# Modulation of Neuropeptide Growth Factor Signalling by Anti-cancer Substance-P Analogues

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A Thesis submitted for the Degree of Doctor of Philosophy

The University of Edinburgh

2005

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# Declaration

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous candidature for a higher degree. All work presented in this thesis, unless acknowledged, was initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Uzma Tufail-Hanif

30/09/05

# Acknowledgements

In the name of Allah, The Compassionate, The Merciful

"Glory to You (Allah), of knowledge we have none, save what you have taught us:
In truth it is You who is perfect in knowledge and wisdom."

(al-Quran: Ch2 v32)

Any knowledge obtained through this study has only been revealed by the Will of Allah, the originator of creation and giver of knowledge. Therefore, I feel that all praise and thanks for the completion of this thesis are primarily due to Him.

I would next like to thank Alison MacKinnon for her invaluable help and support through the course of my time as a PhD student in the lab and particularly for her ongoing support following my maternity leave and during my write-up. Thanks also to Tariq Sethi and Duncan Jodrell. For discussions about experimental methods, thanks to Julie Love, Elizabeth Collis, Yatish Lad, Weng Sie Wong, Al Jimenez and Neil Henderson. Thanks to Kirsten Atkinson for help with plasmid preps for G-protein over-expression. I would also like to thank a few of my past supervisors (Martin Rumsby, Mike Whelan and Andreas Schatzlein) for their encouraging words of advice which helped in my consideration and pursuit of a PhD.

A very big thank you to my husband for putting up with the adverse effects of this PhD on so many things, especially during the six months of writing up. At the moment I can't even imagine a married life where I'm not doing my PhD but I do look forward to it! I could not have done this without you Irfan! Thank you so much for all your support and especially for having to live without me and Hasan when we moved to my mum's house for two months of the write-up. Thanks to my younger sister Saira for taking care of Hasan for the two months we were staying and to my mum and dad for letting us come to stay. It was such a big help, I really would not be submitting this thesis without your help. Thanks to my mum and dad for everything they have done for me which has enabled me to get this far and for

always giving me so much encouragement and support in pursuing my studies....even when it looked like I may never stop being a student! Thanks to my ever-supporting sisters Shana, Rosie and Saira for putting up with the lack of communication on my part as a consequence of being busy with the PhD, and especially for all the times I've not been in touch while writing up. Thanks to my in-laws for putting up with not seeing or hearing from me (and by association, Hasan) very much over the last six months. Thanks also to my relatives and friends for their prayers to help me to get this thesis finished while understanding my difficulty in keeping in touch.

Last but not least, a special thank-you to my darling son Hasan Yahya for going to nursery while I wrote my thesis. For all the times I could not be with you, I apologise but I want you to know that I missed you too and was thinking about you!

# List of Abbreviations

| | |
|---|---|
| **aa** | Amino acid |
| **ADTRS** | Agonist directed trafficking of receptor signalling |
| **ADH** | Antidiuretic hormone |
| **Akt** | Protein kinase B |
| **Ala** | Alanine |
| **Anoikis** | Detachment induced apoptosis (from the Greek for homelessness) |
| **APS** | Ammonium persulphate |
| **Arg** | Arginine |
| **ATP** | Adenosine triphosphate |
| **AVP** | Arginine$^{8}$-vasopressin |
| **BN** | Bombesin |
| **BLI** | Bombesin-like immunoreactivity |
| **BRP** | Bombesin related peptide |
| **BSA** | Bovine serum albumin |
| **$Ca^{2+}$** | Calcium |
| **CaCl** | Calcium chloride |
| **CAM** | Cell adhesion molecule |
| **cAMP** | Cyclic adenosine monophosphate |
| **CAS** | p130 Crk associated substrate |
| **CCK** | Cholecystokinin |
| **cDNA** | Cloned DNA |
| **CHO** | Chinese hamster ovary |
| **CRUK** | Cancer Research UK |
| **CTC** | Cubic ternary complex |
| **DCC** | L-Dopa decarboxylase |
| **DMEM** | Dulbecco's modified eagles medium |

| | |
|---|---|
| **DMSO** | Dimethylsulphoxide |
| **DTT** | Dithiothreitol |
| **80K/MARCKS** | Myristoylated alanine-rich C kinase substrate |
| **e1** | GPCR extracellular loop 1 |
| **e2** | GPCR extracellular loop 2 |
| **e3** | GPCR extracellular loop 3 |
| **$EC_{50}$** | The concentration of an agonist, which produces 50% of the maximum possible response for that agonist |
| **ECL** | Enhanced chemiluminescence |
| **EGFR** | Epidermal growth factor receptor |
| **ETC** | Extended ternary complex |
| **ERK** | Extracellular regulated kinase |
| **FACS** | Fluorescence activated cell sorter |
| **FAK** | Focal adhesion kinase |
| **FCS** | Foetal calf serum |
| **FSH** | Follicle stimulating hormone |
| **FTS** | S-*trans,trans*-farnesylthiosalicylic acid |
| **FURA-2/AM** | Fura-2-tetraacetoxymethylester AME |
| **G418** | Geneticin |
| **Gln** | Glutamine |
| **Gly** | Glycine |
| **GDP** | Guanine diphosphate |
| **GHRH** | Growth hormone releasing hormone |
| **GMP** | Guanine monophosphate |
| **G-protein** | Guanine nucleotide binding protein |
| **GPCR** | G protein coupled receptor |
| **GRP** | Gastrin releasing peptide |
| **GRPR** | Gastrin releasing peptide receptor |
| **GTP** | Guanine triphosphate |

| | |
|---|---|
| **HBSS** | Hanks balanced salt solution |
| **hCG** | Human chorionic gonadotrophin |
| **HRP** | Horseradish peroxidase |
| **i1** | GPCR intracellular loop 1 |
| **i2** | GPCR intracellular loop 2 |
| **i3** | GPCR intracellular loop 3 |
| **$IC_{50}$** | The concentration of an agonist, which produces 50% of the maximum possible inhibitory response for that agonist |
| **IGF** | Insulin-like growth factor |
| **IGF-1R** | Insulin-like growth factor receptor type 1 |
| **JNK** | c-Jun N-terminal kinase |
| **KSHV** | Kaposi's sarcoma-associated herpes virus |
| **$K_d$** | Dissociation constant describes the strength of binding (affinity) between the receptor and ligand |
| **$K_i$** | Inhibition constant is the equilibrium dissociation constant for binding of the unlabelled drug |
| **Leu** | Leucine |
| **LH** | Luteinizing hormone |
| **LPA** | Lysophosphatidic acid |
| **Lys** | Lysine |
| **mAChR** | $M_3$ muscarinic acetylcholine receptor |
| **MAPK** | Mitogen activated protein kinase |
| **MDR** | Multidrug resistant |
| **Met** | Methionine |
| **$MgCl_2$** | Magnesium chloride |
| **mRNA** | Messenger ribonucleic acid |
| **MTT** | 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide |
| **NaCl** | Sodium chloride |

| | |
|---|---|
| **NMB** | Neuromedin B |
| **NRSE** | Neuron-restrictive silencer element |
| **NSCLC** | Non-small cell lung cancer |
| **$PA_2$** | Antagonist potency |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **pcDNA** | plasmid cDNA |
| **PBS** | Phosphate buffered saline |
| **Phe** | Phenylalanine |
| **PI3-K** | Phosphatidylinositol 3-kinase |
| **PKC** | Protein kinase C |
| **PKD** | Protein kinase D |
| **PLC** | Phospholipase C |
| **PMA** | Phorbol $^{12}$-myristate $^{13}$-acetate |
| **PMSF** | Phenyl methane sulfonyl fluoride |
| **Pro** | Proline |
| **PTx** | Pertussis toxin |
| **ROS** | Reactive oxygen species |
| **RPM** | Revolutions per minute |
| **RTK** | Receptor tyrosine kinase |
| **SCLC** | Small cell lung cancer |
| **SDS** | Sodium dodecyl sulphate |
| **SH2** | Src-homology 2 |
| **SP** | Substance P |
| **SP-D** | Substance P analogue D |
| **SP-G** | Substance P analogue G |
| **STI** | Soybean Trypsin Inhibitor |
| **TBST** | Tris buffered saline containing Tween-20 |
| **TM** | Transmembrane region |
| **Trp** | Tryptophan |
| **TSH** | Thyroid stimulating hormone |

| | |
|---|---|
| **USF** | Upstream stimulatory factor |
| **$V_{1A}R$** | Vasopressin receptor subtype 1A |
| **VP** | Vasopressin |

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# Abstract

Widespread metastases are characteristic of the most aggressive form of lung cancer, small cell lung cancer (SCLC). Although initially sensitive to treatment by radio- and chemotherapy, SCLC develops chemoresistance so the 2-year survival rate remains less than 5%. The aberrant proliferation of SCLC is sustained by multiple autocrine and paracrine growth loops involving calcium mobilising neuropeptides such as vasopressin (AVP) and gastrin releasing peptide (GRP). The expression of these neuropeptides and their receptors are a hallmark of the disease and present an important target for therapeutic intervention. Analogues of substance-P, including [D-Arg$^{1}$,D-Phe$^{5}$,D-Trp$^{7,9}$,Leu$^{11}$]-substance-P (SP-D) and [Arg$^{6}$,D-Trp$^{7,9}$,N$^{me}$Phe$^{8}$]-substance-P (6-11) (SP-G), are novel anti-cancer agents which inhibit the growth of SCLC cells. Investigations into the mechanism of action of substance- P analogues on bombesin receptors revealed that in addition to blocking bombesin-induced mitogenesis and signal transduction they also have agonist activity. This unique pharmacological activity of 'biased agonism' may be central to the growth inhibitory effects of these agents.

