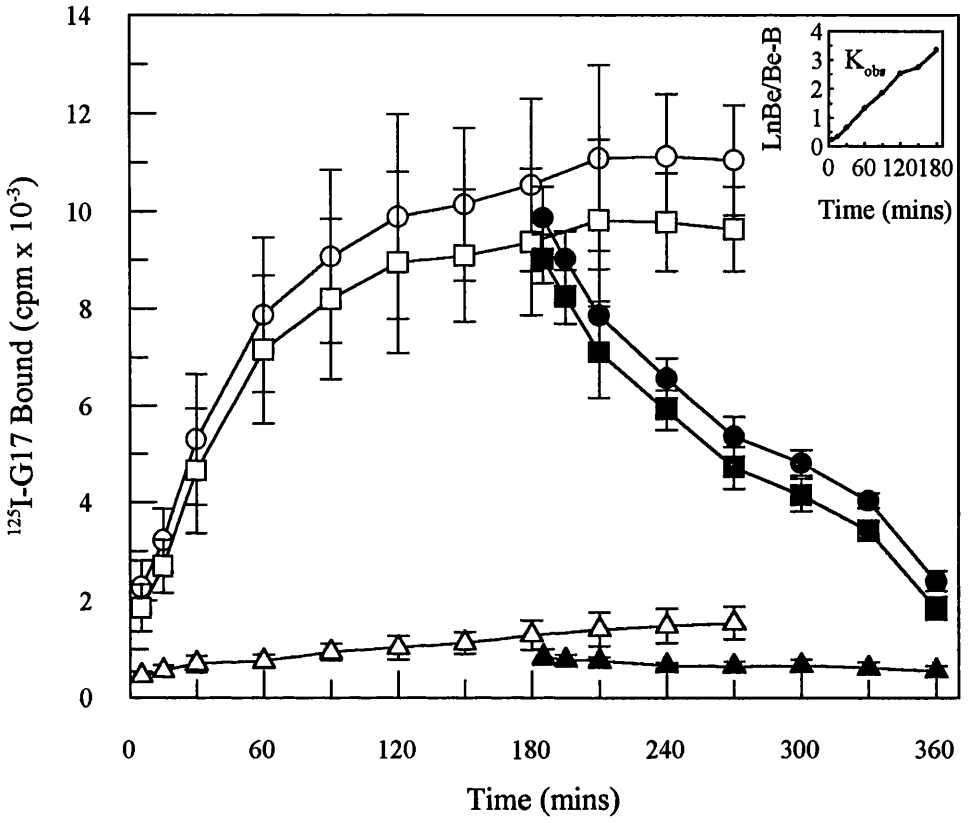

Figure 3.2 **AR42J whole cell association and dissociation time courses with ^{125}I-G17**

Cells were incubated either with 0.029nM ^{125}I-G17 alone (-○-) or in the presence of 0.029uM G17 (-△-) at 22°C. Specific binding (-□-) is total binding (-○-) minus non-specific binding (-△-). At 180 mins, 10uM G17 was added to the incubation medium to dissociate ^{125}I-G17 from binding sites. Dissociation results are denoted by total(-●-), specific (-■-) and non-specific binding (-▲-) with each point the mean (+/-SD) of four experiments performed in duplicate. Inset is a graphical representation of the association rate constant where the slope of the line is the observed association rate constant (K_{obs}).

3.1.3 Effect of increasing ^{125}I-G17 concentrations

Increasing concentrations of ^{125}I-G17 were added to 1 x 10^6 AR42J cells and incubated for 180mins at 22°C in assay buffer. Specific binding increased linearly with increasing ^{125}I-G17 concentration, but did not reach saturation because of the large quantity of radiolabel which would have been required due to the high concentration of cells per tube. Scatchard analysis was not performed as extrapolation of the data would have been required therefore invalidating the results. A concentration of 0.029nM ^{125}I-G17 (Figure 3.3) gave a level of specific binding between 10-20% of total radioactivity added and therefore was used in subsequent experiments.

3.1.4 Effect of different incubation buffers

Addition of BSA to the incubation buffer significantly ($p < 0.01$) decreased the non-specific binding by 30% resulting in an increase in specific binding of 17% (Figure 3.4).

A significant increase in specific binding was observed when 50mM Hepes (buffer 3) was used rather than 50mM phosphate (buffer 1) (Figure 3.5). Addition of magnesium chloride to 50mM phosphate (buffer 2) did not significantly increase specific binding but addition of magnesium chloride to 50mM Hepes (buffer 4) caused a 58% increase in specific binding. Addition of calcium chloride to 50mM Hepes did not result in as large an increase in specific binding. Addition of both magnesium chloride and calcium chloride to Hepes buffer (buffer 6) did not result in a further increase in specific and therefore buffer 4 (50mM Hepes + 10mM $MgCl_2.6H_2O$ + 0.1% BSA) was chosen for subsequent experiments.

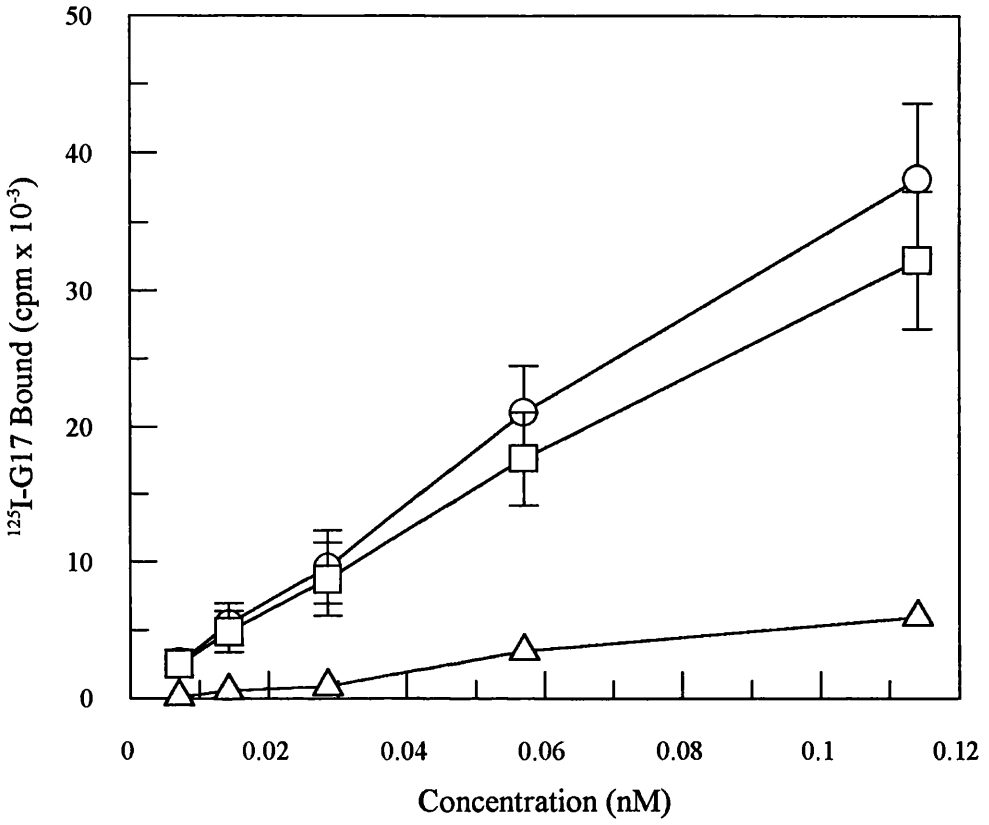

Figure 3.3 **Effect of increasing ^{125}I-G17 concentration on binding to AR42J cells**

Cells were incubated at 22°C for 180mins in the absence (-○-) or presence (-△-) of 1000 fold excess of unlabeled G17. Specific binding (-□-) was total binding (-○-) minus non-specific binding (-△-) and each point is the mean (+/-SD) of four experiments performed in duplicate.

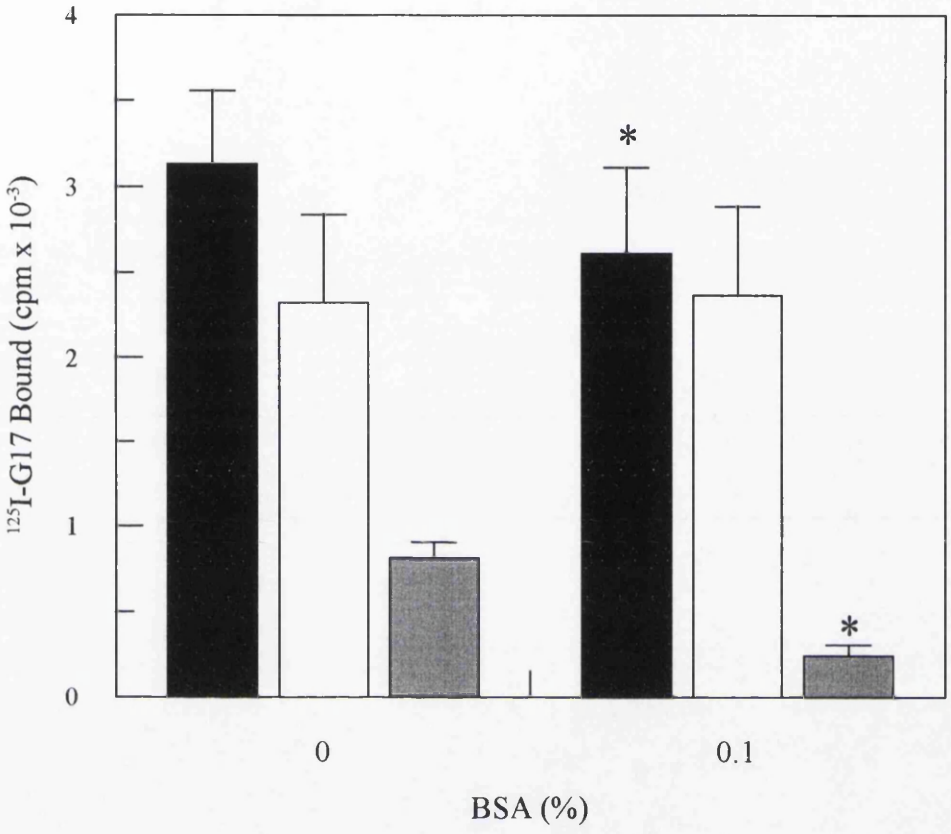

Figure 3.4 **Effect of addition of BSA to incubation buffer on ^{125}I-G17 binding to AR42J cells**

Cells were incubated in 50mM Hepes, 10mM $MgCl_2.6H_20$ +/- 0.1% BSA for 60mins at 22°C and results are the mean (+SD) of total (■), specific (□) and non-specific (▩) binding from three experiments performed in duplicate.
* denotes level of significance ($p<0.01$) when compared with corresponding binding from buffer without BSA.

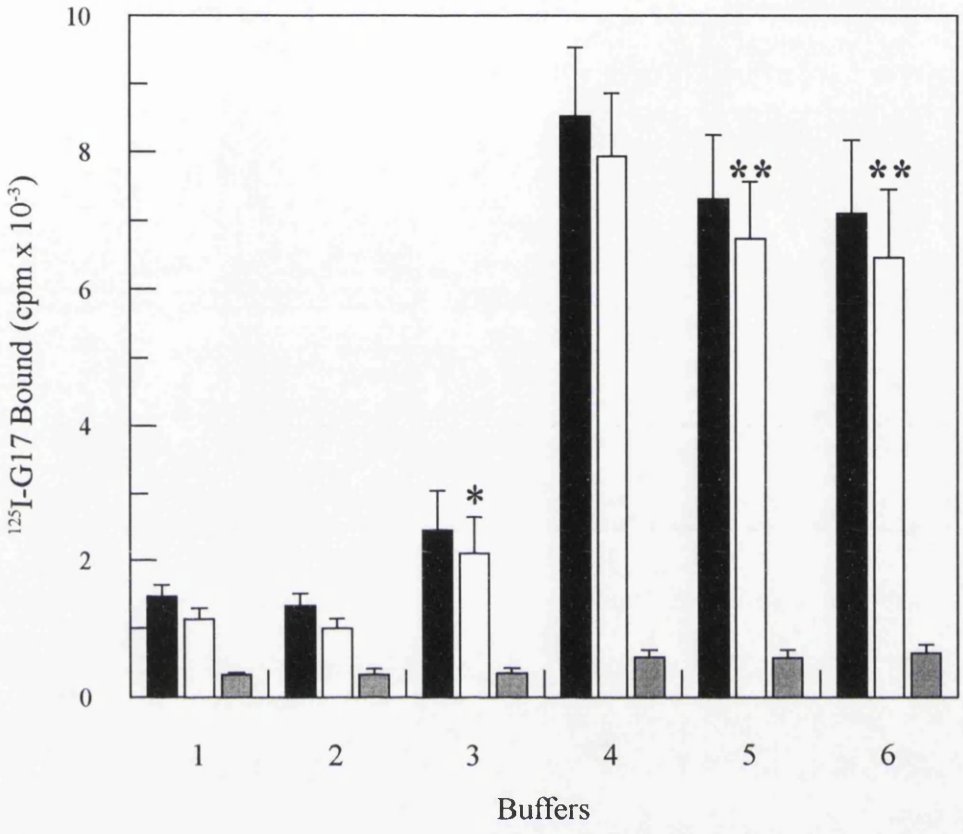

Figure 3.5 **Effect of different incubation buffers on ^{125}I-G17 binding to AR42J cells**

AR42J cells were incubated with different buffers for 180mins at 22°C. Each bar represents the mean (+SD) of total (■), specific (□) and non-specific (▩) binding from six experiments performed in duplicate.
* and ** denote levels of significance ($p<0.01$) in comparison to buffer 1 and 4 respectively. Buffers were as follows :

1 50mM phosphate + 0.1% BSA
2 50mM phosphate + 10mM $MgCl_2.6H_20$ + 0.1% BSA
3 50mM Hepes + 0.1% BSA
4 50mM Hepes + 10mM $MgCl_2.6H_20$ + 0.1% BSA
5 50mM Hepes + 10mM $CaCl_2$ + 0.1% BSA
6 50mM Hepes + 10mM $MgCl_2.6H_20$ + 10mM $CaCl_2$ + 0.1% BSA

3.1.5 Effect of pH

The pH of the 50mM Hepes, 10mM $MgCl_2.6H_2O$, 0.1% BSA buffer was adjusted to 6.5, 7.0, 7.5 and 8.0. Optimum binding was obtained after 180mins at 22°C at a pH between 6.5 and 7.0 (Figure 3.6) and decreased to half the maximum specific binding at pH8.0. Specific binding at pH 6.5 was not significantly different from pH 7.0 and so the more physiological pH of 7.0 was chosen for subsequent experiments.

3.1.6 Effect of incubation temperature

At an assay incubation temperature of 4°C, specific binding increased linearly with increasing time between 2.5 and 90 mins, after which a plateau was reached and steady state maintained for a further 180 mins (Figure 3.7). Binding at 22°C followed a similar pattern with specific binding increased by 50% compared with that at 4°C. Steady state was achieved after 180 mins incubation and maintained for 90 mins. A rise in specific binding at 37°C was not observed and at time points between 2.5 and 90 mins a rapid decline in specific binding was observed. An incubation temperature of 22°C was therefore considered optimum and all subsequent experiments were performed in an ambient temperature water bath to achieve this.

3.1.7 Effect of separation method

Following incubation of cells (1 x 10^6/tube) at 22°C for 180 mins, the reaction was terminated by either centrifuging at 13000g for 2 mins in a microcentrifuge, filtering through a Brandell cell harvester onto filters pre-soaked in ice-cold assay buffer containing 0.5% BSA or filtering through a Millipore filter onto filters pre-soaked in ice-cold assay buffer containing 0.5% BSA (Figure 3.8). Each of the conditions involved

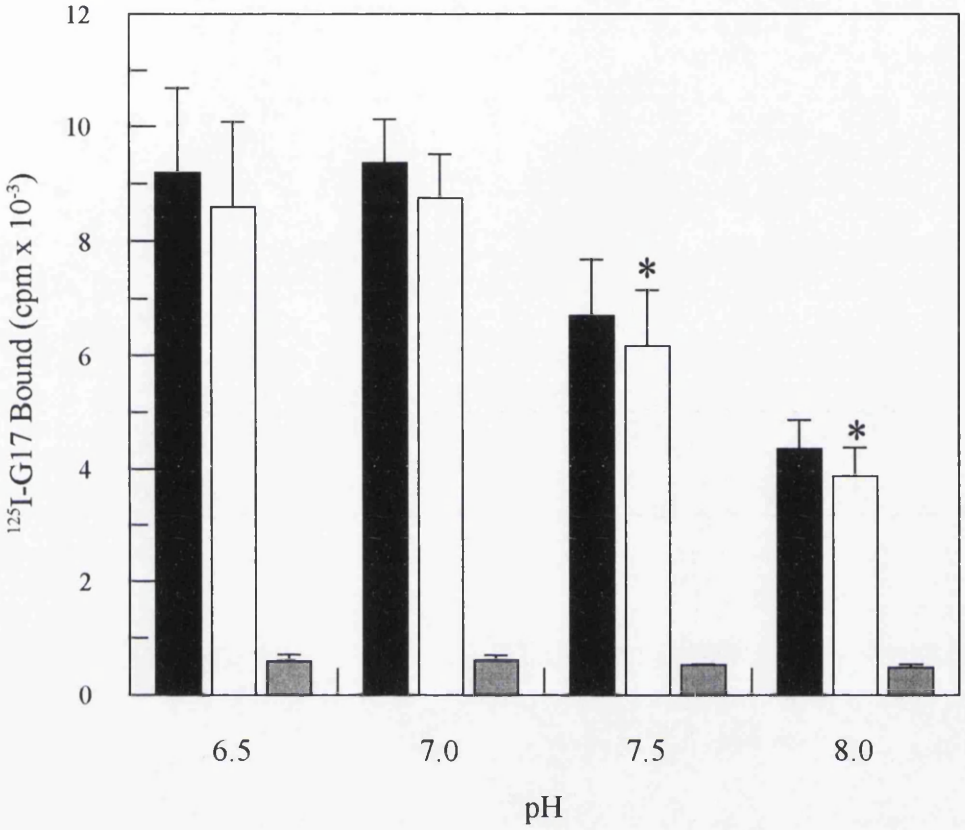

Figure 3.6 **Effect of buffer pH on ^{125}I-G17 binding to AR42J cells**

Effect of pH of incubation buffer (50mM Hepes, 10mM $MgCl_2.6H_20$, 0.1% BSA) on ^{125}I-G17 binding to AR42J cells. Reaction was incubated at 22°C for 180mins and each bar represents the mean (+SD) total (■), specific (□) and non-specific (▩) binding from six experiments performed in duplicate.
* denotes level of significance ($p<0.01$) in comparison to corresponding binding with pH 6.5.

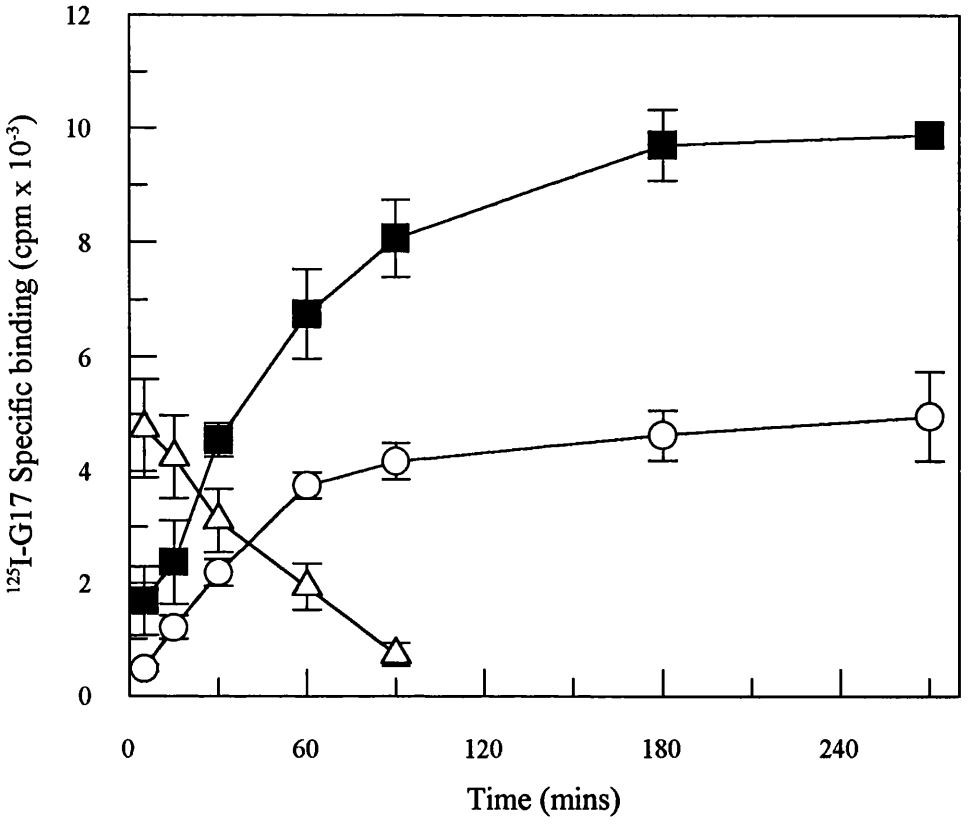

Figure 3.7 **Effect of different incubation temperatures on the time course of specific ^{125}I-G17 binding to AR42J cells**

Effect of different incubation temperatures, 4°C (○), 22°C (■) and 37°C (△), on the time course of ^{125}I-G17 specific binding to AR42J cells. Each point is the mean (+/-SD) of six experiments performed in duplicate.

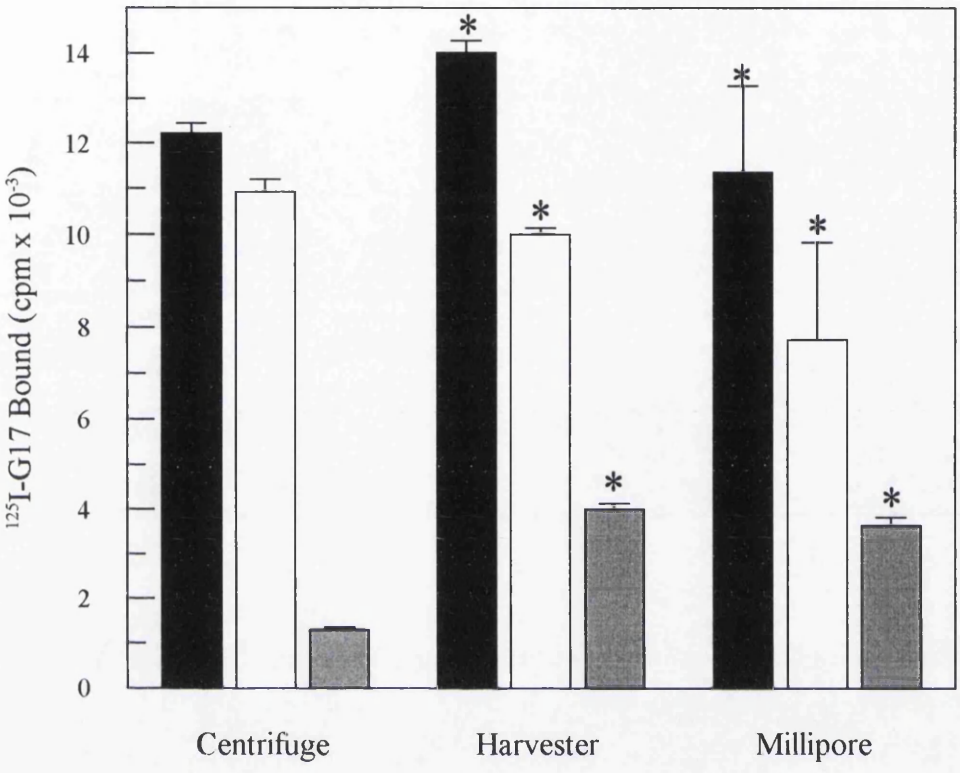

Figure 3.8 **Effect of different separation methods on ^{125}I-G17 binding to AR42J cells**

Following incubation of cells for 180 mins at 22°C, reaction tubes were either centrifuged at 13000g for 2mins, filtered with a brandell harvester onto ice cold filters or filtered with a millipore filter onto ice cold filters. Each bar represents the mean (+SD) total (■), specific (□) and non-specific (▩) binding of two experiments performed in triplicate.

* denotes level of significance ($p<0.01$) when compared to corresponding binding with the centrifugation method.

two washes with ice-cold assay buffer to reduce non-specific binding and the pellet or filter was counted in a gamma counter for 1 min. Although total binding was higher with the Brandell harvester and Millipore methods, the specific binding was not increased due to the significant increase ($p<0.01$) in non-specific binding compared to the centrifugation method. Therefore centrifugation was the separation method of choice for future assays.

3.2 Competition assays

3.2.1 Effect of gastrin/CCK_B receptor agonists on binding of ^{125}I-G17 to AR42J cells

To assess the affinity of gastrin/CCK_B receptor agonists for gastrin/CCK_B receptor sites on AR42J cells, competition curves were analysed using a least squares curve fitting program (LIGAND). Inhibition of ^{125}I-G17 binding to AR42J cells by G17, G34, CCK8S and CCK8 was statistically best fit by a single site model (Figure 3.9). Scatchard analysis of cold G17 displacement data revealed a single binding site with a K_D of 0.3nM and maximal capacity of 24fmols/10^6 cells. Specific ^{125}I-G17 binding was displaced from AR42J cells by G17 with an IC_{50} of 0.4nM, but CCK8S was the most potent competitor with an IC_{50} of 0.25nM and 1.6 fold higher potency than G17. The rank order of potency was CCK8S > G17 > G34 > CCK8 with IC_{50} values for G34 and CCK8 of 0.58nM and 1.6nM respectively (Table 3.1). The rank order of affinities for the gastrin/CCK_B receptor on AR42J cells is CCK8S $\leq$ G17 $\leq$ G34 $\leq$ CCK8 with Ki/K_D values of 0.23nM, 0.3nM, 0.52nM and 1.5nM respectively.

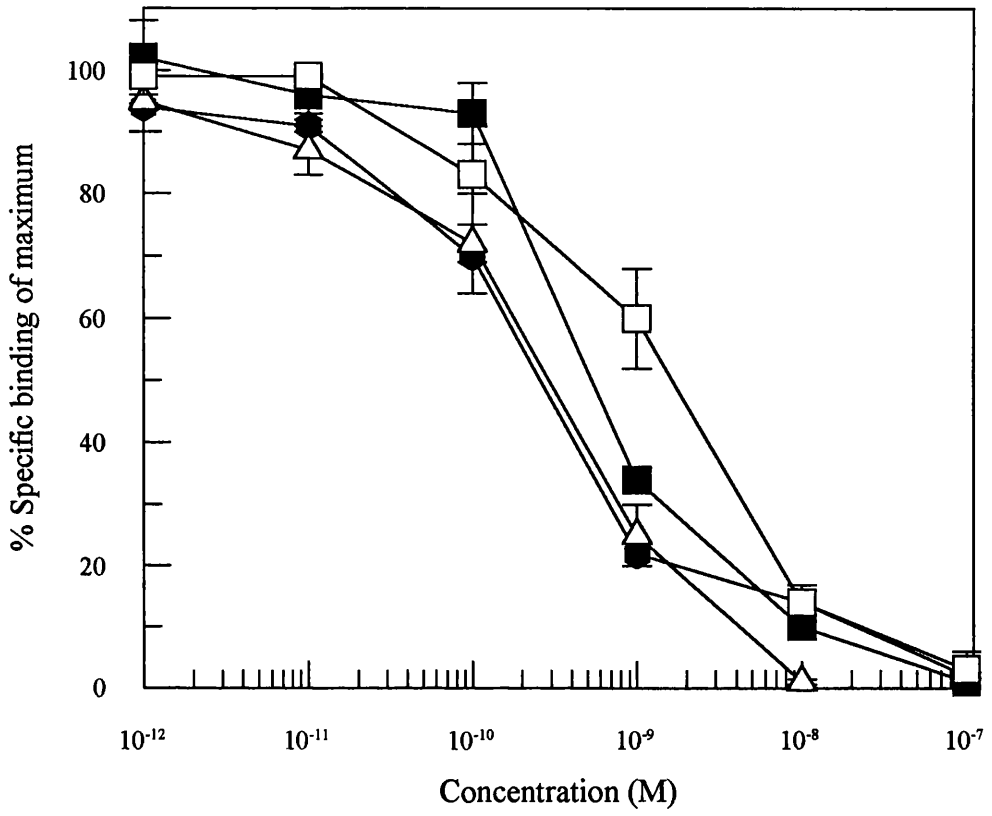

Figure 3.9 **Comparison of agonist displacement of ^{125}I-G17 binding to AR42J cells**

Displacement of ^{125}I-G17 binding to AR42J cells by increasing concentrations of agonists CCK8S (-●-), G17 (-△-), G34 (-■-) and CCK8 (-□-). Each point is the mean (+/-SD) of at least three experiments performed in duplicate.

CLASSIFICATION	LIGAND	IC_{50} (nM)	K_D / Ki (nM)
AGONISTS	CCK8S	0.25	0.23
	G17	0.4	0.3
	G34	0.58	0.52
	CCK8	1.6	1.5
ANTAGONISTS	L740093	0.02	0.018
	L365260	4.7	4.3
	CAM1028	5.0	4.5
	L364718	230	209
	CR1409	4530	4118
	CR1505	15000	13624

Table 3.1 **Table of half maximal values and affinities from inhibition curves of receptor agonists/antagonists to the gastrin/CCK_B receptor on AR42J cells**

3.2.2 Effect of gastrin/CCK_B and CCK_A receptor antagonists on binding of ^{125}I-G17 to AR42J cells

All the receptor antagonists inhibited ^{125}I-G17 binding to AR42J cells (Figures 3.10, 3.11, 3.12) with the following order of potency, L740093 > L365260 > CAM1028 > L364718 > CR1409 > CR1505. Data was analysed by LIGAND (Table 3.1) and the log-logit plot of displacement data was found to be linear indicating binding to one site. A relatively new gastrin/CCK_B receptor antagonist tested was CAM1028 which displaced gastrin binding to the gastrin/CCK_B receptor with an IC_{50} of 5nM. The second novel gastrin/CCK_B receptor antagonist analysed was L740093 which caused detectable inhibition of binding of ^{125}I-G17 at 1pM, half maximal inhibition (IC_{50}) at 0.02nM and complete inhibition at 1nM. L365260 was 235 fold less potent with an IC_{50} of 4.7nM and complete inhibition at 0.1uM. Devazepide (L364718) was the most potent of the the CCK_A receptor antagonists with an IC_{50} value of 0.2uM. Lorglumide (CR1409) was the more potent of the two proglumide derivatives analysed with an IC_{50} of 4.5uM in comparison to loxiglumide (CR1505) with an IC_{50} of 15uM. The inhibitory constants (Ki) for gastrin/CCK_B receptor antagonists (Table 3.1) followed the same pattern as for IC_{50} values with L740093 being the most potent with a 17 fold higher affinity for the gastrin/CCK_B receptor on AR42J cells.

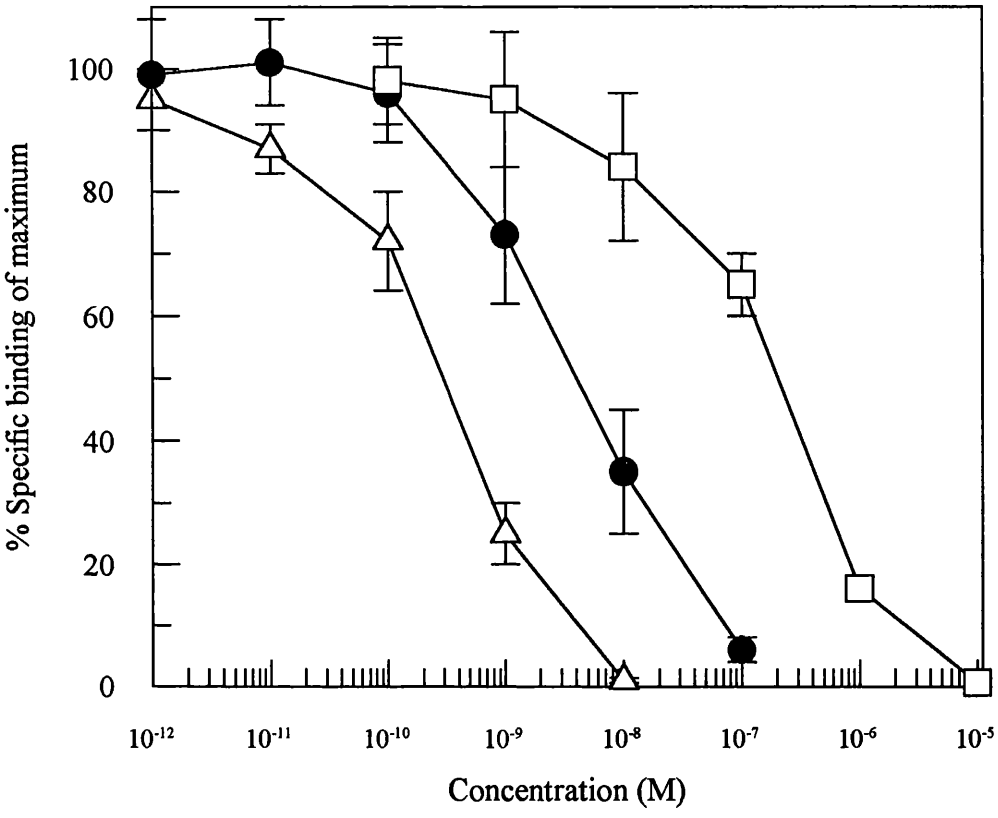

Figure 3.10 **Comparison of antagonists with G17 displacement of ^{125}I-G17 binding to AR42J cells**

Displacement of ^{125}I-G17 binding to AR42J cells with increasing concentrations of G17 (-△-), and CCK_B and CCK_A receptor antagonists L365260 (-●-), and L364718 (-□-) respectively. Each point is the mean (+/-SD) of at least four experiments performed in duplicate.

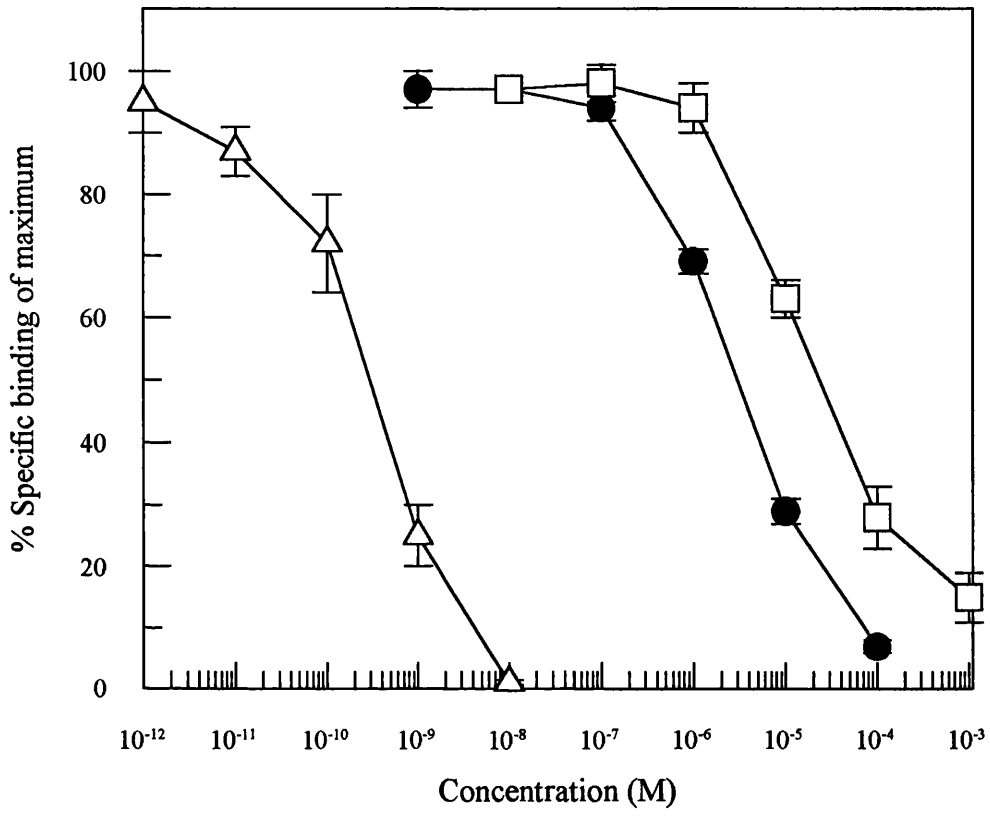

Figure 3.11 **Comparison of antagonists with G17 displacement of ^{125}I-G17 binding to AR42J cells**

Displacement of ^{125}I-G17 binding to AR42J cells with increasing concentrations of G17 (-△-), and proglumide derivatives, CR1409 (-●-), and CR1505 (-□-). Each point is the mean (+/-SD)of at least four experiments performed in duplicate.