The aim of this study was to determine whether these agents exhibit 'biased agonist' activity at receptors other than the bombesin/GRP receptor and investigate factors influencing their ability to modulate neuropeptide signalling. Model cell systems consisting of CHO-K1 cell-lines stably expressing GRP or $V_{1A}$ receptors were therefore established and the effect of SP-D and SP-G tested. Expression of GRP and $V_{1A}$ receptors led to the development of a transformed phenotype as cells showed increased cloning efficiency and survival in soft-agar and suspension growth respectively. GRP and $V_{1A}$ receptor expressing cells were less adherent, more migratory and not contact inhibited. Neuropeptide receptor stimulation provided some protection from the cytotoxic effects of etoposide suggesting a role in chemoresistance. Substance-P analogues inhibited normal and anchorage-independent growth of receptor expressing cells. In receptor binding studies on

GRP and $V_{1A}$ receptor expressing cells, analogue inhibited radioligand binding non-competitively. Transfected GRP and $V_{1A}$ receptors effectively coupled to $G\alpha_q$ to increase intracellular calcium and the analogues were effective antagonists of this response. Neuropeptide and analogues stimulated ERK activity in GRP and $V_{1A}$ receptor expressing cells. Activation of ERK by neuropeptide was rapid and transient while analogue induced activation was delayed and sustained. Analogue-stimulated ERK activity was pertussis toxin sensitive whereas neuropeptide-stimulated ERK activation was not. In addition, analogue induced ERK activity was blocked by inhibition of EGF receptor kinase. This indicates that SP-D and SP-G facilitate receptor coupling to G-protein $G_i/G_o$ subunits for subsequent calcium-independent ERK activation via EGFR transactivation. Stable cell-lines expressing different levels of $V_{1A}$ receptor were used to examine the effect of altering the ratio of receptor to G-protein on the ability of the analogues to direct receptor signalling. There appeared to be little receptor reserve for calcium and ERK responses stimulated by neuropeptide as the efficacy of the response increased with increasing receptor numbers. In contrast, analogue-induced ERK activation occurred with a higher receptor reserve for activated $G_i$ as the magnitude of the response did not increase between medium and high expressing cells. Chimeric $V_{1A}$ receptors containing the second ($V_{1i}2$) or third intracellular ($V_{1i}3$) loop of the $V_2$ receptor were used to investigate the influence of substance-P analogues on G-protein selectivity. Both receptors were still capable of binding AVP and SP-G but had altered ability to activate PLC and ERK. The second intracellular loop of V1AR was essential for AVP-stimulated PLC and ERK activation but not for SP-G-induced ERK activation. This confirms that the effects of the agents cause an alteration in the receptor-G-protein coupling domains of receptors. These findings demonstrate that substance-P analogues are biased agonists of receptors other than GRP receptors, activating downstream signals which differ from those stimulated by the natural agonist through promoting an alternative agonist state of the receptor. This pathway

selectivity combined with the receptor specificity of different substance-P analogues offers great potential for the tailored treatment of neuropeptide-dependent tumours.

# Chapter 1

# Introduction

Numerous physiological processes, such as cell growth, selective cell death, embryogenesis, tissue repair and immune responses, involve tight regulation of cell proliferation. In fully developed organisms, the differentiated cells of many tissues and organs are generally maintained in a non-proliferating condition. When the need arises, a milieu of growth factors and extracellular matrix proteins interact with the cell in a regulated manner to resume proliferation. In contrast, the proliferation of cancerous cells is unrestrained due to the acquisition of partial or complete independence from mitogenic signals present in the extracellular environment (Cross & Dexter, 1991; Westermark & Heldin, 1991). Studies of human tumours and animal models suggest that this is a consequence of a multistage process involving genetic alterations which drive the progressive transformation of normal cells into malignant ones (Hanahan & Weinberg, 2000). Such genetic alterations in tumour cell genomes are demonstrated across multiple sites and over a spectrum which ranges from subtle point mutations to severe changes in chromosome complement (Blume-Jensen & Hunter, 2001).

Generally, a minimum of six successive genetic changes are believed to collectively dictate malignant growth and are manifest in the accepted hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg, 2000). Cancer cells can secrete growth factors which stimulate proliferation through activating receptors present in the cell membrane in an autocrine or paracrine manner. Alterations in the number or structure of receptors and changes in the activity of intracellular signalling pathways can therefore

contribute to the escape from normal regulatory controls (Sager, 1989). An increasing number of oncogenes are being found to represent growth factors, their receptors and post-receptor signalling molecules. This has fuelled intense investigation into growth factors and their associated signal transduction mechanisms in an effort to understand their role in mediating cancer cell growth and provide novel targets for controlling this devastating disease.

It has emerged that cell proliferation can be stimulated through the activity of two major types of growth factors; one involves polypeptide growth factors binding to receptors with intrinsic tyrosine kinase activity (Aaronson, 2005) while the other involves hormones binding to receptors coupled to guanine nucleotide binding regulatory proteins (G-proteins) (Gutkind, 1998b). The intracellular signalling cascades activated by the growth factors form an extensive network and considerable cross-talk can occur between pathways. Multiple, independent pathways can therefore act in a synergistic manner to stimulate cell growth (Rozengurt, 1986; Lowes *et al*, 2002).

Neuropeptides, such as bombesin, gastrin and substance-P, are found in the neural and neuroendocrine cells of the gastrointestinal tract and nervous system (Walsh, 1987). These small regulatory peptides are more commonly known for their role as fast-acting neurotransmitters in the central nervous system where they are synthesised and stored in pre-synaptic neurones. They also function both systemically and locally in the peripheral neuroendocrine system as autocrine and paracrine regulators. This group of structurally diverse signalling molecules play a crucial role in the regulation of exocrine and endocrine secretion, smooth muscle contraction, pain transmission, fluid homeostasis, blood pressure and inflammation as well as food intake, body temperature and behavioural response (Rozengurt, 2002). Over the last two decades, the discovery that these peptides are also potent cellular growth factors for both normal and cancerous cells (Table 1.1)

**Table 1.1. Neuropeptides mitogenic for normal and cancerous cells**

| Neuropeptide | Normal cells | Cancer cells |
|---|---|---|
| **Angiotensin** | Cardiac and lung fibroblasts; hepatic stellate cells; intestinal epithelial cells; mesangial cells; smooth muscle | Pancreatic; adrenocortical; breast epithelial |
| **Bombesin** | 3T3 fibroblasts; airway epithelium | SCLC; prostate; glioblastoma; renal cell carcinoma; colon |
| **Bradykinin** | 3T3 fibroblasts; mesangial cells | SCLC; prostate |
| **Cholecystokinin** | Pancreas | SCLC; pancreatic; colon |
| **Endothelin** | 3T3 and cardiac fibroblasts; endothelial; keratinocytes; astrocytes; smooth muscle | Ovarian and cervical carcinoma; Kaposi's sarcoma; prostate; melanoma |
| **Galanin** | Sensory neurons | SCLC |
| **Gastrin** | Enterochrommafin cells; gastric mucosa; colon epithelium | SCLC; colon |
| **Neurotensin** | Adrenal cortex; intestinal epithelial cells | Pancreatic; SCLC; prostate; astrocytes; colon |
| **Neuropeptide Y** | Olfactory neurons; smooth muscle | Breast cancer |
| **Substance-P** | Fibroblasts; smooth muscle; endothelial cells; corneal epithelial cells; astrocytes; T lymphocytes | Astrogliomas |
| **Vasopressin** | 3T3 fibroblasts; mesangial cells; hepatic stellate cells | SCLC; breast; pheochromocytoma |
| **Vasoactive Intestinal Peptide** | 3T3 fibroblasts; keratinocytes; brain development | Breast; SCLC; prostate; colon; urinary bladder; neuroblastoma |

(Table from Rozengurt, 2002)

has made them the subject of further interest. Hence it has become apparent that the mitogenic effects of neuropeptides are central to a variety of normal and abnormal processes which include development, inflammation, and malignant transformation (Woll & Rozengurt, 1990). Neuropeptides have been demonstrated to function as autocrine growth factors for a number of cancers including lung, breast, prostate and pancreas (Wang *et al*, 1996c; Moody *et al*, 2003). In addition, neuropeptides and their receptors have been found to be the principal driving force behind the clinically aggressive small cell lung cancer (SCLC), providing a paradigm for neuropeptide-driven tumourigenesis (Cuttitta *et al*, 1985; Moody & Cuttitta, 1993).

## 1.1 Lung Cancer

Each year, more than a quarter of a million people in the UK are diagnosed with cancer. The most common fatal malignancy is lung cancer, which causes over 40,000 deaths each year in the UK (World Health Organisation mortality statistics 2000). Whilst the incidence of lung cancer in men has begun to fall, the incidence in women is continuing to increase (Osann, 1998; Peto *et al*, 2000). Lung cancer has been the commonest cause of cancer death in British men for many years and it has now overtaken breast cancer as the commonest cause of cancer death in British women. Despite medical and surgical intervention, there has been little change in the survival rate for cancer with approximately 90% of affected patients dying within one year of diagnosis. Cigarette smoke carcinogens are the major cause of most lung cancers (Hecht, 1999). However, it is anticipated that an increasing number of future cases will be unrelated to smoking and presumably due to environmental pollution instead (Sethi, 1997).

Molecular studies have shown that genetic damage to the smoking exposed respiratory epithelium persists for decades after smoking cessation (Fong *et al*, 2003). The cells are thought to begin to harbour genetic damage after prolonged exposure to cigarette smoke since changes have been detected in morphologically normal cells from smokers. Consistent with a multi-step model of carcinogenesis, it is believed that 10-20 genetic mutations are required to produce a lung cancer cell from a normal one (Sethi, 1997).

### 1.1.1. Classification of Lung Cancer

Lung carcinomas, which originate from the respiratory epithelium, are classified into two groups according to histological type. Approximately 75% of all cases are non-small cell lung cancer (NSCLC) and include squamous cell carcinomas, adenocarcinomas and large-cell carcinomas while the remaining 25% are small-cell lung cancers (SCLCs).

Around thirty percent of lung cancers are squamous cell carcinomas (SQCC), which arise from metaplastic squamous epithelial cells. SQCCs, adenosquamous and mucoepidermoid carcinomas display features of squamous differentiation to varying degrees, ranging from well to poorly differentiated (Maggiore *et al*, 2004). The markers of squamous differentiation which characterise these cell-lines include involucrin, transglutaminase activity, higher molecular weight keratins and cornified envelope formation. In addition, all of these tumour types demonstrate EGF receptor (EGFR) upregulation (Ullmann *et al*, 2004).

Thirty percent of lung tumours are adenocarcinomas; previously considered to arise from a number of cell types such as surface epithelium (ciliated and mucus producing cells) which generate mucin-containing or mucin-secreting tumours, and the progenitor cells of the peripheral airways (Clara cells and Type II pneumocytes) which result in peripheral airway cell tumours (Gazdar & McDowell, 1988). Peripheral airway tumours include the adenocarcinoma subtypes, bronchioloalveolar and papillary, and often display the morphological features of

lepidic and papillary growth. The recent development of a mouse model of lung tumourigenesis by Kim *et al* (2005) has led to the isolation of a population of stem cells from which adenocarcinomas are likely to arise. This invaluable model will allow the detailed study of non-small cell lung cancer development and, through a better understanding of the role of stem cells in this process, should aid the design of better intervention strategies for treating human lung cancer (Berns, 2005).

Ten percent of lung tumours are classified as large cell, and usually diagnosed by exclusion of the other 3 types of lung cancer. Like adenocarcinomas, large cell tumours are preferentially located in the periphery of the lung.

SCLCs were commonly thought to arise from pulmonary neuroendocrine cells and some evidence suggested a stem cell origin (Gazdar & Carney, 1985). They are now believed to be derived mainly from epithelial cells that line the larger airways (Garber *et al*, 2001). SCLCs can be histologically classified into three sub-groups as follows (Hirsch *et al*, 1988):

(1) Small cell carcinoma- includes most of the tumours previously included in the oat cell/ intermediate subtypes and more than 90% of untreated SCLC fall into this category.

(2) Mixed small cell/large cell carcinoma- contains a spectrum of cell types ranging from typical SCLC to larger cells having prominent nucleoli and resembling large cell carcinoma.

(3) Combined small cell carcinomas- typical SCLC elements mixed with areas of differentiated squamous cell or adenocarcinoma.

Neuroendocrine carcinomas represent a spectrum of disease and SCLC, which is associated with a poor prognosis, is at one extreme. Bronchial carcinoids are at the other extreme and have a more favourable outlook following surgical resection (Harpole *et al*, 1992). Between the two extremes is a sub-type called well-differentiated neuroendocrine carcinoma of the lung. Like SCLC, it occurs primarily in smokers but metastasises less frequently and has a 5 year survival rate greater than 50%. However, the pathological diagnosis of well-differentiated

neuroendocrine tumours from SCLC is difficult due to the extreme similarities between the cell-types.

Patients with mixed tumours have been observed, with cells simultaneously displaying features of both SCLC and NSCLC phenotypes. For example, neuroendocrine features are displayed by 25% of NSCLC tumours and correlate with greater initial sensitivity to radio- and chemo-therapy. However, such tumours are also faster growing and have a worse prognosis than adenocarcinomas lacking neuroendocrine features. Clinical evidence has also demonstrated that a potential for transitions between tumour types also exists e.g. from SCLC to NSCLC phenotype. Each of the four major types of lung cancer reflect the phenotypic features of cells present in the normal bronchial epithelium. Therefore, it has been considered that such transitions may mimic the normal cellular transitions which take place in the bronchial epithelium and be mediated by persistent genetic abnormalities. It has thus been proposed that a common differentiation pathway present in the bronchial epithelium may give rise to the four types of lung carcinoma (Mabry *et al*, 1991). The possibility has been demonstrated *in vitro* through a model cell system of the transition from a SCLC to NSCLC phenotype (Wang *et al*, 1996a). The oncogene-induced transition led to an undifferentiated phenotype with the loss of neuroendocrine markers, reduction in GRP secretion, increase in EGFR expression and the acquisition of drug resistance patterns reminiscent of NSCLC.

## 1.1.2. Lung Cancer Treatment & Prognosis

### *1.1.2.1. Non-Small Cell Lung Cancers*

Long term survival in NSCLC patients can be up to 70% depending on stage. Localised carcinomas are potentially curable and therefore NSCLCs with no or few metastases at the time of presentation are subject to surgical resection. Following surgical resection alone, 5 year survival rates of 55-67% have been reported (Mountain, 1997). Patients with un-operable disease of similar stage are treated with

radiotherapy and this has resulted in 5 year survival rates of 13-39% (Rowell & Williams, 2001). However, the majority of patients with NSCLC are not surgical candidates at the time of diagnosis. For these patients, minimally invasive interventional radiology procedures can improve survival, reduce pain, and improve quality of life. In patients with extensive stage disease, a good performance status, haemoglobin levels ≥11 g/dL and age ≥47 years confers a survival advantage (Albain *et al*, 1991a).

Systemic chemotherapy can also be used and has provided short-term relief to patients with advanced stage disease. However, a chemotherapeutic approach is not routine at present and is viewed as being successful only in a small minority of patients. Traditionally, NSCLC tumours have commonly been regarded as being less sensitive to chemotherapy than untreated SCLC tumours. This could be attributed to the previous lack of efficacy of agents such as cyclophosphamide, methotrexate, and adriamycin in NSCLC compared with their effectiveness against SCLC. Second generation cytotoxic drugs, such as cisplatin, ifosamide, mitomycin, vinblastine and etoposide, have since demonstrated significant responses in NSCLC (Rintoul & Sethi, 2002a). Nonetheless, uncertainty over the benefit of chemotherapy in NSCLC has generated an apparent deadlock in survival rates which could potentially be improved through more wide-spread use of these second generation cytotoxic drugs. The NSCLC Collaborative Group carried out a meta-analysis to evaluate the effect of cytotoxic therapy on survival in 1995 which indicated that combination therapy containing cisplatin provided a significant survival advantage in early stage disease when used in combination with either radio- or chemo-therapy (NSCLC Collaborative Group, 1995).

Adverse prognostic factors associated with NSCLC survival include large tumour size, erb-2 oncoprotein expression levels, mutation of the K-ras gene, vascular invasion and tumour angiogenesis (Albain *et al*, 1991a; Harpole *et al*, 1995). Overall, the prognosis for NSCLC patients is unfavourable.

### 1.1.2.2. Small-cell Lung Cancers

SCLCs are invariably associated with smoking. SCLC is considered to be a systemic disease and is the most aggressive form of lung cancer. The majority of patients present with extensive disease, characterised by thoracic disease involving more than one hemithorax and/or widespread metastases. The occurrence of limited disease involving only one hemithorax is less common. SCLC follows an aggressive course and, although initially sensitive to radio- and chemotherapy, the 5-year survival rate remains 3-8% (Rintoul & Sethi, 2002a). SCLC, once diagnosed, is not normally operable as patients present with metastatic disease.

Most SCLC tumours are sensitive to the cytotoxic effects of chemotherapeutic drugs and initially demonstrate high response rates. Around 15-20 cytotoxic agents have been shown to have major activity against SCLC and various regimens produce similar responses (Hong *et al*, 1989). However, it has been found that combinations containing etoposide are well tolerated and highly active (Jett *et al*, 1990). At present, a cisplatin/etoposide combination is the most commonly, but not exclusively, used regimen in the UK (Rintoul & Sethi, 2002a).

Combination chemotherapy is more effective in limited SCLC than single agent treatment, and intensive doses are more effective than those which only produce mild toxic effects. The optimum duration of chemotherapy for limited disease patients has not been defined, although no significant improvement in survival occurs beyond 3-6 months of treatment (Murray *et al*, 1993; Johnson *et al*, 1996). Combined chemotherapy with simultaneous chest irradiation provides a survival advantage of 5.4% at 3 years (Pignon *et al*, 1992). The average survival of patients with limited disease is 14-20 months (Osterlind *et al*, 1986).

Extensive stage SCLC is treated using multiple chemotherapeutic agents, usually in combination with radiotherapy. For these patients however, radiotherapy does not provide an additive survival advantage and is used primarily for symptomatic relief from primary tumour and control of metastatic disease (Twelves *et al*, 1990). Many extensive disease patients have a poor performance status compared with limited stage patients and are less able to tolerate aggressive treatment regimens. Low-dose,

single agent regimens have been developed for these patients which is not as effective as normal treatment and thus associated with a poorer prognosis (James *et al*, 1996; Souhami *et al*, 1997). The average survival of patients with extensive disease is 8-13 months (Osterlind *et al*, 1986).

The main drawback with SCLC is that despite initial sensitivity to chemotherapy, patients invariably relapse with chemoresistant disease and such patients have an exceptionally poor prognosis with median survival 2-3 months. SCLC patients who survive beyond 2 years from initial diagnosis are more likely to die from other forms of lung cancer and this can retrospectively be attributed to an original mis-diagnosis. Limited success has been obtained through second-line chemotherapy with oral etoposide, etoposide/ cisplatin, cyclophosphamide/ doxorubicin/ vincristin, lomustine/ methotrexate and topotecan (Evans *et al*, 1985; Johnson *et al*, 1990; Greco, 1993). Nonetheless, relapse and chemoresistance are an inevitable outcome irrespective of treatment modality. With no significant improvements in the treatments available for SCLC being obtained of late, the overall prognosis for SCLC patients is dishearteningly poor. Consequently, there is a striking need for new therapeutic approaches to the treatment of this disease and this could be forthcoming through a greater understanding of the molecular and cellular biology of SCLC.

## 1.1.3. Small Cell Lung Cancer

### *1.1.3.1. SCLC Characteristics*

SCLC 'oat' cells are classically characterised by indistinct or absent nucleoli and sparse cytoplasm. The cells are 2-3 times the size of mature lymphocytes and their nuclei are darkly stained. Small dense-core granules are visible in the cytoplasm and are the manifestation of peptide secretion. This is a hallmark of the neuroendocrine properties observed in SCLC and less frequently in other lung carcinomas.

When cultured, SCLC cell-lines grow as tightly clustered spheroids in suspension, form colonies in soft agarose and can form tumours in nude mice. The cell population doubling time ranges from 32-72 hours for various cell-lines.

SCLC cells clearly possess neuroendocrine biochemistry and have been found to express numerous peptide hormones, which stimulate the aberrant growth of these tumours and that are also responsible for many of the clinical symptoms associated with SCLC (Sethi & Woll, 1995). The cells have also been shown to express a number of bio-markers of neuroendocrine differentiation such as L-Dopa decarboxylase (DCC), neuron specific enolase (NSE), creatine kinase BB isoenzyme and chromogrannin A. The endocrine markers L-Dopa Decarboxylase and NSE are also found expressed in approximately 25% of NSCLC adenocarcinomas. Although these cells have chemosensitivity, they also grow faster and have a worse prognosis than other adenocarcinomas (Gazdar & McDowell, 1988).

SCLC cell-lines have been sub-classified into two distinct groups according to heterogeneity in neuroendocrine marker expression (Carney *et al*, 1985). Classic SCLC cell-lines express high levels of all 4 bio-markers whereas variant SCLC cell-lines express only some of these markers. For example, L-Dopa carboxylase is absent in 10% of SCLC cell-lines. Morphological variants represent 20% of SCLC cell-lines and grow as loose aggregates with increased growth rates and cloning efficiencies.

Variant cell-lines grow more rapidly in culture, have higher cloning efficiency in soft agar and are more tumourigenic in nude mice than classic cell-lines. In addition, variant cells are larger, less differentiated and relatively radio- and chemo-resistant. These cell-lines are thought to be representative of mixed small cell/ large cell SCLC tumours and are associated with a poor prognosis. In some cases, but not all, the clinical development of chemoresistance can be associated with a conversion from classic to variant SCLC phenotype. In other cases, since only 10% of SCLC patients have tumours demonstrating large cell admixture histology, other mechanisms may

underlie the transition from chemo-responsive to non-responsive SCLC. Overall, the majority of SCLC cell-lines are of a classic phenotype and are thought to maintain this phenotype during chemotherapy and the development of chemoresistance. In the present study, the classical SCLC cell-line NCI-H345 is used which grows well in culture and expresses neuropeptide receptors (Sethi *et al*,1992).

A number of longitudinal studies have identified a positive correlation between extensive disease and the presence of endocrine markers (Carney, 1991). The levels of NSE, which is a highly specific marker for neurons, are elevated in extensive disease. Sequential determinations of NSE levels are of value in monitoring the response to chemotherapy and detection of relapse. Similarly, Chromogrannin A, BB isoenzyme of creatine kinase and carcinoma embryonic antigen have also demonstrated a prognostic value and correlate significantly with tumour response to cytotoxic therapy and subsequent relapse. Furthermore, a longitudinal study involving cell-lines before and after the development of radio- and chemo-therapy resistance demonstrated an increase in neurosecretory granules and differentiation in SCLC cells after chemotherapy (Brambilla *et al*, 1991). In contrast, another study of cell-lines and biopsies from treated patients, as well as long-term untreated SCLC cultures, demonstrated large cells of de-differentiated phenotype and loss of neuroendocrine markers (Bepler *et al*, 1987).