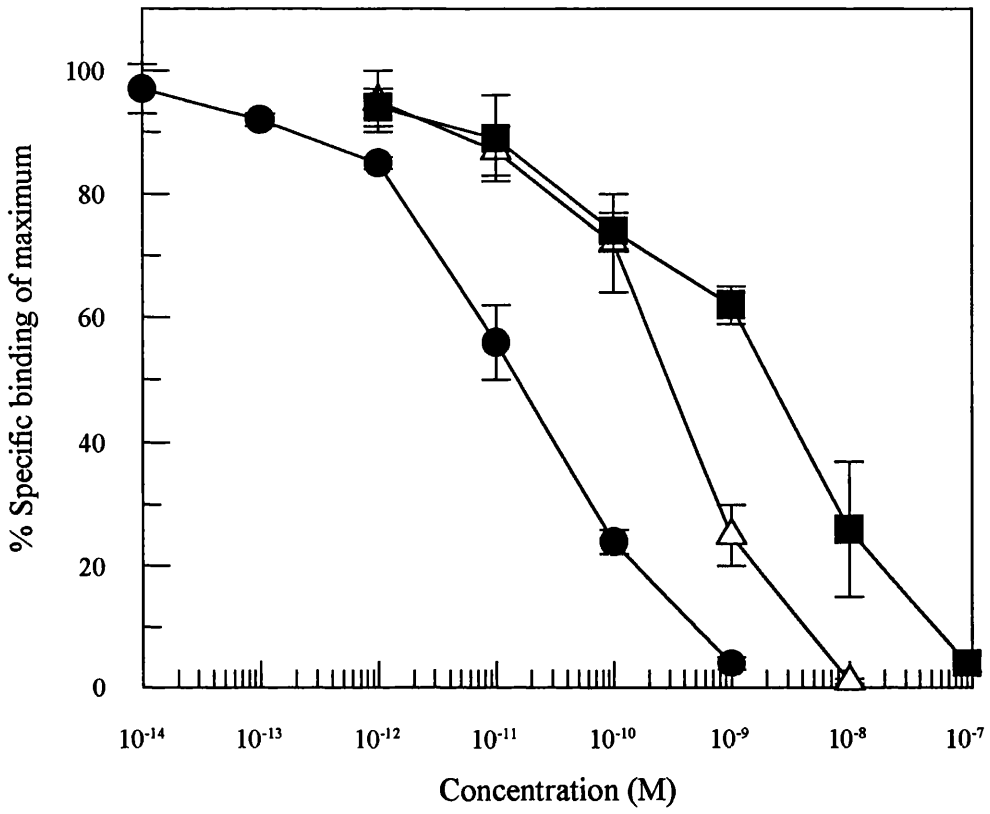

Figure 3.12 **Comparison of new antagonists with G17 displacement of ^{125}I-G17 binding to AR42J cells**

Displacement of ^{125}I-G17 binding to AR42J cells with increasing concentrations of G17 (-△-), and CCK_B receptor antagonists L740093 (-●-), and CAM1028 (-■-). Each point is the mean (+/-SD) of at least four experiments performed in duplicate.

4 DISCUSSION

The basic binding assay protocol is straightforward and the reasons for choice of conditions for the final protocol are discussed here in detail.

It was important that an appropriate model was selected to best mimic the ultimate cell membranes to be analysed. In this instance the rat pancreatic cell line, AR42J, was considered to be the most suitable control model since it had previously been found to possess both CCK_A and gastrin/CCK_B receptors on its cell surface (Scemama *et al* 1989) which had been characterised with various ligands (Seva *et al* 1990a; Watson *et al* 1991; Blackmore and Hirst 1992). Human colorectal carcinoma cell lines HT29, LoVo and DLD1 were analysed *in vitro* for gastrin/CCK_B receptor status but significant specific ^{125}I-G17 binding was not observed (data not shown). Of the human cell lines tested, only LoVo cells were previously shown to exhibit high affinity gastrin/CCK_B receptors, but this was after growing them as xenografts in mice (Watson SA, personal communication). The gastrin/CCK_B receptor status of the human colorectal carcinoma cell line DLD1 is not known. A human gastric tumour cell line, MKN45, grown as xenografts in nude mice also exhibited high affinity (K_D of 1.2nM) gastrin/CCK_B receptors (Watson *et al* 1992b). Although optimisation assays may have benefited from inclusion of gastrin/CCK_B receptor positive human carcinoma cell lines, a lack of facilities for growing animal xenografts prevented their use in this thesis.

The radioligand, ^{125}I-G17 was used in preference to ^{125}I-CCK8 since the ultimate goal of this thesis was to measure gastrin/CCK_B receptors in human tissues. Gastrin-17 is selective for gastrin/CCK_B receptors and can be radiolabelled to give a high specific

activity. The requirements for scatchard analysis, 10-90% receptor saturation with radiolabel could not be met as large quantities of radioligand would have been required to achieve saturation due to the high number of cells used per tube and this was prohibitively expensive. The use of fewer cells/tube was not an option as assay precision was adversely affected.

In an attempt to simulate genuine physiological conditions *in vitro*, various buffers have been used in radioligand binding assays. Some groups have used Kreb-Hanseleit solution as a physiological buffer (Scemama *et al* 1987; Szecowka *et al* 1985), while others used cell culture media such as minimal eagles medium (Watson *et al* 1992a). The cell culture medium RPMI 1640 was used initially in the binding assay described in this thesis as this was the medium that the AR42J cells were grown in. Comparison of RPMI 1640 with 50mM Hepes buffer, showed binding to be increased by approximately 50% with the Hepes buffer (data not shown) and therefore further optimisation of the binding buffer excluded RPMI 1640 and cells were resuspended in Hepes buffer. Of the buffers tested (Figure 3.5) it was found that 50mM Hepes, 10mM $MgCl_2.6H_2O$, 0.1% BSA, gave the highest specific binding. Addition of magnesium chloride was found to enhance binding of the radioligand as did calcium chloride but in combination binding did not surpass that of magnesium chloride alone. This is in agreement with Innis and Snyder (1980) who found enhanced binding of ^{125}I-CCK8 to CCK receptors in the presence of magnesium ions. The addition of magnesium ions is known to promote agonist binding to G-proteins by favouring the high affinity agonist receptor-G-protein complex (Hulme and Birdsall 1992) and this may explain the results found here. Addition of 0.1% BSA to the assay buffer (Figure 3.6) caused a significant

reduction ($p < 0.01$) in non-specific binding and was therefore included in the final buffer.

Variations in pH within a range 6.5-8.0 caused a significant decrease in specific binding from pH 7.0 to 8.0. Although binding at pH 7.0 was not significantly different from pH 6.5, pH 7.0 was chosen as the most physiologically relevant pH. A pH of 7 was prefered for competition experiments with gastrin/CCK_B receptor agonists/antagonists, also required to facilitate comparisons with published data. Steigerwalt and Williams (1981) found that ^{125}I-CCK33 binding to guinea-pig pancreatic acini was optimal at pH 5.5. Praissman and Brand (1991) demonstrated using autoradiographical methods that maximum specific ^{125}I-G17 binding to the human gastric body was observed at pH 6.5.

Separation of bound from free radioligand in the binding assay was evaluated by the methods of filtration and centrifugation. The advantages of using filtration are that it is rapid and convenient with a high throughput. Total binding with filtration was significantly higher than with centrifugation, but non-specific binding was also significantly greater therefore specific binding was in fact decreased in comparison to centrifugation. As non-specific binding was also a potential problem in the final assay which was to be used for human tissue membrane preparations which contain a heterologous population of cells, the most suitable method for separation was centrifugation.

The present study found that although a cell number of < 1 x 10^6cells/tube was necessary to give specific binding of less than 10% of total added, it was necessary to use 1 x 10^6 cells/tube due to the requirement of a pellet which could be washed with ease. This resulted in specific binding of 17% of total added. Any cell number less than this and washing of the pellet formed during termination of the reaction by centrifugation caused problems with assay precision because of the removal of cells with aspiration of the supernatant.

It is important to establish the time needed for equilibrium binding to be achieved under different experimental conditions. Steady state must be reached before dissociation experiments can be performed. Dissociation experiments where 10uM G17 was added to the incubation medium revealed a half life of 55mins for ^{125}I-G17. This is not in agreement with Scemama and co-workers who reported a half life of 8.5mins for dissociation of ^{125}I-G17 from gastrin/CCK_B receptors on AR42J cells (Scemama *et al* 1987). This discrepancy may be due to differences in incubation temperature used since Scemama *et al* performed their experiments at 37°C rather than 22°C, which is likely to result in faster kinetics.

This idea is supported by the finding in this study that a temperature of 37°C was impractical due to the rapid association rate measured in seconds rather than minutes. Kleveland and Waldum (1986) showed the same pattern of binding at 30°C for ^{125}I-G17 with rat fundic plasma membranes. Svoboda *et al* (1982) demonstrated internalisation of the ligand-receptor complex of CCK_A receptors such that at 37°C, binding was too rapid

for a reliable assay to be developed. For these reasons a temperature of 22°C was chosen over and 37°C.

Gastrin-17 was shown to displace ^{125}I-G17 binding to AR42J cells with an IC_{50} of 0.4nM. Binding was displaced over a 10-90% displacement range and the slope of the displacement curve was unity indicating competitive agonist binding. Scatchard analysis revealed a single high affinity binding site with a K_D of 0.3nM and Bmax of 24fmols/10^6 cells (Table 3.1). This is in agreement with Watson *et al* who found a K_D for the gastrin/CCK_B receptor of 0.46nM and Bmax of 55fmols/10^6 cells on AR42J cells (Watson *et al* 1992b). Dissociation constants for the gastrin/CCK_B receptor vary widely throughout the literature with values between 0.46-4.6nM and maximal capacities between 55-94fmols/10^6 cells in AR42J cells. Differences may be due to different assay conditions and/or equilibrium kinetics. In the present study the K_D and Bmax are both lower than those quoted in the literature and this may be due to assay temperatures of 22°C. Log-logit plots of the data were also found to be linear, demonstrating a ligand binding to single class of binding site. Values for inhibition (Ki) constants (Table 3.1) were determined from inhibition curves of the ability of various antagonists to inhibit ^{125}I-G17 to AR42J cells by the Cheng-Prusoff correction method (Cheng and Prusoff 1973).

Competition studies (Figure 3.9) with different agonists of the gastrin/CCK family showed similar potencies, with the exception of non-sulphated CCK8, in inhibiting ^{125}I-G17 binding to AR42J cells. This is in agreement with Scemama and co-workers (Scemama *et al* 1987) who also demonstrated equal potency between G17 and CCK8S

but observed a two fold lower potency with CCK8 as compared with the present system where CCK8 was five fold lower in ability to inhibit binding. A report by the same group in 1989 found CCK8 was as efficient as G17 and pentagastrin at inhibiting binding in AR42J cells with IC_{50} values of 1.5, 1.1 and 1.2nM respectively under different experimental conditions to the current study, (Scemama *et al* 1989).

Proglumide, a weak gastrin receptor antagonist, (Rovati 1968) requires micromolar quantities *in vitro* and *in vivo* to cause inhibition of gastrin-stimulated growth and is the only gastrin receptor antagonist which has reached phase III clinical trials in patients with advanced colorectal and gastric cancer (Harrison *et al* 1990). However, there was no effect on either patient survival or tumour growth (Morris *et al* 1990). Proglumide inhibits growth effects of gastrin in the murine colon cancer cell line MC26 and with the same cells grown *in vivo* as xenografts thereby prolonging survival of tumour bearing mice (Singh *et al* 1987; Beauchamp *et al* 1985). The proglumide derivatives CR1409, (lorglumide) and CR1505, (Loxiglumide) were analysed in the present study. Half maximal concentrations for CR1409 and CR1505 were 4.5uM and 13uM respectively and were comparable to those obtained by Seva *et al* with AR42J cells (3uM and 13uM respectively). Watson *et al* reported IC_{50} values for CR1409 of 4uM (Watson *et al* 1992a). The newly developed gastrin/CCK_B receptor antagonist, CAM1028 (Hughes *et al* 1990), which is structurally similar to the gastrin/CCK_B receptor antagonist C1-988 (Parke-Davis, personal communication) was found to have equal potency with L365260 with an IC_{50} of 5nM. The gastrin/CCK_B receptor antagonist L740093, a 1,4 benzodiazepine derivative inhibited ^{125}I-G17 binding to AR42J cells with an IC_{50} of 0.02nM. The only previous study to date on this compound reported an

IC_{50} of 0.04nM for binding to the gastrin/CCK_B site on guinea-pig gastric glands (Patel *et al* 1994) which was more than 210 times more potent than the parent compound L365260 (IC_{50} = 8.5nM). In the present study L740093 was found to be 235 fold more potent in inhibiting binding than L365260, with IC_{50} values of 0.02nM and 4.7nM respectively.

This chapter has described in detail an optimised radioligand binding assay which has been characterised using a panel of agonists and antagonists. The values attained for the dissociation affinity constant, maximal capacities and half maximal concentrations for competing ligands correlate with those reported previously in the literature confirming the validity of the gastrin/CCK_B receptor binding properties of AR42J cells found using this assay. This optimised assay is therefore suitable for use as a control from which to ascertain whether preparation of AR42J plasma membranes affects the number, affinity and/or characteristics of the gastrin/CCK_B receptors thus far identified.

CHAPTER 4 GASTRIN/CCK$_B$ RECEPTORS IN AR42J CELL MEMBRANES

1 INTRODUCTION

The whole cell AR42J radioligand binding assay was optimised as described in Chapter three. The next step was to use this as a basis with which to optimise and evaluate a similar assay using membranes prepared from the same cell line, ensuring that no receptor properties were lost due to membrane preparation.

One of the main advantages of using gastrin/CCK_B receptor positive tumour cell lines to prepare plasma membranes is that cells can be grown *in vitro* to produce large quantities of homogeneous material. Many groups have studied AR42J cells because of their gastrin/CCK_B receptor properties (Seva *et al* 1990a,1990b; Scemama *et al* 1987; Blackmore and Hirst 1992; Watson *et al* 1991), but to date only one report has characterised gastrin/CCK_B receptors on AR42J plasma membranes (Lambert *et al* 1991). Nevertheless, in this paper full optimisation of the radioligand binding methodology was not reported, although membranes were shown to have 80% gastrin/CCK_B receptors with affinities for CCK8S and G17 of 1nM and 4nM respectively and 20% CCK_A receptors with affinities of 1nM and 1uM for CCK8S and G17 respectively.

Characterisation of gastrin/CCK_B receptors on AR42J plasma membranes is important as original cell receptor properties should be retained after membrane preparation. Characterisation of these receptors in AR42J membranes is also required for comparison of gastrin/CCK_B receptor status from plasma membranes prepared from human colonic and gastric carcinoma and corresponding normal tissue.

Studies with AR42J plasma membranes may also provide important data on storage conditions (i.e. temperature, length of time in storage and requirements for protective agents) which preserve receptor structure and function since receptors of interest may be destroyed in the process of membrane preparation.

Ultimately it must be realised that although studies with animal and human cell lines yield important information about the gastrin/CCK_B receptor, caution must be exercised about their clinical relevance. Direct study of plasma membranes from human gastrointestinal normal mucosa and tumours in conjunction and comparison with AR42J plasma membranes as control are needed.

1.1 Objectives

The object of this study was to establish a reliable and sensitive assay capable of detecting and characterising gastrin/CCK_B receptors on cell membranes.

2 METHODS

Materials and general methods are in Chapter 2

2.1 Kinetic assays

Association kinetics were determined by incubating membranes as described in Chapter 2, section 3.7.1, at increasing time points from 5 to 270mins to ensure steady state had been reached. Dissociation kinetics were studied by incubating membranes with radioligand until steady state was attained prior to excess unlabeled G17 being added and residual binding measured at time increments between 2.5 and 180mins. Specific binding was defined as the total binding minus that in the tubes containing excess G17.

2.2 Optimisation assays

These assays included the optimisation of membrane concentration, ^{125}I-G17 concentration, incubation buffer, pH and temperature. The basic protocol was as described in radioligand binding with 100ug/tube of fraction II and fraction I membranes incubated with 0.029nM ^{125}I-G17 and each of the individual conditions under study varied accordingly. Non-specific binding was defined as binding in the presence of 0.029uM G17.

2.3 Membrane storage

Storage time and buffer were tested for optimal ^{125}I-G17 binding to AR42J membranes. Membranes were prepared fresh on day zero and each of three aliquots diluted either in whole cell assay buffer 1, membrane assay buffer 2 or human tissue membrane assay buffer 3 (Chapter 2, section 2) at a concentration of 2mg/ml. Membranes (100ug/tube) were incubated on day zero with 0.029nM ^{125}I-G17 for 180mins at 22°C. The remaining aliquots of membrane from the corresponding buffers were stored at -70°C for up to 14 days. Binding as described above in Section 2.2 was measured on days 1 (18 hours), 2, 7 and 14 after freezing.

Storage temperature was examined by storage of membranes (2mg/ml) in membrane assay buffer 2 at 4°C, -20°C and -70°C. Binding was determined on the day of membrane preparation and was designated the experimental control. Binding was re-examined after storage of membranes at respective temperatures for 18-24 hours.

2.4 Cell disruption

In the present study, the initial method of cell disruption was optimised since a number of different methods have been used throughout the literature for the measurement of receptors. These include freeze-thawing in liquid nitrogen (Scemama *et al* 1987; Tahairi-Jouti *et al* 1992), manual homogenisation (Szecowka *et al* 1985) and mechanical homogenisation (Preston *et al* 1993).

In an initial study to determine the optimal method of cell disruption, a number of methods were examined. These included homogenisation with a polytron at a setting of two for three fifteen second bursts, sonicating with a probe sonicator for three fifteen second bursts at an amplitude of thirty microns, freeze-thawing in liquid nitrogen, manual homogenisation in a teflon-in-glass homogeniser, sonication in a bath sonicator and lysing of cells in hypotonic buffer. Disruption of the cells was monitored using the trypan blue exclusion test, with subsequent examination of the membranes under a light microscope.

2.5 Competition assays

The gastrin/CCK_B receptor on AR42J membranes was characterised using the agonists G17, G34, CCK8S and CCK8. The gastrin/CCK_B receptor was further characterised using the gastrin/CCK_B receptor antagonists L365260, L740093 and CAM1028, and the CCK_A receptor antagonist L364718. All competition assays were performed with 0.029nM ^{125}I-G17 (final concentration) and increasing concentrations (10^{-16}-10^{-5}M) of the corresponding agonists and antagonists for 180mins at 22°C in the assay buffer.

3 RESULTS

3.1 Assay optimisation

3.1.1 Comparison of different cell disruption methods

The probe sonicator produced the best overall disruption of cells in comparison to the other methods which did not completely disrupt all cells. Mechanical homogenisation using a polytron is commonly used to disrupt cells but it produced considerably more heat than the other methods tested and therefore may have caused problems with the stability of receptors on membrane fragments. Membranes prepared using the probe sonicator appeared the most uniform and therefore this was chosen as the method of cell disruption.

3.1.2 Effect of membrane concentration on binding of ^{125}I-G17 to AR42J membranes

Increasing concentrations of AR42J fraction II and fraction I membranes, between 6.25ug/tube and 150ug/tube were incubated with ^{125}I-G17 (0.029nM, final concentration) for 180mins at 22°C. Specific binding (Figure 4.1) was shown to be linear within this range, although membranes from fraction I had a sharper incline and therefore required less protein per tube to reach the same level of specific binding as fraction II membranes. The concentration of membranes for both fraction II and fraction I used for subsequent experiments was 100ug/tube, which gave specific binding of less than 19% of total radiolabel added. This concentration was also found to be the minimum necessary for the formation of a pellet of reasonable size.

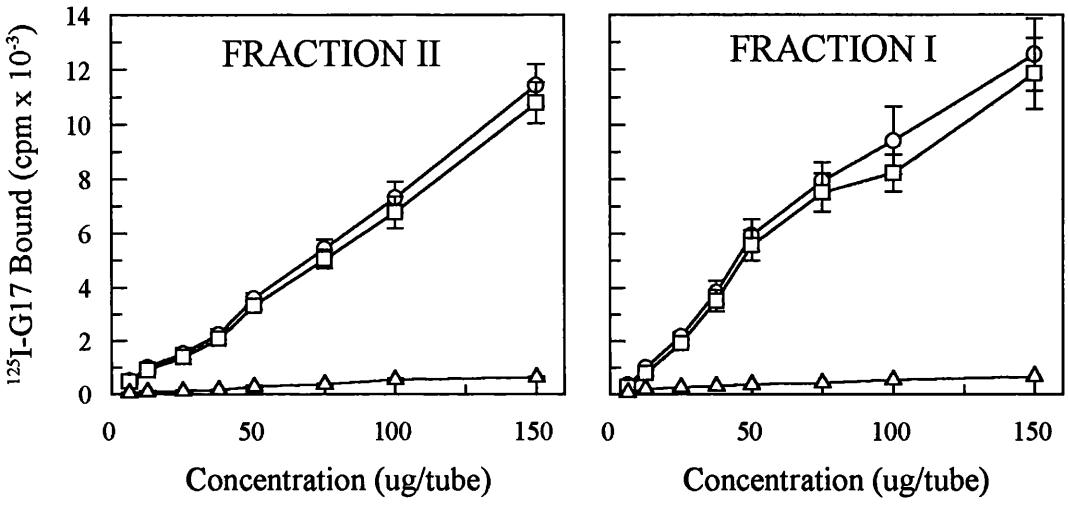

Figure 4.1 **Effect of increasing membrane concentration on ^{125}I-G17 binding to AR42J membranes**

Binding of ^{125}I-G17 to increasing concentrations of AR42J membranes at 22°C for 180mins. Total binding (-○-); non-specific binding (-△-) in the presence of 1000 fold excess unlabeled G17 ; specifc binding (-□-). Each point is the mean (+/-SD) of four experiments performed in duplicate.

3.1.3 Effect of incubation time on binding of ^{125}I-G17 to AR42J membranes

Binding reached a plateau after 180mins for both fraction II and fraction I membranes, confirming the reaction had reached steady state. The *observed* association rates, K_{obs} for AR42J membranes were calculated using non linear regression program by LIGAND (Figure 4.2 and 4.3 inset, K_{obs} is the slope of the line). Non-specific binding changed little throughout the duration of the assay and was less than 9% of total binding at 270mins of incubation for both membrane fractions. Addition of 10uM unlabelled G17 into the incubation medium at 180mins when the reaction was at steady state caused dissociation of bound radioactivity. Dissociation rates (K_{-1}) were calculated using LIGAND and from equation 4 (Chapter 1, section 7), the association rate was calculated. The dissociation constant (K_{+1}) was calculated from equation 5 (Chapter 1, section 7). Dissociation rates for both AR42J fraction II and I membranes were 0.12nM and 0.097nM respectively.

3.1.4 Effect of increasing ^{125}I-G17 concentrations

Increasing ^{125}I-G17 concentrations were added to 100ug AR42J membrane fractions II or I for 180mins at 22°C in assay buffer (Figure 4.4). Specific binding increased linearly with increasing ^{125}I-G17 concentrations with both fraction II and I but did not reach saturation. Scatchard analysis was regarded as invalid due to the extrapolation of data required since the reaction did not reach saturation.

3.1.5 Effect of addition of protease inhibitors to incubation buffer

Membrane fractions II and I were incubated with different buffers with and without protease inhibitors (Figure 4.5). Addition of 1uM soya bean trypsin inhibitor to the buffer significantly increased specific binding by 36-55% ($p < 0.01$). Binding with buffer 3,

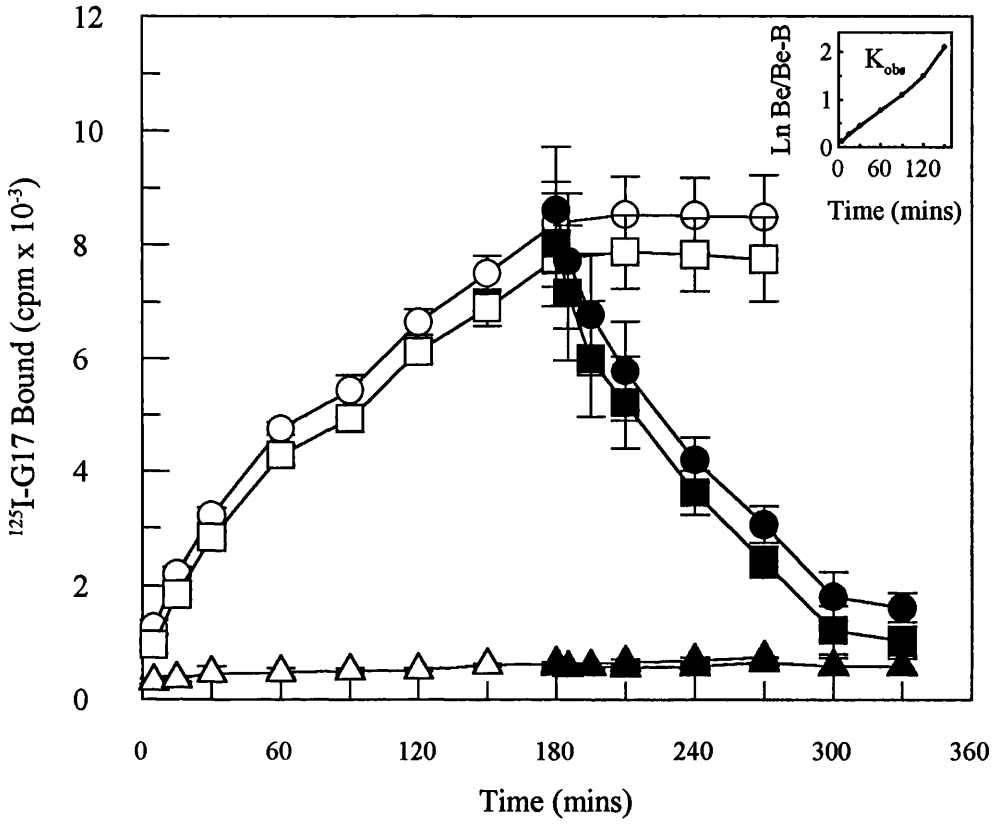

Figure 4.2 **AR42J fraction II membrane association and dissociation time courses with ^{125}I-G17**

Membranes (100ug/tube) were incubated either with 0.029nM ^{125}I-G17 alone (-○-) or in the presence of 0.029uM G17 (-△-) at 22°C. Specific binding (-□-) is total binding (-○-) minus non-specific binding (-△-). At 180 mins, 10uM G17 was added to the incubation medium to dissociate ^{125}I-G17 from binding sites. Dissociation results are denoted by total (-●-), specific (-■-) and non-specific binding (-▲-) with each point the mean (+/-SD) of four experiments performed in duplicate. Inset is a graphical representation of the association rate constant where the slope of the line is the observed association rate constant (K_{obs}).

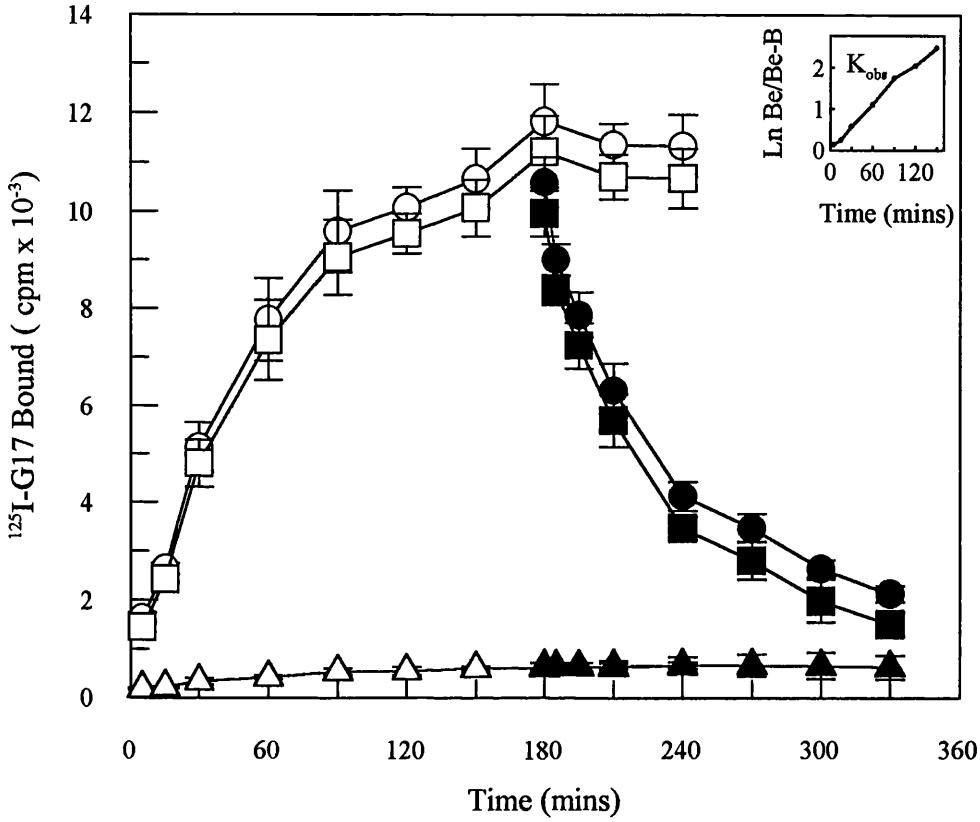

Figure 4.3 **AR42J fraction I membrane association and dissociation time courses with ^{125}I-G17**

Membranes (100ug/tube) were incubated either with 0.029nM ^{125}I-G17 alone (-○-) or in the presence of 0.029uM G17 (-△-) at 22°C. Specific binding (-□-) is total binding (-○-) minus non-specific binding (-△-). At 180 mins, 10uM G17 was added to the incubation medium to dissociate ^{125}I-G17 from binding sites. Dissociation results are denoted by total (-●-), specific (-■-) and non-specific binding (-▲-) with each point the mean (+/-SD) of four experiments performed in duplicate. Inset is a graphical representation of the association rate constant where the slope of the line is the observed association rate constant (K_{obs}).

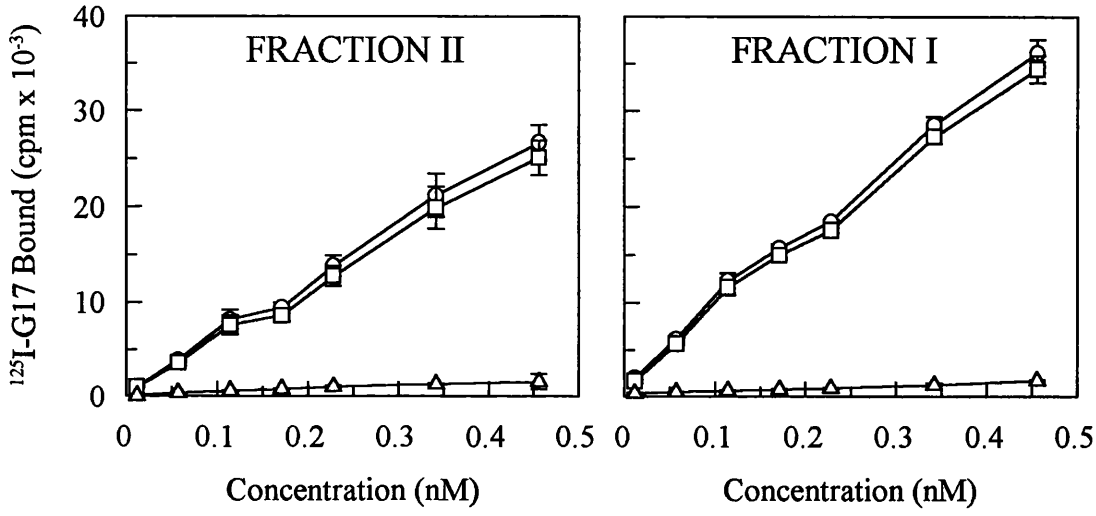

Figure 4.4 **Effect of increasing ^{125}I-G17 concentration on binding to AR42J membranes**

Membranes were incubated at 22°C for 180mins in the absence (-○-) or presence (-△-) of 1000 fold excess of unlabeled G17. Specific binding (-□-) was total binding (-○-) minus non-specific binding (-△-) and each point is the mean (+/-SD) of four experiments performed in duplicate.

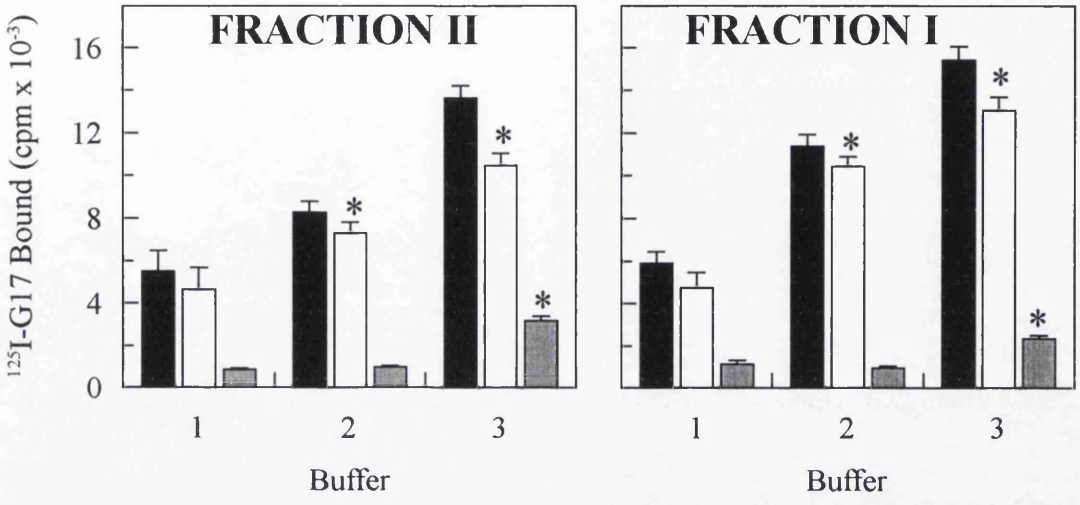

Figure 4.5 **Effect of protease inhibitors on ^{125}I-G17 binding to AR42J membranes**

AR42J membranes were incubated with different buffers for 180mins at 22°C. Each bar represents the mean (+SD) of total (■), specific (□) and non-specific (▩) binding from four experiments performed in duplicate.
* denotes the level of significance ($p<0.01$) when compared to corresponding binding with buffer 1. Buffers were as follows :

1 50mM Hepes + 10mM $MgCl_2.6H_20$ + 0.1% BSA
2 50mM Hepes + 10mM $MgCl_2.6H_20$ + 1uM SBTI + 0.1% BSA
3 50mM Hepes + 10mM $MgCl_2.6H_20$ + 1uM SBTI + 1uM Bestatin + 1uM bacitracin + 1mM aprotinin + 1.5mM DL-dithiothreitol + 1uM PMSF + 10% glycerol + 0.1% BSA

human membrane assay buffer (Chapters 2, section 2.5) increased binding by 20-30% in comparison with buffer 2. Although the human membrane assay buffer may have been the most appropriate to use in experiments involving AR42J membranes, the experiment including buffer 3 was performed at the end of the study. Therefore buffer 2 was used as the assay buffer for measurement of gastrin/CCK_B receptors on AR42J membranes.

3.1.5 Effect of pH

The AR42J membrane assay buffer (50mM Hepes, 10mM $MgCl_2.6H_2O$, 1uM soya bean trypsin inhibitor, 0.1% BSA) was prepared at pH 6.5, 7.0, 7.5 and 8.0. Optimum binding was obtained after 180mins at 22°C for both membrane fractions II and I at pH.6.5. Binding then steadily decreased to pH 8.0 (Figure 4.6). As there was no statistical difference between pH 6.5 and 7.0, to allow for comparison of results with the whole cells, the more physiological pH of 7.0 was chosen for subsequent experiments.

3.1.6 The effect of temperature on ^{125}I-G17 binding to AR42J membranes at steady state.

Specific binding was higher at 22°C than at 4°C or 37°C, similar to the results observed for whole cell preparations (Figure 4.7). Specific binding was proportionally greater in fraction I than in fraction II membranes, although both followed the same general pattern.

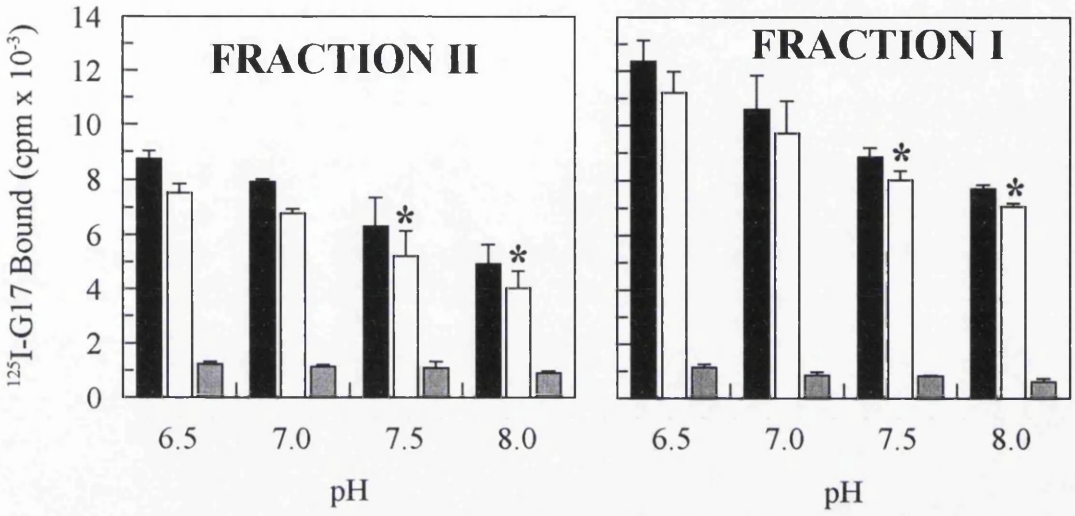

Figure 4.6 **Effect of buffer pH on ^{125}I-G17 binding to AR42J membranes**

Membranes were incubated in buffer (50mM Hepes, 10mM $MgCl_2.6H_20$, 1uM SBTI, 0.1% BSA) at pH 6.5-8.0 for 180mins at 22°C. Each bar represents the mean (+SD) total (■), specific (□) and non-specific (▩) binding from at least three experiments performed in duplicate.

* denotes level of significance ($p<0.01$) when compared to corresponding binding with pH 6.5.

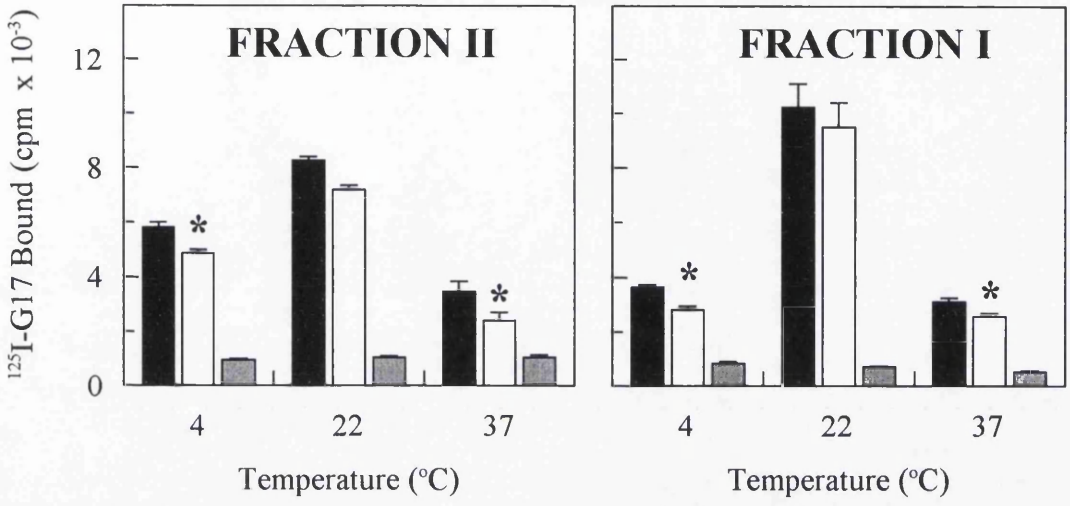

Figure 4.7 **Effect of different incubation temperatures on ^{125}I-G17 binding to AR42J membranes**

Membranes were incubated at temperatures of 4°C, 22°C and 37°C for 180mins and each bar represents the mean (+SD) total (■), specific (□) and non-specific (▩) binding from four experiments performed in duplicate.
* denotes level of significance ($p<0.01$) when compared to corresponding binding at 22°C.

3.2 Membrane storage

3.2.1 Effect of storage time and protective agents on fraction II AR42J membranes

Fraction II AR42J membranes were stored at -70°C in buffer containing either no protective agents, or with 10% glycerol (v/v) or 10% sucrose (w/v). Binding was analysed after storage at various time points over a period of fourteen days (Figure 4.8). On the day of membrane preparation (O), radioligand binding was measured at 22°C for 180mins in buffer without either glycerol or sucrose. This was used as the control. After freezing for 18 hours at -70°C (day1), membranes stored without glycerol or sucrose showed a sharp drop (40%) in specific binding of radiolabel, whereas the specific binding of membranes stored in glycerol or sucrose was not significantly decreased. Membranes stored without glycerol or sucrose formed a gelatin-like clot which may have contributed to reduced binding due to loss of protein.

Specific binding in membranes stored without protection decreased steadily from day one to day fourteen by which time only 15% of the original specific binding was retained. A significant decrease in binding with membranes stored in buffer with glycerol and sucrose occurred by day seven, when there was a sharp drop in specific binding to 50% of that of day one. Non-specific binding was significantly less using the buffer containing glycerol compared with sucrose and therefore subsequent experiments used membranes which had been stored for a maximum of two days at -70°C in buffer containing 10% glycerol.

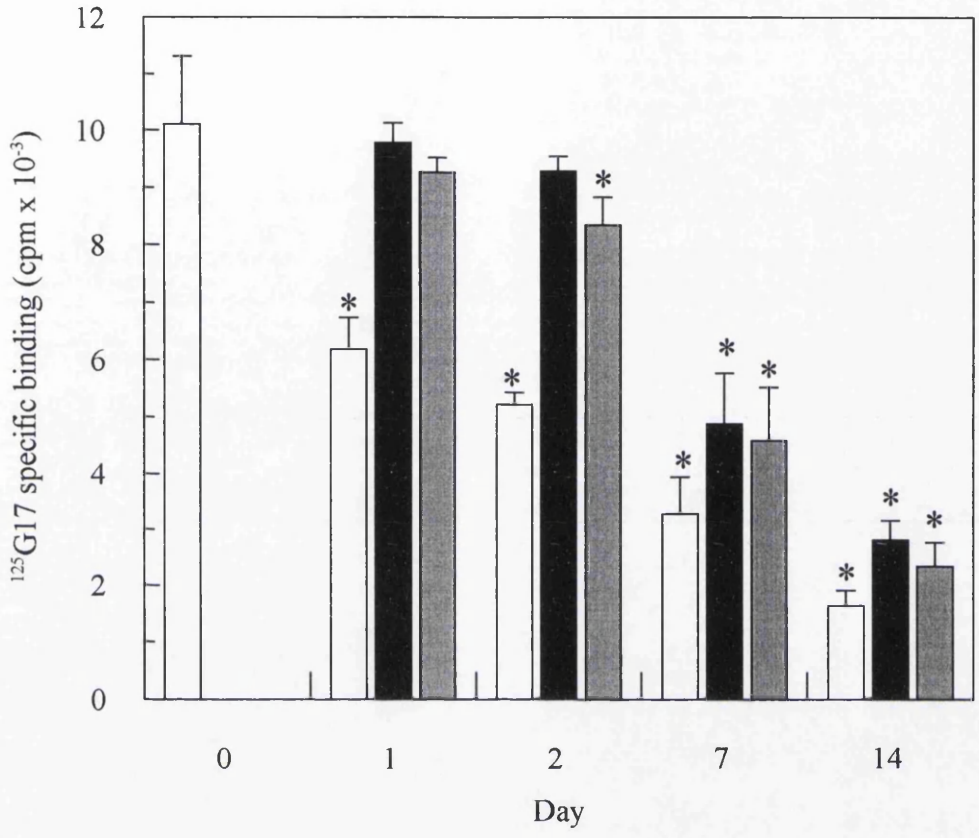

Figure 4.8 **Effect of storage time on ^{125}I-G17 binding to fraction II membrane with addition of either glycerol or sucrose**

Membranes (2mg/ml) were stored in aliquots at -70°C with and without buffer containing either 10% glycerol or 10% sucrose for a period of two weeks. Day of preparation was denoted (0). Each bar represents the mean (+SD) specific binding for buffer 1 (□), buffer 2 (■) and buffer 3 (▩) from three experiments performed in duplicate.

* denotes the level of significance ($p<0.01$) when compared to day zero. Buffers were as follows :

1 50mM Hepes + 10mM $MgCl_2.6H_20$ + 1uM SBTI + 0.1% BSA
2 50mM Hepes + 10mM $MgCl_2.6H_20$ + 1uM SBTI + 10% glycerol + 0.1% BSA
3 50mM Hepes + 10mM $MgCl_2.6H_20$ + 1uM SBTI + 10% sucrose + 0.1% BSA

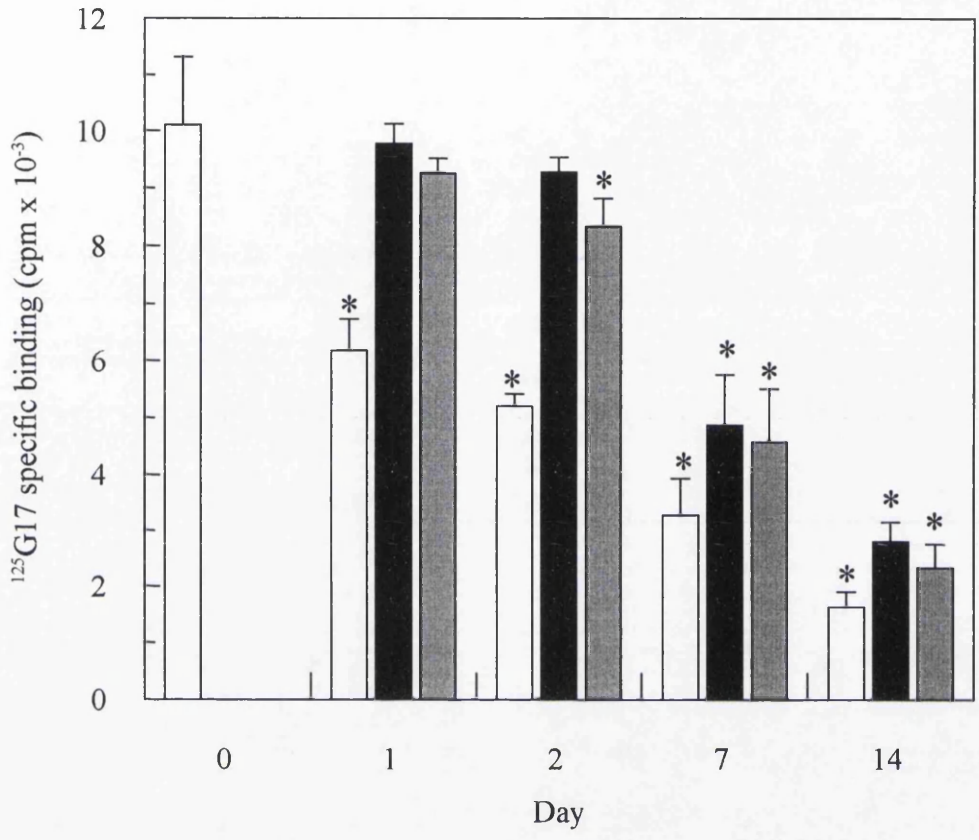

Figure 4.8 **Effect of storage time on ^{125}I-G17 binding to fraction II membrane with addition of either glycerol or sucrose**

Membranes (2mg/ml) were stored in aliquots at -70°C with and without buffer containing either 10% glycerol or 10% sucrose for a period of two weeks. Day of preparation was denoted (0). Each bar represents the mean (+SD) specific binding for buffer 1 (□), buffer 2 (■) and buffer 3 (▩) from three experiments performed in duplicate.

* denotes the level of significance ($p<0.01$) when compared to day zero. Buffers were as follows :

1 50mM Hepes + 10mM $MgCl_2.6H_20$ + 1uM SBTI + 0.1% BSA
2 50mM Hepes + 10mM $MgCl_2.6H_20$ + 1uM SBTI + 10% glycerol + 0.1% BSA
3 50mM Hepes + 10mM $MgCl_2.6H_20$ + 1uM SBTI + 10% sucrose + 0.1% BSA

3.2.2 Effect of storage temperature on ^{125}I-G17 binding to fraction II AR42J membranes

AR42J fraction II membranes were stored in buffer containing 10% glycerol as a protective agent at 4°C, -20°C and -70°C immediately after preparation. Binding performed at 24 hours showed specific binding to be significantly decreased at 4°C and -20°C but not at -70°C in comparison to the freshly prepared membranes analysed on the day of preparation (Figure 4.9). It was noted that storage at any temperature lowered non-specific binding. Subsequently membranes were stored at -70°C.

3.3 Competition assays

3.3.1 Effect of gastrin/CCK$_B$ receptor agonists on binding of ^{125}I-G17 to AR42J membranes

To assess the affinity of gastrin receptor agonists for the gastrin/CCK$_B$ receptor site, competition curves were analysed using a least squares curve fitting program (LIGAND). Inhibition of ^{125}I-G17 binding to AR42J membranes fraction II by G17, G34, CCK8S and CCK8 were statistically best fit by a single binding site model (Figure 4.10). Scatchard analysis of cold G17 displacement for fraction II membranes data revealed a single binding site with a K_D of 2nM and maximal capacity of 1160fmols/mg protein. The half maximal (IC_{50}) value for G17 displacement of ^{125}I-G17 from AR42J fraction II membranes was 1.6nM with CCK8S the most potent agonist with an IC_{50} of 0.3nM which was 20 fold higher potency than G17. The order of potency being CCK8S > G17 > G34 > CCK8 with IC_{50} values for G34 and CCK8 of 2nM and 3nM respectively (Table 4.1). Inhibition constants (Ki) were calculated and results are summarised in Table 4.2. The order of affinity for the gastrin/CCK$_B$ receptor on AR42J fraction II

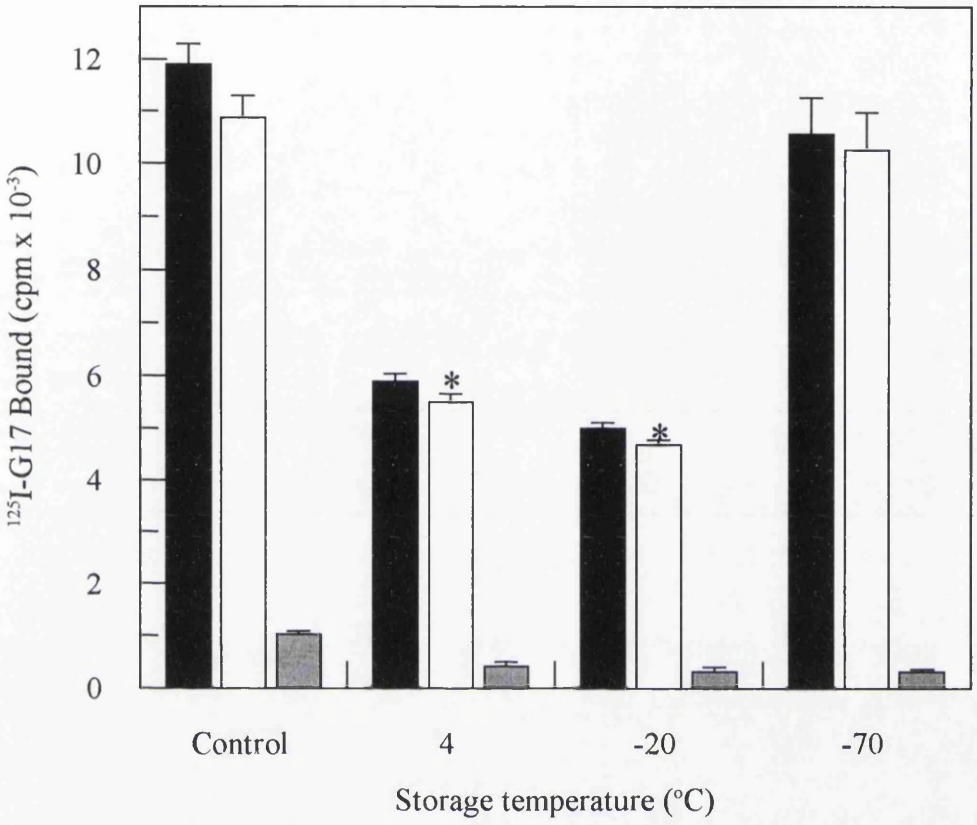

Figure 4.9 **Effect of storage temperature on ^{125}I-G17 binding to AR42J fraction II membranes**

Membranes were stored in 2mg/ml aliquots at 4°C, -20°C and -70°C in membrane buffer containing 10% glycerol. Binding of ^{125}I-G17 to membranes on the day of preparation prior to storage is denoted as control. Each bar represents the mean (+SD) of total (■), specific (□) and non-specific (▩) binding from four experiments performed in duplicate.

* denotes the level of significance ($P<0.01$) when compared to corresponding binding with control.

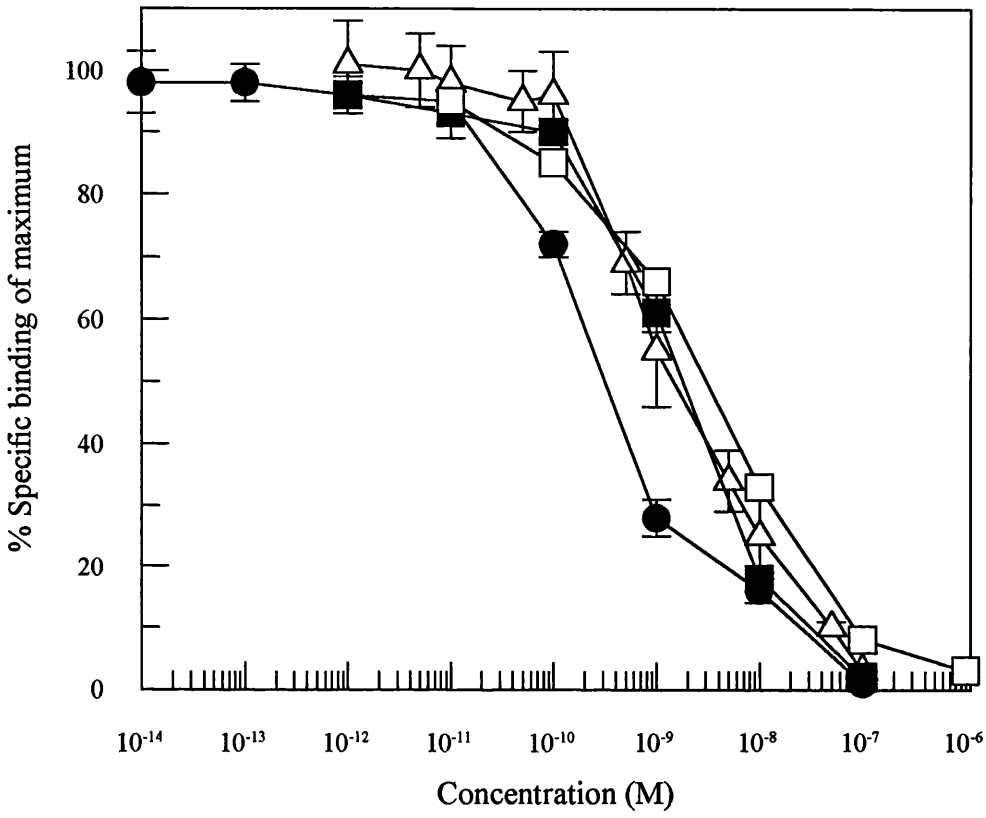

Figure 4.10 **Comparison of agonist displacement of ^{125}I-G17 binding to AR42J fraction II membranes**

Displacement of ^{125}I-G17 binding to AR42J membranes with increasing concentrations of agonists CCK8S (-●-), G17 (-△-), G34 (-■-) and CCK8 (-□-). Each point is the mean (+/-SD) of at least three experiments performed in duplicate.

CLASSIFICATION	LIGAND	IC_{50} FRACTION II (nM)	IC_{50} FRACTION I (nM)
AGONISTS	**CCK8S**	**0.3**	
	G17	**1.6**	**0.87**
	G34	**2.0**	
	CCK8	**3.0**	
ANTAGONISTS	**L740093**	**0.03**	
	CAM1028	**5.6**	
	L365260	**22**	**10**
	L364718	**202**	**131**

Table 4.1 **AR42J membrane half maximal (IC_{50}) data for the gastrin/CCK_B receptor**

CLASSIFICATION	LIGAND	K_D / Ki FRACTION II (nM)	K_D / Ki FRACTION I (nM)
AGONISTS	CCK8S	0.29	
	G17	2.0	1.0
	G34	1.67	
	CCK8	3.2	
ANTAGONISTS	L740093	0.029	
	CAM1028	5.5	
	L365260	22	9.7
	L364718	196	127

Table 4.2 **Gastrin/CCK_B receptor affinities from inhibition curves with receptor agonists/antagonists on AR42J membranes**

membranes was CCK8S > G34 > G17 > CCK8 with Ki/K_D values of 0.29nM, 1.67nM, 2nM and 3.2nM respectively. AR42J fraction I membranes were only incubated with increasing concentrations of G17 which gave an IC_{50} of 0.87nM. Scatchard analysis of cold G17 displacement data revealed a K_D of 1nM and maximal capacity of 720fmols/mg protein.

3.3.2 Effect of gastrin/CCK_B and CCK_A receptor antagonists on binding of ^{125}I-G17 to AR42J membranes

All antagonists inhibited ^{125}I-G17 binding (Figures 4.11 and 4.12) to fraction II membranes with the following order of potency, L740093 > L365260 > CAM1028 > L364718. The gastrin/CCK_B receptor antagonist L740093, showed half maximal inhibition at 0.03nM. The gastrin/CCK_B receptor antagonists CAM1028 and L365260 were 200 and 700 times less potent respectively than L740093 with respective IC_{50} values of 5.6nM and 22nM. The CCK_A receptor antagonist L364718 showed half maximal inhibition at 0.2uM. Similarly for fraction I membranes, the order of potency was L365260 > L364718 with IC_{50} values of 10nM and 0.13uM (Figure 4.13) and respective affinites (Ki) for the gastrin/CCK_B receptor of 9.7nM and 0.127uM respectively.

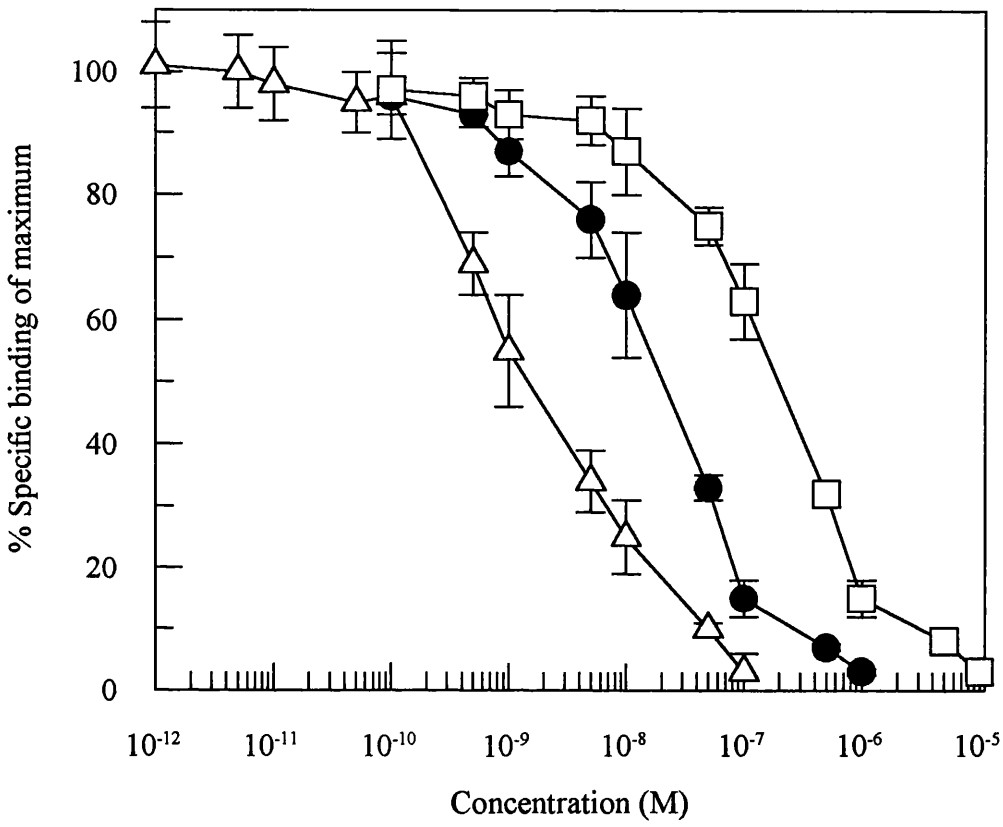

Figure 4.11 **Comparison of antagonists with G17 displacement of ^{125}I-G17 binding to AR42J fraction II membranes**

Displacement of ^{125}I-G17 binding to AR42J fraction II membranes with increasing concentrations of G17 (-△-), and CCK_B and CCK_A receptor antagonists L635260 (-●-), and L364718 (-□-) respectively. Each point is the mean (+/-SD) of four experiments performed in duplicate.

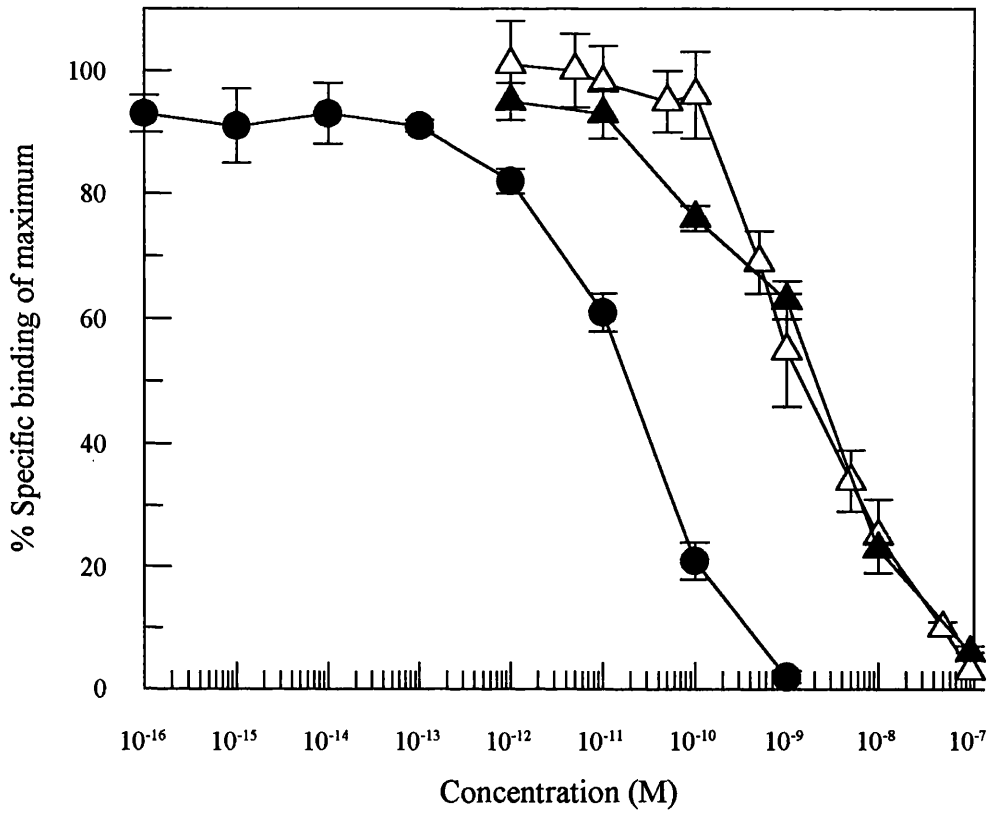

Figure 4.12 **Comparison of new antagonists with G17 displacement of ^{125}I-G17 binding to AR42J fraction II membranes**

Displacement of ^{125}I-G17 binding to AR42J membranes with increasing concentrations of G17 (-△-), and CCK_B receptor antagonists L740093 (-●-), and CAM1028 (-▲-). Each point is the mean (+/-SD), of at least three experiments performed in duplicate.

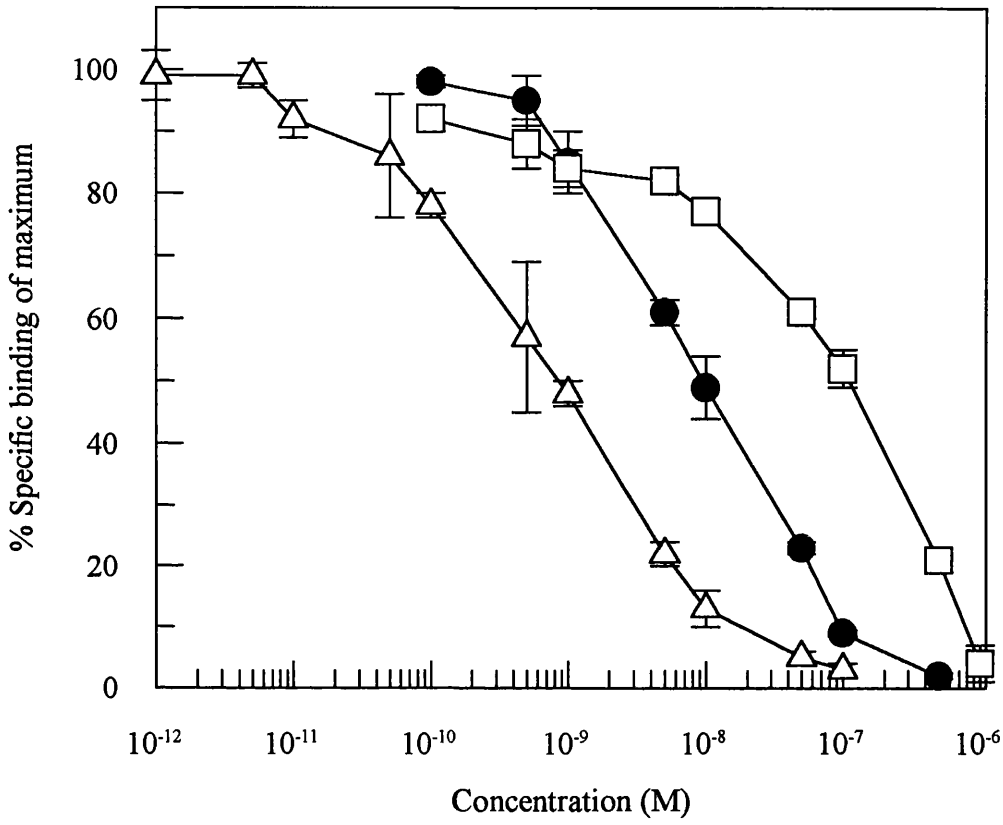

Figure 4.13 **Comparison of antagonists with G17 displacement of ^{125}I-G17 binding to AR42J fraction I membranes**

Displacement of ^{125}I-G17 binding to AR42J fraction I membranes with increasing concentrations of G17 (-△-), and CCK_B and CCK_A receptor antagonists L365260(-●-), and L364718 (-□-) respectively. Each point is the mean (+/-SD) of four experiments performed in duplicate.

4 DISCUSSION

The aim of this study was to assess whether gastrin receptors which had previously been measured and characterised on whole AR42J cells were in any way diminished or altered by the process of membrane preparation. Since tissue membrane preparation is known to be problematic, optimisation of the radioligand binding methodology for AR42J plasma membranes was of critical importance since the information obtained was to be used to aid the development of an assay capable of measuring and characterising gastrin/CCK_B receptors present on membranes prepared from human tissue.

Fraction I membranes were shown to have similar but not identical binding properties to that of fraction II membranes. Examination of these two fractions with gastrin receptor agonists and antagonists revealed similar but not identical affinities for the gastrin/CCK_B receptor thereby suggesting that fraction I membranes were perhaps from a different source or perhaps in a different state. It is possible that fraction I membrane receptors were from internal cell organelles derived from cells that had lysed during the thirty minute period prior to centrifugation and membrane preparation.

A higher concentration of fraction II membranes was required to reach the same level of specific binding as with fraction I membranes. As the protein content measures not only the gastrin receptor but also other membrane proteins in the membrane fragments prepared, it is possible that fraction I membranes were 'purer' than fraction II membranes. The reason for this could be that they were derived from different sources or that equivalent membranes contained more receptors. Scatchard analysis of cold G17 displacements demonstrates that the maximal binding capacity of fraction I membranes

was less than that of fraction II membranes by 440fmol/mg protein suggesting the former argument. It is also possible that the two fractions are of different receptor states or affinities and this is supported by AR42J membrane receptor affinity data.

Kinetic studies showed that fraction I membranes had a one and a half times faster association rate than fraction II membranes and consequently a faster dissociation rate. This again suggests a higher affinity receptor/receptor state. The dissociation half lives were similar for fraction II and fraction I membranes and were 31mins and 25mins respectively. This was much faster than observed with whole cells which had a half life of 55mins. This observation may be due to sequestering of the ligand in intact cells which would not be apparent with the membranes. Additionally, there may be a change in the conformational structure of the receptor on whole cells due to activation of second messenger systems.

As was observed with the whole cells, increasing radiolabel increased specific binding in a linear manner. Again no saturation of the membranes with ^{125}I-G17 was reached and so Scatchard analysis was not performed since extrapolation of the data is invalid and may yield inaccurate affinities and receptor densities.

Conditions for optimisation of the membrane assay were based on the results obtained during optimisation of the whole cell assay. Additional experiments were required to determine optimal conditions for storage of the plasma membranes once prepared. Since the nature of the preparation releases proteolytic enzymes which was not a problem with the whole cell assay, there was an added requirement for protease

inhibitors. The whole cell buffer was used as a control (Figure 4.5, buffer 1), from which to study addition of protease inhibitors. The most useful inhibitor in AR42J receptor assays was considered to be soya bean trypsin inhibitor, since the AR42J cells are pancreatic in origin and are known to secrete zymogen granules rich in trypsin and chymotrypsin. Addition of 1uM soya bean trypsin inhibitor increased specific binding for both membrane fractions by 60-70%, an increase which was not observed with AR42J whole cells (data not shown). This confirms that proteases' are active and released by cell disruption.

Membrane fractions II and I showed identical patterns of binding over the pH range 6.5-7.0 Maximum specific binding at was found at pH 6.5, similar to that of AR42J whole cells. Normal rat pancreatic CCK_A receptors have an acidic pH optimum of 5.5 for ^{125}I-CCK33 and a pH optimum of 6.0 for ^{125}I-CCK8 (Steigerwalt and Williams 1981). They noted that the change in pH did not alter the affinity of the CCK receptor but did increase the receptor density by increasing accessibility of receptors otherwise unavailable for binding.

Kinetics for the AR42J whole cells were previously shown to be optimal at a temperature of 22°C. Due to limited membrane availability, kinetics were not studied but binding was assessed at steady state (i.e.180mins). Significantly higher specific binding was observed at a temperature of 22°C than either 4°C or 37°C after 180mins, therefore subsequent experiments were performed at 22°C.

The abilities of agonists and antagonists to displace specific ^{125}I-G17 binding was compared with both membrane fractions and with whole cells as this is the most sensitive way to ensure receptor properties are maintained between whole cells and membrane preparations. Mathematical derivation of kinetic experiments produced similar dissociation constants for fraction II and I membranes of 0.12nM and 0.097nM respectively. Affinities derived from kinetic analysis of membrane fractions were six fold lower affinity than for gastrin/CCK_B receptors on AR42J whole cells (K_D of 0.02nM).

Fraction II and I membranes were displaced by the natural agonist G17, the CCK_B receptor antagonist L365260 and CCK_A receptor antagonist L364718. The respective affinities for G17 were slightly higher with fraction I membranes which had a K_D of 1nM compared to fraction II membranes which had a K_D of 2nM. The affinities calculated from displacement and kinetic experiments should correspond, but as has been shown values are significantly different between the two analysis. Affinity for gastrin binding to AR42J whole cells (K_D of 0.3nM) was up to six times greater than binding to AR42J fraction II and I membranes. The CCK_B receptor antagonist L365260 displaced specific ^{125}I-G17 binding to fraction II membranes with a half maximal concentration of 22nM in comparison to 10nM and 4.7nM for fraction I membranes and AR42J cells respectively. The CCK_A receptor antagonist L364718 displaced specific ^{125}I-G17 binding to fraction II membranes with a half maximal concentration of 202nM in comparison to 131nM and 230nM for fraction I membranes and AR42J cells respectively. The IC_{50} values were corrected using the Cheng-Prusoff equation and affinity inhibition constants are summarised in Table 4.2.

Fraction II membranes were further characterised with competition assays using the agonists CCK8S, CCK8 and G34 and the new antagonists CAM1028 and L740093. The order of potency of the ligands was similar to that found for AR42J whole cells, L740093 > CCK8S > G17 > G34 > CCK8 > CAM1028 > L365260 > L364718. Half maximal values for each ligand tested were found to be similar to those from AR42J whole cells. Sulphation of CCK8 increased its affinity for the gastrin/CCK_B receptor seven fold in comparison to desulphated G17 and six fold in comparison with desulphated CCK8. The same order of potency was reported by Huang and colleagues, with the addition of sulphated G17(G17II) which demonstrated a higher affinity than CCK8S, G17 and CCK8 for the gastrin/CCK_B receptor on guinea-pig pancreatic acini (Huang *et al* 1989).

In summary, this chapter has demonstrated that gastrin/CCK_B receptors are measurable on plasma membranes prepared from AR42J whole cells. The membrane fractions retain the broad characteristics and properties of the gastrin/CCK_B receptors on whole cells. Freezing of the membranes does not alter this after storage at -70°C in the presence of glycerol for short periods of time only (i.e. < 2 days). This data provides the ground work on which to develop an assay to detect and characterise membranes prepared from fresh and frozen human colonic and gastric, tumour and normal tissues.

CHAPTER 5 GASTRIN/CCK$_B$ RECEPTORS IN NORMAL AND NEOPLASTIC COLORECTAL TISSUES

1 INTRODUCTION

Gastrointestinal cancers have been shown to respond trophically *in vitro* and *in vivo* to gastrin, and this action is most likely to be mediated through binding to gastrin/CCK_B receptors.

The first demonstration of gastrin/CCK_B receptors on colonic cells was in 1985 (Singh *et al* 1985) when high affinity gastrin/CCK_B receptors (K_D of 0.4-0.55nM) were found on crude membrane fractions from normal rat colonic mucosa. High affinity receptors (K_D of 0.25-0.6nM) were found to be present on membranes from one human (LoVo) and one murine (MC-26) colon cancer cell line whereas another human colon cancer cell line, HT29 showed little specific binding (Singh *et al* 1985). In contrast to the report by Singh *et al*, specific gastrin binding to the human colorectal cell line, HT29 was demonstrated by McRae *et al* in 1986. The cell line bound ^{125}I-G17 and ^{3}H pentagastrin when cells were grown in serum free medium and pentagastrin stimulated growth of these cells.

Recently, Frucht *et al* (1992) observed gastrin/CCK_B receptor expression in only one out of ten human colon cancer cell lines analysed. Six of these cell lines were derived from secondary instead of primary tumours and only one concentration of G17 was used in the binding study (100pM). Also CCK binding was observed in three out of ten lines but as competition assays with CCK receptor antagonists were not performed it is unclear whether the binding represented CCK_A or gastrin/CCK_B receptors. This low gastrin/CCK_B receptor expression in human tumour cell lines may be due to methodological problems, in particular the length of time in culture. Watson *et al*,

(1989b) found that human colorectal cell lines lost their gastrin responsiveness when passaged more than 250 times. This was assessed by tritiated thymidine uptake. This suggests that the presence of the gastrin/CCK_B receptor could be dynamic and may alter after increased passage times.

Eggstein *et al* (1991) showed that growth of the human colonic cell line SW403 was increased by gastrin *in vitro*, and this effect was reversed by the gastrin/CCK_B receptor antagonist benzotript. When SW403 cells were grown as solid tumours in nude mice, pentagastrin stimulated both proliferation and ornithine decarboxylase (ODC) activity *in vivo*. Stimulation of growth in SW403 cells by gastrin was accompanied by an increase in ODC activity which was blocked by DMFO, an irreversible inhibitor of ODC. However, epidermal growth factor induced proliferation did not increase ODC activity. In contrast, a previous report by Seidel *et al* (1985) failed to show ODC stimulation by gastrin in the normal rat mucosa. This may be due to differences between normal and malignant tissue and/or species differences.