### *1.1.3.2. SCLC Neuroendocrinology*

As mentioned previously, the neuroendocrine nature of SCLC is the most prominent feature of this clinically aggressive cancer. The first neuropeptide discovered to be produced by SCLCs was bombesin/GRP (Wood *et al*, 1981; Moody *et al*, 1981), and since then the number of peptide hormones and growth factors found to be secreted by SCLCs has been expanding (Table 1.2). Most SCLCs produce only a subset of these products, although a minority of SCLCs tumours appear capable of synthesising a variety of these hormones (Sethi & Woll, 1995).

Many of these products are synthesised as pro-hormones, and specific post-translational modification is required for biological activity (Quinn *et al*, 1991). The ectopic hormone production underlies some of the paraneoplastic syndromes which SCLC patients present with. For example, adrenocorticophic factor (ACTH) secretion causes ectopic Cushing's Syndrome, while the release of vasopressin and atrial natriuretic protein (ANP) contribute to dilutional hyponatraemia (Gross *et al*, 1993). A number of these products have also been detected in NSCLCs and normal lung tissue (Wood *et al*, 1981; Becker, 1985).

A great deal of investigation has been undertaken into the role of ectopic hormone secretion in SCLC tumour biology. It was postulated that SCLC cells may express receptors for many of these peptides, enabling the peptides to function as autocrine growth factors for SCLCs. The presence of functional receptors was demonstrated through assessing the ability of some 32 neuropeptides and hormones to mobilise intracellular calcium as a measure of receptor-ligand interaction (Table 1.3) (Woll & Rozengurt, 1990). Heterogeneity in the expression of individual receptors was found to exist amongst the cell-lines and each of the five cell-lines responded to at least two different agents (Sethi & Woll, 1995). It was further demonstrated that nanomolar concentrations of the calcium mobilising neuropeptides were able to increase the cloning efficiency of SCLC cells under serum-free conditions. This showed that SCLC growth could be sustained by a network of autocrine and paracrine interactions involving multiple neuropeptides. Numerous studies have focussed on elucidating the signal transduction mechanisms underlying the mitogenic effects of neuropeptides, with a view to discovering prospective targets for therapeutic intervention.

**Table 1.2. Peptides and hormones secreted by SCLC**

| Neuropeptides | Growth Factors |
|---|---|
| Atrial natriuretic peptide | Adrenocorticotrophic hormone |
| Calcitonin | Chorionic gonadotrophin |
| Calcitonin gene-related peptide | Estradiol |
| Cholecystokinin | Follicle stimulating hormone |
| Endothelin | Granulocyte colony-stimulating factor |
| Gastrin | Growth hormone releasing hormone |
| GRP/bombesin | Insulin-like growth factor-I |
| Neuromedin B | Insulin-like growth factor-I binding protein |
| Neurotensin | Parathyroid hormone |
| Opioid peptides | Stem cell factor |
| Oxytocin | |
| Prolactin | |
| Serotonin | |
| Somatostatin | |
| Substance K | |
| Substance-P | |
| Vasoactive intestinal peptide | |
| Vasopressin | |

(Table and references for each individual hormone in Sethi & Woll (1995)

**Table 1.3. The effect of neuropeptide and peptide hormones on calcium mobilisation in SCLC cells**

| Effective | Non-effective |
|---|---|
| Bradykinin | Adrenocorticotrophic hormone |
| Cholecystokinin | Calcitonin |
| Gastrin | Chorionic gonadotrophin |
| Galanin | Endothelin |
| Bombesin | Follicle stimulating hormone |
| Gastrin Releasing Peptide | Growth hormone releasing hormone |
| Neurotensin | Gastrointestinal peptide |
| Vasopressin | Neuropeptide-Y |
| | Parathyroid hormone |
| | Substance K |
| | Substance-P |

(Adapted from Woll & Rozengurt (1990))

## 1.2. Neuropeptides

### 1.2.1. Neuropeptide receptors

Neuropeptides mediate their cellular effects through binding extracellularly to G-protein coupled receptors (GPCRs). The human genome encodes more than 1000 members of this superfamily, making it the largest group of cell surface receptors. Neuropeptides are ligands for about 20% of the GPCRs, 55% have classic transmitters or compounds like prostaglandin E2 and anandamide as ligands, and 25% have no known ligands (Hokfelt *et al*, 2003). GPCRs associate with and activate heterotrimeric G-proteins to stimulate or inhibit the formation of second messengers and modulate ion channels (Gether, 2000). The receptors are also capable of directly

interacting with other effector molecules, including receptor serine/threonine kinases, tyrosine kinases, protein tyrosine phosphatases and adaptor proteins (Pierce *et al*, 2002). As shown in Figure 1.1 all GPCRs are predicted to adopt a structure comprising of seven transmembrane α-helical segments of 20-25 amino acid residues with three extracellular loops, three intracellular loops, an extracellular N terminus and cytoplasmic C terminus each of varying length (Ji & Grossman, 1998). GPCR domains involved in ligand binding include transmembrane (TM) regions, the N-terminus and the extracellular loops joining transmembrane domains (Gether & Kolbika, 1998). Intracellularly, the second and third cytoplasmic loops and the C-terminal region (which can form a 'pseudo-loop' through membrane tethering at a conserved palmitoylation site) are thought to be critical for interaction with G-proteins (Wess, 1997b; Erlenbach & Wess, 1998).

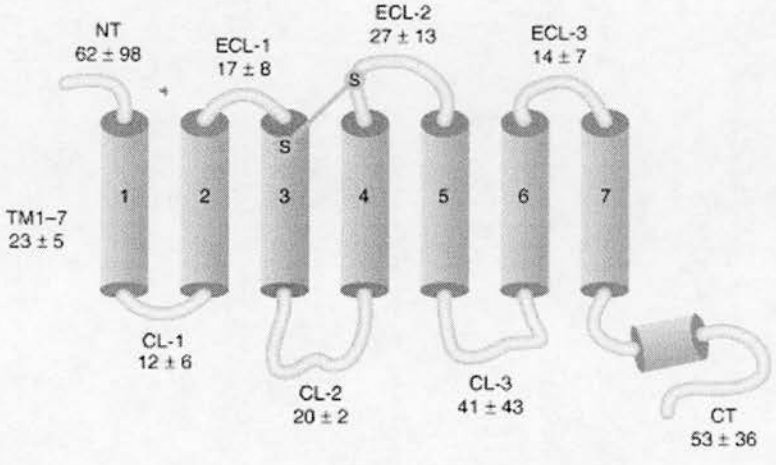


**Figure 1.1. GPCR secondary structure.** The disulfide bond that links TM3 and ECL2 is conserved in 91.8% of GPCRs but absent in 7.6% members of the rhodopsin family, 2% of the secretin receptor family, 8% of the glutamate receptor family and 7.4% of the frizzled receptor family. Abbreviations: CL, cytoplasmic loop; CT, cytoplasmic tail; ECL, extracellular loop; GPCR, G-protein-coupled receptor; NT, N terminus; TM, transmembrane helix. Numbers indicate the numbers of residues in each region (mean±S.D). (Karnik *et al*, 2003)

Ligands are thought to be initially attracted towards the receptor through electrostatic interactions with specific receptor residues, effectively increasing the local concentration of ligand and subsequent binding probability. Further non-covalent interactions with the receptor facilitate a conformational change in the spatial organisation of receptors' transmembrane bundle. This subsequently affects the conformation of the intracellular loops, uncovering previously masked G-protein binding sites, and results in the formation of a high affinity ligand-receptor-G-protein complex (Gutkind, 1998b). The ensuing G-protein activation leads to the initiation of downstream signalling. The process of signal transfer by the receptor from the cell exterior to the interior can thus be viewed as a three-step process: (1) signal generation (ligand binding), (2) transmembrane signal transduction (conformational change in receptor), (3) signal transfer to cytoplasmic signal molecules (G-protein activation).

#### *1.2.1.1. The GPCR superfamily*

Members of the GPCR superfamily are grouped into subfamilies A, B and C according to amino acid sequence homology (Gether, 2000). Sequences within each family generally share over 25% sequence identity in the transmembrane core region, and a distinctive set of highly conserved residues and motifs. The largest subfamily, family A, comprises rhodopsin-like GPCRs (Figure 1.2) which are activated by a wide range of structurally unrelated ligands binding within a pocket formed by transmembrane (TM) regions 3, 5, 6 and 7 (e.g. rhodopsin, β2-adrenoreceptor and many neuropeptides) or to extracellular regions of the receptor (e.g. glycoprotein receptors for FSH, LH) (Jensen & Spalding, 2004). Gastrointestinal peptides activate receptors of the smaller subfamily B through binding to both extracellular and transmembrane regions of the receptor. Family C contains the metabotropic-like receptors for glutamate, calcium and various taste molecules, which bind exclusively to the extracellular amino-terminal domain. Thus, different modes of ligand binding exist across the various sub-classes of GPCRs and this is a

reflection of the configuration of the ligand binding pocket. Key residues present in the transmembrane helices may contribute to the formation of the binding pocket which is dictated by the spatial organisation of the receptor in the membrane.

### *1.2.1.2. Heterotrimeric G-proteins*

The heterotrimeric G-proteins, which associate with GPCRs, are important for maintaining the specificity and temporal characteristics of the cellular responses to signals (Rudolph *et al*, 1996). They are composed of α, β and γ subunits and demonstrate heterogeneity through the 17 α subunits, five β subunits and 14 γ subunits which have been cloned and identified to date. G-proteins are generally referred to according to the function of the α subunits and are grouped into four distinct classes according to the sequence similarity of these subunits: $G_q$ proteins activate phospholipase Cβ; $G_s$ proteins stimulate adenylyl cyclase; $G_i$ proteins inhibit adenylyl cyclase and activate G-protein-coupled inwardly rectifying potassium (GIRK) channels; $G_{12}$ and $G_{13}$ regulate the activity of small GTPases (Pierce *et al*, 2002).