While reports have demonstrated the *in vitro* gastrin responsiveness of fresh human tumour tissue (Watson *et al* 1989b) and used this as a marker of gastrin/CCK_B receptor status, very few have directly demonstrated specific high affinity gastrin/CCK_B binding sites. The first study to demonstrate specific gastrin/CCK_B receptors on primary colon cancer tissue was by Rae-Venter *et al* in 1980. Membranes were partially purified, by discontinuous sucrose gradients, from eight human colon cancer biopsies and three normal colonic mucosa. Membranes were assayed in the presence of 100pM ^{125}I-G17 at 20°C for two hours. Non-specific binding was defined as binding of ^{125}I-G17 in the

presence of excess unlabeled G17. Seven out of eight (87.5%) colon tumours expressed gastrin/CCK_B receptors with a K_D 0.4-0.6nM and receptor density of 0.5-1.3fmols/mg protein. Competition studies were performed with pentagastrin, CCK8 and caerulein, a CCK-like peptide all competed for gastrin/CCK_B receptor binding sites

The same group went on to publish a more detailed study in 1989, in which freshly resected colorectal tumours and healthy normal mucosa were examined for gastrin/CCK_B receptor status (Upp *et al* 1989). Gastrin binding assays were performed on crude membranes prepared from frozen sections. They found thirty-eight of the sixty-seven (56.7%) cancers had high affinity receptors (K_D < 1nM) and seven with low affinity receptors (K_D >1nM). Positive tumours exhibited only a single class of high affinity sites with K_D's of 0.1-0.3nM and gastrin/CCK_B receptor contents between 1.5-50fmol/mg membrane protein. Twenty of the thirty-eight people with gastrin/CCK_B receptor positive tumours had receptor densities above 10fmol/mg of protein. No correlation between gastrin/CCK_B receptor content and patient age, sex, serum CEA concentration or degree of differentiation was found. The mean receptor density of Dukes' A or B tumours was twice that of Dukes' stage C or D lesions. The content of gastrin receptors in normal colon mucosa was determined in fifty-nine out of the sixty-seven patients studied. Similar high affinity gastrin/CCK_B receptors to the colon cancers were found in the normal mucosa in thirty-two patients and low affinity in nine other patients. A further twenty-two normal samples had no detectable gastrin/CCK_B receptors. There was a highly significant correlation between the presence of gastrin/CCK_B receptors on normal mucosa and corresponding tumours. Other studies by this group have also identified high affinity gastrin/CCK_B receptors in membranes

prepared from freshly resected human colorectal tumours (Upp *et al* 1989; Chicone *et al* 1989).

In contrast, the only negative report to date was by Kumamoto *et al* in 1989 who reported high affinity gastrin/CCK_B receptors in human fundic mucosa (K_D of 1.6nM and receptor density of 15fmol/mg protein) but were unable to demonstrate significant gastrin/CCK_B receptors in normal colonic mucosa of two patients undergoing surgery for colorectal cancer.

1.1 Objective

The object of this study was to assess whether membranes prepared from human colorectal cancers expressed measurable high affinity gastrin/CCK_B receptors using a radioligand binding assay. This assay had been used to measure high affinity gastrin/CCK_B receptors in AR42J whole cells and crude plasma membrane preparations. Therefore it was assumed that if gastrin/CCK_B receptors were present on colonic tumour and normal tissue, this assay should permit their detection.

2 METHODS

Materials and general methods can be found in Chapter 2.

2.1 Statistical analysis

In each case, a hypothesis test was performed to determine whether there was evidence to reject the hypothesis that the data were normally distributed. This was based on the correlation between the observations and their 'normal scores' (Minitab Reference Manual 1994).

If there was no evidence to reject the hypothesis that the data were normally distributed then the mean values in all groups were compared using a two sample t-test. If there was evidence to reject the hypothesis of normality then the median values in each group were compared using the non-parametric Mann Whitney test. Statistical significance was set at $p < 0.05$.

3 RESULTS

3.1 Patient Data

The study involved thirty-three patients who presented with primary colonic carcinoma. Eleven patients had tumours in either the caecum or ascending colon (Table 5.1, page1). The remaining twenty-two patients had primary tumours in either the distal colon or rectum (Table 5.1, page 2 and 3). All tumours were histologically defined as adenocarcinoma with varying levels of de-differentiation and categorised according to the Dukes' stage of cell invasion.

Initials	Age	Sex	Tumour site	Histology	Different -iation	Dukes' stage
D.H.	83	F	caecum	adenocarcinoma	poor	C
G.McK	71	M	caecum	adenocarcinoma	poor	D
J.B.	84	M	caecum	adenocarcinoma	moderate	B
R.H.	79	M	caecum	mucinous adenocarcinoma	moderate	B
R.P.	86	M	caecum	adenocarcinoma	moderate	B
R.S.	68	M	caecum	adenocarcinoma	moderate	B
P.G.	74	M	caecum	adenocarcinoma	moderate	C
M.R.	75	F	caecum	adenocarcinoma	well	B
F.M.	74	F	ascending	adenocarcinoma	moderate	B
M.Cl.	62	M	ascending	adenocarcinoma	moderate	C
J.Ca.	66	M	ascending	adenocarcinoma	well	B

Table 5.1 **Colorectal patient details** (page one of three)

Initials	Age	Sex	Tumour site	Histology	Different -iation	Dukes' stage
I.E.	46	M	descending	adenocarcinoma	moderate	B
I.D.	67	F	sigmoid	adenocarcinoma	poor	C
P.S.	56	F	sigmoid	adenocarcinoma	poor	C
E.L.	56	F	sigmoid	adenocarcinoma	poor	D
H.B.	66	M	sigmoid	adenocarcinoma	poor	D
J.Co.	70	M	sigmoid	adenocarcinoma	moderate	B
J.M.	63	M	sigmoid	adenocarcinoma	moderate	B
T.T.	87	M	sigmoid	adenocarcinoma	moderate	B
B.T.	44	M	sigmoid	adenocarcinoma	moderate	C
A.B.	84	F	rectosigmoid	adenocarcinoma	moderate	C
M.Mcl.	48	M	rectosigmoid	adenocarcinoma	moderate	C

Table 5.1 (continued) **Colorectal patient details** (page two of three)

Initials	Age	Sex	Tumour site	Histology	Different -iation	Dukes' stage
I.M.	64	F	rectum	adenocarcinoma	poor	D
J.H.	47	M	rectum	adenocarcinoma	moderate	A
G.A.	67	F	rectum	adenocarcinoma	moderate	B
J.A.	70	M	rectum	adenocarcinoma	moderate	B
M.Y.	71	F	rectum	adenocarcinoma	moderate	B
T.W.	63	M	rectum	adenocarcinoma	moderate	B
J.Cum.	46	M	rectum	adenocarcinoma	moderate	C
J.K.	67	M	rectum	adenocarcinoma	moderate	C
L.H.	58	M	rectum	adenocarcinoma	moderate	C
A.Cr.	53	F	rectum	adenocarcinoma	moderate	D
M.Mor.	56	M	rectum	adenocarcinoma	moderate	D

Table 5.1 (continued) **Colorectal patient details** (page three of three)

3.2 Comparison of different methods of membrane preparation

3.2.1 Frozen tissues

(i) Cryostat method

Three patient samples, taken at random, were tested using the cryostat method for preparing crude membranes (Appendix III, table 1). Tissue sections of 20um were sliced from frozen tissues using a cryostat, followed by subsequent membrane preparation as described in Chapter 2, section 3.5. The patient tissues tested using this method were G.McK., P.S. and M.McL. Radioligand binding was examined using increasing ^{125}I-G17 concentrations of 0.1, 0.25 and 0.5nM (final concentration in the tube). The results showed little or no specific binding with either the normal membranes or the tumour membranes (Figures 5.1 and 5.2). For example at 0.5nM ^{125}I-G17 concentration, M.Mcl. tumour membranes gave 11% specific binding that was still only a fractional 0.06% specific binding of total added. In most cases there was insufficient tissue to allow multipoint saturation curves to be performed and therefore the figures are shown as histograms to emphasise that most of the binding was non-specific in nature. With the exception of patient P.S. normal tissue, total binding in both normal and tumour tissue increased linearly with increasing radiolabel as did non-specific binding. Relatively high specific binding (45%) was observed with patient P.S. normal membranes (Figure 5.1) at a concentration of 0.1uM ^{125}I-G17, but specific binding was not observed at higher ^{125}I-G17 concentrations. In each patient, comparison of normal and tumour total binding, it was noted that total binding in the tumour was two to three fold greater than the total binding in normal membranes.

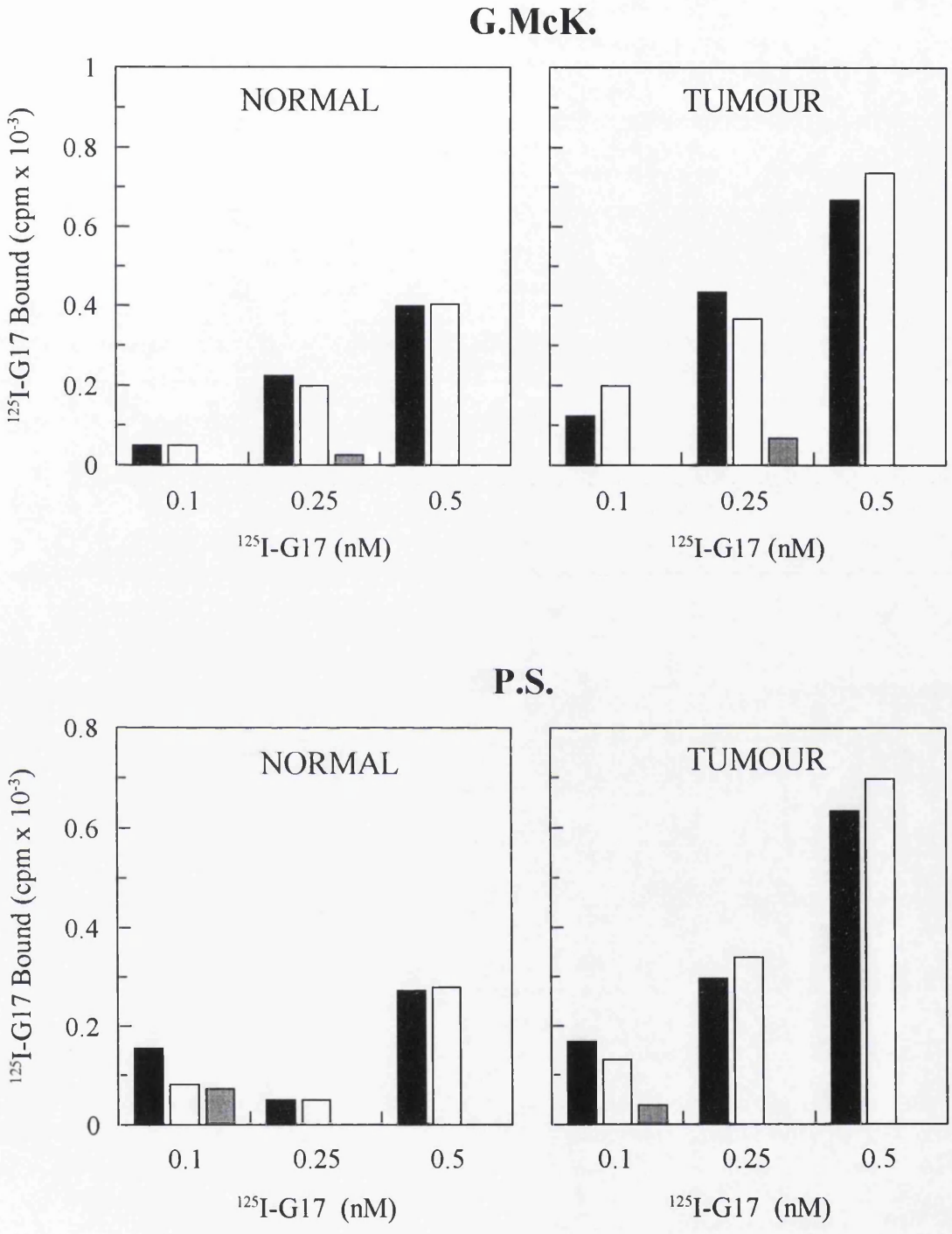

Figure 5.1 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to normal and tumour membranes of patients G.McK. and P.S. Specific binding (▩) is total (■) minus non-specific binding (□).**

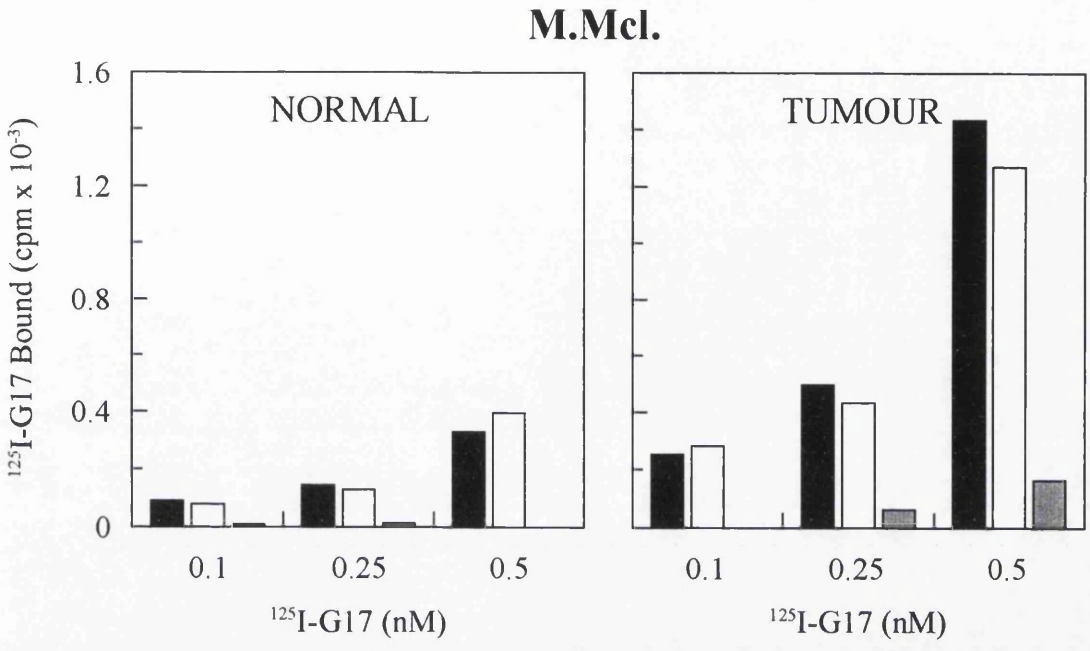

Figure 5.2 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to normal and tumour membranes of patient M.Mcl. Specific binding (▩) is total (■) minus non-specific binding (□).**

(ii) Pulverised method

Tissue samples from twenty-two patients (Appendix III, table 2, pages 1 to 4) were analysed following preparation of membranes by the pulverised method as described in Chapter 2, section 3.5. All patient tissues were immediately snap frozen in liquid nitrogen. These tissues had not been previously immersed in the human collection buffer (Chapter 2, section 2.5) and had been stored at -70°C for varying periods of time up to three years. Six patients were tested with increasing ^{125}I-G17 concentrations between 0.1 and 0.5nM (Figures 5.3 to 5.5), while a further sixteen patients were screened using 0.125nM ^{125}I-G17 (Figures 5.6 and 5.7) only due to the expense of the radiolabel and in some cases lack of tissue. In general, total binding was found to be higher than was observed with membranes prepared using the cryostat method. Specific binding was no greater than 50% of total binding and 0.007% of total added. Total binding varied greatly between different patients for both normal and tumour membranes. In all but one (T.W.) of the twenty-two patients studied by this method, there was a two to three fold increase in total binding between normal and tumour total binding.

3.2.2 Fresh tissues

(i) Homogenisation method

Tumour and normal tissue samples from eight patients (Appendix III, table 3, pages 1 to 3) were immersed in the human collection buffer (Chapter 2, section 2.5), immediately upon resection. Membranes were prepared as quickly as possible (generally within 30mins of resection) by the homogenisation method as described in Chapter 2, section 3.6. In general total binding was found to be increased in comparison to the previous membrane preparation methods of cryostat sectioning and pulverisation

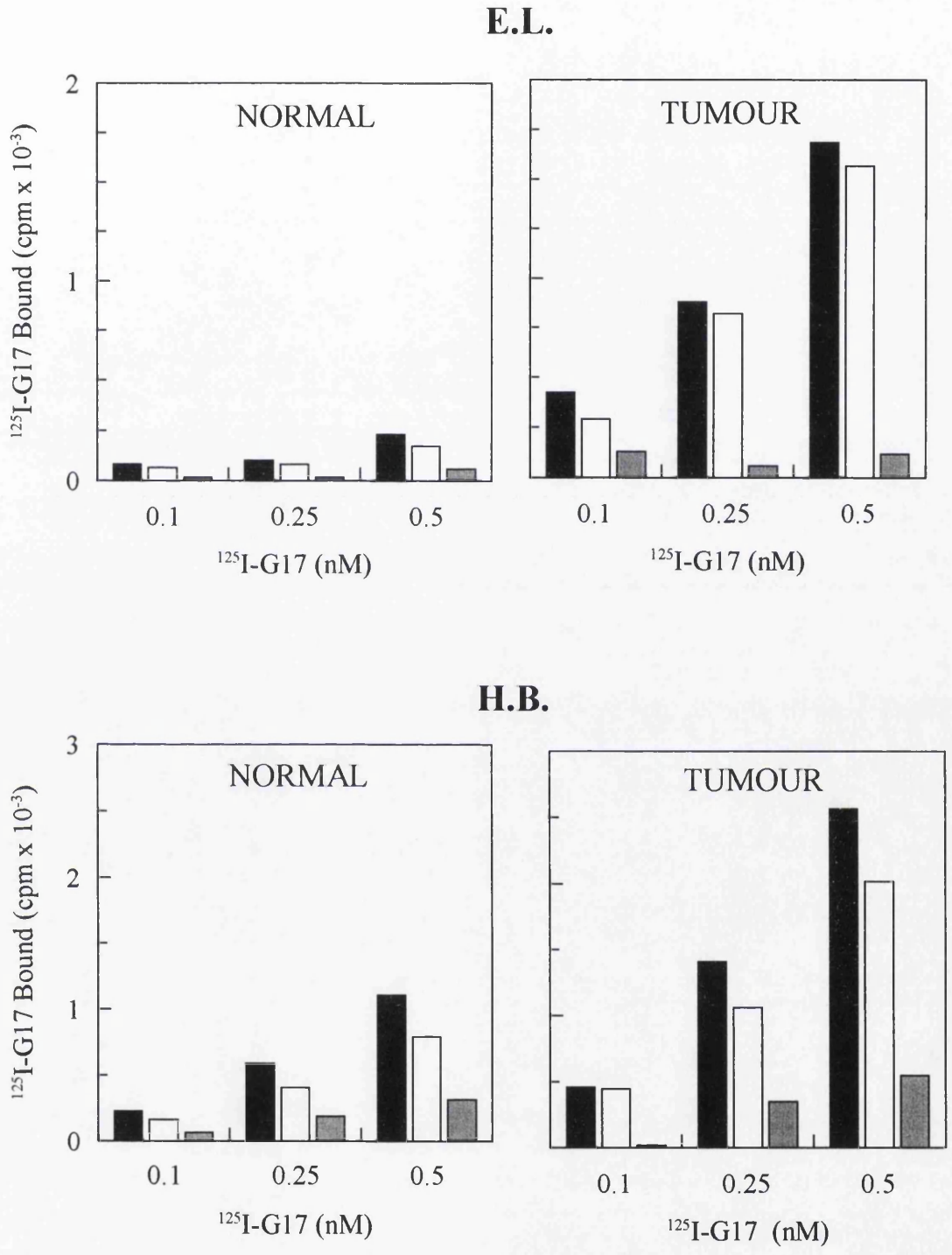

Figure 5.3 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to normal and tumour membranes of patients E.L. and H.B. Specific binding (▩) is total (■) minus non-specific binding (□).**

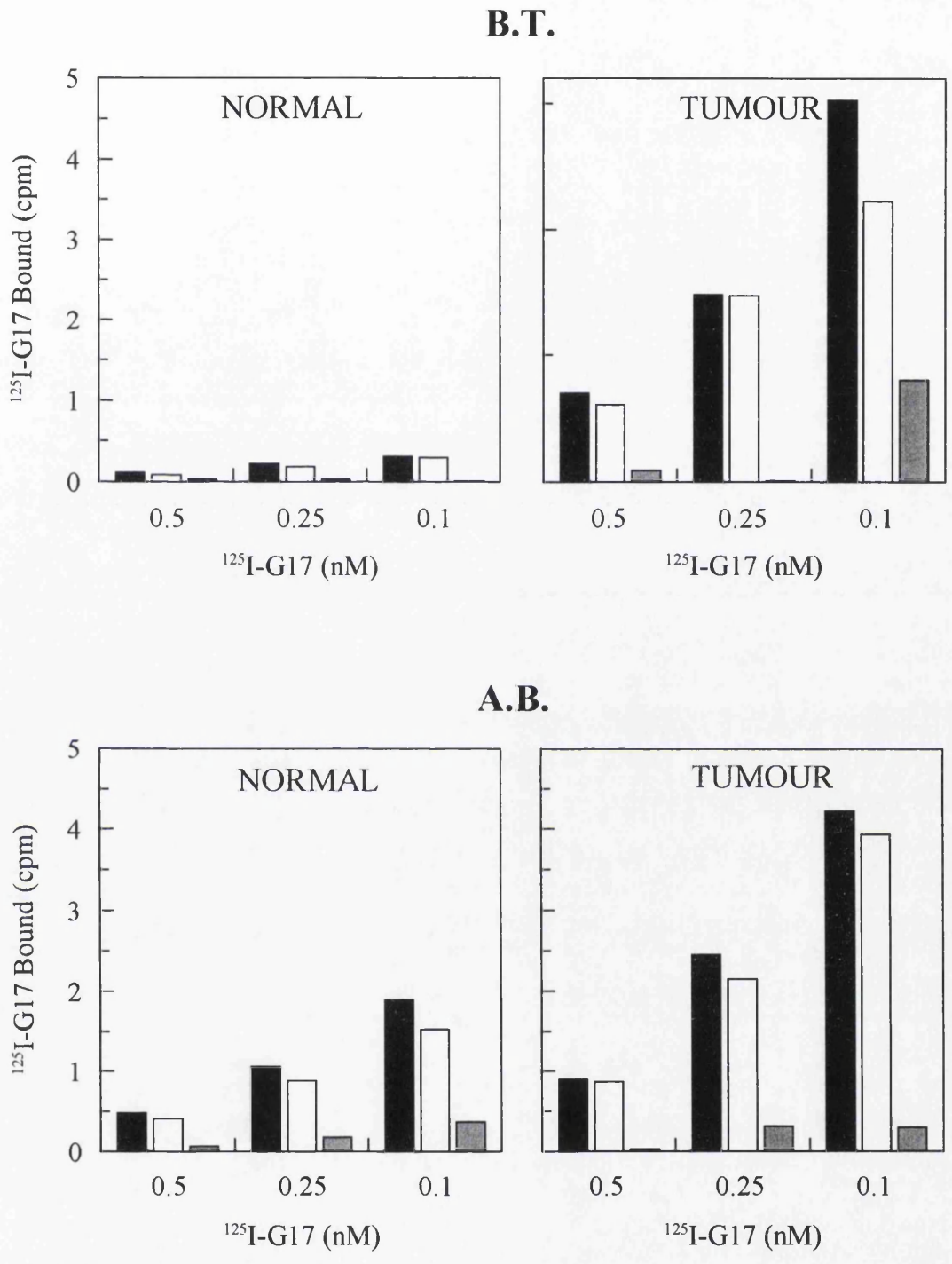

Figure 5.4 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to normal and tumour membranes of patients B.T. and A.B. Specific binding (▨) is total (■) minus non-specific binding (□).**

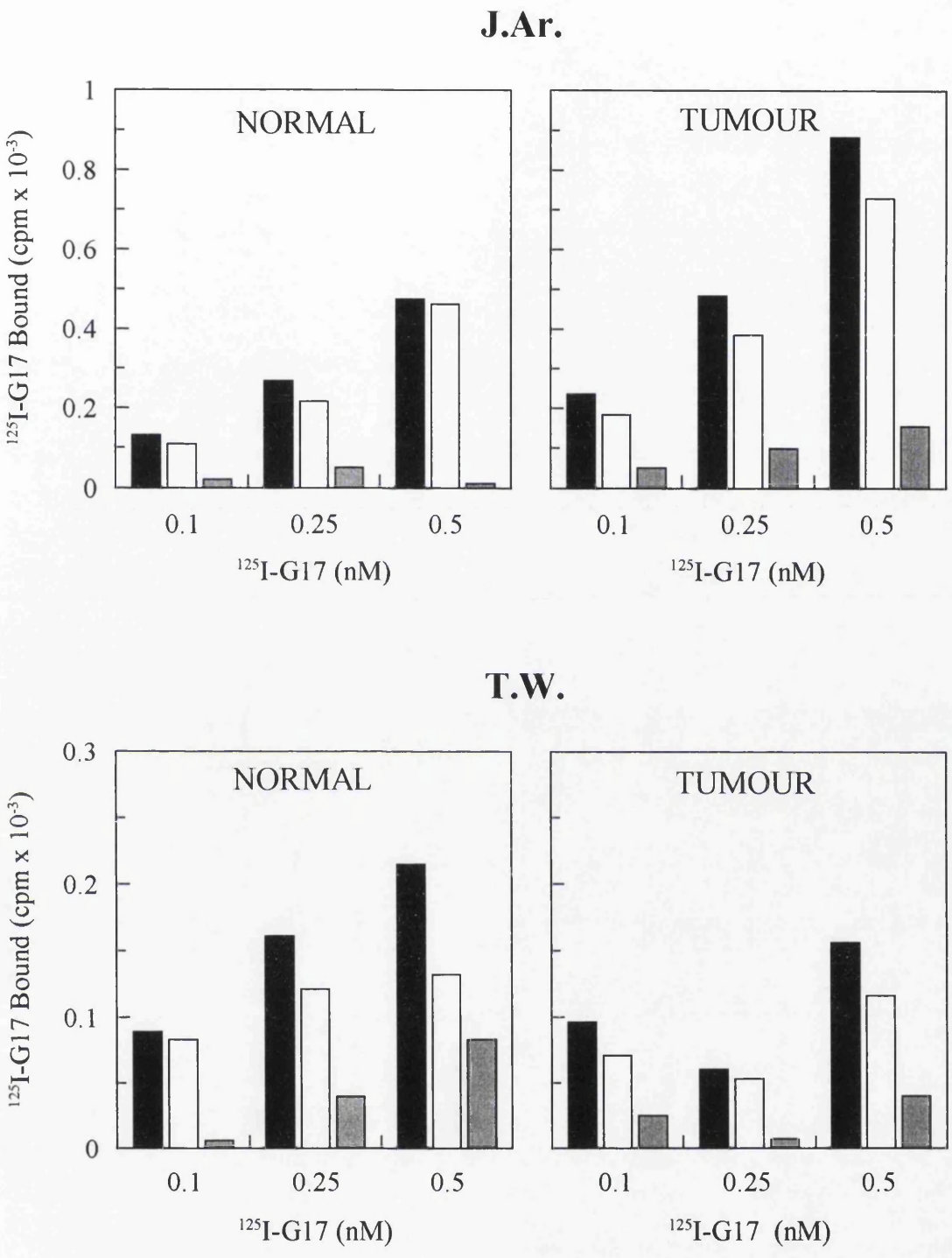

Figure 5.5 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to normal and tumour membranes of patients J.Ar. and T.W. Specific binding (▩) is total (■) minus non-specific binding (□).**

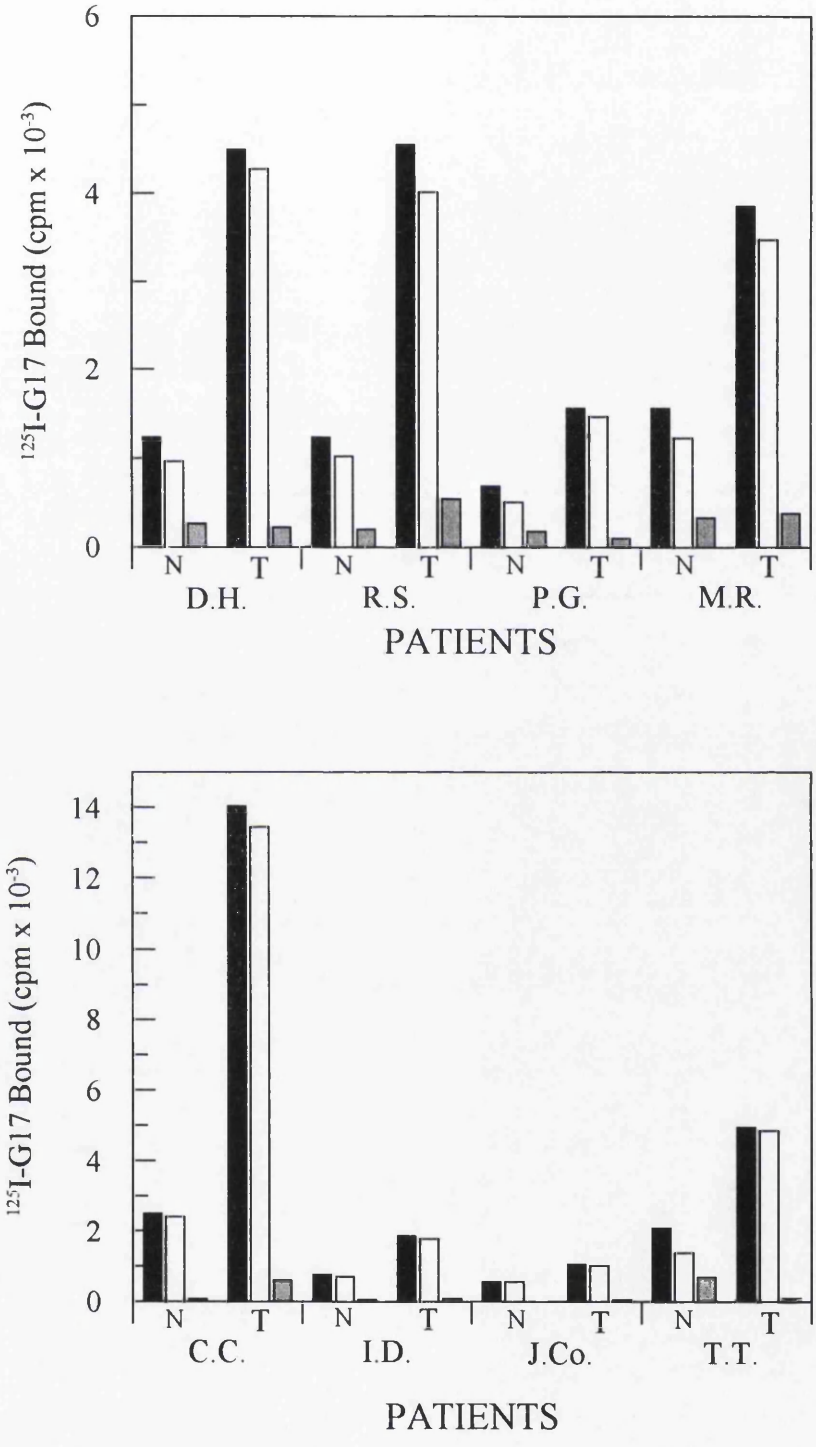

Figure 5.6 **Effect of radiolabel concentration (0.125nM) on ^{125}I-G17 binding to normal (N) and tumour (T) membranes of patients D.H., R.S., P.G., M.R., C.C., I.D., J.Co., and T.T. Specific binding (▒) is total (■) minus non-specific binding (□).**

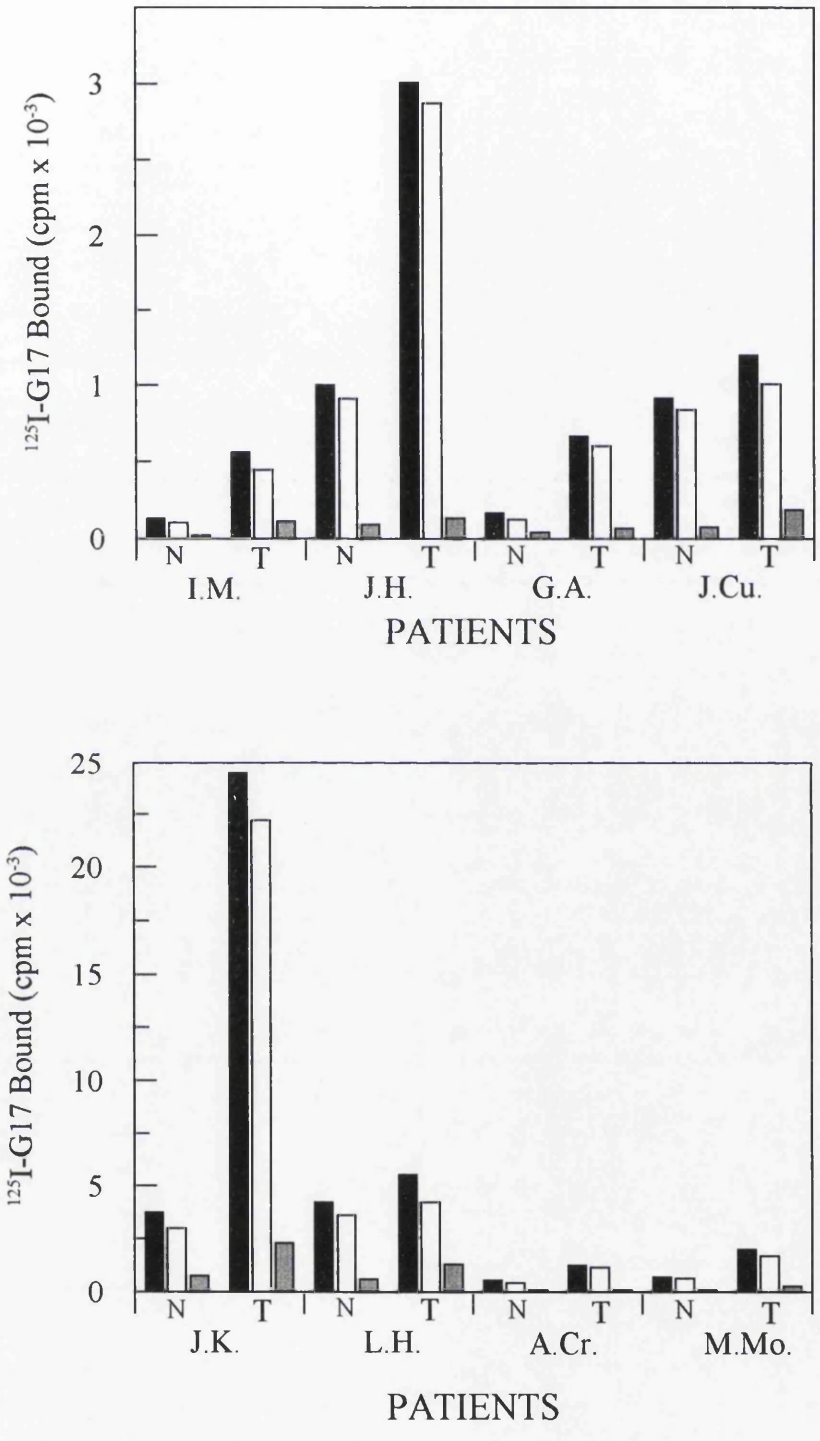

Figure 5.7 **Effect of radiolabel concentration (0.125nM) on ^{125}I-G17 binding to normal (N) and tumour (T) membranes of patients I.M., J.H., G.A., J.Cu., J.K., L.H., A.Cr. and M.Mo. Specific binding (▩) is total (■) minus non-specific binding (□).**

(Figures 5.8-5.12). Increasing ^{125}I-G17 concentrations were used to analyse all of the patients normal and tumour membranes and although specific binding was more consistent and reproducible than the cryostat method , binding still only reached 0.45% of the radioactivity added. Displacement of specific binding to tumour or normal membranes from patients R.P., M.Cl., M.Y., J.M. and I.E. were unsuccessful at a radiolabel concentration of 0.5nM and increasing unlabeled G17 concentrations between 10^{-12}-10^{-5}M (data not shown).

3.3 Comparison of gastrin/CCK$_B$ receptor status with tumour site, differentiation and Dukes' stage

Tumour site, differentiation and Dukes' stage were compared using total binding and the ratio of tumour/normal total binding from thirty-two patients. Total binding in tumour membranes was consistently two to three fold higher than the corresponding normal membranes. Total ^{125}I-G17 bound in all three groups was not normally distributed (see methods) and so non-parametric Mann Whitney tests were used to analyse the data. In contrast, the ratio of tumour/normal ^{125}I-G17 total bound in all three groups were normally distributed and therefore data was analysed using a Two-sample t-test.

Eleven patient tumours were situated in the left colon and twenty-one tumours in the right colon. Median levels for ^{125}I-G17 total bound (Figure 5.13, graph A) in the left colon were not significantly different from levels in the right colon ($p = 0.2042$, Mann Whitney). The mean levels for the ratio of tumour/normal ^{125}I-G17 total bound (Figure 5.13, graph B) in the left colon were not significantly different from the mean total binding in the right colon ($p = 0.3134$, Two sample t-test).