The domain of the Gα subunit which is involved in binding and hydrolysing GTP is structurally identical to the superfamily of GTPases that includes small G-proteins (e.g. Rho, Rac, Arf) and elongation factors (Kjeldgaard *et al*, 1996). The β and γ subunits form a functional dimer that is not dissociable except by denaturation. Membrane attachment is proposed to occur via sites of lipid modification present in the N-terminal region of the α-subunit and the C-terminal region of the γ subunit which are in close proximity in the heterotrimer (Resh, 1996). G-proteins are inactive in the GDP-bound, heterotrimeric state and are activated by receptor catalysed guanine nucleotide exchange resulting in GTP binding to the α subunit (Rudolph *et*

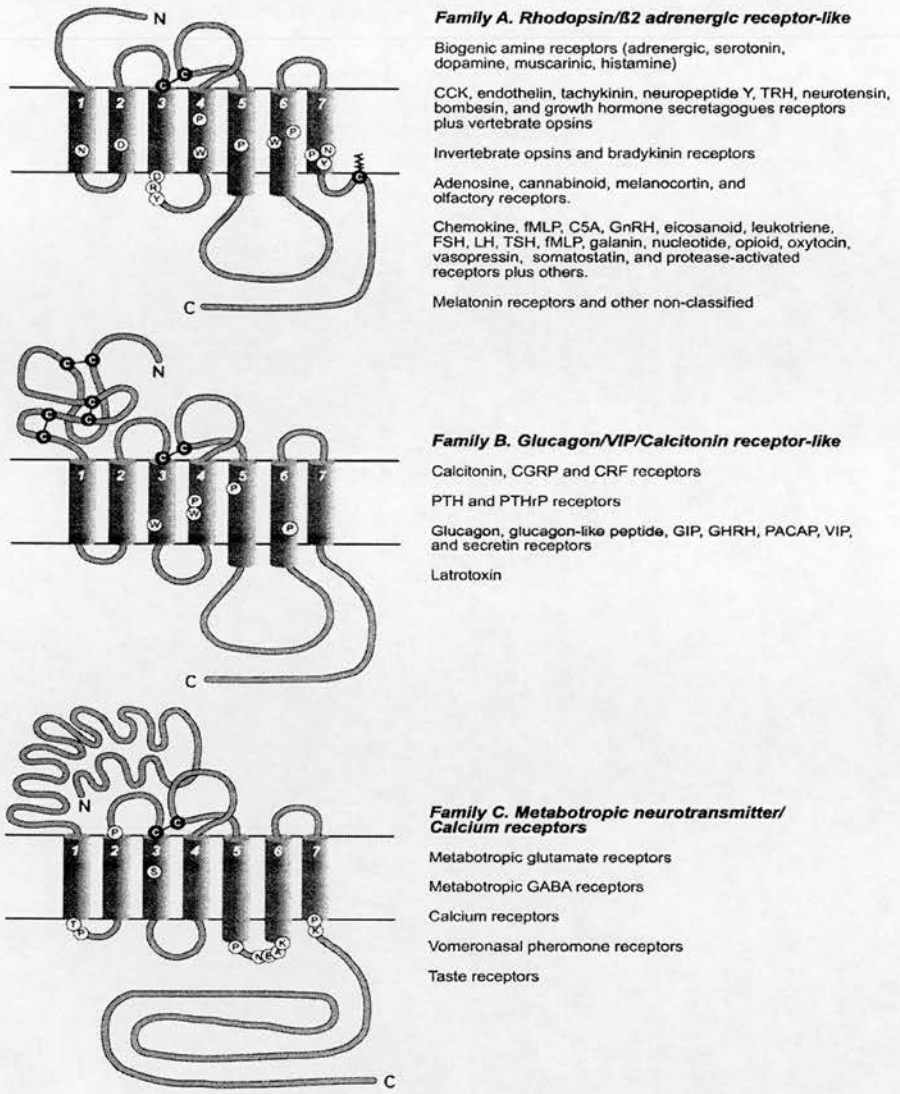


**Figure 1.2. The GPCR superfamily.** A *snake diagram* for a prototypical member of each subfamily is shown with highly conserved residues (*black letter in white circles*) and disulphide bonds between conserved cysteine residues (*white letters in black circles*) . **Family A** receptors *(upper panel)* can phylogenetically be divided into six subgroups as indicated. Family A receptors are characterized by a series of highly conserved key residues and a majority of the receptors have a palmitoylated cysteine in the carboxy-terminal tail causing formation of a putative fourth intracellular loop. **Family B** receptors *(middle panel)* are characterized by a long amino terminus containing several cysteines presumably forming a network of disulfide bridges. The conserved prolines are different from the conserved prolines in the A receptors and the DRY motif at the bottom of TM 3 is absent. **Family C** receptors *(lower panel)* are characterized by a very long amino terminus (~600 amino acids). The amino-terminal domain is thought to contain the ligand-binding site. Except for two cysteines forming a putative disulfide bridge, the C receptors do not have any of the key features characterizing A and B receptors. A unique characteristic of the C receptors is a very short and highly conserved third intracellular loop. (Gether, 2000)

*al*, 1996). GTP binding reduces the affinity of Gα for Gβγ which leads to dissociation of the GTP-bound α subunit from Gβγ subunits. In the GTP-bound, active conformation, a new surface is formed on Gα-GTP subunits which enables them to interact with effectors with 20-100 fold higher affinity than in their GDP-bound state. The 'active' Gα-GTP and the free Gβγ subunits subsequently initiate cellular responses by altering the activity of intracellular effector molecules (Dhanasekaran *et al*, 1998). The cellular response is terminated when the intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP which facilitates the re-association of Gα-GDP with Gβγ.

In general, the G-protein subunits $G\alpha_q$, $G\alpha_i$ and $G\alpha_s$ preferentially couple to different families of receptors (Birnbaumer, 1992). However, many GPCRs are able to activate more than one sub-type of G-protein and thereby modulate multiple intracellular signals. For example, the thryotropin receptor is able to activate all three families of G-proteins (albeit each with differing efficacy) whereas the $\alpha_2$-adrenergic receptor couples to both $G\alpha_s$ and $G\alpha_i$ (Eason & Liggett, 1995). Since homologous receptors display similar coupling specificity, a receptor's conformation (particularly the intracellular regions which recognise G-proteins) is thought to dictate the G-protein selectivity of distinct receptor sub-types (Albert & Robillard, 2002).

### *1.2.1.3. GPCR activation and downstream signalling initiation*

The diversity of GPCR binding modes for agonists suggests that there could be multiple ways of propagating the activation of GPCRs. However, given the ability of the receptors to activate the same intracellular signaling pathways through the same classes of G proteins it is thought that the fundamental underlying mechanisms of GPCR activation may have been conserved during evolution (Gether, 2000).

The most extensively investigated GPCRs are members of the largest subfamily of GPCRs, the rhodopsin-family. This subfamily is also the most relevant to the study here since the receptors for many neuropeptides, including bombesin and vasopressin, are also members. The process of receptor activation is thus described according to the molecular insights this subfamily provides. Bovine rhodopsin is the only member of the GPCR superfamily for which a crystal structure has been solved, and this confirms the existence of an anti-clockwise bundle of transmembrane helices (viewed from extracellular side) that are connected by loops of varying lengths (Palczewski *et al*, 2000). The transmembrane helices are tightly packed in this arrangement through the formation of extensive hydrogen bonds between residues of the same TM as well as others. Conserved cysteine residues in extracellular loop 1 and TM3 form a disulphide bond, provide stability through constraining the loops and the receptor. This effectively positions extracellular loop 2 in such a way that the transmembrane core is inaccessible for ligand-binding. In addition, ionic interactions occur between conserved residues of the TM regions. Overall, important conformational constraints imposed by intramolecular interactions are believed to preferentially maintain the receptor in an inactive conformation. The process by which the constraining intramolecular interactions are released through ligand binding is not fully understood but thought to be responsible for the conformational changes which result in receptor activation and G-protein binding. Specific movements of the TM helices are believed to form an essential part of the switch from the inactive to the active conformation. The bovine rhodopsin structure has been used as a guide to structure-function analysis of other GPCRs and has shown that the separation of the TM3 and TM6 in GPCR activation is common (Karnik *et al*, 2003). It has also been postulated that a key element of receptor activation involves the conserved Asp-Arg-Tyr (DRY) motif which is located at the interface of TM3 and the second intracellular loop. The invariably conserved arginine (ArgIII:26) located within this motif has been postulated to be constrained in the hydrophilic binding pocket (formed by conserved polar residues in TM1, TM2 and TM7) through ionic interaction with the adjacent residue

(AspIII:25) (Gether, 2000). Protonation of AspIII:25 through agonist binding is thought to disrupt this interaction and so enable AspIII:26 to shift out of the polar pocket (Gether & Kolbika, 1998). This subsequently leads to the cytoplasmic exposure of previously buried sequences in the second and third intracellular loops, facilitating G-protein interaction and the induction of GDP release from the G-protein. Site-directed mutagenesis studies have shown that altering key residues leads to increased agonist-independent receptor activity, demonstrating their importance in the receptor activation process (Bihoreau *et al*, 1993). Such studies of constitutively active mutant (CAMR) receptors have been crucial to the understanding of how receptor activation relates to downstream signalling.

### *1.2.1.4. Ligand-selective receptor conformations*

GPCRs with known native ligands have been successfully targeted for therapeutic intervention, with >50% of drugs on the market acting as either surrogate activators (agonists) or inhibitors (antagonists) of GPCR activity. Central to the drug discovery process has been the development of various pharmacological models to describe the efficacies of different GPCR ligands.

A simple model was initially used to describe the formation of the high affinity agonist-receptor-G-protein complex which initiates downstream signalling. The two-state model is based on the ability of a receptor to adopt an inactive and an active conformation and to activate a single G-protein (De Lean *et al*, 1980). According to this model, a receptor exists in equilibrium of functionally distinct conformations: inactive **R**, which does not interact with G-proteins and active **R***, which activates G-proteins and initiates downstream signalling events. The level of basal receptor activity, in the absence of ligands, is determined by the equilibrium between **R** and **R***. The intrinsic efficacy of a ligand (effectiveness in stimulating a response) is determined by its ability to alter this equilibrium. Thus, agonists bind to and stabilise the active **R*** form of the receptor, whereas antagonists bind to and

stabilise the inactive conformation, thus reducing the formation of **R***. The receptor activity induced by various other types of GPCRs ligands, e.g. partial agonists and inverse agonists, can also be accommodated by this model. For example, a partial agonist stimulates a partial response because it less effectively stabilises the active state (therefore less effectively stimulates GDP/GTP exchange) than does the native (full) agonist.

Constitutive GPCR activity can also occur in the absence of agonist binding and is due to spontaneous isomerisation of GPCRs from the inactive to the active state (Seifert & Wenzel-Seifert, 2002). This increases the basal G-protein activity in comparison to the absence of GPCR. The 'extended' ternary complex model accommodates the ability of a receptor to spontaneously form active states, and the 'cubic' ternary complex model (Figure 1.3) builds upon this by allowing the inactive state of the receptor to also interact with G-proteins (Kenakin, 2002b). These models are used to describe the efficacies of different agonists for stabilising distinct receptor states which can be influenced by an agonist's affinity for a particular receptor state. Given that a single receptor can couple to more than one G-protein, traditional receptor theory as proposed by Furchgott (1966) would predict that an agonist would have similar efficacies for different downstream signalling pathways. However, there are a number of reports that show that differential effector activation by agonists that cannot be explained by this mechanism (Berg *et al*, 1998). For example $\alpha_{2A}$ receptor agonists show different efficacies for $G_i$-mediated adenylate cyclase inhibition and $G_S$-mediated adenylyl cyclase stimulation in transfected CHO cells (Brink *et al*, 2000), and the neurotensin receptor (NTS1) agonists EISA-1 and neuromedin N show reverse potency orders for $G_q$- and $G_S$-mediated responses (Skrzydelski et al., 2003).