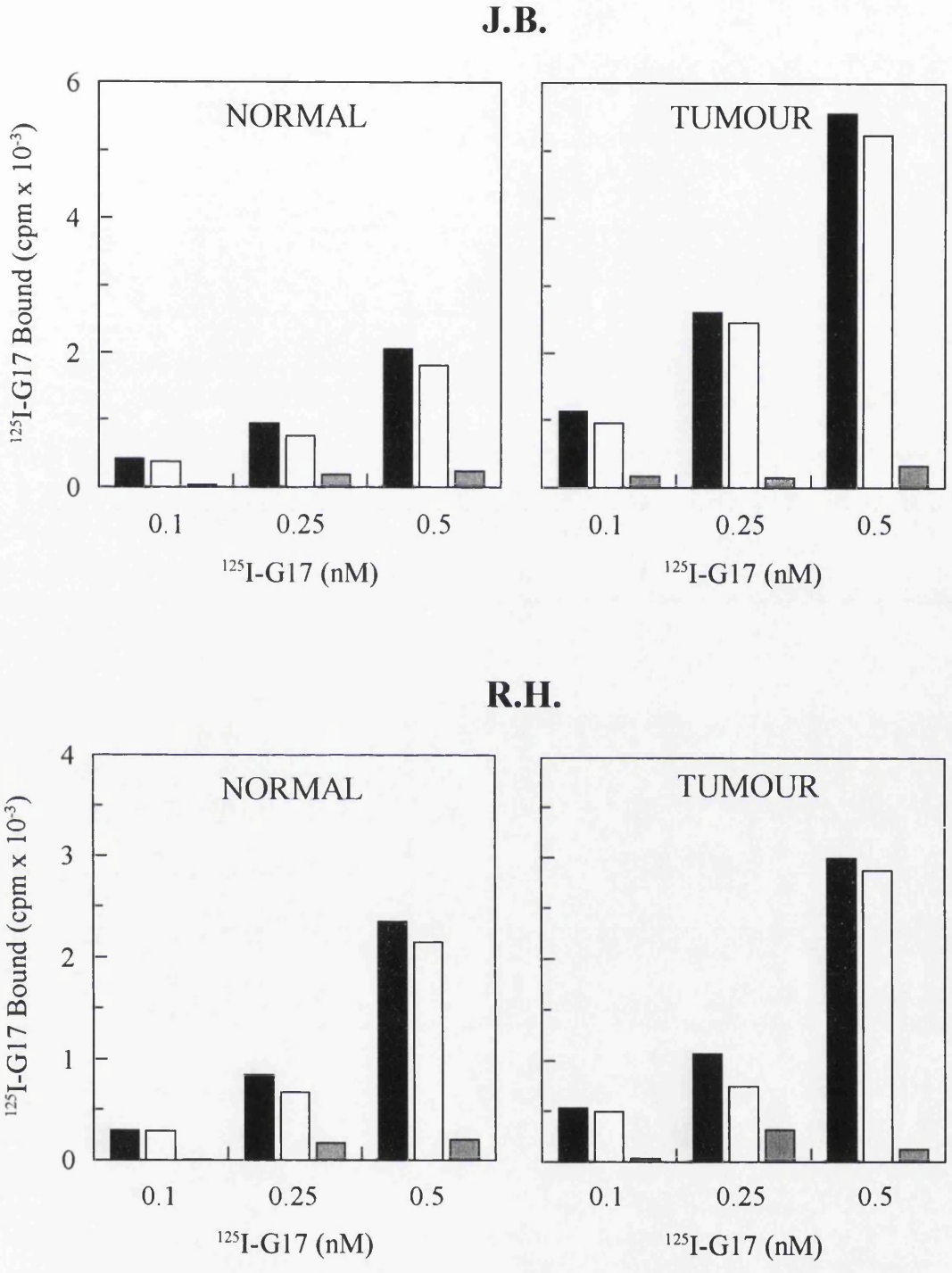

Figure 5.8 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to normal and tumour membranes of patients J.B. and R.H. Specific binding (▨) is total (■) minus non-specific binding (□).**

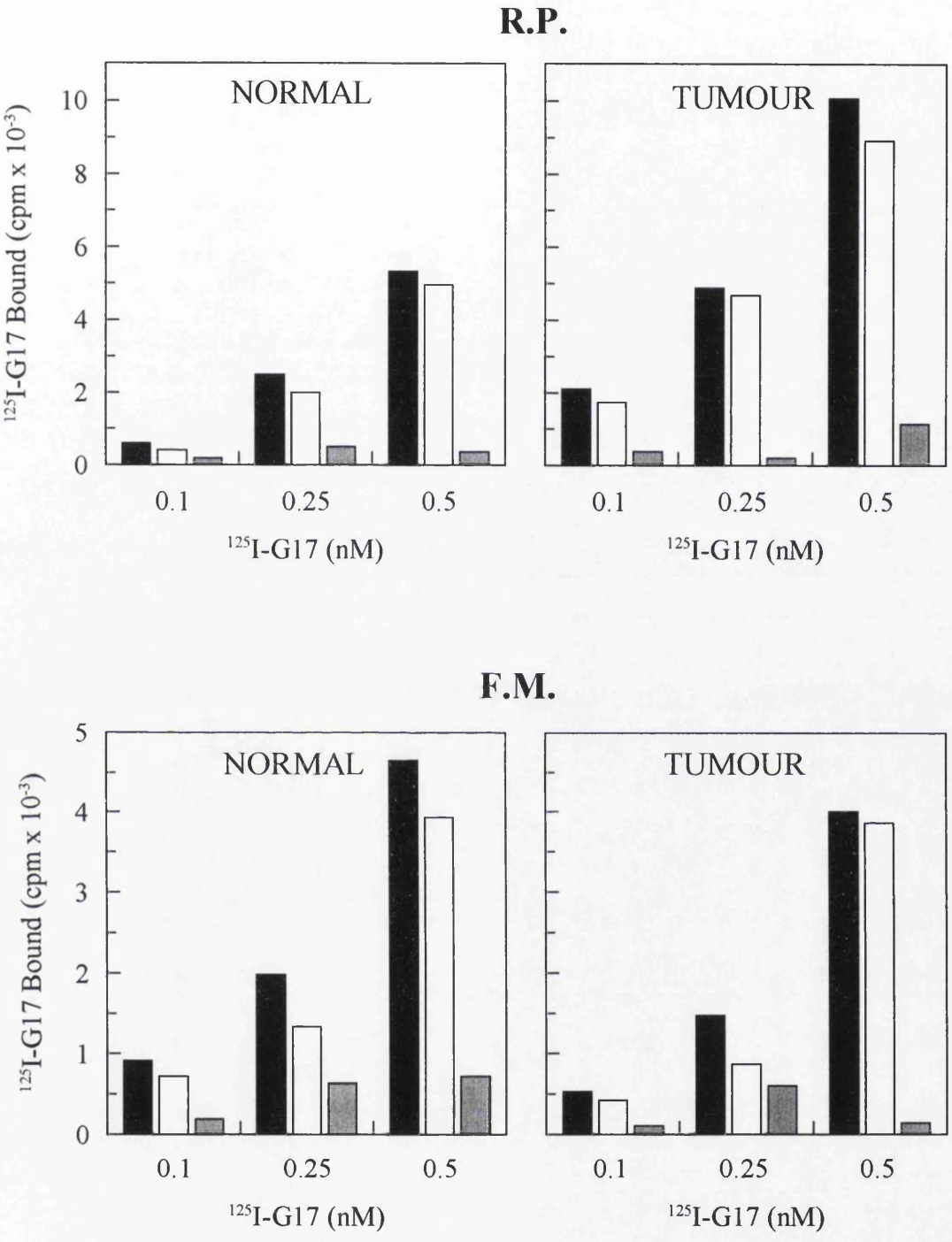

Figure 5.9 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to normal and tumour membranes of patients R.P. and F.M. Specific binding (▨) is total (■) minus non-specific binding (□).**

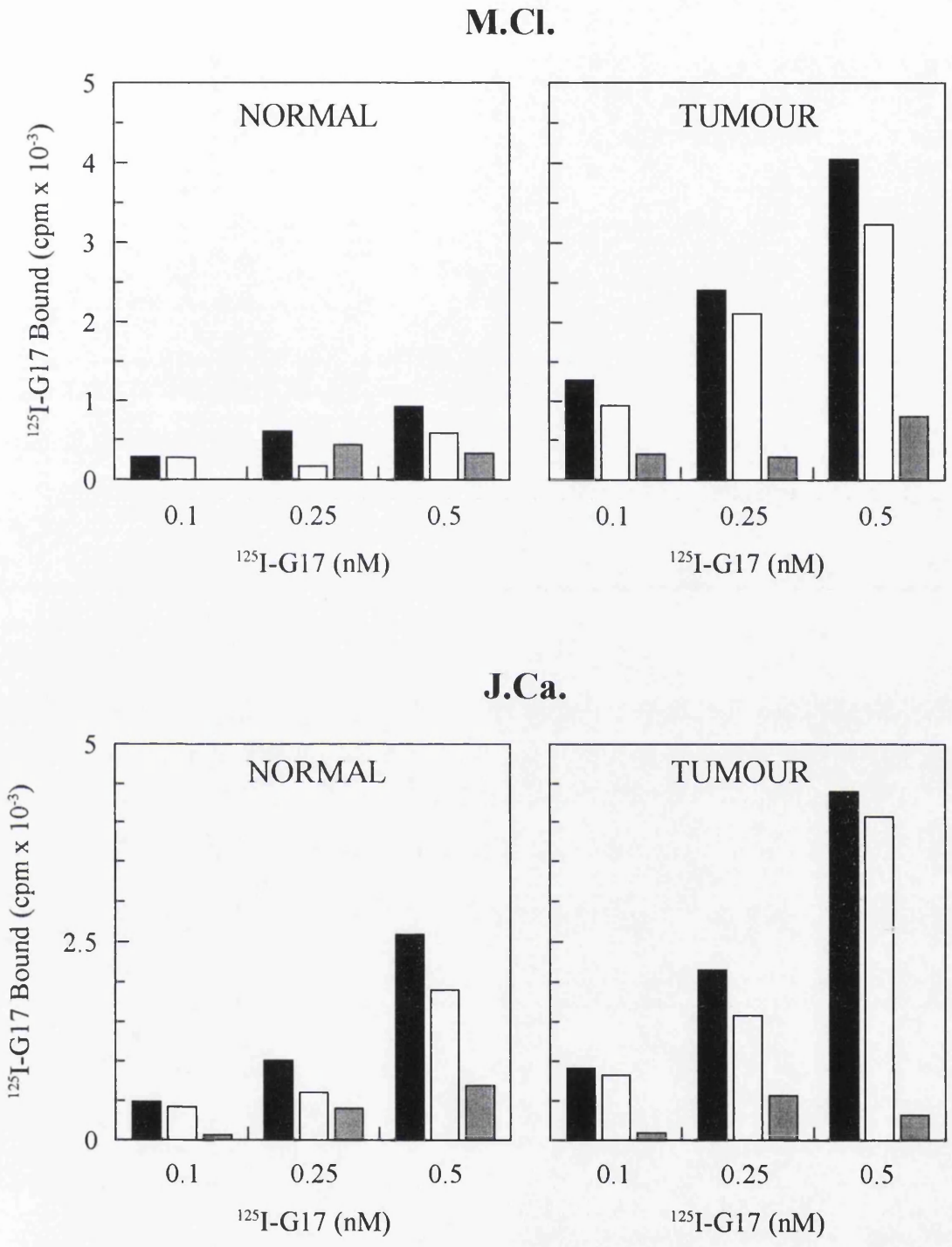

Figure 5.10 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to normal and tumour membranes of patients M.Cl. and J.Ca. Specific binding (▨) is total (■) minus non-specific binding (□).**

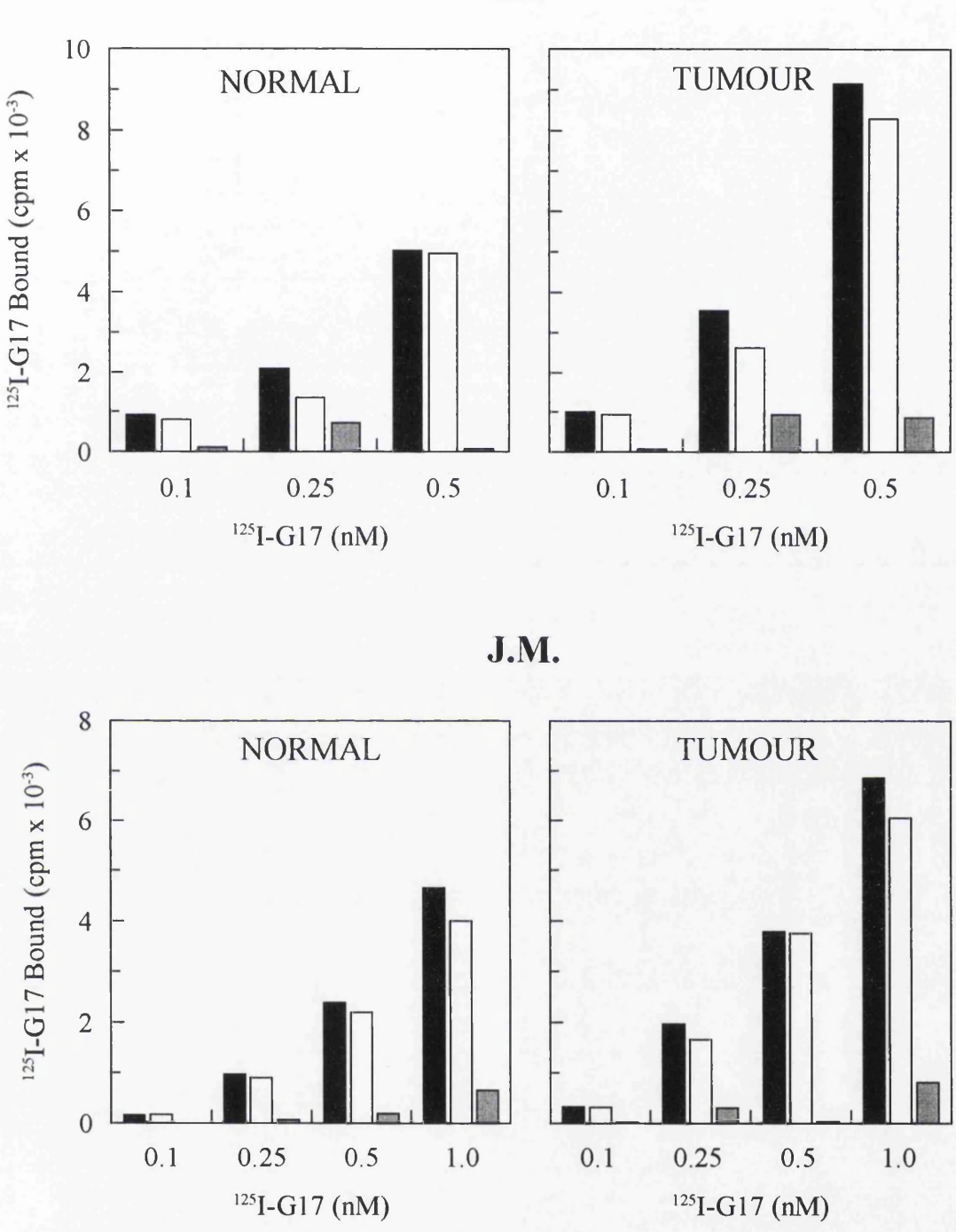

Figure 5.11 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to normal and tumour membranes of patients I.E. and J.M. Specific binding (▨) is total (■) minus non-specific binding (□).**

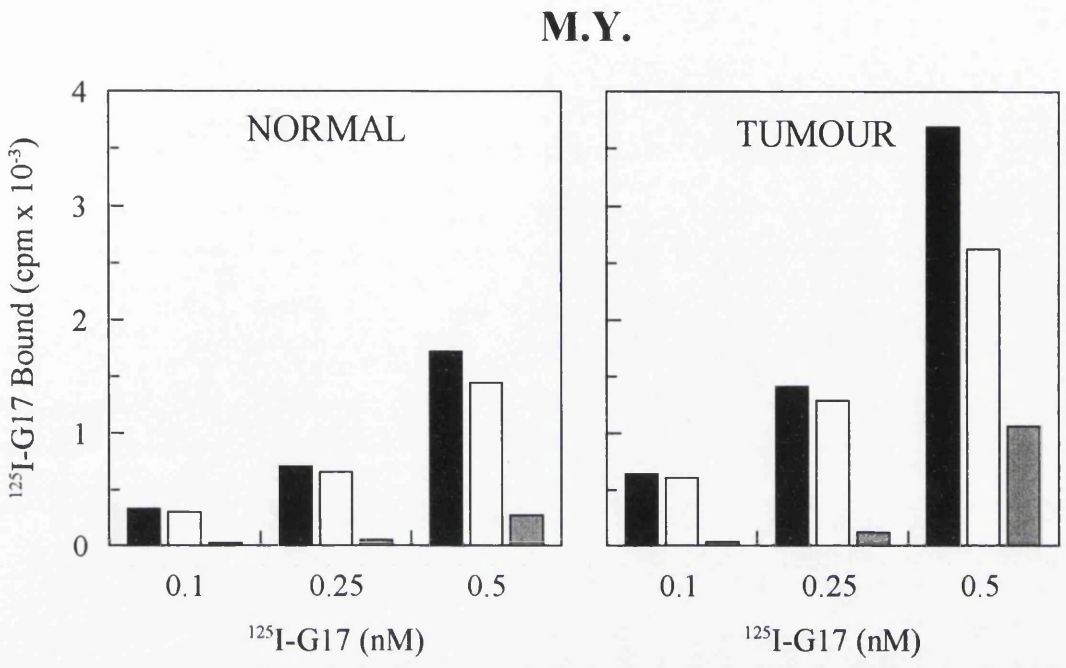

Figure 5.12 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to normal and tumour membranes of patient M.Y. Specific binding (▩) is total (■) minus non-specific binding (□).**

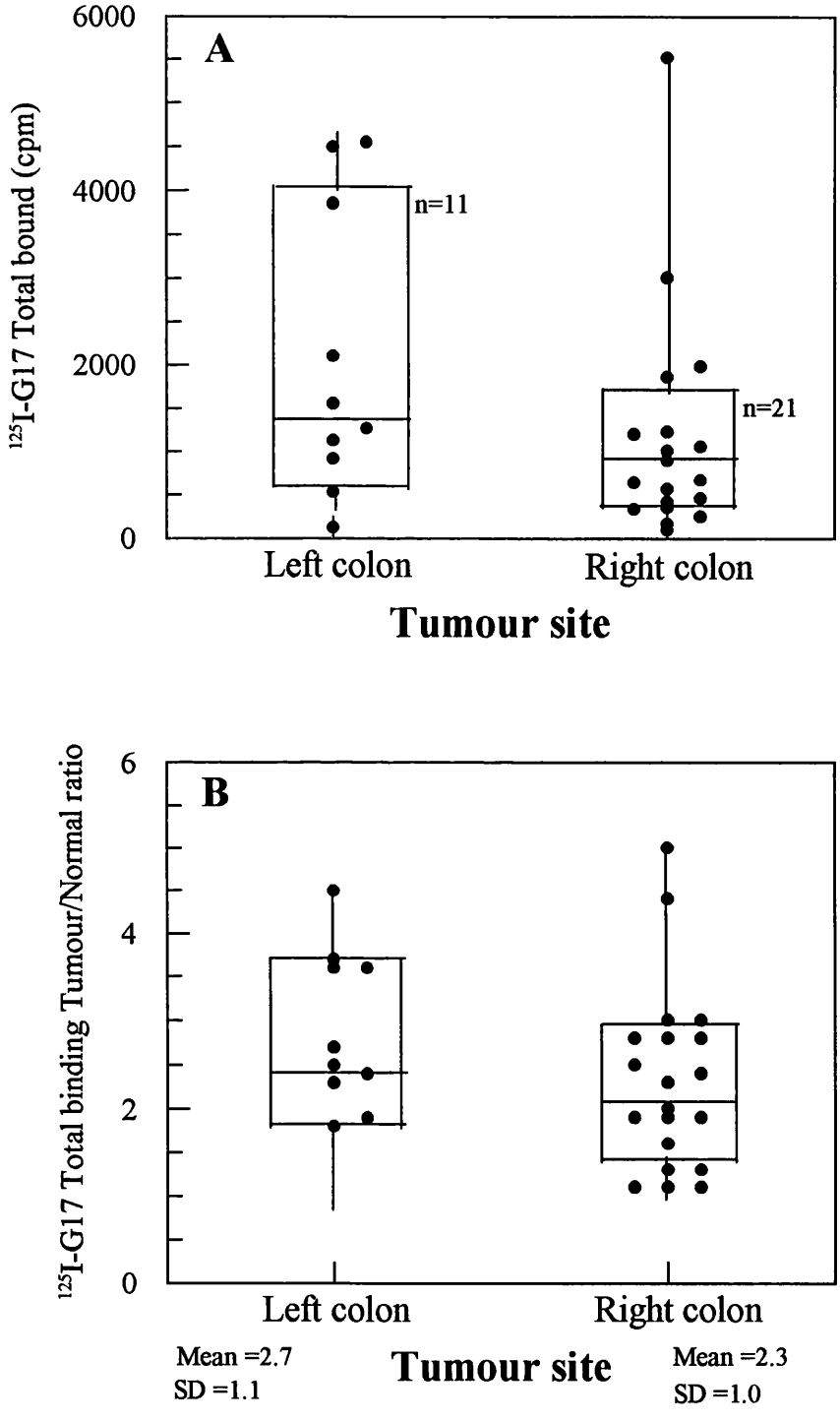

Figure 5.13 **A. Comparison of tumour site of left and right colon versus ^{125}I-G17 total bound (cpm). B. Comparison of tumour site versus the ratio of tumour : normal ^{125}I-G17 total bound. Dots are individual patients displayed with medians, interquartiles and confidence intervals.**

	Total binding		Ratio of tumour : normal total binding	
Tumour site	**Median**	**IQ Range**	**Median**	**IQ Range**
Left colon (n =11)	1269	532-3856	2.5	1.9-3.6
Right colon (n =21)	883	386-1546	2.0	1.45-2.8

Table 5.2 **Comparison of total ^{125}I-G17 bound (cpm) and the ratio of tumour : normal total ^{125}I-G17 bound according to tumour site in colorectal patients. IQ = interquartile.**

Seven tumours in the study were poorly differentiated, twenty-three were moderately differentiated and only two were well differentiated. Median values of total ^{125}I-G17 binding (Figure 5.14, graph A) were not significantly different when compared between the first two groups (p = 0.1855, Mann Whitney). The mean values for the ratio of tumour/normal total ^{125}I-G17 binding were marginally higher in patients with poorly differentiated tumours (p = 0.09, Two sample t-test) in comparison with moderately differentiated tumours (Figure 5.14, graph B). Since only two well differentiated tumours were involved in the study they were not compared.

Finally the Dukes' stage was compared and classes A and B were grouped together as were classes C and D. Fifteen tumours were either stage A or B and seventeen were either stage C or D. Median values for total ^{125}I-G17 binding (Figure 5.15, graph A) were not significantly different when the two groups were compared (p = 0.6326, Mann Whitney). The mean ratios of tumour/normal total ^{125}I-G17 binding (Figure 5.15, graph B) were not significantly different in patients with A and B types in comparison to C and D stages (p = 0.1656, Two sample t-test).

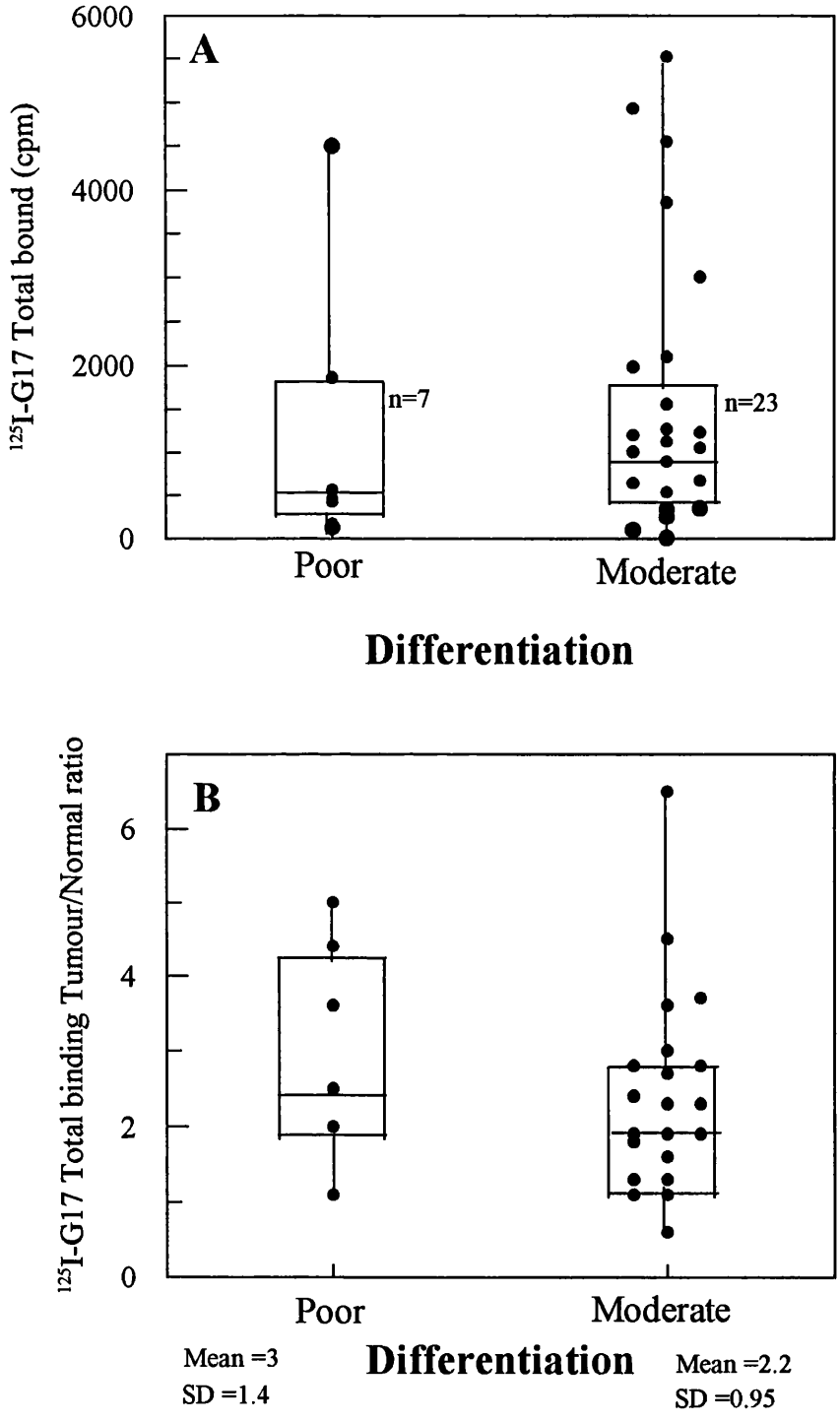

Figure 5.14 **A. Comparison of the level of tumour differentiation versus ^{125}I-G17 total bound (cpm). B. Comparison of tumour differentiation versus the ratio of tumour : normal ^{125}I-G17 total bound. Dots are individual patients displayed with medians, interquartiles and confidence intervals.**

	Total binding		Ratio of tumour : normal total binding	
Different-iation	**Median**	**IQ Range**	**Median**	**IQ Range**
Poor (n =7)	458	170-1862	2.5	2.0-4.4
Moderate (n =23)	1054	532-1987	1.9	1.5-2.8

Table 5.3 **Comparison of total ^{125}I-G17 bound (cpm) and the ratio of tumour : normal total ^{125}I-G17 bound according to differentiation in colorectal patients. IQ = interquartile.**

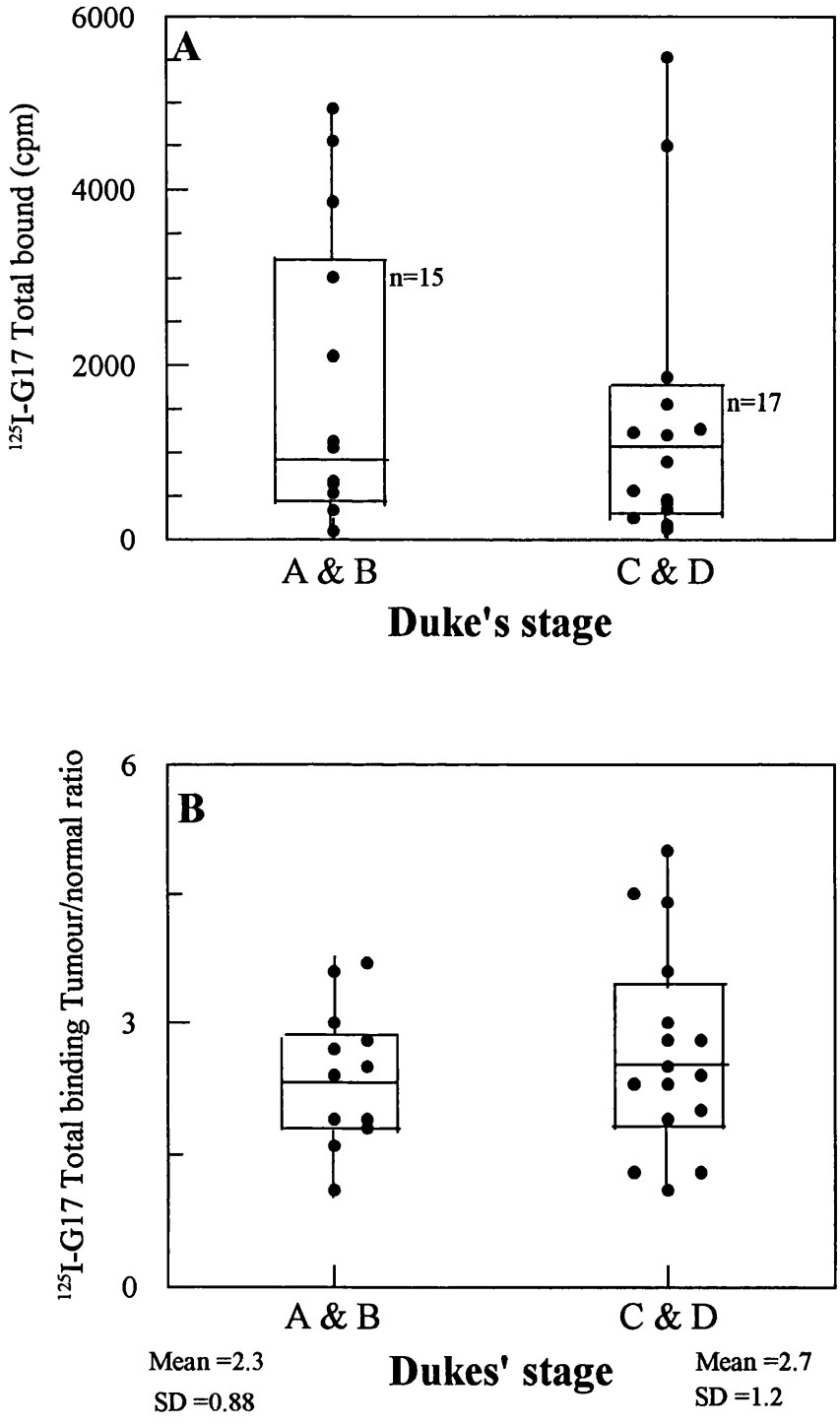

Figure 5.15 **A. Comparison of Dukes' stage A&B and C&D versus ^{125}I-G17 total bound (cpm). B. Comparison of Dukes' stage versus the ratio of tumour : normal ^{125}I-G17 total bound. Dots are individual patients displayed with medians, interquartiles and confidence intervals.**

	Total binding		Ratio of tumour : normal total binding	
Dukes' stage	**Median**	**IQ Range**	**Median**	**IQ Range**
A & B (n =15)	969	532-3220	2.15	1.75-2.85
C & D (n =17)	1048	367-1786	2.45	1.925-3.45

Table 5.4 **Comparison of total ^{125}I-G17 bound (cpm) and the ratio of tumour : normal total ^{125}I-G17 bound according to tumour stage in colorectal patients. IQ = interquartile.**

4 DISCUSSION

While gastrin/CCK_B receptors have been identified in normal and malignant colonic cells from a number of different species (Singh *et al* 1985; Guo *et al* 1990; Watson *et al* 1992a) little is known about these receptors in primary human colorectal tumours.

The aim of this study was to detect and characterise high affinity gastrin/CCK_B receptors in human colorectal cancer and normal tissue membrane preparations using ^{125}I-G17 in a radioligand binding assay. The technique was proven to be satisfactory following optimisation using both AR42J whole cell and membrane preparations, on which high affinity gastrin/CCK_B receptors were measured reliably and consistently. In contrast to this, membranes prepared from both normal and malignant mucosa of the colon were found to show little or no specific binding of ^{125}I-G17. The low specific binding observed with some patient tissues was not displaceable even with high concentrations of unlabeled G17. In an attempt to determine why no specific binding was observed with these membrane preparations, various methods of membrane preparation were explored.

The main technique used for membrane preparation, pulverisation under liquid nitrogen, has been successfully employed previously in the preparation of gastrointestinal tumours for study of high affinity gastrin/CCK_B receptors (Upp *et al* 1989), as well as other tumours including gastrin releasing peptide (Preston *et al* 1993) and oestrogen receptors (Singh *et al* 1993). After pulverisation, the resulting powder was subjected to the same membrane preparation as that of freshly homogenised tissues (Chapter 2,

section 3.6). Even less overall total binding with little or no specific binding was observed with the cryostat method (Appendix III, table 1).

There are a number of reasons why the tumours may have given very little specific binding which could not be displaced with competing ligands, including the possibility that gastrin/CCK_B receptors were not present on the colonic tissue analysed. This is in contradiction to the study by Upp *et al* who demonstrated the presence of gastrin/CCK_B receptors on two thirds of human colonic tumours. It is also possible that the method of storage may have contributed to receptor loss or degradation. This is unlikely to be the sole cause of receptor loss in the present study since of the thirty-three patient tissues analysed, nine were examined for gastrin/CCK_B receptor status immediately following resection. Additionally, tissues from the Upp *et al* study were only snap frozen in liquid nitrogen.

Whilst it is quite possible that none of these tissues contained gastrin/CCK_B receptors, results from Upp *et al* and further studies by the same group would suggest it was statistically unlikely that thirty-three samples analysed consecutively would be gastrin/CCK_B receptor negative (Upp *et al* 1989; Chicone *et al* 1989). It is possible that Upp *et al* may have had access to all or most of the tumour tissue, whereas restricted amounts of tumour tissue was collected in this study, and gastrin/CCK_B receptors may have been present on parts of the tissues not collected. To date, the study by Upp *et al* is the only substantial study on gastrin/CCK_B receptors in colorectal tumours and the only group to publish positive results. Another group (Kumamoto *et al* 1989) reported negative findings using ^{125}I-G17 in a radioligand binding assay, although the study

included only two normal/tumour colonic tissues. However, the same group using single point assays with 400pM ^{125}I-G17 detected a high degree of specific binding in human gastric fundic mucosa with K_D of 1.6nM and receptor capacity of 15fmol/mg protein. Other groups have used 'gastrin-responsiveness' as an indirect marker of the presence of gastrin/CCK_B receptors in fresh primary tumours (Watson *et al* 1988, 1989b) or established human carcinoma cell lines (Frucht *et al* 1992). Again, in these indirect studies, only a proportion of the cell lines tested exhibited high affinity gastrin/CCK_B receptors. Watson *et al* screened 31 colorectal tumours and showed that 35% of colorectal cancers which were 'gastrin responsive' contained tumour cells which responded to physiological concentrations of gastrin (Watson *et al* 1989b).

If the method of membrane preparation or storage conditions are not accountable for complete loss of receptors, the assay conditions used are the next obvious consideration. Failure to detect gastrin/CCK_B receptors in colorectal membranes are unlikely to be caused by the chosen incubation conditions such as buffer, pH and temperature since the binding protocol used was similar to that of Upp and colleagues (1989). The human assay buffer increased specific binding by approximately 30% in comparison to AR42J membrane assay buffer in the membrane assay, therefore protease inhibitors should be sufficient to inhibit proteolytic receptor damage. Upp *et al* iodinated [Leu^{15}]-gastrin-17 using either Iodogen, Enzymobead or Chloramine T techniques (Singh *et al* 1985) whereas the present study used a commercially available iodinated [Tyr^{15}]-gastrin-17 (NEN-Dupont), prepared using a modification of the Hunter and Greenwood method (1962). The radiolabel used in this study was ^{125}I-[Tyr^{15}]-gastrin17 as opposed to ^{125}I-[Leu^{15}]-gastrin17 and although specific activity of both radioligands

were similar, the method of iodination may have contributed to the lack of detection of gastrin/CCK_B receptors in the present study.

An autocrine mechanism for gastrin binding in tumour cells has been suggested by several groups (Hoosein *et al* 1990; Watson *et al* 1991; Blackmore and Hirst 1992). It is possible that gastrointestinal tumours may secrete their own gastrin and therefore it is feasible that the high local concentration of gastrin may result in a low affinity state of the receptor. Also in the binding assay radiolabeled gastrin could then have to compete with gastrin produced by the tumour cells themselves. Some groups have reported elevated serum gastrin concentrations in colorectal cancer patients (Smith *et al* 1988; Charnley *et al* 1992; Seitz *et al* 1992) but this remains debatable with other studies finding no difference in comparison to controls (Suzuki *et al* 1988; Creutzfeldt and Lambert 1991; Yapp *et al* 1992; Kikendall *et al* 1992; Penman *et al* 1994). However increased local gastrin concentrations at the tumour site may increase growth of tumours which are not normally responsive to physiological gastrin concentrations. The gastrin/CCK_B receptor may only require to be in a low affinity state because of high locally produced concentrations of gastrin and could explain the differences between normal high affinity endocrine cells and tumour cells.

The overall trend in the data presented is that total binding in tumours was found to be two-three fold greater per mg of protein than that in normal mucosa from the same patient. This may suggest that gastrin/CCK_B receptors are present in low numbers and/or very low affinity states which are difficult to detect using the current assay methodology. It is also possible that gastrin may bind with low affinity to a 'specific receptor', other

than the gastrin/CCK_B receptor, present in tumours thereby explaining the 2/3 fold greater total binding in tumours compared to normal tissues. This would also explain the low specific binding of total added which is not displaceable with gastrin/CCK_B receptor antagonists. The existence of a third class of gastrin/CCK_B receptors (CCK_C or CCK_G) which binds CCK and gastrin with the same affinity remains unclear at present. However, gastrin/CCK_B receptor antagonists do not inhibit gastrin stimulation via the hypothesised third class of receptor (Bold *et al* 1994; Singh *et al* 1995; Imdahl *et al* 1995).

The low levels of specific binding seen in several patients (Figures 5.8, 5.9 and 5.12) may represent binding to low affinity sites which were noted to be found in 10% of patients from the Upp *et al* study. It is also increasingly recognised that CCK receptors and gastrin/CCK_B receptors may exist in multiple affinity states (including a very low affinity state) and move between states by poorly understood mechanisms (Yu *et al* 1990; Talkad *et al* 1994; Huang *et al* 1994). The physiological relevance of these low affinity states is currently unknown. A study by Chang *et al* showed that GTP analogues can decrease affinity of radiolabeled CCK for the CCK receptor on pancreatic membranes but had no effect on the affinity of the radiolabeled CCK_A antagonist L364718 (Chang *et al* 1986).

Alternatively, low numbers of gastrin/CCK_B receptors may be present within the tumours due to the loss of mechanisms controlling maturation of gastrin precursors into mature gastrin. Recently several groups have presented evidence which has supported a trophic role for pro-gastrin derived glycine extended intermediates (Seva *et al* 1994; Nègre *et al* 1994; Kaise *et al* 1994, 1995). Seva and colleagues have also presented

preliminary results showing that the G-Gly receptor is present on the human colorectal cell lines HT29 and LoVo (Seva *et al* 1995) and high concentrations of glycine-extended gastrin-17 are mitogenic for a gastrin responsive human colon cancer (DLD1) cell line *in vitro* (Singh *et al* 1994).

Tumour site, differentiation and Dukes' stage for the thirty-three patients in the study were correlated with ^{125}I-G17 total binding and the ratio of tumour/normal tissue total binding. Tumours arising from the caecum and ascending colon were classed as the right colon and tumours from the descending, sigmoid, rectosigmoid and rectal sites were grouped together as the left colon. There was no correlation between tumour site and level of either total binding or the ratio of tumour/normal total binding. In addition, there was no significant difference between total ^{125}I-G17 binding in poor or moderately differentiated patient tumours. A marginal increase was observed in the ratio of tumour/normal binding with poorly differentiated tumours compared to moderately differentiated tumours. No significant difference between the level of tumour cell invasion (Dukes'stage) and total binding was observed in any tissue membranes analysed.

In a study by Upp *et al*, a significant correlation was found between tumours situated on the left and right side of the colon and gastrin/CCK_B receptor density (Upp *et al* 1989). They also reported no difference between moderate and mucinous tumours. However they did demonstrate that if normal tissues did not express gastrin/CCK_B receptors then neither did corresponding tumour tissues. Therefore the normal mucosa may be of importance in the determination of the patients gastrin/CCK_B receptor status. In the same report , colon tumours with no lymph node or distant metastasis

(Dukes' stage A&B) had a significantly higher gastrin/CCK_B receptor density than more advanced tumours (Dukes' stage C&D).

In conclusion, high affinity gastrin/CCK_B receptors were not found on any normal or malignant human colorectal tissues analysed despite being consistently and reliably measured in AR42J cells and membranes. It is possible that gastrin/CCK_B receptors are present in either low numbers or in a low affinity state which were not detectable with the current methodology.

CHAPTER 6 GASTRIN/CCK$_B$ RECEPTORS IN NORMAL AND NEOPLASTIC HUMAN GASTRIC TISSUE

1 INTRODUCTION

In contrast to the studies of gastrin/CCK_B receptors in the colon, more is known about these receptors in the stomach and gastrin/CCK_B receptors have been widely studied and characterised in animal models using radioligand binding techniques.

In 1976, Lewin and co-workers were the first to demonstrate specific binding of radiolabeled gastrin to the rat gastric fundic mucosa (Lewin *et al* 1976). Although binding was observed on both membranes and isolated cells, the binding was of low affinity (K_D = 9nM). A tritiated radiolabel was used in this study and consequently specific acivity was low (60Ci/mmol). This may have contributed to the detection of only low affinity gastrin/CCK_B receptors.

The gastrin/CCK_B receptor radioligand binding assay optimised by Takeuchi *et al* used a high specific activity label (^{125}I-G17) (~2000Ci/mmol) with crude plasma membranes from the rat oxyntic mucosa (Takeuchi *et al* 1979). High affinity gastrin/CCK_B receptors were detected (K_D = 0.4nM). These receptors were subsequently characterised by another group using a variety of ligands (Johnson *et al* 1985).

Other groups have since reported high affinity gastrin/CCK_B receptors in the stomach of various animal species. Specific high affinity binding of ^{125}I-leu-G17 to isolated canine fundic cells was described by Soll *et al* in 1984. Cells were separated using elutriation and binding was shown to correlate with parietal cell content. Other investigators have demonstrated high affinity oxyntic gastrin/CCK_B receptor binding sites

for gastrin in the rat (Singh *et al* 1985), rabbit (Magous and Bali 1982) and guinea-pig (Ramani and Praissman1989).

Gastrin has been shown to promote growth in some gastric cancer cell lines grown either *in vitro* or *in vivo* (Ishizuka *et al* 1992; Watson *et al* 1989a, 1989b; Singh *et al* 1985; Ochiai *et al* 1985) and gastrin/CCK_B receptors have been characterised on these cells (Sethi and Rozengurt 1992; Ishizuka *et al* 1992; Watson *et al* 1989a).

Gastrin/CCK_B receptors have also been reported on cancer cell lines from a variety of species including several human gastric cancer cell lines. This is important as human tumour cell lines may provide information which is more pharmacologically and clinically relevant. The human gastric cancer cell line TMK-1, has been shown to respond trophically to 10nM gastrin as assessed by [^{3}H] thymidine uptake. This process appears to be mediated through a high affinity gastrin/CCK_B receptor on the cell membrane (Ochiai *et al* 1985). Weinstock and Baldwin (1988) examined five human gastric cancer cell lines using whole cells in radioligand binding studies. They used isolated canine parietal cells as positive controls with K_D's of 1.7 and 0.2nM for ^{125}I-met G17 and ^{125}I-leu G17 respectively. However, the affinity constants for the human gastric tumour cell lines ranged between 0.2 and 1.3uM i.e. gastrin/CCK_B receptor affinities of approximately 1000 times less than parietal cells.

A report by Matsushima and colleagues found no gastrin/CCK_B receptor gene expression using RT-PCR in either human gastric carcinoma cell lines or adenocarcinoma tissues (Matsushima *et al* 1994). In contrast, a recent abstract by Clerc *et al* found high levels of gastrin/CCK_B receptor mRNA in two out of four extracts of human gastric

carcinoma tissues using the same technique (Clerc *et al* 1995). However, when these tissues were examined by radioligand binding techniques, specific binding of radiolabeled gastrin was not found in crude membrane preparations.

Human gastric cancer cell lines have been screened using a single saturating dose of ^{125}I-G17. The gastric cell line AGS was found to be strongly positive for specific gastrin binding sites, with an affinity (K_D < 1nM) similar to normal rat fundic cells (Singh *et al* 1985). Seven AGS clones were established and four were positive for gastrin binding sites (>12 fmols). Of the others, one was found to be negative and two exhibited gastrin binding sites of less than 3.3 fmols. Whether gastrin is trophic for all AGS clones to the same extent or dependent on the level of gastrin receptors present on the cell lines is not known.

Radioligand binding studies were performed on human scirrhous gastric carcinomas by Kumamoto *et al* (1988). Using membrane preparations and ^{125}I-G17, four out of five carcinomas with specific binding between 1.1-18.2fmols/mg protein. The presence of gastrin receptors was more frequent in the poorly differentiated scirrhous carcinomas (Borrman type IV) than in other gastric adenocarcinomas examined (Borrman type II or III). A further study by the same group in 1989 demonstrated specific ^{125}I-G17 binding to human gastric fundic mucosa with a K_D of 1.6nM and receptor capacity of 15fmol/mg protein.

Loss of responsiveness to gastrin has been shown in established cell lines on repeated subculture *in vitro* (Watson *et al* 1988). Gastrin responsiveness could be

retained by transplanting cells into nude mice and growing *in vivo* before re-establishment *in vitro*. The same group also reported that several human gastric cell lines lacked any mitogenic response to gastrin at passage >250 *in vitro* when compared to freshly derived primary gastric tumours, where ~50% were gastrin responsive (Watson *et al* 1989b). This variation in ability of gastrin to induce mitogenic effects may be due to up and down regulation of receptors. Therefore cautious interpretation of results is required before gastrointestinal tumour cell lines can be declared 'gastrin/CCK_B receptor negative'.

1.1 Objective

The object of this study was to demonstrate high affinity gastrin/CCK_B receptors on membrane preparations from the normal human body region of the stomach and to determine if these receptors were present in corresponding gastric tumours.

2 METHODS

Materials and methods can be found in Chapter 2.

3 RESULTS

3.1 Patient data

Four out of nine tumours and corresponding normal tissues from either body and/or antral sites were collected in the human collection buffer stated previously (Table 6.1). These tissues were collected immediately upon resection and stored on ice, in the collection buffer, until dissection by a pathologist. Tissues from the remaining five patients were collected from theatre and snap frozen in liquid nitrogen without immersion in the human collection buffer (Table 6.1).

3.2 Patient results

3.2.1 Patient tissues collected and assayed fresh in human collection buffer

Three out of four patient gastric body membranes (Table 6.2) tested, expressed high affinity gastrin/CCK_B receptors with K_D's and Bmax's between 0.2-1.1nM and 28-76fmol/mg protein respectively. Specific ^{125}I-G17 binding was displaceable with G17, G34, L365260 and L364718. The minimum time between resection and immersion in collection buffer on ice was never more than five minutes, except for patient A.M. This may be the reason why gastrin/CCK_B receptors were not detected on gastric body membranes from this patient. Antral tissue also collected from two of the four patients was not found to have detectable gastrin/CCK_B receptors.

Initials	Age	Sex	Normal tissue site	Tumour site	Differentiation
1.Patient tissues collected and assayed fresh in human collection buffer					
J.N.	68	M	body	antrum	poor
J.M.	51	M	body/antrum	oesophageal	poor
A.M.	55	M	body	antrum	poor
G.S.	82	M	body/antrum	oesophageal	poor
2. Patient tissues collected and frozen without human collection buffer prior to assay					
F.S.	68	F	body/antrum	oesophageal	poor
R.B.	70	M	antrum	body	moderate
A.H.	75	M	body	antrum	well
C.R.	70	F	antrum	body	poor
S.H.	64	M	antrum	body	poor

Table 6.1 **Gastric patient details**

INITIALS	Bmax (fmol/mg)	K_D (nM)		Ki (nM)	
		G17	**G34**	**L365260**	**L364718**
G.S.	44	0.2	0.48	2.6	229
J.M.	76	1.1	-	14	96
J.N.	28	0.4	-	0.88	163
F.S.	21	0.7	-	-	-

Table 6.2 **Gastrin/CCK_B receptor affinity constants and receptor capacities for gastric patient body membranes**

(i) Patient J.N.

Patient JN, a 68 year old male, had a poorly differentiated antral gastric tumour and so only body tissue was obtained from this patient at resection. Body and tumour membranes were tested using increasing concentrations of radiolabel, but only body membranes gave significant specific binding (Figure 6.1). Association time courses were performed with body and tumour membrane preparations (Figure 6.2). Association of ^{125}I-G17 to body membranes reached a plateau at 5mins and remained at this level for a further 55mins. Total binding for tumour membranes was less than 50% that of body membranes (Figure 6.2) and no specific binding was observed at any time point. Specific binding of ^{125}I-G17 to body membranes was displaced by G17, L365260 and L364718 (Figure 6.3). LIGAND analysis of the displacement data revealed a single binding site with K_D of 0.4nM and Bmax 28fmols/mg of protein. Half maximal (IC_{50}) values for G17, L365260 and L364718 were found to be 0.46, 1.4 and 260nM respectively. The corrected inhibitory constants (Ki) for receptor antagonists L365260 and L364718 were calculated as 0.88nM and 163nM respectively (Table 6.2).

(ii) Patient J.M.

Body, antral and tumour tissues were obtained for patient JM, a 51 year old male who also presented with a poorly differentiated gastro-oesophageal tumour. Again, significant specific binding of ^{125}I-G17 was observed only in body membranes (Figure 6.4). Unfortunately, only small amounts of tissue were obtained and therefore experiments on this patient were limited to a single displacement assay. Specific binding of ^{125}I-G17 to body membranes was displaced by G17, L365260 and L364718 (Figure 6.5). LIGAND analysis of the displacement data again revealed a single binding site with K_D of 1.1nM

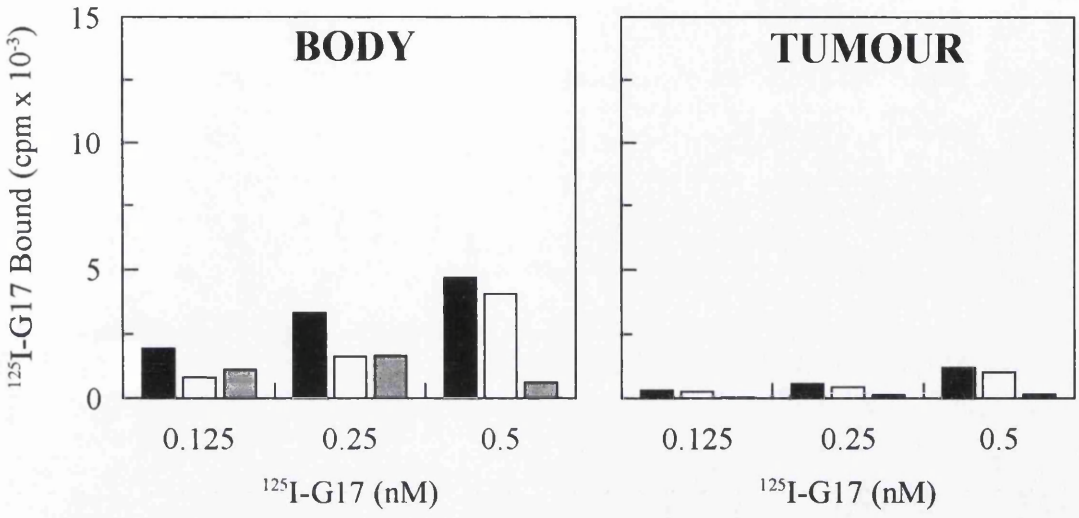

Figure 6.1 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to membranes from patient J.N.**

Membranes (100ug/tube) were incubated for 15mins at 22°C with increasing concentrations of ^{125}I-G17. Specific binding (▩) is total (■) minus non-specific binding (□).

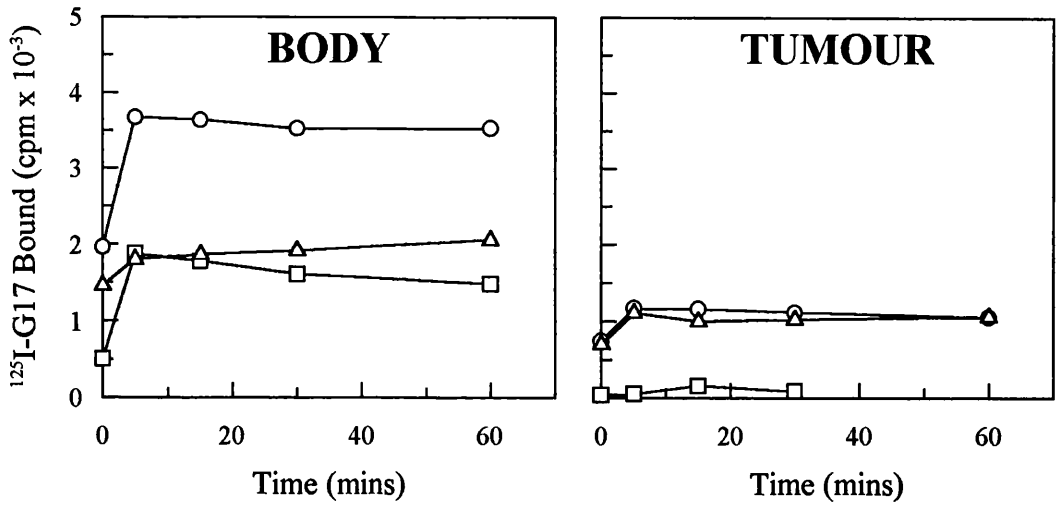

Figure 6.2 **Association time-courses on membranes from patient J.N.**

Membranes (100ug/tube) were incubated with 0.25nM ^{125}I-G17 for measurement of total binding (-O-) at 22°C. Non-specific binding was measured in the presence of 0.25uM G17 (-Δ-) and specific binding (-□-) was calculated as total minus non-specific.Results are the mean of one experiment performed in duplicate.

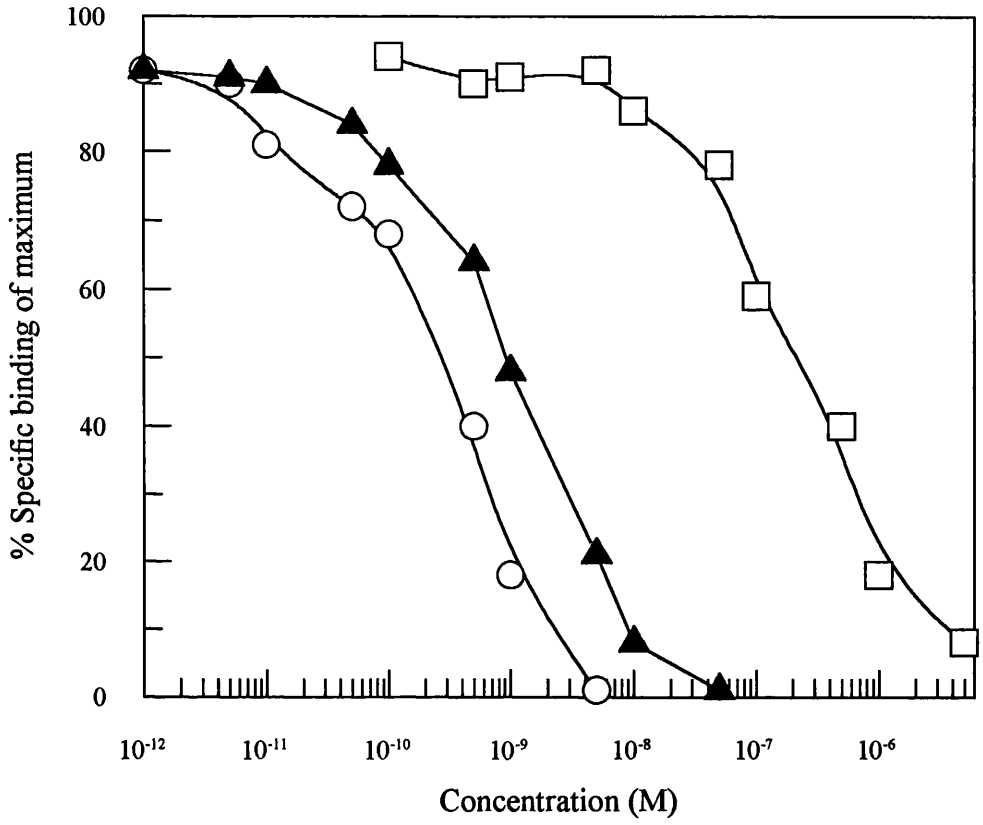

Figure 6.3 **Displacements on body membranes from patient J.N.**

Displacement of ^{125}I-G17 binding to J.N. body membranes (100ug/tube) by increasing concentrations of G17 (-○-) and by CCK_B and CCK_A receptor antagonists L365260 (-▲-) and L364718 (-□-) respectively. Each point is the mean of two experiments performed in duplicate.

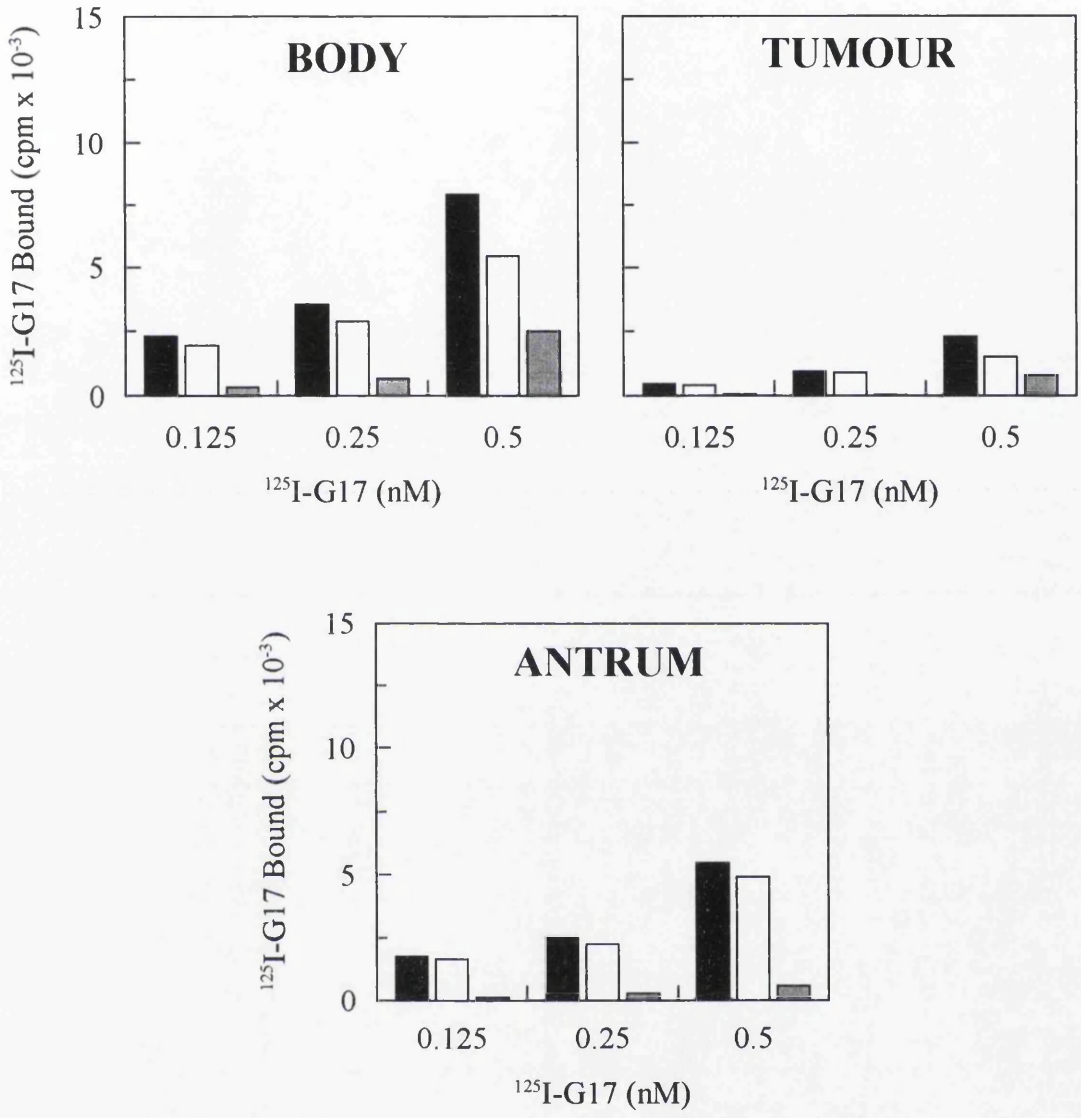

Figure 6.4 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to membranes from patient J.M.**

Membranes (100ug/tube) were incubated for 15mins at 22°C with increasing concentrations of ^{125}I-G17. Specific binding (▩) is total (■) minus non-specific binding (□).

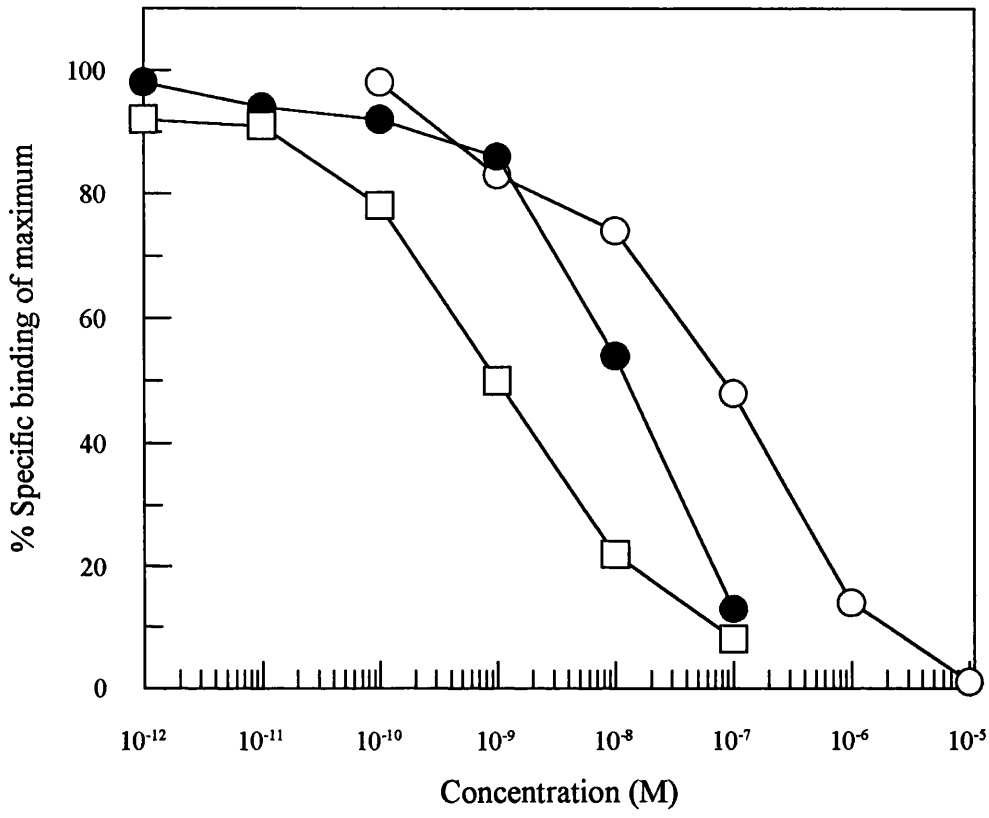

Figure 6.5 **Displacements on body membranes from patient J.M.**

Displacement of ^{125}I-G17 binding to J.M. body membranes (100ug/tube) by increasing concentrations of G17 (-□-) and by CCK_B and CCK_A receptor antagonists L365260 (-●-) and L364718 (-○-) respectively. Each point is the mean of one experiment performed in duplicate.

and Bmax of 76fmols/mg protein. Half maximal (IC_{50}) values for G17, L365260 and L364718 were found to be 1.1, 17 and 120nM respectively. The corrected inhibitory constants (Ki) for the receptor antagonists L365260 and L364718 were calculated as 14nM and 96nM respectively (Table 6.2).

(iii) Patient A.M.

Patient AM was a 55 year old male who also had a poorly differentiated antral gastric tumour and so only body tissue was collected at the time of resection. After resection, tumour and corresponding gastric tissue was not immediately immersed in collection buffer but left at room temperature for more than 20mins. This was considered to be significant since gastrin/CCK_B receptors were not detected on the body membranes. Very little binding and no specific binding was found with either body or tumour membranes. Radiolabel concentration was increased to a final concentration of 0.5nM in the tube (data not shown) but this still did not yield any further results for this patient.

(iv) Patient G.S.

Patient GS, an 82 year old male, had a poorly differentiated gastro-oesophageal tumour and so tissues from the body and antral regions of the stomach were plentiful. Membranes prepared from each region were tested using increasing concentrations of radiolabel (Figure 6.6). Only membranes from the body gave significant specific binding (36%). Association time course experiments were subsequently performed with all three membrane preparations (Figure 6.7). The gastric body membrane association maintained a plateau between 30 and 100mins. The association time course with tumour membranes

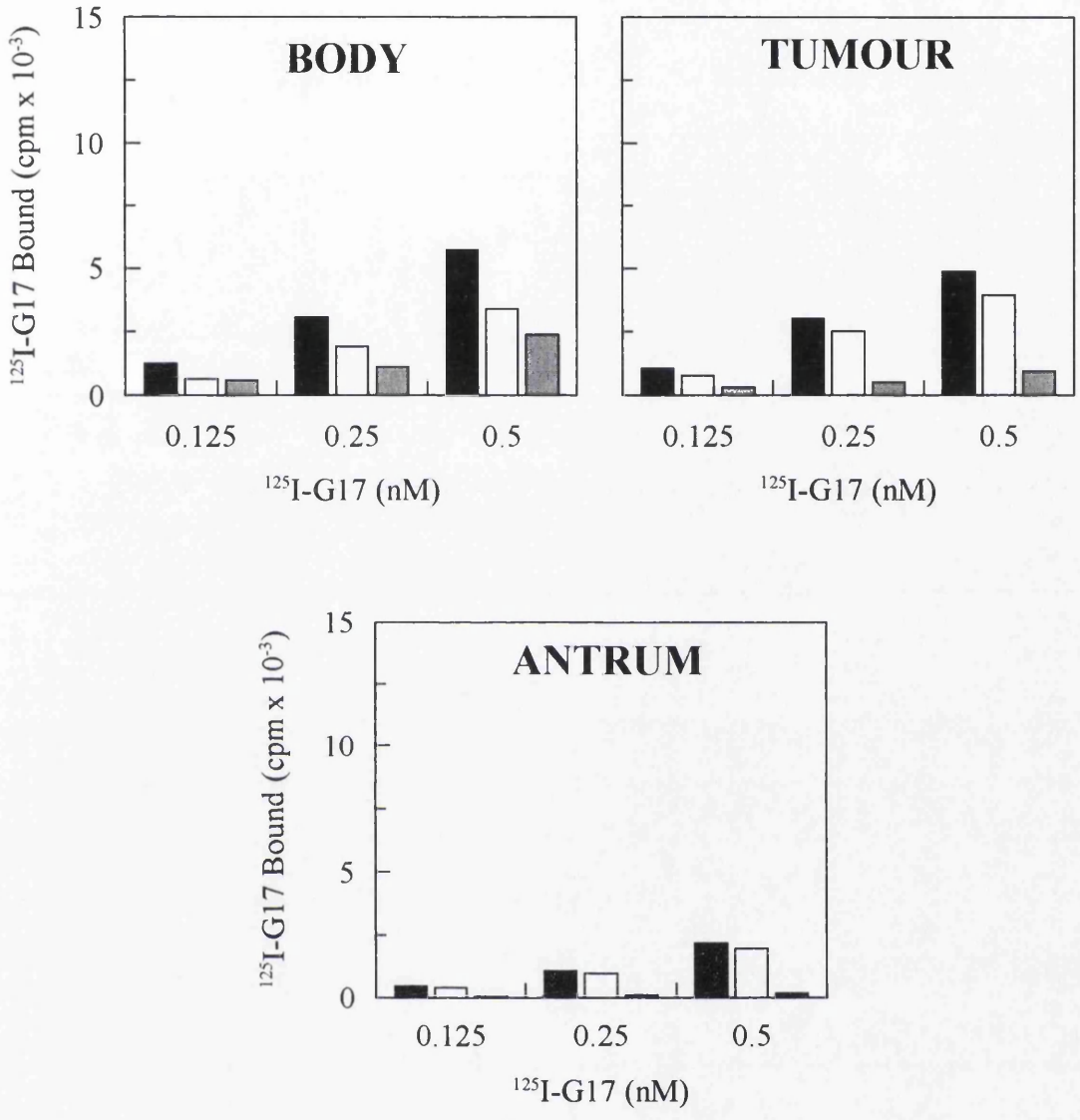

Figure 6.6 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to membranes from patient G.S.**

Membranes (100ug/tube) were incubated for 90mins at 22°C with increasing concentrations of ^{125}I-G17. Specific binding (▩) is total (■) minus non-specific binding (□).

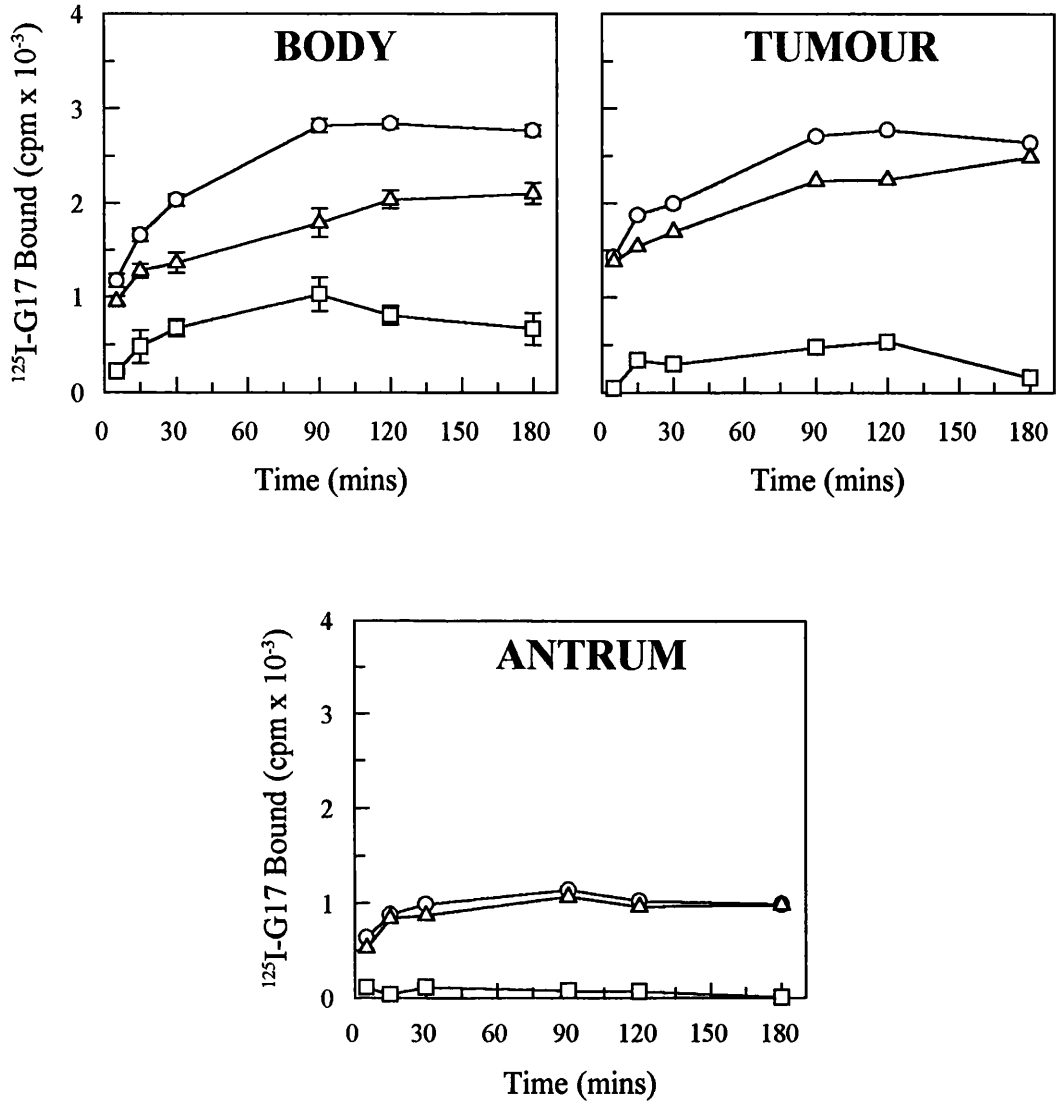

Figure 6.7 **Association time-courses on membranes from patient G.S.**

Membranes (100ug/tube) were incubated with 0.25nM ^{125}I-G17 for measurement of total binding (-O-) at 22°C. Non-specific binding was measured in the presence of 0.25uM G17 (-△-) and specific binding (-□-) was calculated as total minus non-specific. Results are the mean (+/-SD) of three experiments performed in duplicate for body membranes and one experiment performed in duplicate for antral and tumour membrane results.

was found to plateau between 15 and 120mins thereafter decreasing sharply. Antral membranes showed no significant specific binding.

The agonists G17 and G34 and CCK_B/CCK_A receptor antagonists L365260 and L364718 respectively, all displaced ^{125}I-G17 from membranes prepared from the body tissue (Figure 6.8). LIGAND analysis of the displacement data revealed a single binding site with K_D of 0.2nM and Bmax of 44fmols/mg protein. Binding to a single site was shown using a log-logit plot of the G17 displacement data which was linear with an IC_{50} value of 0.34nM. Specific ^{125}I-G17 binding to body membranes was displaced by G34, L365260 and L364718, with IC_{50} values of 0.55nM, 3.2nM, 260nM respectively. Inhibition constants (Ki's) for patient G.S. are summarised in Table 6.2. The order of affinity for the gastrin/CCK_B receptor was G17 >G34 > L365260 >>L364718, with K_D/Ki values of 0.2nM, 0.48nM, 2.6nM and 229nM respectively.

Although specific binding was obtained with G.S. tumour membranes, displacement of total binding was not observed using a range of concentrations of G17 between 10^{-13}-10^{-6}M (data not shown).

3.2.3 Patient tissues collected and frozen without human collection buffer

Five patient tissues were collected without human collection buffer before snap freezing in liquid nitrogen for periods of one-three years prior to assay. Only two out of five patient normal tissues were collected from the gastric body regions (Table 6.1) and of these only patient FS was found to have detectable gastrin/CCK_B receptors on body membranes.