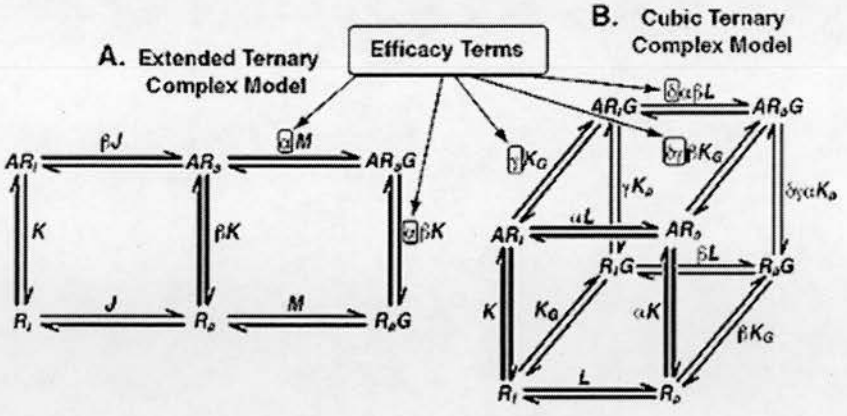


**Figure 1.3. Models of GPCRs.** (*A*) Extended Ternary Complex (ETC) model: The receptor exists in an inactive ($R_i$) and active ($R_a$) state; G proteins (mediating physiological response) bind only to the active-state Ra. The addition of a ligand [A] results in three corresponding ligand-bound species with varying propensities to form the active state and different affinities for G proteins. (*B*) Cubic Ternary Complex (CTC) model: as for the ETC model, with the addition that the inactive state of the receptor can also form a complex with the G protein (but does not signal). The rate constant *K* adds a description of the quality of the ligand-receptor complex, in terms of what characteristics it imparts to the ability of the G protein to exchange GDP and GTP. Figure adapted from Kenakin (2002b).

In addition, experimentally derived data can not be explained by the idea of a single receptor active state underlying agonist efficacy. Studies of constitutively active receptor mutants (CAMR) and the use of insurmountable antagonists have suggested that intermediate receptor conformational states can exist (Vauquelin & van Liefde, 2005). In addition, different agonists may trigger distinct effector pathways through a single receptor by dictating its preference for certain G-proteins. 'N-state receptor models', in which the receptor is assumed to adopt N states that may be inactive (**R**) or active (**R***, **R****, **R*****,,,**)** have been developed to accommodate these findings (Brink *et al*, 2000). According to these, a ligand can bind to a variety of receptor conformations according to the relative affinity of the ligand for those conformations; enriching certain receptor conformations and depleting others. Each ligand-selective conformation can potentially couple to a different G-protein subtype as a consequence of differential ligand-specific affinity

factors ($\alpha,\gamma,\delta$ in Figure 1.3) influencing the efficacy of coupling to G-proteins. Such factors can be incorporated into a three-state receptor model (Figure 1.4.) representing the interaction of one receptor (two active, one inactive state) and two G-proteins. The nature of the ligand determines the efficacy of the ligand, which dictates the affinity of the ligand-bound receptor for G-protein. The affinity of an activated receptor for G-protein is thus altered in the presence of ligand according to the efficacy of the ligand. Therefore, the efficacy with which one such ligand-selective receptor conformation couples to a particular G-protein species can differ from that of a different ligand-selective receptor state. This would account for the ability of a ligand to produce multiple stimuli (have multiple intrinsic efficacies) upon interaction with a receptor and reveals a potential for differential regulation of each signalling pathway coupled to the receptor (Clarke, 2005).

The concept of agonist-directed trafficking of receptor signalling ('ADTRS' which is also known as 'agonist trafficking' or 'biased agonism') is based upon this idea of distinct receptor states differentially activating G-proteins (Kenakin, 1995). The hypothesis of ADTRS proposes that when a receptor signals though two or more independent signal transduction pathways, the relative efficacies of a series of agonists may differ for the pathways (Kenakin, 1995). Central to ADTRS is a requirement for the independent pathways activated by a receptor to diverge at the receptor/G-protein level (e.g. receptor activates two G-proteins independently, leading to the transduction of the stimulus through two separate effector pathways) (Leff *et al*, 1997). Thus, based upon the capacity of ligands to promote unique, ligand selective receptor conformations, a ligand could theoretically activate each different pathway with varying efficacy. Evidence in support of this notion has started to accumulate (Watson *et al*, 2000; Cussac *et al*, 2002; Newman-Tancredi *et al*, 2002; Martin *et al*, 2002; Skrzydelski *et al*, 2003), which suggests that ligand-directed signalling to different cellular effector pathways using biased agonists may be a real possibility. This could provide the next effective level of agonist selectivity for drug

development strategies beyond agonist affinity for selective receptor subtypes (Kenakin, 1997b).

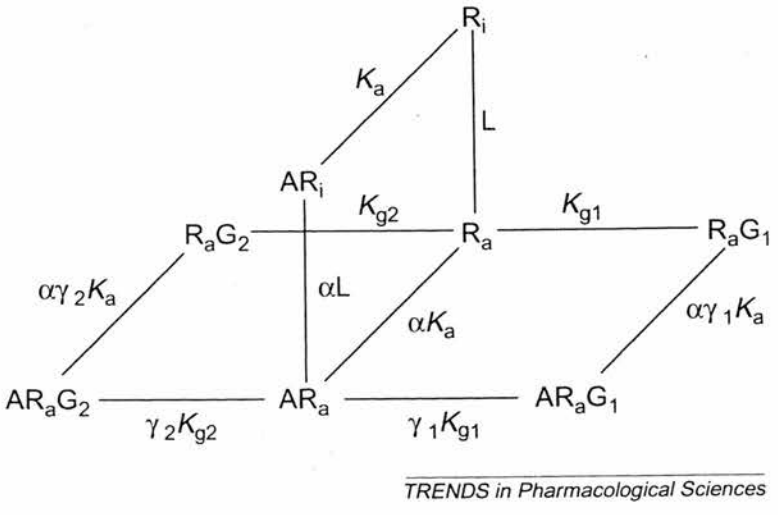


**Figure 1.4. Extended ternary complex model for the interaction between a single receptor and two different G-proteins.** The receptor undergoes transition from an inactive ($R_i$) to an active ($R_a$) state and the activated receptor ($R_a$) can interact with two separate G-proteins according to a scheme where A is the ligand and G is the G-protein. Transition between states is governed by $K_a$ (the affinity of the ligand for $R_i$); $\alpha K_a$ (affinity of ligand for $R_a$); $K_g$ (affinity of $R_i$ for G in absence of A); $\gamma K_g$ (affinity of $R_a$ for G in presence of A); L (natural constitutive activity of receptor). In general, the response in such a system will be controlled by the relative concentrations of the two G-protein types, the capability (efficacy) of the ligand to induce receptor coupling to each G-protein ($\gamma 1$ and $\gamma 2$), the ability of ligand to induce the receptor active state ($\alpha$) and the natural constitutive activity of the receptor (L). (Kenakin, 2003a)

### *1.2.1.5. GPCRs and cancer*

As mentioned previously, site directed mutagenesis of GPCRs can produce constitutively active receptors. However, the first GPCR isolated through a screen for fibroblast transforming potential was not found to contain any activating

mutations, indicating that some other mechanism may underlie its growth promoting ability (Young *et al*, 1986). Cellular transformation simply through ectopic expression of 5-$HT_{2C}$ and muscarinic acetylcholine $M_1$, $M_3$ and $M_5$ receptors demonstrated that wild-type GPCRs could be tumourigenic in an agonist-dependent manner (Julius *et al*, 1989; Gutkind *et al*, 1991). Agonist-dependent transformation by $\alpha_{1B}$-adrenoceptors was rendered agonist-independent through mutation, indicating that analogous mutations occurring spontaneously in this class of receptor proteins could play a key role in the induction or progression of neoplastic transformation (Allen *et al*, 1991). Such naturally occurring, constitutively activating mutations have been identified in thyroid-stimulating hormone receptors in 30% of hyperfunctioning human thyroid adenomas and in a minority of differentiated thyroid carcinomas (Arvanitakis *et al*, 1997). This suggests a direct link between GPCRs and human cancer and, although not common, activating mutations have also been detected in a few other GPCRs (Table 1.2). It is interesting to note that the naturally occurring mutations identified so far have been found in GPCRs which all belong to the rhodopsin-like GPCR subfamily (subfamily A).

Activating mutations of GPCRs in human neoplasias have been identified relatively rarely but more commonly the persistent stimulation of GPCRs by tumour-secreted agonists has been implicated. A prime example of this is the autocrine and/or paracrine mechanism of action of the neuropeptides secreted by small-cell lung cancer (SCLC) cells which also express the cognate receptors (Sethi & Woll, 1995). A number of the same neuropeptide/GPCR combinations have also been implicated in colon adenomas and carcinomas and gastric hyperplasia and carcinomas (Gutkind, 1998b). In particular, GRP receptors have been implicated in prostate cancer and $CCK_1$ and $CCK_2$ receptors in pancreatic hyperplasia and carcinoma (Marinissen & Gutkind, 2001). Several anti-cancer strategies are thus aimed at targeting these autocrine and paracrine growth loops.

Functional GPCRs are also encoded by the genome of several DNA viruses. Kaposi's sarcoma-associated herpes virus (KSHV) encodes a constitutively active GPCR that has transforming potential (Sodhi *et al*, 2000) and has recently been shown to contribute to the angiogenic response characteristic of Kaposi's sarcoma lesions by up-regulating expression of vascular endothelial growth factor (VEGF) (Xu *et al*, 1993). A number of other GPCRs (e.g. for LPA, interleukin 8, thrombin, sphingosine-1-phosphate) have similarly been found to play a key role in tumour angiogenesis (Marinissen & Gutkind, 2001). This has made GPCRs attractive targets for affecting tumour growth through inhibition of new blood vessel formation.

At least ten of the 17 G$\alpha$-subunits cloned so far have demonstrated oncogenic potential (Dhanasekaran *et al*, 1998) and mutations that affect the intrinsic GTPase activity of $G\alpha_s$ (*gsp* oncogene) and $G\alpha_{i2}$ (*gip2* oncogene) have been identified in various tumours (Table 1.4). The *gep* oncogene isolated from Ewing's sarcoma has potent fibroblast transforming ability in NIH 3T3 cells and encodes a wild-type form of $G\alpha_{12}$. Although activating mutations of the $G_{12/13}$ or $G_q$ family have not been identified in human cancers, breast, colon and prostate adenocarcinoma cell-lines have been found to express high levels of wild-type $G\alpha_{12/13}$.

G-protein $\alpha$-subunits and GPCRs are thus implicated in aberrant cell growth and provide novel targets for the therapeutic treatment of cancer.