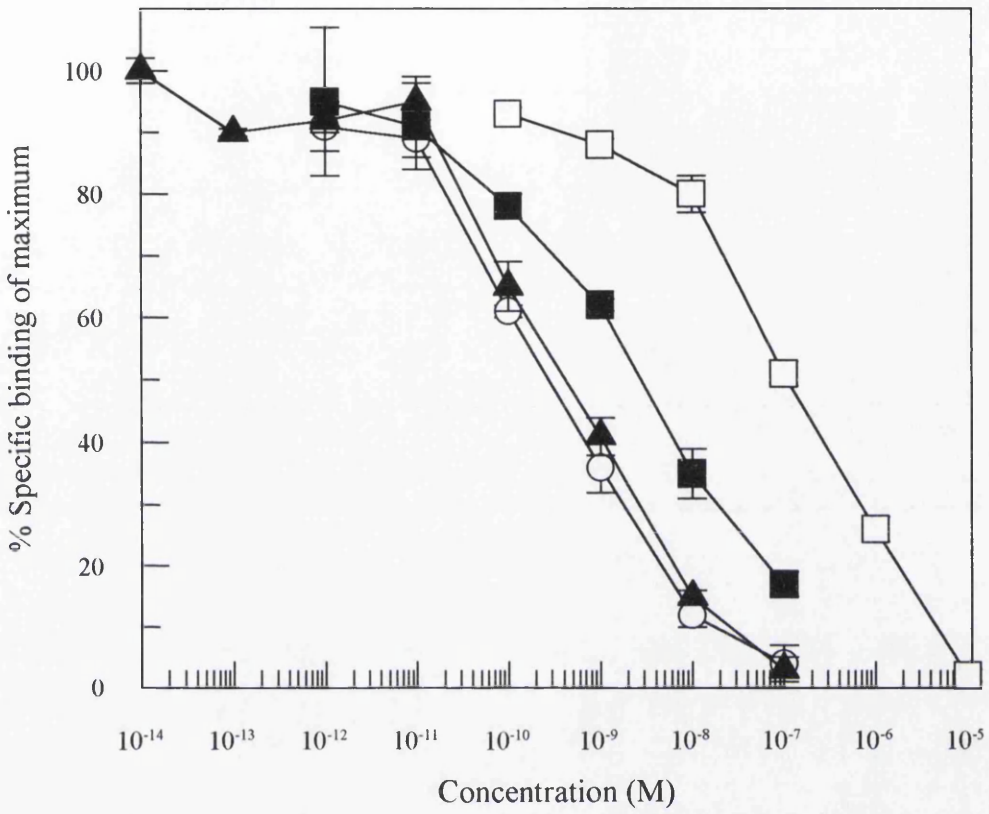

Figure 6.8 **Displacements on body membranes from patient G.S.**

Displacement of ^{125}I-G17 binding to G.S. body membranes (100ug/tube) by increasing concentrations of agonists G17 (-○-) and G34 (-▲-), and by CCK_B and CCK_A receptor antagonists L365260 (-■-) and L364718 (-□-) respectively. Each point is the mean (+/-SD)of three experiments performed in duplicate.

(i) Patient F.S.

Only one patient F.S., showed significant specific binding on body membranes (Figure 6.9). G17 displaced ^{125}I-G17 specific binding on body membranes with an IC_{50} value of 0.5nM (Figure 6.10). LIGAND analysis of the displacement data revealed a single binding site with K_D of 0.7nM and Bmax of 21fmols/mg of protein (Table 6.2). Although ^{125}I-G17 binding on antral and tumour membranes was observed (Figure 6.9), only 10-16% was specifically bound.

(ii) Patients A.H., R.B., C.R. and S.H.

Specific ^{125}I-G17 binding to patient A.H. body membranes was 21% (Appendix IV, table 13). However, the concentration of ^{125}I-G17 was high (0.5nM), therefore multi-point saturation experiments were not performed (Figure 6.11). Specific ^{125}I-G17 binding between 11-32 % was observed with membranes prepared from antral tissue from patients R.B. and S.H (Figure 6.11 and 6.12). Again the concentration of ^{125}I-G17 was high (0.5nM), therefore multi-point saturation experiments were not performed. All four patient tumour membranes tested showed no specific ^{125}I-G17 binding (Figure 6.11 and 6.12).

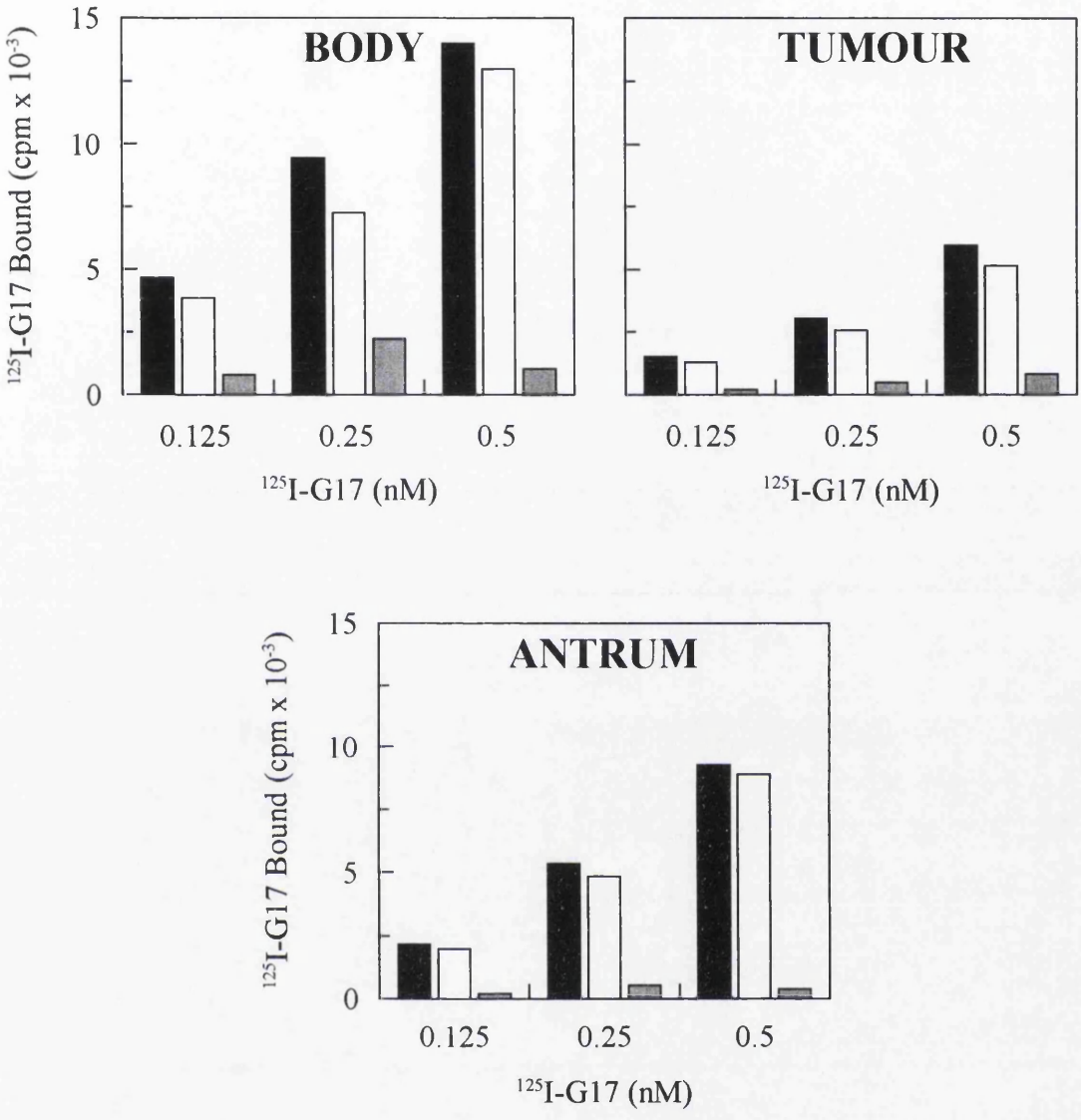

Figure 6.9 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to membranes from patient F.S.**

Membranes (100ug/tube) were incubated for 15mins at 22°C with increasing concentrations of ^{125}I-G17. Specific binding (▩) is total (■) minus non-specific binding (□).

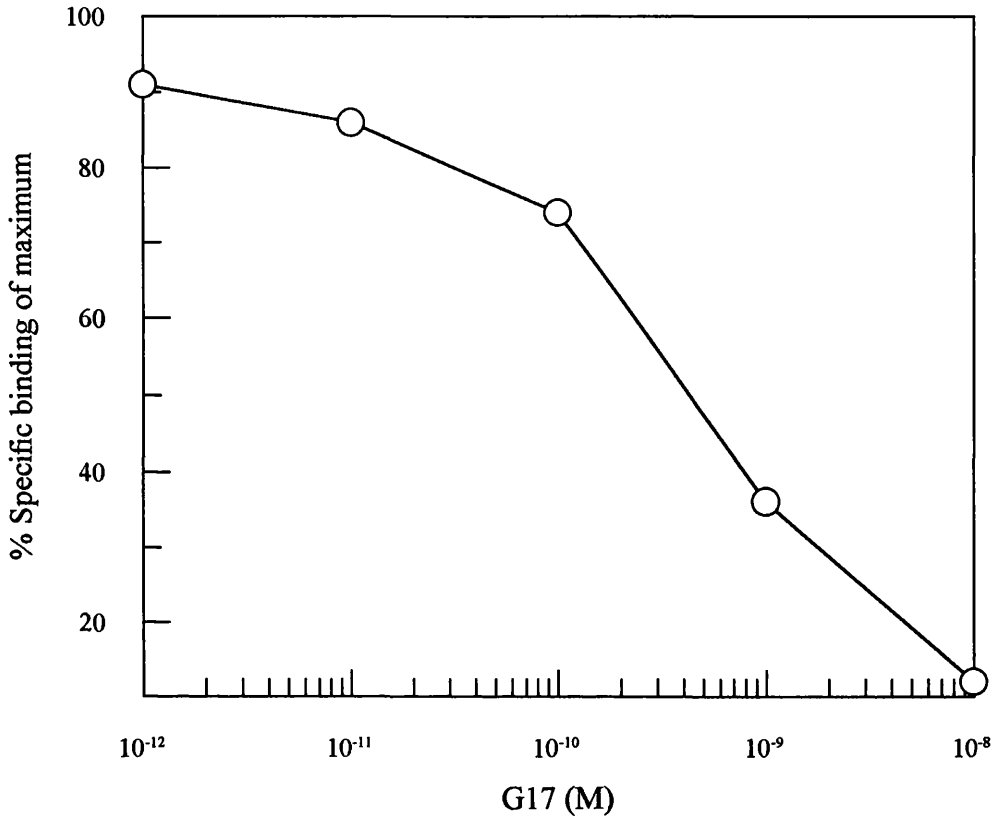

Figure 6.10 **Displacement on body membranes from patient F.S.**

Displacement of ^{125}I-G17 binding to F.S. body membranes (100ug/tube) by increasing concentrations of G17 (-o-). Each point is the mean of one experiment performed in duplicate.

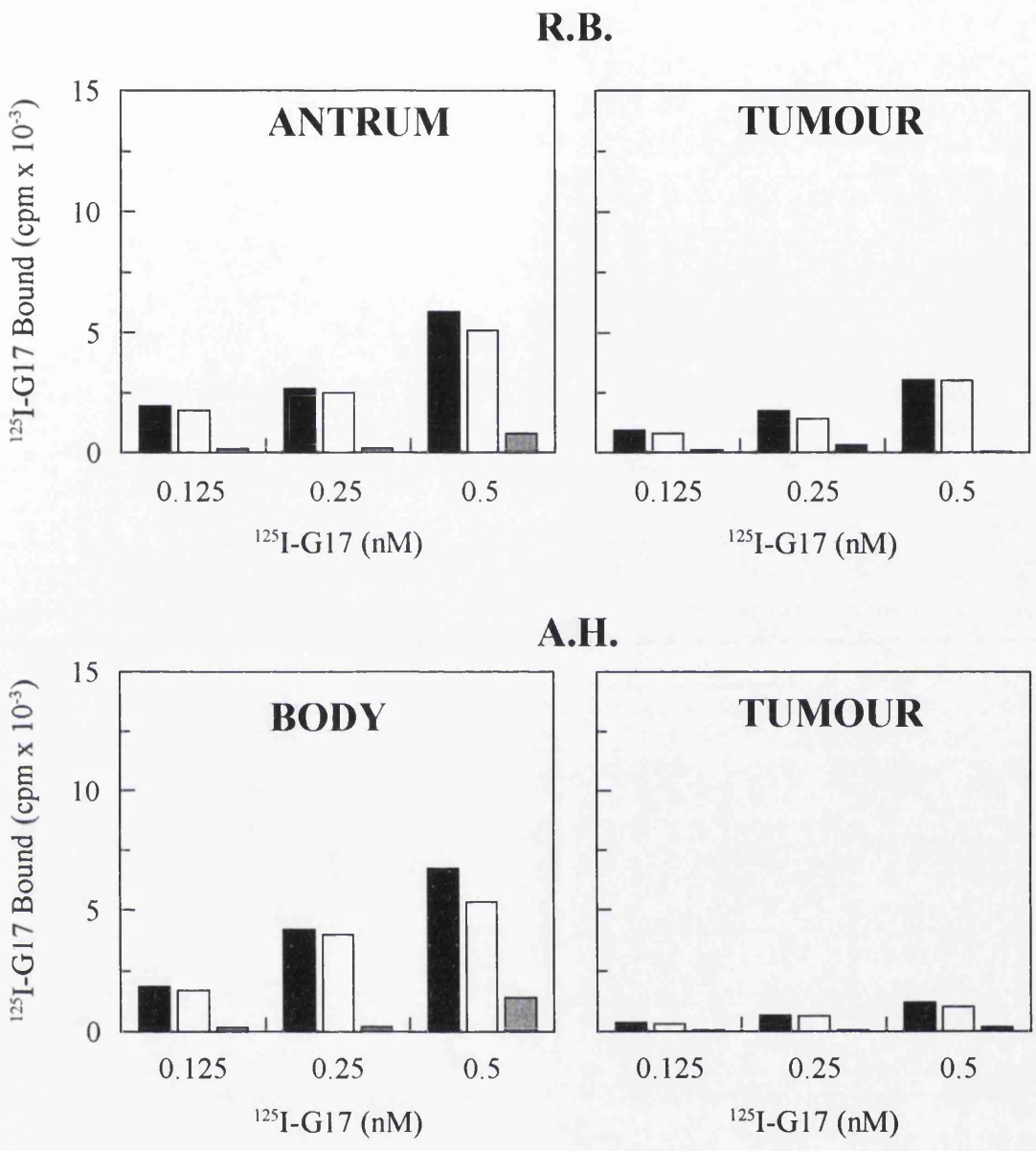

Figure 6.11 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to membranes from patients R.B. and A.H.**

Membranes (100ug/tube) were incubated for 15mins at 22°C with increasing concentrations of ^{125}I-G17. Specific binding (▩) is total (■) minus non-specific binding (□).

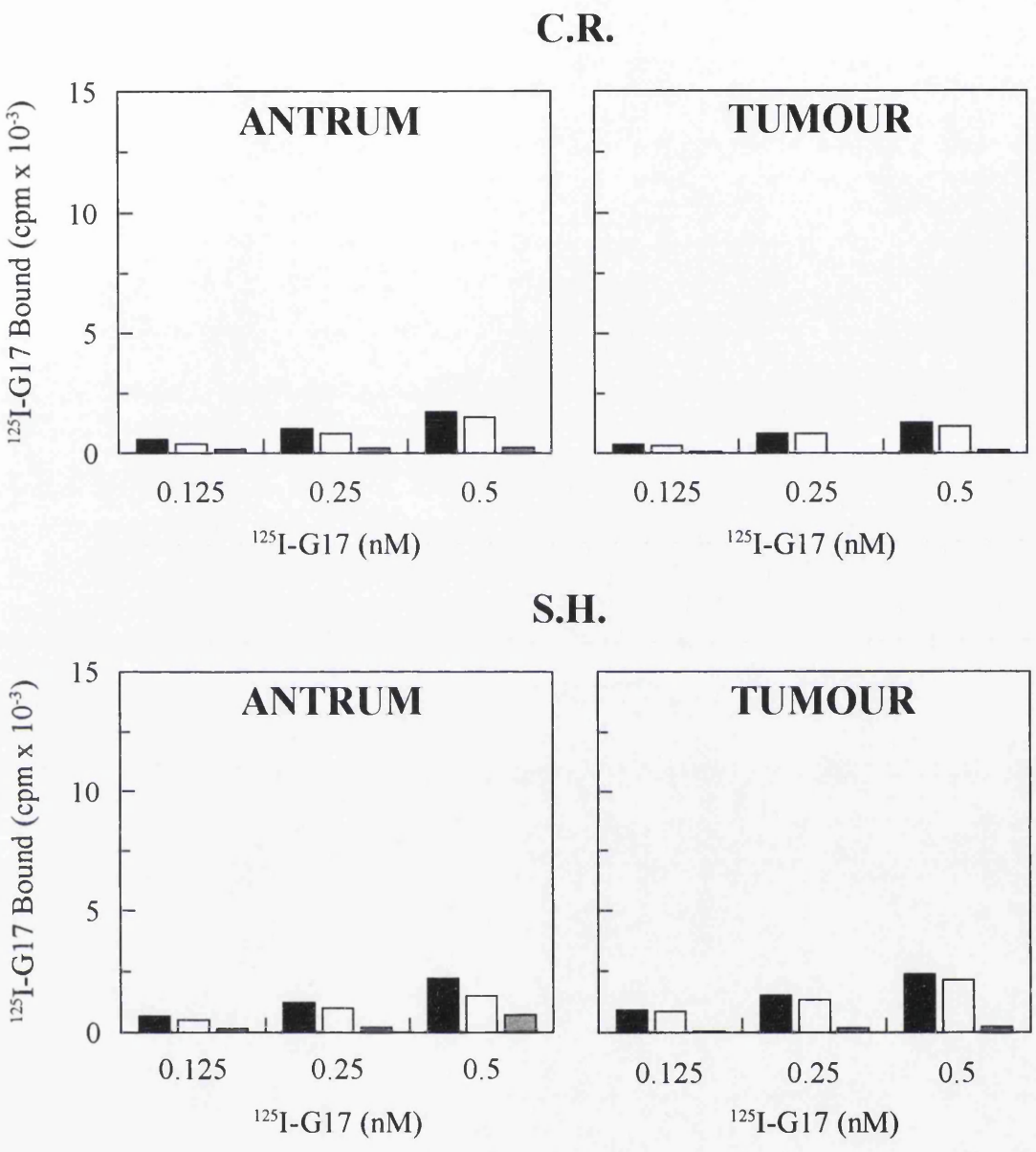

Figure 6.12 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to membranes from patients C.R. and S.H.**

Membranes (100ug/tube) were incubated for 15mins at 22°C with increasing concentrations of ^{125}I-G17. Specific binding (▩) is total (■) minus non-specific binding (□).

4 DISCUSSION

The aim of this study was to use a radioligand binding assay to detect and characterise gastrin/CCK_B receptors in human gastric tumours and normal antral or body tissues. Praismann and Brand reported evidence of two gastrin binding sites in the human oxyntic mucosa (Praismann and Brand 1991). Frozen sections were incubated with ^{125}I-G17 at 22°C and binding was quantitated by autoradiography. Scatchard analysis of saturation data revealed an ultra high affinity binding site with K_D of 8.61pM and a second high affinity site, K_D of 0.34nM with receptor densities of 28 and 450fmols/mg protein respectively. Characterisation with receptor antagonists was not reported. Kumamoto and colleagues demonstrated specific ^{125}I-G17 binding to gastric fundic mucosa from a duodenal ulcer patient (Kumamoto *et al* 1989). In contrast to Praismann and Brand, they reported only one binding site with a dissociation constant of 1.1nM and receptor capacity of 15fmols/mg protein. A previous paper by the same group also demonstrated specific ^{125}I-G17 binding to eight human gastric tumour tissues, although details of affinities were not given and characterisation was not performed (Kumamoto *et al* 1988).

In the previous study (Chapter 5), gastrin/CCK_B receptors were not detected in any membranes prepared from colorectal tumours or corresponding normal tissue using the current assay methodology. In contrast, four out of six patient's gastric body membranes were found to have high affinity gastrin/CCK_B receptors (K_D 0.2-1.1nM) with receptor densities above 20fmol/mg protein and corresponded to results observed by Kumamoto *et al.* Specific ^{125}I-G17 binding to membranes was displaceable with gastrin/CCK_B receptor agonists G17 and G34 and CCK_B/CCK_A receptor antagonists

L365260 and L364718 respectively. The findings of Praismann and Brand were not confirmed in this study, as log-logit plots were all found to be linear indicating binding to a single high affinity site. Specific ^{125}I-G17 binding to patient G.S. body membranes was displaced with G17, G34, L365260 and L364718 and gastrin/CCK_B receptor affinities were 0.2nM, 0.48nM, 2.6nM and 229nM respectively. These followed a similar order of potency as for AR42J fraction II membranes which were previously shown to have affinities for the gastrin/CCK_B receptor of 2nM, 1.67nM, 22nM and 196nM respectively. G.S. body membrane G17 displacement results were the mean of three experiments in duplicate whereas other ligand displacements were only perfomed once and this may have contributed to experimental error in comparison to results obtained in displacements with AR42J membranes. This may also explain variations in dissociation constants for gastrin/CCK_B receptor positve body membranes (K_D of 0.2-1.1nM).

Binding with patient A.M. was not observed and the fact that so little total binding was obtained suggested that there may have been almost complete degradation of any gastrin/CCK_B receptors present due to the time lapse between resection and immersion in human collection buffer which in this case was much longer than with other patients. This was important for subsequent collection of human tissue. In addition, the gastrin/CCK_B receptor may have a short half-life and periods of ischaemia due to the unavoidable clamping of the blood supply during surgery may also cause receptor loss.

Other patient body membranes (J.M. and J.N.) which were collected fresh from theatre and stored in the human collection buffer, were found to exhibit high affinity gastrin receptors (K_D 0.4-1.1nM) which were similar to dissociation constants previously

determined on AR42J fraction II membranes (K_D 1.6nM). Displacements with the gastrin/CCK_B receptor antagonist L365260 were calculated to have inhibitory dissociation constants (Ki) of 14 and 0.88nM for J.M. and J.N. body membranes respectively. The displacement values did not correspond with those for AR42J fraction II membranes (Ki = 22nM) but the results were taken from only one experiment performed in duplicate. Displacements with the CCK_A receptor antagonist L364718 also produced different results between body membranes of different patients (Ki = 96-229nM). L364718 displacement results from patients G.S. and J.N.were closer to results obtained from AR42J membranes (Ki = 200nM) and it must be noted the result from patient J.M. was obtained after only one experiment in duplicate.

From the patient tissues collected and stored without the human collection buffer, only one, patient F.S. demonstrated specific high affinity binding of gastrin to body membranes (K_D = 0.7nM) which corresponded to affinities found in AR42J fraction II membranes. None of the other membranes tested for this group showed any significant specific binding, although three out of the five normal tissues collected were from the antrum and no body tissues were obtained as the tumour was situated in the corpus of the stomach. The only other patient in this group where body tissue was collected was A.H. Specific ^{125}I-G17 binding was detected only at a concentration of 0.5nM ^{125}I-G17 and so further multi-point saturations were not performed. The fact that the tumour site in patients R.B., C.R. and S.H. is located in the corpus combined with the lack of specific binding would also indicate that there may be altered processing or loss of gastrin/CCK_B receptor during carcinogenesis.

Receptor capacities for gastrin/CCK_B receptor postive patients were similar (Bmax 21-76fmols/mg protein) and correlated with reported values for gastrin/CCK_B receptors on colonic normal and cancer tissue membranes (Upp *et al* 1989). Receptor densities from patients were approximately ten times less than those on AR42J membranes (Chapter 4) which may also explain the lack of overall total binding.

No specific binding of ^{125}I-G17 was detectable in any of the antral membranes analysed. Specific binding was observed only in G.S. tumour membranes (Figure 6.2) but binding was not displaceable with G17 concentrations between 10^{-14}-10^{-6}M. This may suggest that there was specific binding to low affinity gastrin/CCK_B binding sites which could not be detected using the current radioligand binding assay. Specific binding was not observed in any of the other tumour membranes tested but it was noted that the overall total binding was greater than that in corresponding antral membranes.

Autocrine production of gastrin in gastric tumour cells has also been postulated (Watson *et al* 1992b). In a study with the gastric carcinoma cell line MKN45G, ninety-seven percent of the cells stained positively with an anti-gastrin antibody. In addition the cell line did not respond trophically to exogenously adminstered gastrin-17 *in vitro* (Watson *et al* 1991). This may support the theory that low affinity gastrin/CCK_B receptors are present in gastric cancer cells since autocrine production of gastrin may produce high local concentrations and become less responsive to exogenously adminstered gastrin which reduces receptor affinity. In the present study there may be low affinity gastrin/CCK_B receptors present on the G.S. tumour membranes which were not further characterised.

Advances in molecular cloning of the gastrin/CCK_B receptor have produced some insight into the possible presence of the receptor in gastrointestinal cancer tissues. A recent report detailed evidence of increased gastrin/CCK_B receptor mRNA expression in two out of four gastric cancer extracts (Clerc *et al* 1995). It is possible that the tumours tested in this study did not have any gastrin/CCK_B receptors at all, but the statistics from the study by Clerc *et al* would suggest that some tumour cells may have expressed mRNA for the receptor. This could then indicate a possible defect in the translation of the message and may explain undetectable specific binding in the tumour membranes tested.

In conclusion, high affinity gastrin/CCK_B receptors have been detected and characterised in the gastric body of patients who had undergone surgery for gastric carcinoma. Four out of six patient gastric body tissues exhibited gastrin/CCK_B receptors. Affinities for gastrin were found to be in the nanomolar range and corresponded with those found in AR42J fraction II membranes which served as a control for this study.

CHAPTER 7 FINAL DISCUSSION AND CONCLUSIONS

The object of this programme of work was to develop a radioligand binding assay capable of detecting gastrin/CCK_B receptors in human gastrointestinal tissues. The initial detection and characterisation of high affinity gastrin/CCK_B receptors in AR42J cells and membranes was investigated in order to standardise the radioligand binding assay. This was considered important since the assay would ultimately be used for the detection of high affinity gastrin/CCK_B receptors in human gastrointestinal tissues.

Gastrin/CCK_B receptors were accurately and reliably measured using an optimised AR42J whole cell assay and crude membranes prepared from AR42J cells were found to retain similar receptor binding properties. High affinity gastrin/CCK_B receptors were characterised and following freezing of membranes at -70°C receptor affinity was retained for a limited period.

AR42J whole cells were shown to have an affinity of 0.3nM for the gastrin/CCK_B receptor. Interestingly, fraction I membranes were shown to have a much higher affinity (K_D =1nM) for the gastrin/CCK_B receptor than fraction II membranes (K_D = 2nM). In fact, the order of IC_{50} values for each agonist/antagonist analysed demonstrated the same order of potency i.e. whole cells $\geq$ fraction I membranes $\geq$ fraction II membranes. This suggests that the gastrin/CCK_B receptor is in one affinity state in AR42J whole cells which is different from either AR42J fraction I or II membranes. Moreover, the higher affinity seen with fraction I membranes also suggests that receptors may be in a different state from fraction II membranes which is possible since they were derived from different sources. Differences in affinity state may also reflect a degree of receptor damage.

High affinity gastrin/CCK_B receptors in tissues from the human gastric body were successfully detected and characterised in four out of six gastric body tissues analysed. There was no evidence of high affinity gastrin/CCK_B receptors in any tumour tissues tested. However, the presence of low affinity gastrin/CCK_B receptors on these tissues has not been ruled out.

Results from analysis of gastrin/CCK_B receptor status in normal gastric body tissues would suggest that high affinity gastrin/CCK_B receptors are not present on gastric tumour tissues or in either normal or tumour colonic tissues. As previously discussed, it is possible that gastrin/CCK_B receptors are present on these tissues but exist in a low affinity state which may explain the low level of specific binding observed in some tumour tissues. In addition, there is accumulating evidence in support of an autocrine growth mechanism in GI tumour cells which may be mediated by gastrin/CCK_B receptors (Hoosein *et al* 1989; Watson *et al* 1988, 1991; Baldwin and Zhang 1992; Reimy-Heintz *et al* 1993).

High affinity gastrin/CCK_B receptors were not detected on thirty-three colonic cancers and corresponding normal mucosa analysed consecutively. It is unlikely that the lack of specific gastrin/CCK_B receptors in colonic cancer and normal tissues is due to a methodological problem as receptors were readily detected on gastric body tissue samples using the same assay methodology. Upp *et al* found gastrin/CCK_B receptors in approximately two thirds of membranes from human normal and tumour colon but this is the only group to publish positive results (Upp *et al* 1989). Other investigators have either used established human tumour cell lines (Frucht *et al* 1992) or *in vitro*

'gastrin-responsiveness' as an indirect measure of the presence of gastrin receptors in fresh human tumours (Watson *et al* 1988; 1989b). However, these studies showed that only a proportion of tumours or cell lines possessed gastrin/CCK_B receptors or responded to gastrin. While only a proportion of colonic tumours may possess functional gastrin/CCK_B receptors, it seems statistically unlikely that all thirty-three tumours examined should be receptor negative. It is also possible that receptors were present and were not detected. Differences between the current study and that of Upp *et al* include the use of a different radiolabeled gastrin. The same group iodinated their own gastrin instead of using the commercially available iodinated gastrin-17 (NEN Dupont) which was used in the current study. However, since specific ^{125}I-G17 binding was demonstrated on crude membranes from human gastric body tissues, it would appear that the biological activity of the radiolabel was unaffected.

Colorectal tumours were found to have significantly higher total binding compared with corresponding normal colonic mucosa. This increase was generally two to three fold higher; an effect observed in all but one patient sample. These results may suggest ^{125}I-G17 binding to low affinity gastrin/CCK_B receptors, the existence of which is now increasingly recognised. Studies by several different groups have reported multiple CCK and gastrin receptor states (including a very low affinity) which move between states by poorly understood mechanisms (Yu *et al* 1990; Talkad *et al* 1994; Huang *et al* 1994). The clinical relevance of these receptor states is currently unknown.

The emergence in the past few years of the cloning and sequencing of the human gastrin/CCK_B receptor gene has suggested that the gastrin/CCK_B receptor is present on a

fraction of gastrointestinal tumour cells but the presence of gastrin receptor mRNA in these cells may not automatically mean expression of a cell surface receptor. The lack of specific receptor sites in any of the tumour tissues analysed in these studies, combined with literature evidence of the presence of the gastrin/CCK_B receptor gene expression in some GI tumour cells, may be the result of conformational change in the gastrin/CCK_B receptor (i.e. to a low affinity state) during carcinogenesis.

Alternative approaches to radioligand binding in the measurement of gastrin/CCK_B receptors such as detection with anti-gastrin receptor antibodies or by RT-PCR with probes may provide valuable information. Following the recent isolation and sequencing of the genes for CCK_A and CCK_B receptors from human and other species (Wank *et al* 1992a, 1992b; Psiegna *et al* 1992; Kopin *et al* 1992; Miyake *et al* 1994), one approach might be examination of receptor mRNA expression by *in situ* hybridisation or Northern analysis. However, caution should be observed with interpretation of results obtained from such studies since a recent report by Clerc *et al* observed high levels of gastrin/CCK_B receptor mRNA in two out of four extracts of human gastric carcinoma tissues using PCR (Clerc *et al* 1995), but when these tissues were examined using radioligand binding, specific binding of radiolabeled gastrin was not found in crude membrane preparations. This may indicate a possible defect in the translation of the message and may explain undetectable specific binding in the tumour membranes tested.

Alternatively, immunohistochemical studies using specific antibodies to the gastrin/CCK_B receptor may be useful. Only one such antibody, raised to canine parietal cells is currently available (Mu *et al* 1987), but further antibodies may become available in the near future.

While these approaches have the advantage of giving information about the receptor at the cellular level they are poorly quantitative and do not allow pharmacological receptor characterisation. Radioligand binding offers the best quantitative method for studying gastrin/CCK_B receptors in normal and tumour tissues, but may be even more productive in combination with immunocytochemistry and *in situ* hybridisation. The latter two techniques could provide useful screening methods for the presence of gastrin/CCK_B receptors in tumour tissues from the human gastrointestinal tract since they have the added bonus of cryopreservation of receptors and signal amplification. It is important that gastrin/CCK_B receptor are unequivocally shown to be present on these tissues and are characterised before the growing number of gastrin/CCK_B receptor antagonists can be considered as possible treatments for patients with gastric and colorectal cancer.

Finally recent reports have emerged focusing on the trophic effects of pro-gastrin and glycine-extended intermediates which are thought to operate via a receptor other than the gastrin/CCK_B receptor (Seva *et al* 1995; Kaise *et al* 1995). Examination of the growth mechanisms and the presence of these receptors on GI tumours may help to elucidate other growth mechanisms in hormonally controlled GI tumour cells. As yet the clinical relevance of these receptors in human GI tumour cells is not known but several

groups have shown that progastrin derived glycine extended intermediates may possess trophic properties in AR42J cells (Seva *et al* 1994; Nègre *et al* 1994; Kaise *et al* 1994).

This area merits further investigation with a requirement to characterise the glycine extended G17 receptor.