**Table 1.4. G-proteins and GPCRs in tumourigenesis**

| Activating Mutations | | Type of Tumour |
|---|---|---|
| **G proteins** | $G\alpha_s$ | Thyroid toxic adenomas; thyroid carcinomas; growth hormone secreting pituitary adenomas; McCune-Albright syndrome |
| | $G\alpha_{i2}$ | Ovarian sex cord tumours; adrenal cortical tumours |
| **GPCRs** | TSH receptor | Thyroid adenoma; thyroid carcinoma |
| | FSH receptor | Ovarian sex cord tumours; ovarian small cell carcinoma |
| | LH receptor | Leydig cell hyperplasia; male precocious puberty |
| | $CCK_2$ receptor | Colorectal cancer |
| | $Ca^{2+}$-sensing receptor | Autosomal dominant hypocalcaemia; neoplasms |
| **Autocrine and paracrine activation** | | |
| Neuromedin B receptor | | Small-cell lung carcinoma (SCLC) |
| Neurotensin receptor | | SCLC; prostate cancer |
| Gastrin receptor | | SCLC; gastric cancer |
| Cholecystokinin receptors | | SCLC; pancreatic hyperplasia; pancreatic carcinoma; gastrointestinal cancer |
| Vasopressin receptors | | SCLC |

(Adapted from Marinissen & Gutkind, 2001)

## 1.3 Neuropeptide Growth Factor Signalling

Much of the early work of screening neuropeptides for growth promoting activity was carried out using Swiss 3T3 mouse embryonic fibroblasts, establishing that the cells expressed numerous mitogenic GPCRs. Therefore, the intracellular signalling pathways which neuropeptides use to stimulate cellular growth have been extensively investigated using this cell-line (Rozengurt & Mendoza, 1985a; Rozengurt *et al*, 1985b; Rozengurt, 1986). Bombesin has been an attractive model peptide for elucidating the signal transduction mechanism of neuropeptides for a number of reasons. In serum-free medium, DNA synthesis can be initiated by bombesin in the absence of other growth promoting agents (Rozengurt & Sinnett-Smith, 1983). In contrast with other neuropeptide growth factors which are mitogenic only in synergistic combinations, this shows that bombesin has the ability to act as a sole mitogen (Rozengurt, 1986). In addition, the receptors for bombesin-like peptides have been well characterised at the molecular level (Cardona *et al*, 1992; Moody & Cuttitta, 1993; Akeson *et al*, 1997; Donohue *et al*, 1999; Jian *et al*, 1999). A number of the GPCRs for mitogenic neuropeptides (Table 1.1), including those for bombesin and vasopressin, predominantly couple to the $G_{q/11}$ sub-class of heterotrimeric G-proteins for the transduction of neuropeptide signals (Offermanns, 2003). The early signals and molecular events induced by bombesin have thus provided a paradigm for the study of other mitogenic neuropeptides and illustrate the activation and interaction of a variety of signalling pathways (Seckl & Rozengurt, 1995).

### 1.3.1. Bombesin

Bombesin is a 14 amino acid peptide and was first isolated from the skin of the frog *Bombina bombina*. Numerous bombesin-like peptides have since been identified from various species and are classified into the three sub-families: bombesin-related

peptides, GRP and neuromedin B (Figure 1.5). The 27 amino acid gastrin releasing peptide (GRP), first isolated from porcine stomach, was originally thought to be the mammalian homologue of bombesin. However, an amphibian GRP was later isolated and suggests that a mammalian bombesin may yet exist (Nagalla *et al*, 1992). It has been demonstrated that a minimum of 7 C-terminal amino acids are required for the biological effects of bombesin-like peptides (Heimbrook *et al*, 1988).

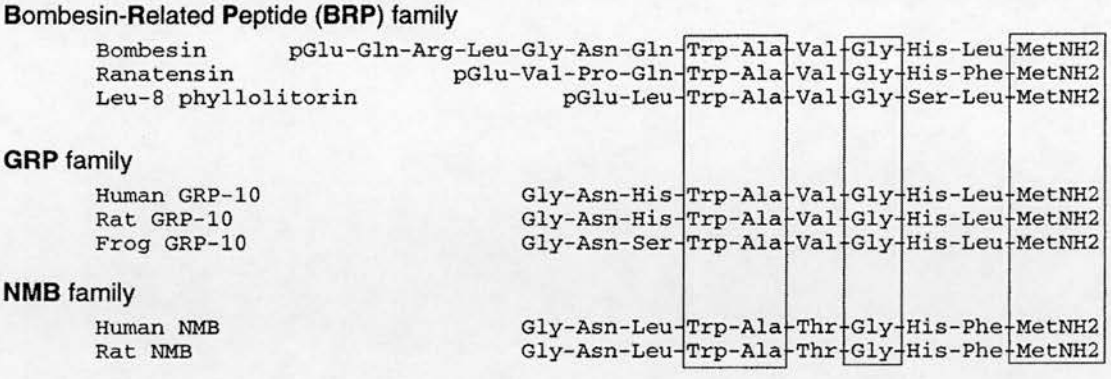


**Figure 1.5. The three subfamilies of bombesin-like peptides.** Representative members of each subfamily are shown. Bombesin, ranatensin, and phyllolitorin are amphibian, GRP and NMB are from the species shown. GRP-10 is the COOH-terminal decapeptide of GRP and contains full biological activity of GRP. (Nagalla *et al*, 1996).

The human GRP gene is located on chromosome 18 (18q21) (Naylor *et al*, 1987), whereas the human neuromedin B lies on the long arm of chromosome 15 (Krane *et al*, 1988). Bombesin-like immunoreactivity (BLI) has been demonstrated in mammalian gastrointestinal tract tissues, brain, spinal cord and lung. Bombesin-like peptides are most commonly known for their functions as neurotransmitters in the central nervous system and as secretory agents in the gut (Panula *et al*, 1984). Infused GRP, with a plasma half-life of 2.8 minutes, can stimulate the secretion of gastrin, pancreatic polypeptide, insulin, glucagon, cholecystokinin and gastric inhibitory peptide which results in amylase and gastric acid secretion (Knigge *et al*, 1984).

The GRP receptor isolated from Swiss 3T3 membranes was found to be a glycosylated transmembrane protein with an apparent molecular weight of MW 75,000-85,000 (Sinnett-Smith *et al*, 1990) and later cloned and proven to belong to the GPCR family (Battey *et al*, 1991). Four subtypes of receptors for bombesin-like peptides have been cloned and characterized to date: a GRP-preferring subtype (GRPR or BB1), a neuromedin B receptor (NMBR or BB2), and a third subtype whose ligand is still unknown (BRS-3R or BB3) (Wada *et al*, 1991; Fathi *et al*, 1996). A fourth class of bombesin receptors (designated BB4) appears to constitute the receptors for the bombesin-related peptides as they have higher affinity for bombesin than GRP (Cutz *et al*, 1981). Receptors cloned from various sources show similar pharmacological properties; GRPR has high affinity for GRP and bombesin but low affinity for NMB whereas NMBR has high affinity for NMB, moderate affinity for bombesin but low affinity for GRP.

The human GRP-R and NMB-R genes are localised on chromosome X (Xp22) and chromosome 6 (6q21-ter) respectively (Iwabuchi *et al*, 2003). Overlapping distribution of GRPR and NMBR has been observed in the gastrointestinal tract and in many parts of the brain. The GRPR is particularly abundantly expressed in the hypothalamus, suggesting roles in regulating pituitary hormone secretion and food intake. GRP infusion has been shown to stimulate the secretion of ACTH, corticotropin and endorphin in human subjects (Knigge *et al*, 1987).

### *1.3.1.1. Signal Transduction Pathways activated by Bombesin*

Bombesin stimulates the growth of Swiss 3T3 cells in a concentration and time-dependent manner (Rozengurt & Sinnett-Smith, 1983). The cascades of intracellular signalling events which follow BN/GRP binding to the GRPR to stimulate DNA synthesis are described (illustrated in Figure 1.6).

BN/GRP binding to the GRP receptor chiefly promotes the activation of $G_q$ proteins (Jian *et al*, 1999). One of the earliest events following $G_q$-protein activation is the mobilisation of calcium from intracellular stores which results in a transient increase in cytosolic calcium and calcium efflux (Rozengurt & Mendoza, 1985a). This is due to the interaction of active $G\alpha_q$ with its cellular effector phospholipase C β (PLC-β). PLC-β becomes activated and catalyses the hydrolysis of phosphatidyl inositol 4,5-bisphosphate ($PIP_2$), resulting in the formation of second messengers inositol 1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG) (Berridge, 1993). $IP_3$ binds to its receptor present in the endoplasmic reticulum and stimulates the rapid release of calcium from intracellular stores. The change in intracellular calcium levels is one of the synergistic signals promoting DNA synthesis (Charlesworth & Rozengurt, 1994).

The activation of protein kinase C (PKC) by DAG also contributes to the growth promoting ability of bombesin (Nishizuka, 1995). There are eleven known PKC isoforms which are differentially expressed in cells and tissues and each has distinct functions at different subcellular locations (Dekker *et al*, 1995). One of the major PKC substrates is myristoylated alanine-rich C kinase substrate (80K/MARCKS) and bombesin increases phosphorylation of this substrate (Zachary *et al*, 1986a). Calcium-dependent calmodulin binding to 80K/MARCKS sequesters this substrate from the membrane. The effective downregulation of 80K/MARCKS causes a reversal of high level 80K/MARCKS expression which is induced when cells cease proliferation and is thus implicated in the control of cell proliferation (Blackshear, 1993). It has also become apparent that PKC has a central role in transducing neuropeptide signals into the activation of serine/threonine (Ser/Thr) protein kinases such as mitogen-activated protein kinase (MAPK/ERK), $p70^{S6K}$ and protein kinase D, although the pathways leading to such activation remain incompletely understood.