APPENDIX I

Cell concentration (1 x 10^{-6}cells/tube)	Total +/-SD (cpm)	Non-specific +/-SD	Specific +/-SD (cpm)	% Specific binding of total added
0.2	1675+/-99	<50*	1625+/-99	3
0.4	3485+/-408	106+/-50	3379+/-426	7
0.6	5302+/-252	185+/-37	5117+/-258	10
0.8	7793+/-109	509+/-30	7248+/-110	14
1.0	9453+/-164	670+/-74	8782+/-168	17
2.0	11416+/-704	1146+/-116	10270+/-639	20
2.4	12206+/-923	1259+/-93	10947+/-859	21

n = 6 experiments in duplicate
*** Counts below gamma counter background**

Table 1 **Effect of increasing cell concentrations on ^{125}I-G17 binding to AR42J cells.**

Time (mins)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)	% Specific binding of total added
5	2279+/-532	429+/-81	1850+/-479	4
15	3221+/-644	521+/-108	2701+/-543	6
30	5301+/-357	645+/-171	4656+/-294	9
60	7881+/-586	720+/-141	7161+/-524	15
90	9079+/-773	881+/-170	8199+/-646	17
120	9892+/-892	943+/-245	8949+/-857	18
150	10150+/-564	1038+/-225	9100+/-358	18
180	10529+/-529	1287+/-45	9241+/-495	18
210	10922+/-546	1411+/-89	9511+/-601	19
240	11089+/-458	1484+/-38	9605+/-482	19
270	11263+/-459	1543+/-36	9719+/-456	19

n = 5 experiments in duplicate

Table 2 **AR42J cell association time course**

Time (mins)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)	% Specific binding of total added
182	11125+/-645	909+/-227	10215+/-602	20
185	9870+/-642	839+/-160	9032+/-506	18
195	9040+/-566	787+/-106	8254+/-555	16
210	7874+/-947	758+/-76	7116+/-948	14
240	6580+/-412	666+/-59	5914+/-413	11
270	5367+/-409	647+/-107	4720+/-437	9
300	4814+/-262	666+/-123	4149+/-336	8
330	4031+/-153	613+/-120	3418+/-182	7
360	2401+/-202	557+/-99	1845+/-232	4

n = 6 experiments in duplicate

Table 3 **AR42J cell dissociation time course**

[^{125}I-G17] (nM)	Total added +/-SD (cpm)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Specific binding of total added
0.0070	**12231+/-958**	**2639+/-918**	**2505+/-926**	**20**
0.0142	**24391+/-2290**	**5423+/-1520**	**4867+/-1503**	**20**
0.0285	**47662+/-4080**	**9624+/-2695**	**8731+/-2678**	**18**
0.0570	**100001+/-9444**	**21065+/-3458**	**17626+/-3453**	**18**
0.1140	**196250+/-10404**	**38146+/-5465**	**32202+/-5036**	**16**

n = 5 experiments in duplicate

Table 4 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to AR42J cells**

Buffer	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)	% Specific binding of total added
1	1458+/-171	329+/-37	1128+/-161	2
2	1333+/-177	334+/-80	1000+/-135	2
3	2448+/-591	348+/-80	2101+/-550	4
4	8517+/-1016	587+/-99	7930+/-926	15
5	7316+/-928	572+/-117	6744+/-823	13
6	7108+/-1059	644+/-115	6464+/-992	13

n = 6 experiments in duplicate

Buffers

1 50mM Phosphate + 10mM $MgCl_2.6H_2O$ + 0.1% BSA

2 50mM Phosphate + 0.1% BSA

3 50mM Hepes + 0.1% BSA

4 50mM Hepes + 10mM $MgCl_2.6H_2O$ + 0.1% BSA

5 50mM Hepes + 10mM $CaCl_2$+ 0.1% BSA

6 50mM Hepes + 10mM $MgCl_2.6H_2O$ + 10mM $CaCl_2$ + 0.1% BSA

Table 5 **Effect of different buffers on ^{125}I-G17 binding to AR42J cells**

pH	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)	% Specific binding of total added
6.5	9188+/-1494	598+/-111	8590+/-1487	17
7.0	9362+/-766	611+/-84	8751+/-761	17
7.5	6706+/-984	531+/-12	6175+/-987	12
8.0	4355+/-503	482+/-53	3873+/-504	8

n = 6 experiments in duplicate

Table 6 **Effect of pH on ^{125}I-G17 binding to AR42J cells**

Time (mins)	4°C Specific +/-SD (cpm)	22°C Specific +/-SD (cpm)	37°C Specific +/-SD (cpm)
5	486+/-61	486+/-61	4749+/-864
15	1206+/-210	1206+/-210	4244+/-728
30	2186+/-238	2186+/-238	3118+/-567
60	3741+/-228	3741+/-228	1932+/-409
90	4169+/-320	4169+/-320	734+/-202
180	4632+/-439	4632+/-439	
270	4957+/-786	4957+/-786	

n = 6 experiments in duplicate

Table 7 **Effect of incubation temperature on ^{125}I-G17 binding to AR42J cells**

Concentration (M)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Binding of Maximum +/-%SD
1×10^{-8}	737+/-75	105+/-50	1+/-0.4
1×10^{-9}	2431+/-294	1799+/-307	25+/-5
1×10^{-10}	5766+/-840	5135+/-830	72+/-8
1×10^{-11}	6938+/-1265	6306+/-1237	87+/-4
1×10^{-12}	7476+/-1330	6844+/-1301	95+/-5
CONTROL	7859+/-1473	7227+/-1442	

n = 7 experiments in duplicate

Table 8 **G17 displacement of ^{125}I-G17 binding to AR42J cells**

Concentration (M)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Binding of Maximum +/-%SD
1×10^{-7}	1028+/-121	99+/-34	2+/-0.6
1×10^{-8}	1783+/-240	854+/-135	14+/-2
1×10^{-9}	2085+/-452	1325+/-99	22+/-2
1×10^{-10}	5082+/-259	4153+/-155	70+/-1
1×10^{-11}	6328+/-323	5400+/-219	91+/-1
1×10^{-12}	6490+/-420	5561+/-317	94+/-0.6
CONTROL	6865+/-410	5936+/-306	

n = 3 experiments in duplicate

Table 9 **CCK8S displacement of ^{125}I-G17 binding to AR42J cells**

Concentration (M)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Binding of Maximum +/-%SD
1×10^{-7}	834+/-70	97+/-54	1+/-0.6
1×10^{-8}	1360+/-142	623+/-153	10+/-2
1×10^{-9}	2875+/-393	2139+/-432	34+/-2
1×10^{-10}	6619+/-1023	5883+/-1079	93+/-5
1×10^{-11}	6796+/-1155	6060+/-1210	96+/-3
1×10^{-12}	7184+/-1038	6447+/-1077	102+/-6
CONTROL	7075+/-1169	6339+/-1216	

n = 4 experiments in duplicate

Table 10 **G34 displacement of ^{125}I-G17 binding to AR42J cells**

Concentration (M)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Binding of Maximum +/-%SD
1×10^{-7}	1524+/-273	443+/-436	3+/-3
1×10^{-8}	2387+/-319	1107+/-292	14+/-3
1×10^{-9}	6087+/-685	4807+/-747	60+/-8
1×10^{-10}	7886+/-683	6605+/-807	83+/-8
1×10^{-11}	9188+/-740	7907+/-524	99+/-0.4
1×10^{-12}	9179+/-712	7898+/-542	99+/-2
CONTROL	9243+/-643	7962+/-535	

n = 4 experiments in duplicate

Table 11 **CCK8 displacement of ^{125}I-G17 binding to AR42J cells**

Concentration (M)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Binding of Maximum +/-%SD
1×10^{-7}	1018+/-110	374+/-125	6+/-2
1×10^{-8}	2962+/-633	2314+/-648	35+/-10
1×10^{-9}	5485+/-648	4840+/-646	74+/-11
1×10^{-10}	7016+/-812	6363+/-750	97+/-8
1×10^{-11}	7286+/-1030	6687+/-986	101+/-7
1×10^{-12}	7225+/-1132	6573+/-1080	99+/-9
CONTROL	7305+/-1309	6672+/-1254	

n = 6 experiments in duplicate

Table 12 **L365260 displacement of ^{125}I-G17 binding to AR42J cells**

Concentration (M)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Binding of Maximum +/-%SD
$1 X 10^{-5}$	633+/-72	38+/-36	0.6+/-0.7
$1 X 10^{-6}$	1639+/-152	1043+/-96	16+/-2
$1 x 10^{-7}$	4879+/-508	4301+/-432	66+/-5
$1 x 10^{-8}$	6061+/-566	5465+/-535	84+/-12
$1 x 10^{-9}$	6820+/-889	6225+/-835	95+/-11
$1 x 10^{-10}$	7009+/-788	6413+/-698	98+/-7
CONTROL	7184+/-1057	6588+/-966	

n = 6 experiments in duplicate

Table 13 **L364718 displacement of ^{125}I-G17 binding to AR42J cells**

Concentration (M)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Binding of Maximum +/-%SD
$1 X 10^{-4}$	1488+/-210	431+/-67	7+/-1
$1 X 10^{-5}$	2855+/-477	1798+/-462	29+/-2
$1 X 10^{-6}$	5346+/-707	4289+/-747	69+/-2
$1 x 10^{-7}$	6885+/-966	5828+/-964	94+/-2
$1 x 10^{-8}$	7055+/-1045	5998+/-907	97+/-2
$1 x 10^{-9}$	7094+/-1187	6037+/-1186	97+/-3
CONTROL	7257+/-1064	6200+/-1076	

n = 4 experiments in duplicate

Table 14 **Lorglumide (CR1409) displacement of ^{125}I-G17 binding to AR42J cells.**

Concentration (M)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Binding of Maximum +/-%SD
$1 X 10^{-3}$	2025+/-131	900+/-264	15+/-4
$1 X 10^{-4}$	2827+/-329	1702+/-457	28+/-5
$1 x 10^{-5}$	4954+/-179	3829+/-465	63+/-3
$1 x 10^{-6}$	6838+/-256	5713+/-342	94+/-4
$1 x 10^{-7}$	7051+/-775	5927+/-862	98+/-3
$1 x 10^{-8}$	7031+/-909	5906+/-1002	97+/-1
CONTROL	7203+/-919	6078+/-1012	

n = 4 experiments in duplicate

Table 15 **Loxiglumide (CR1505) displacement of ^{125}I-G17 binding to AR42J cells**

Concentration (M)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Binding of Maximum +/-%SD
1×10^{-7}	1272+/-237	200+/-105	4+/-1.4
1×10^{-8}	2333+/-896	1261+/-785	26+/-11
1×10^{-9}	3947+/-874	2876+/-815	62+/-3
1×10^{-10}	4453+/-849	3381+/-807	74+/-3
1×10^{-11}	5193+/-1403	4171+/-1327	89+/-7
1×10^{-12}	5422+/-1213	4351+/-1159	94+/-3
CONTROL	5701+/-1239	4749+/-1147	

n = 4 experiments in duplicate

Table 16 **CAM1028 displacement of ^{125}I-G17 binding to AR42J cells.**

Concentration (M)	Total +/-SD (cpm)	Specific +/-SD (cpm)	% Binding of Maximum +/-%SD
$1 X 10^{-9}$	1194+/-155	357+/-90	4+/-1
$1 X 10^{-10}$	2983+/-273	2145+/-252	24+/-2
$1 x 10^{-11}$	5832+/-558	4994+/-597	56+/-6
$1 x 10^{-12}$	8385+/-368	7548+/-330	85+/-1
$1 x 10^{-13}$	9099+/-399	8262+/-375	92+/-1
$1 x 10^{-14}$	9505+/-73	8668+/-133	97+/-4
CONTROL	9750+/-368	8913+/-330	

n = 3 experiments in duplicate

Table 17 **L740093 displacement of ^{125}I-G17 binding to AR42J cells**

APPENDIX II

Concentration (ug/tube)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)	% Specific binding of total added
6.25	513 +/-82	52 +/-3	461 +/-84	1
12.5	1015 +/-111	109 +/-22	906 +/-127	2
25	1527 +/-222	131 +/-18	1396 +/-24	3
37.5	2249 +/-198	170 +/-12	2078 +/-194	4
50	3608 +/-188	283 +/-59	3324 +/-138	7
75	5455 +/-341	383 +/-33	5071 +/-351	10
100	7325 +/-572	545 +/-57	6780 +/-580	13
150	11431 +/-768	651 +/-24	10780 +/-743	20

n = 4 experiments in duplicate

Table 1 **Effect of increasing fraction II AR42J membrane concentration on ^{125}I-G17 binding**

Concentration (ug/tube)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)	% Specific binding of total added
6.25	**364 +/-40**	**91 +/-26**	**273 +/-29**	**0.5**
12.5	**1012 +/-44**	**209 +/-97**	**803 +/-100**	**2**
25	**2179 +/-146**	**267 +/-98**	**1912 +/-133**	**4**
37.5	**3819 +/-431**	**309 +/-98**	**3509 +/-410**	**7**
50	**5946 +/-586**	**377 +/-140**	**5569 +/-570**	**11**
75	**7937 +/-681**	**425 +/-77**	**7511 +/-701**	**15**
100	**9420 +/-1242**	**554 +/-34**	**8231 +/-675**	**16**
150	**12544 +/-1313**	**684 +/-67**	**11860 +/-1295**	**23**

n = 4 experiments in duplicate

Table 2 **Effect of increasing fraction I AR42J membrane concentration on ^{125}I-G17 binding**

Time (mins)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)	% Specific binding of total
5	1275+/-117	311+/-45	963+/-160	2
15	2200+/-120	357+/-43	1843+/-85	4
30	3212+/-137	455+/-129	2832+/-117	6
60	4752+/-108	474+/-81	4278+/-184	9
90	5442+/-268	509+/-42	4933+/-240	10
120	6646+/-224	519+/-26	6126+/-20	12
150	7501+/-308	600+/-32	6900+/-319	14
180	8381+/-517	624+/-38	7757+/-492	15
210	8534+/-662	655+/-57	7879+/-642	16
240	8515+/-659	688+/-58	7827+/-639	15
270	8486+/-738	739+/-8	7747+/-738	15

n = 4 experiments in duplicate

Table 3 **AR42J fraction II membrane association time course**

Time (mins)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)	% Specific binding of total added
180	8611 +/-1103	588 +/-25	8023 +/-1088	16
185	7717 +/-1187	559 +/-53	7157 +/-1185	15
195	6780 +/-1065	584 +/-55	5996 +/-1029	12
210	5777 +/- 877	560 +/- 74	5217 +/-819	11
240	4191 +/- 398	581 +/-91	3610 +/-379	7
270	3055 +/-316	648 +/-93	2407 +/-266	5
300	1807 +/-425	588 +/-139	1219 +/-426	2
330	1614 +/-253	581 +/-144	1033 +/-257	2

n = 4 experiments in duplicate

Table 4 **AR42J fraction II membrane dissociation time course**

Time (mins)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)	% Specific binding of total added
5	1626 +/-121	185 +/-21	1441 +/-110	3
15	2638 +/-106	218 +/-19	2420 +/-95	5
30	5144 +/-522	339 +/-59	4805 +/-117	10
60	7776 +/-843	419 +/-43	7356 +/-818	15
90	9585 +/-837	531 +/-67	9054 +/-776	19
120	10075 +/-409	542 +/-86	9532 +/-407	20
150	10659 +/-612	596 +/-51	10063 +/-577	21
180	11822 +/-757	615 +/-92	11206 +/-717	23
210	11348 +/-432	649 +/-60	10699 +/-452	22
240	11330 +/-629	664 +/-68	10666 +/-600	22

n = 4 experiments in duplicate

Table 5 **AR42J fraction I membrane association time course**

Time (mins)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)	% Specifc binding of total added
180	10588 +/-514	637 +/-84	9951 +/-472	21
185	9005 +/-319	634 +/-53	8370 +/-303	17
195	7872 +/-464	635 +/-87	7236 +/-462	15
210	6323 +/-550	631 +/-133	5692 +/-548	12
240	4130 +/-298	662 +/-175	3468 +/-240	7
270	3475 +/-288	670 +/-220	2805 +/-393	6
300	2642 +/-180	661 +/-264	1981 +/-436	4
330	2126 +/-165	624 +/-251	1502 +/-247	3

n = 4 experiments in duplicate

Table 6 **AR42J fraction I membrane dissociation time course**

[^{125}I-G17] (nM)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)
0.0029	**1002 +/-72**	**50 +/-0**	**952 +/-72**
0.014	**3878 +/-281**	**335 +/-42**	**3542 +/-259**
0.029	**8182 +/-1016**	**590 +/-29**	**7592 +/-1023**
0.043	**9415 +/-592**	**801 +/-53**	**8614 +/-582**
0.057	**13839 +/-1084**	**1044 +/-150**	**12794 +/-1079**
0.086	**21195 +/-2261**	**1304 +/-74**	**19890 +/-2195**
0.114	**26710 +/-1816**	**1581 +/-79**	**25128 +/-1796**

n = 3 experiments in duplicate

Table 7 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to AR42J fraction II membranes**

[^{125}I-G17] (nM)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)
0.0029	**1979 +/-237**	**353 +/-40**	**1626 +/-198**
0.014	**6141 +/-136**	**502 +/-10**	**5638 +/-144**
0.029	**12274 +/-823**	**658 +/-46**	**11615 +/-826**
0.043	**15731 +/-441**	**773 +/-36**	**14958 +/-476**
0.057	**18482 +/-265**	**913 +/-54**	**17569 +/-293**
0.086	**28605 +/-829**	**1255 +/-173**	**27350 +/-660**
0.114	**36129 +/-1420**	**1693 +/-47**	**34436 +/-1409**

n = 3 experiments in duplicate

Table 8 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to AR42J fraction I membranes**

Buffer	Membrane fraction	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)
1	I	5877+/-552	1134+/-195	4743+/-727
2	I	11375+/-544	947+/-95	10428+/-474
3	I	15432+/-638	2355+/-156	13077+/-631
1	II	5496+/-971	848+/-71	4648+/-1032
2	II	8282+/-516	990+/-63	7292+/-516
3	II	13642+/-556	3177+/-205	10465+/-576

n = 4 experiments in duplicate

Buffers

1. 50mM Hepes + 10mM $MgCl_2.6H_20$ + 0.1% BSA, pH 7.0
2. 50mM Hepes + 10mM $MgCl_2.6H_20$ + 1uM SBTI + 0.1% BSA, pH7.0
3. 50mM Hepes + 10mM $MgCl_2.6H_20$ + 1uM SBTI + 1uM Bacitracin + 1uM Bestatin + 1uM PMSF + 1.5mM DTT + 1mM Aprotinin + 10% glycerol + 0.1% BSA, pH7.0

Table 9 **Effect of protease inhibitors on ^{125}I-G17 binding to AR42J membranes**

pH	Membrane fraction	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)
6.5	I	12351+/-794	1152+/-109	11199+/-782
7.0	I	10570+/-1281	872+/-95	9698+/-1199
7.5	I	8849+/-336	832+/-24	8017+/-327
8.0	I	7710+/-142	634+/-107	7076+/-102
6.5	II	8745+/-292	1229+/-97	7515+/-341
7.0	II	7921+/-97	1137+/-76	6785+/-173
7.5	II	6304+/-1032	1095+/-227	5209+/-929
8.0	II	4937+/-701	902+/-73	4035+/-631

Fraction I **n = 4 experiments in duplicate**
Fraction II **n = 3 experiments in duplicate**

Table 10 **Effect incubation buffer pH on ^{125}I-G17 binding to AR42J membranes**

Temp (°C)	Membrane fraction	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)
4	I	3638+/-101	842+/-65	2796+/-153
22	I	10212+/-902	722+/-10	9491+/-901
37	I	3110+/-138	538+/-41	2573+/-100
4	II	5820+/-189	959+/-36	4861+/-159
22	II	8256+/-148	1053+/-50	7204+/-160
37	II	3461+/-393	1055+/-93	2406+/-301

n = 4 experiments in duplicate

Table 11 **Effect of incubation temperature on ^{125}I-G17 binding to AR42J membranes**

Day	Addition	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)
0*	-	11143+/-1141	1041+/-81	10101+/-1214
1	-	6736+/-552	545+/-127	6191+/-556
1	Glycerol	10167+/-356	390+/-65	9776+/-355
1	Sucrose	9714+/-271	454+/-59	9260+/-261
2	-	5761+/-107	537+/-107	5223+/-211
2	Glycerol	9769+/-293	489+/-109	9280+/-261
2	Sucrose	8797+/-470	450+/-58	8347+/-477
7	-	3570+/-719	287+/-112	3283+/-654
7	Glycerol	5168+/-946	290+/-74	4878+/-890
7	Sucrose	4857+/-955	272+/-34	4585+/-938
14	-	1804+/-234	164+/-62	1638+/-270
14	Glycerol	2928+/-335	121+/-54	2807+/-349
14	Sucrose	2469+/-385	126+/-46	2343+/-422

n = 3 experiments in duplicate

*** Membranes used on day of preparation**

Table 12 **Effect of storage time and protective agents on ^{125}I-G17 binding to AR42J membranes**

Temperature (°C)	Total +/-SD (cpm)	Non-specific +/-SD (cpm)	Specific +/-SD (cpm)
*	11896+/-398	1023+/-56	10873+/-431
4	5896+/-148	417+/-82	5506+/-154
-20	4998+/-108	321+/-82	4677+/-100
-40	5225+/-118	290+/-23	4935+/-95
-70	10568+/-687	313+/-51	10254+/-727

n = 4 experiments in duplicate

*** Membranes used without freezing**

Table 13 **Effect of AR42J fraction II membrane storage temperature on ^{125}I-G17 binding**

Concentration (M)	Fraction II % Binding of maximum +/-%SD	Fraction I % Binding of maximum +/-%SD
1×10^{-7}	3+/-3	3+/-1
5×10^{-8}	10+/-1	5+/-1
1×10^{-8}	25+/-6	13+/-3
5×10^{-9}	34+/-5	22+/-2
1×10^{-9}	55+/-9	48+/-2
5×10^{-10}	69+/-5	57+/-12
1×10^{-10}	96+/-7	78+/-2
5×10^{-11}	95+/-5	86+/-10
1×10^{-11}	98+/-6	92+/-8
5×10^{-12}	100+/-6	99+/-2
1×10^{-12}	101+/-6	100+/-4

n = 4 experiments in duplicate

Table 14 **G17 displacement of ^{125}I-G17 binding to AR42J membranes**

Concentration (M)	Fraction II % Binding of maximum +/-%SD	Fraction I % Binding of maximum +/-%SD
$1 X 10^{-6}$	3+/-0.5	-
$5 X 10^{-7}$	7+/-0.5	2+/-0.5
$1 X 10^{-7}$	15+/-3	9+/-0.5
$5 X 10^{-8}$	33+/-2	23+/-1
$1 X 10^{-8}$	64+/-10	49+/-5
$5 X 10^{-9}$	76+/-6	61+/-2
$1 X 10^{-9}$	87+/-4	85+/-5
$5 X 10^{-10}$	93+/-2	95+/-4
$1 X 10^{-10}$	96+/-3	98+/-1

n = 4 experiments in duplicate

Table 15 **L365260 displacement of ^{125}I-G17 binding to AR42J membranes**

Concentration (M)	Fraction II % Binding of maximum +/-%SD	Fraction I % Binding of maximum +/-%SD
1×10^{-5}	3+/-2	-
5×10^{-6}	8+/-0.5	-
1×10^{-6}	15+/-3	4+/-3
5×10^{-7}	32+/-2	21+/-2
1×10^{-7}	63+/-7	52+/-3
5×10^{-8}	75+/-3	61+/-1
1×10^{-8}	87+/-7	77+/-2
5×10^{-9}	92+/-4	82+/-1
1×10^{-9}	93+/-4	84+/-3
5×10^{-10}	96+/-3	88+/-4
1×10^{-10}	97+/-8	92+/-2

n = 4 experiments in duplicate

Table 16 **L364718 displacement of ^{125}I-G17 binding to AR42J membranes**

Concentration (M)	CCK8S % Binding of maximum +/-%SD	G34 % Binding of maximum +/-%SD	CCK8 % Binding of maximum +/-%SD
$1 X 10^{-6}$	-	-	3+/-1
$1 X 10^{-7}$	2+/-1	1+/-0.5	19+/-6
$1 X 10^{-8}$	13+/-1	16+/-2	35+/-5
$1 X 10^{-9}$	41+/-3	28+/-3	70+/-5
$1 X 10^{-10}$	90+/-2	72+/-2	88+/-3
$1 X 10^{-11}$	93+/-4	95+/-2	95+/-4
$1 X 10^{-12}$	96+/-3	96+/-2	97+/-7
$1 X 10^{-13}$	-	98+/-3	-
$1 X 10^{-14}$	-	98+/-5	-

n = 4 experiments in duplicate

Table 17 **Agonist displacement of ^{125}I-G17 binding to AR42J fraction II membranes**

Concentration (M)	CAM1028 % Binding of maximum +/-%SD	L740093 % Binding of maximum +/-%SD
$1 X 10^{-7}$	6+/-1	-
$1 X 10^{-8}$	23+/-1	-
$1 X 10^{-9}$	63+/-3	2+/-1
$1 X 10^{-10}$	76+/-2	21+/-3
$1 X 10^{-11}$	93+/-4	61+/-3
$1 X 10^{-12}$	95+/-3	82+/-2
$1 X 10^{-13}$	-	91+/-1
$1 X 10^{-14}$	-	93+/-5
$1 X 10^{-15}$	-	91+/-6
$1 X 10^{-16}$	-	93+/-3

n = 4 experiments in duplicate

Table 18 **Antagonist displacement of ^{125}I-G17 binding to AR42J fraction II membranes**

APPENDIX III

Patient Initials	Tissue	[^{125}I-G17] (nM)	Total binding (cpm)	Non-Specific binding (cpm)	Specific binding (cpm)
G.McK.	normal	0.1	<50	<50	0
		0.25	224	198	26
		0.5	398	403	0
G.McK.	tumour	0.1	123	198	0
		0.25	434	366	68
		0.5	669	735	0
P.S.	normal	0.1	155	82	73
		0.25	<50	<50	0
		0.5	272	279	0
P.S.	tumour	0.1	170	131	39
		0.25	296	339	0
		0.5	633	697	0
M.McL	normal	0.1	90	80	10
		0.25	145	130	15
		0.5	331	396	0
M.McL	tumour	0.1	252	282	0
		0.25	497	433	64
		0.5	1433	1269	164

Table 1 **Colorectal patients normal and tumour results :**
1. Frozen tissue samples - Cryostat method

Patient Initials	Tissue	[^{125}I-G17] (nM)	Total binding (cpm)	Non-Specific binding (cpm)	Specific binding (cpm)
E.L.	normal	0.1	84	66	18
		0.25	101	83	18
		0.5	233	172	61
E.L.	tumour	0.1	423	292	131
		0.25	882	823	59
		0.5	1686	1567	119
H.B.	normal	0.1	225	162	63
		0.25	592	406	186
		0.5	1104	791	313
H.B.	tumour	0.1	458	443	15
		0.25	1406	1059	347
		0.5	2561	2015	546
B.T	normal	0.1	117	89	28
		0.25	222	189	33
		0.5	311	299	12
B.T.	tumour	0.1	348	303	45
		0.25	740	736	4
		0.5	1512	1113	399

Table 2 **Colorectal patient normal and tumour results :**
2. Frozen tissue samples - pulverised method
(page one of four)

Patient Initials	Tissue	[^{125}I-G17] (nM)	Total binding (cpm)	Non-Specific binding (cpm)	Specific binding (cpm)
A.B.	normal	0.1	478	410	68
		0.25	1060	881	179
		0.5	1894	1527	367
A.B.	tumour	0.1	896	870	26
		0.25	2453	2144	309
		0.5	4223	3929	294
J.Ar.	normal	0.1	131	109	22
		0.25	267	215	52
		0.5	474	462	12
J.Ar.	tumour	0.1	235	183	52
		0.25	483	384	99
		0.5	883	729	154
T.W.	normal	0.1	89	83	6
		0.25	161	121	40
		0.5	215	132	83
T.W.	tumour	0.1	96	71	25
		0.25	60	53	7
		0.5	156	116	40

Table 2 (continued) **Colorectal patient normal and tumour results :**
2. Frozen tissue samples - pulverised method
(page two of four)

Initials	Tissue	[^{125}I-G17] (nM)	Total binding (cpm)	Non-Specific binding (cpm)	Specific binding (cpm)
D.H.	normal	0.125	1234	964	270
D.H.	tumour	0.125	4498	4278	220
R.S.	normal	0.125	1226	1022	204
R.S.	tumour	0.125	4552	4013	539
P.G.	normal	0.125	684	503	181
P.G.	tumour	0.125	1556	1459	97
M.R.	normal	0.125	1556	1223	333
M.R.	tumour	0.125	3856	3476	380
C.C.	normal	0.125	2491	2414	77
C.C.	tumour	0.125	14039	13435	604
I.D.	normal	0.125	759	705	54
I.D.	tumour	0.125	1862	1782	80
J.Co.	normal	0.125	562	568	0
J.Co.	tumour	0.125	1054	1005	49
T.T.	normal	0.125	2076	1383	693
T.T.	tumour	0.125	4926	4836	90

Table 2 (continued) **Colorectal patient normal and tumour results :**
2. Frozen tissue samples - pulverised method
(page three of four)

Initials	Tissue	[^{125}I-G17] (nM)	Total binding (cpm)	Non-Specific binding (cpm)	Specific binding (cpm)
I.M.	normal	0.125	128	106	22
I.M.	tumour	0.125	562	448	114
J.H.	normal	0.125	1003	912	91
J.H.	tumour	0.125	3008	2873	135
G.A.	normal	0.125	168	125	43
G.A.	tumour	0.125	669	603	66
J.Cu.	normal	0.125	915	841	74
J.Cu.	tumour	0.125	1200	1009	191
J.K.	normal	0.125	3748	2996	752
J.K.	tumour	0.125	24491	22207	2284
L.H.	normal	0.125	4221	3622	599
L.H.	tumour	0.125	5522	4225	1297
A.Cr.	normal	0.125	526	428	98
A.Cr.	tumour	0.125	1230	1133	97
M.Mo.	normal	0.125	698	624	74
M.Mo.	tumour	0.125	1987	1705	282

Table 2 (continued) **Colorectal patient normal and tumour results**
2. Frozen tissue samples - pulverised method
(page four of four)

Initials	Tissue	[^{125}I-G17] (nM)	Total binding (cpm)	Non-specific binding (cpm)	Specific binding (cpm)
J.B.	normal	0.1	415	376	39
		0.25	945	757	188
		0.5	2057	1818	239
J.B.	tumour	0.1	1126	952	174
		0.25	2616	2458	158
		0.5	5559	5237	322
R.H.	normal	0.1	291	286	5
		0.25	840	673	167
		0.5	2346	2147	199
R.H.	tumour	0.1	532	496	36
		0.25	1067	749	318
		0.5	2996	2872	124
R.P.	normal	0.1	592	406	186
		0.25	2492	1994	498
		0.5	5324	4962	362
R.P.	tumour	0.1	2104	1724	380
		0.25	4886	4680	206
		0.5	10062	8924	1138

Table 3 **Colorectal patient normal and tumour results :**
3. Fresh tissue samples - homogenisation method
(table one of three)

Initials	Tissue	[^{125}I-G17] (nM)	Total binding (cpm)	Non-Specific binding (cpm)	Specific binding (cpm)
F.M.	normal	0.1	912	722	190
		0.25	1975	1336	639
		0.5	4650	3928	722
F.M.	tumour	0.1	532	426	106
		0.25	1470	867	603
		0.5	4006	3862	144
M.Cl.	normal	0.1	282	280	2
		0.25	612	173	439
		0.5	924	592	332
M.Cl.	tumour	0.1	1269	941	328
		0.25	2405	2116	289
		0.5	4049	3239	810
J.Ca.	normal	0.1	491	426	65
		0.25	1012	608	404
		0.5	2588	1896	692
J.Ca.	tumour	0.1	916	824	92
		0.25	2145	1577	568
		0.5	4390	4068	322

Table 3 (continued) **Colorectal patients normal and tumour results :**
3. Fresh tissue samples - homogenisation method
(page two of three)

Initials	Tissue	[^{125}I-G17] (nM)	Total binding (cpm)	Non-specific binding (cpm)	Specific binding (cpm)
I.E.	normal	0.1	922	812	110
		0.25	2091	1361	730
		0.5	5034	4962	72
I.E.	tumour	0.1	1008	934	74
		0.25	3553	2614	939
		0.5	9146	8290	856
J.M.	normal	0.1	162	176	0
		0.25	975	906	69
		0.5	2386	2191	195
		1.0	4652	4000	652
J.M.	tumour	0.1	336	317	19
		0.25	1973	1662	311
		0.5	3783	3748	35
		1.0	6863	6046	817
M.Y.	normal	0.1	328	304	24
		0.25	711	659	52
		0.5	1724	1448	276
M.Y.	tumour	0.1	641	604	37
		0.25	1413	1292	121
		0.5	3690	2628	1062

Table 3 (continued) **Colorectal patients normal and tumour results :**
3. Fresh tissue samples - homogenisation method
(page three of three)

APPENDIX IV

[^{125}I-G17] (nM)	Membrane	Total (cpm)	Non-specific (cpm)	Specific (cpm)	% Specific binding
0.125	**Body**	**1945**	**819**	**1126**	**58**
0.25	**"**	**3322**	**1646**	**1676**	**50**
0.5	**"**	**4682**	**4059**	**623**	**13**
0.125	**Tumour**	**314**	**272**	**42**	**13**
0.25	**"**	**574**	**446**	**128**	**22**
0.5	**"**	**1191**	**1034**	**157**	**13**

Results are representative of two experiments performed in duplicate

Table 1 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to membranes from patient J.N.**

Time (mins)	Membrane	Total (cpm)	Specific (cpm)	% Specific binding
2.5	Body	1963	505	51
5	"	3677	1875	49
15	"	3641	1777	46
30	"	3530	1610	42
60	"	3537	1481	42
2.5	Tumour	748	42	6
5	"	1174	58	5
15	"	1163	161	14
30	"	1119	92	8
60	"	1056	-	-

Results are from one experiment in duplicate

Table 2 **Association time courses for membranes from patient J.N.**

Concentration (M)	~~L365260~~ G17 % Binding of Maximum	L365260 % Binding of Maximum	L364718 % Binding of Maximum
5×10^{-6}	-	-	8
1×10^{-6}	-	-	18
5×10^{-7}	-	-	40
1×10^{-7}	-	-	59
5×10^{-8}	-	1	78
1×10^{-8}	-	8	86
5×10^{-9}	1	21	92
1×10^{-9}	18	48	91
5×10^{-10}	40	64	90
1×10^{-10}	68	78	94
5×10^{-11}	72	84	-
1×10^{-11}	81	90	-
5×10^{-12}	90	91	-
1×10^{-12}	92	92	-

Results are representative of two experiments in duplicate

Table 3 **Displacement of ^{125}I-G17 binding to body membranes from patient J.N.**

[^{125}I-G17] (nM)	Membrane	Total (cpm)	Non-specific (cpm)	Specific (cpm)	% Specific binding
0.125	**Body**	**2286**	**1944**	**342**	**15**
0.25	**"**	**3544**	**2876**	**668**	**19**
0.5	**"**	**7931**	**5448**	**2483**	**31**
0.125	**Antral**	**1762**	**1646**	**116**	**7**
0.25	**"**	**2518**	**2234**	**284**	**11**
0.5	**"**	**5478**	**4891**	**587**	**11**
0.125	**Tumour**	**462**	**413**	**49**	**11**
0.25	**"**	**948**	**902**	**46**	**5**
0.5	**"**	**2308**	**1517**	**791**	**34**

Results are representative of two experiments in duplicate

Table 4 **Effect of increasing radiolabel concentrations on ^{125}I-G17 binding to membranes from patient J.M.**

Concentration (M)	G17 % Binding of Maximum	L365260 % Binding of Maximum	L364718 % Binding of Maximum
1×10^{-5}	-	-	1
1×10^{-6}	-	-	14
1×10^{-7}	9	13	48
1×10^{-8}	35	54	74
1×10^{-9}	50	86	83
1×10^{-10}	78	92	98
1×10^{-11}	91	94	-
1×10^{-12}	92	98	-

Results are from one experiment in duplicate

Table 5 **Displacement of ^{125}I-G17 binding to body membranes from patient J.M.**

[^{125}I-G17] (nM)	Membrane	Total (cpm)	Non-specific (cpm)	Specific (cpm)	% Specific binding
0.125	**Body**	**1246**	**642**	**604**	**48**
0.25	**"**	**3064**	**1930**	**1134**	**37**
0.5	**"**	**5762**	**3400**	**2362**	**41**
0.125	**Antral**	**442**	**401**	**41**	**9**
0.25	**"**	**1062**	**956**	**106**	**10**
0.5	**"**	**2147**	**1963**	**184**	**9**
0.125	**Tumour**	**1062**	**758**	**304**	**29**
0.25	**"**	**3006**	**2494**	**512**	**17**
0.5	**"**	**4866**	**3919**	**947**	**19**

Results are representative of one experiment in duplicate

Table 6 **Effect of increasing radiolabel concentrations on ^{125}I-G17 binding to membranes from patient G.S.**

	Body			Antrum			Tumour		
Time (mins)	**Total** (cpm)	**Specific** (cpm)	**% Specific binding**	**Total** (cpm)	**Specific** (cpm)	**% Specific binding**	**Total** (cpm)	**Specific** (cpm)	**% Specific binding**
5	**1171+/-72**	**220+/-21**	**19+/-1**	**638**	**111**	**17**	**1427**	**48**	**11**
15	**1664+/-68**	**476+/-169**	**23+/-4**	**880**	**38**	**4**	**1876**	**336**	**18**
30	**2037+/-60**	**673+/-86**	**33+/-4**	**992**	**117**	**12**	**1995**	**297**	**15**
90	**2822+/-71**	**1028+/-179**	**36+/-6**	**1139**	**72**	**6**	**2711**	**471**	**17**
120	**2844+/-48**	**805+/-98**	**29+/-3**	**1026**	**67**	**7**	**2776**	**527**	**19**
180	**2769+/-57**	**662+/-167**	**24+/-6**	**987**	**5**	**0.5**	**2643**	**157**	**6**

Body membranes, n = 3 experiements in duplicate; Antral and tumour membranes, n = one experiment in duplicate

Table 7 **Association time courses for membranes from patient G.S.**

Concentration (M)	G17 % Binding of Maximum +/-SD	G34 % Binding of Maximum +/-SD
1×10^{-7}	4+/-3	3+/-2
1×10^{-8}	12+/-2	15+/-1
1×10^{-9}	36+/-4	41+/-3
1×10^{-10}	61+/-1	65+/-4
1×10^{-11}	89+/-3	95+/-4
1×10^{-12}	91+/-4	92+/-2
1×10^{-13}	-	90+/-1
1×10^{-14}	-	100+/-2

n = 3 experiments in duplicate

Table 8 **Agonist displacement of ^{125}I-G17 binding to body membranes from patient G.S.**

Concentration (M)	L365260 % Binding of Maximum +/-SD	L364718 % Binding of Maximum +/-SD
1×10^{-5}	-	2+/-2
1×10^{-6}	-	26+/-4
1×10^{-7}	17+/-1	51+/-1
1×10^{-8}	35+/-2	80+/-2
1×10^{-9}	62+/-2	88+/-7
1×10^{-10}	78+/-3	93+/-12
1×10^{-11}	91+/-1	-
1×10^{-12}	95+/-1	-

n = 3 experiments in duplicate

Table 9 **Antagonist displacement of ^{125}I-G17 binding to body membranes from patient G.S.**

[^{125}I-G17] (nM)	Membrane	Total (cpm)	Non-specific (cpm)	Specific (cpm)	% Specific binding
0.125	**Body**	**4632**	**3847**	**785**	**17**
0.25	**"**	**9448**	**7246**	**2202**	**23**
0.5	**"**	**14008**	**12986**	**1022**	**7**
0.125	**Antral**	**2166**	**1966**	**200**	**9**
0.25	**"**	**5348**	**4814**	**534**	**10**
0.5	**"**	**9262**	**8887**	**375**	**4**
0.125	**Tumour**	**1506**	**1287**	**219**	**15**
0.25	**"**	**3048**	**2546**	**502**	**16**
0.5	**"**	**5962**	**5132**	**830**	**14**

Results are representative of two experiments in duplicate

Table 10 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to membranes from patient F.S.**

Concentration (M)	G17 % Binding of Maximum
1×10^{-7}	12
1×10^{-8}	36
1×10^{-9}	74
1×10^{-10}	86
1×10^{-11}	91
1×10^{-12}	94

Results are from one experiment in duplicate

Table 11 **Displacement of ^{125}I-G17 binding to body membranes from patient F.S.**

[^{125}I-G17] (nM)	Membrane	Total (cpm)	Non-specific (cpm)	Specific (cpm)	% Specific binding
0.125	**Body**	**1867**	**1689**	**178**	**10**
0.25	**"**	**4184**	**3977**	**207**	**5**
0.5	**"**	**6745**	**5350**	**1395**	**21**
0.125	**Tumour**	**347**	**297**	**50**	**14**
0.25	**"**	**670**	**632**	**38**	**6**
0.5	**"**	**1182**	**1008**	**174**	**15**

Results are representative of two experiments in duplicate

Table 13 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to membranes from patient A.H.**

[^{125}I-G17] (nM)	Membrane	Total (cpm)	Non-specific (cpm)	Specific (cpm)	% Specific binding
0.125	**Antral**	**562**	**398**	**164**	**29**
0.25	**"**	**1034**	**824**	**210**	**20**
0.5	**"**	**1744**	**1504**	**240**	**14**
0.125	**Tumour**	**362**	**308**	**54**	**15**
0.25	**"**	**824**	**811**	**13**	**2**
0.5	**"**	**1286**	**1136**	**150**	**12**

Results are representative of two experiments in duplicate

Table 14 **Effect of increasing radiolabel on ^{125}I-G17 binding to membranes from patient C.R.**

[^{125}I-G17] (nM)	Membrane	Total (cpm)	Non-specific (cpm)	Specific (cpm)	% Specific binding
0.125	**Antral**	**663**	**513**	**150**	**23**
0.25	**"**	**1214**	**998**	**216**	**18**
0.5	**"**	**2210**	**1502**	**708**	**32**
0.125	**Tumour**	**902**	**847**	**55**	**6**
0.25	**"**	**1510**	**1332**	**178**	**12**
0.5	**"**	**3157**	**2394**	**763**	**24**

Results are representative of two experiments in duplicate

Table 15 **Effect of increasing radiolabel concentration on ^{125}I-G17 binding to membranes from patient S.H.**

APPENDIX V

Poster

"Development of an assay to detect gastrin receptors in gastrointestinal tumours"
Presented as a poster at the British Society of Gastroenterology in Edinburgh, September 1994.

Presentation

"Development of an assay to detect gastrin receptors in the human gastrointestinal tract"
Oral presentation at the British Society of Gastroenterology in Brighton, March 1996.

Publications

JF Mackenzie, CA Dorrian, ID Penman, VP Gerskowitch, KEL McColl. Development of an assay to detect gastrin receptors in gastrointestinal tumours. **Gut 1994; 35 (suppl) : S73 (abstract)**

JF Mackenzie, CA Dorrian, ID Penman, VP Gerskowitch, KEL McColl. Development of an assay to detect gastrin receptors in the human gastrointestinal tract. **Gut 1996; (suppl) : T146 (abstract)**

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