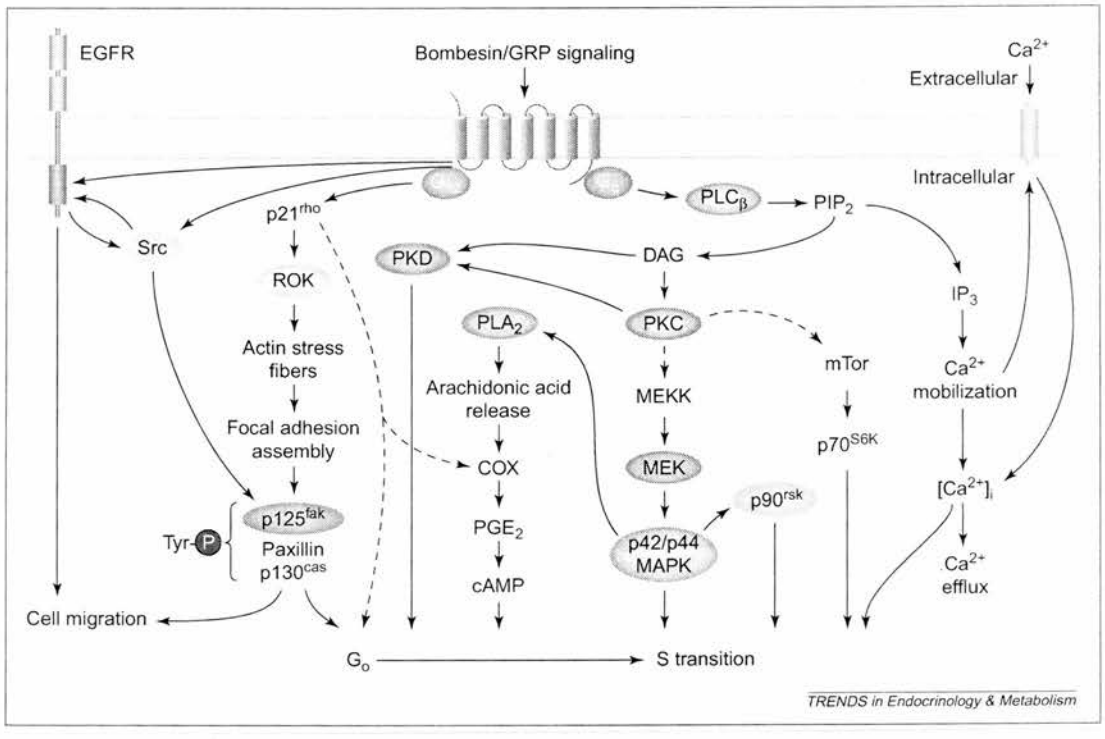


**Figure 1.6. A paradigm of mitogenic GPCR signalling: signal transduction pathways activated by engagement of the bombesin/GRP receptor.** The binding of the ligand (e.g. bombesin) to the cognate GPCR (e.g. the bombesin/GRP preferring receptor) induces activation of the heterotrimeric G proteins of the $G_q$ and $G_{12}$ subfamilies. Signalling through $G_q/G_{11}$ leads to PLC activation, hydrolysis of $PIP_2$, generation of $IP_3$ and DAG and activation of subsequent phosphorylation cascades leading to the activation of ERKs, $p70^{S6K}$ and PKD. These pathways are representative of studies in Swiss 3T3 fibroblasts, SCLC cell lines and pancreatic cancer cells. In other cell types, activation of tyrosine phosphorylation pathways including Src, EGFR and/or Pyk-2 promote Ras-mediated ERK activation via the SOS–Grb2 complex. Signalling through the $G_{12}$ subfamily (comprising $G\alpha_{12}$ and $G\alpha_{13}$) transduces GPCR signals into Rho activation, actin remodelling, assembly of focal adhesions and tyrosine phosphorylation of the focal adhesion-associated proteins FAK, CAS and paxillin, and complex formation between FAK and Src. These pathways are implicated in both cell proliferation and cell migration. (Rozengurt, 2002).

## Bombesin stimulated ERK activation

Extracellular regulated kinases (ERKs) are activated through protein phosphorylation cascades to relay mitogenic signals to the nucleus and this can be mediated by PKC in some cell types (Seufferlein *et al*, 1996a). ERK activation leads

to increased expression of the immediate early response genes (e.g. *FOS, JUN* and *MYC*) and subsequent regulation of cell cycle events (Treisman, 1996). The dual specificity ERK kinase (MEK), which directly phosphorylates ERK1 (p44) and ERK2 (p42) on specific serine and threonine residues, can be activated in a number of ways (Rozengurt, 1998a). MEK can be activated through tyrosine kinase receptor signalling as well as through the activation of $G_i$ and $G_q$ coupled GPCRs, but different mechanisms have been described. Tyrosine kinase receptors (RTKs) activate ERK1/2 through the adaptor complex, SOS-Grb2, which activates the small GTPase Ras ($p21^{ras}$) (Marshall, 1995). ERK1/2 activation by $G_i$–coupled GPCRs has been shown to be mediated by $G\beta\gamma$ subunits and involves the Src-kinase dependent tyrosine phosphorylation of Shc, which recruits the SOS-Grb2 complex for Ras activation (Gutkind, 1998a). In both cases, activated Ras recruits Raf-1 ($p74^{raf-1}$) to the plasma membrane and activates a kinase cascade which involves Raf-1, MEK and ERK1/2.

In Swiss 3T3 cells, bombesin stimulates ERK1/2 activation via $G_q$ in a PKC-dependent but Ras-independent manner (Seufferlein *et al*, 1996a). The precise mechanisms by which PKC isoforms are able to activate ERK1/2 have not been defined. However, studies using recombinant cell systems have revealed that the same GPCR can activate ERK through different pathways according to cell type (Della Rocca *et al*, 1997). Thus, bombesin has been shown to stimulate ERK1/2 via a PKC-independent and Ras-dependent manner in Rat-1 fibroblasts expressing GRPRs (Charlesworth & Rozengurt, 1997). This highlights the importance of cell context in studies of GPCR signalling pathways.

Importantly, GPCRs have been shown to be able to transactivate RTKs such as the EGFR and this is thought to lead to Ras-dependent ERK activation (see Wetzker & Bohmer (2003) for a review on mechanisms underlying such activity). It has recently been demonstrated that bombesin-stimulated EGFR transactivation does not mediate synergistic cell proliferation induced by bombesin and insulin, and that insulin compensates for the requirement for EGFR transactivation in bombesin-induced DNA synthesis (Santiskulvong *et al*, 2004).

## Bombesin stimulated p70 S6 kinase activation

Activation of p70 ribosomal S6 kinase ($p70^{S6K}$) is a common cellular response to mitogenic stimulation and requires phosphorylation of the kinase at multiple sites by several kinases. This leads to increased translation of essential components of the protein synthetic apparatus (Pullen *et al*, 1998). The activation of $p70^{S6K}$ by RTKs is dependent on phosphatidylinositol 3-kinase (PI3-K) activity (Pullen & Thomas, 1997). Bombesin stimulated $p70^{S6K}$ activation is PKC dependent but the mechanism is not fully understood (Withers *et al*, 1997). The drug Rapamycin inhibits $p70^{S6K}$ activation in response to all mitogenic stimuli, including bombesin. Rapamycin blocks the activity of the RAFT1/FRAP/mTOR kinase, which shares homology with the catalytic domain of PI3-K and is able to phosphorylate $p70^{S6K}$ at the rapamycin sensitive-site (Burnett *et al*, 1998). This suggests that RAFT1 and $p70^{S6K}$ activity are involved in the mitogenic effects of bombesin. The effect of rapamycin on mitogenesis is reduced when Swiss 3T3 cells are stimulated with both bombesin and insulin. This suggests that $p70^{S6}$ is a signalling intermediate of one of the parallel pathways leading to DNA synthesis rather than an obligatory point of convergence in mitogenic signalling (Withers *et al*, 1997).

## Bombesin stimulated Protein Kinase D activation

As shown, PKC is central to the transduction of neuropeptide signals into protein kinase cascades but the precise downstream targets of specific PKC isoforms remain undefined. Protein kinase D (PKD) is a Ser/Thr kinase with distinct structural and enzymological properties (Valverde *et al*, 1994). Activation of PKD requires phosphorylation at multiple sites and is stimulated by bombesin through a PKC dependent pathway (Yuan *et al*, 2000). PKD activation has been shown to potentiate neuropeptide-stimulated DNA synthesis by increasing the duration of ERK

signalling (Sinnett-Smith *et al*, 2004). In addition, it has been demonstrated that $G\alpha_{13}$ contributes to PKD activation through a Rho- and protein kinase C-dependent signalling pathway (Yuan *et al*, 2001) showing that PKD activation is mediated by both $G\alpha_q$ and $G\alpha_{13}$ in response to bombesin receptor stimulation.

## Bombesin stimulated Tyrosine Kinase Activation

Unlike the RTKS, GPCRs lack intrinsic tyrosine kinase activity. Nonetheless, bombesin stimulates the rapid tyrosine phosphorylation of a number of substrates (Zachary *et al*, 1991b; Tallett *et al*, 1996). Other neuropeptides, including vasopressin, also have similar effects (Zachary *et al*, 1991b; Sinnett-Smith *et al*, 1993).

The non-receptor protein tyrosine kinase Focal Adhesion Kinase (FAK/$p125^{FAK}$) is one of the most prominent of neuropeptide-stimulated tyrosine phosphorylation substrates (Zachary *et al*, 1992). FAK localises to focal adhesions and FAK-mediated signal transduction is implicated in embryonic development, cell migration, proliferation, apoptosis and metastasis (Seckl & Rozengurt, 1993). FAK phosphorylation facilitates the binding of Src family kinases which leads to further FAK phosphorylation (Parsons & Parsons, 1997). This induces the formation of a FAK signalling complex that activates multiple effector pathways. Neuropeptide-induced phosphorylation of the focal adhesion proteins paxillin and $p130^{cas}$ (CAS) promotes assembly of a FAK signalling complex (Zachary *et al*, 1993). Phosphorylated CAS forms a complex with the proto-oncogene c-Crk, which has Src homology binding domains SH2 and SH3, and this is thought to regulate c-Crk activity (Rosen *et al*, 1995). The kinase is able to regulate the activity of the small GTPase Rap1, which has been shown to stimulate mitogenesis in Swiss 3T3 cells, through binding to the guanine nucleotide exchange factor C3G (Cassamassima & Rozengurt, 1997). The importance of tyrosine phosphorylation in neuropeptide-stimulated mitogenesis is demonstrated through the inhibition of bombesin induced DNA synthesis by tyrosine kinase inhibitors (Seckl & Rozengurt, 1993).

Bombesin-stimulated tyrosine phosphorylation occurs via a pathway which is independent of the $G_q$-mediated PLC pathway. It has been demonstrated that FAK phosphorylation does not require calcium mobilisation and is largely independent of PKC (Sinnett-Smith *et al*, 1993). Bombesin stimulates focal adhesion assembly and stress fiber formation in Swiss 3T3 cells (Buhl *et al*, 1995). FAK tyrosine phosphorylation is thought to occur downstream of focal adhesion assembly and actin re-organisation since disruption of the actin cytoskeleton prevents bombesin-mediated FAK phosphosphorylation (Rozengurt, 1995). Activation of the small GTPase Rho induces focal adhesion assembly, stress fiber formation and nuclear signalling and also leads to FAK phosphorylation (Tapon & Hall, 1997). Inhibition of Rho activity using C3 exoenzyme from *C. botulinum* inhibits bombesin-induced FAK, paxillin and CAS phosphorylation suggesting that neuropeptide-stimulated FAK phosphorylation is downstream of Rho activation (Rankin *et al*, 1994). Members of the $G_{12}$ G-protein subfamily, $G\alpha_{12}$ and $G\alpha_{13}$, induce Rho activation and constitutively active subunits have been shown to stimulate Rho-dependent cytoskeletal responses as well as FAK, paxillin and CAS phosphorylation (Buhl *et al*, 1995; Needham & Rozengurt, 1998). Thus, neuropeptide receptors such as the bombesin receptor are thought to also be able to couple to $G_{12}$ proteins to mediate their effects.

## Bombesin stimulated Arachidonic Acid release and Prostaglandin sythesis

Although bombesin stimulates DNA synthesis in the absence of other factors, vasopressin is mitogenic for Swiss 3T3 cells only in synergistic combination with other factors, e.g. insulin (Rozengurt & Sinnett-Smith, 1983). Since independent neuropeptide-stimulated pathways apparently act synergistically to promote DNA synthesis, it is considered that the ability of bombesin to act as a sole mitogen may be due to the activation of a unique pathway not stimulated by vasopressin. Along this line, bombesin induces the release of arachidonic acid into the extracellular medium whereas vasopressin does not (Millar & Rozengurt, 1990). Arachidonic acid

release has been shown to occur through ERK-mediated Phospholipase $A_2$ ($PLA_2$) activation in many cell types (Clark *et al*, 1995). A role for arachidonic acid in mitogenesis is demonstrated through the potentiation of vasopressin-induced mitogenesis by externally applied arachidonic acid (Millar & Rozengurt, 1990). In addition, it has been found that arachidonic acid released by bombesin-stimulated cells is converted to E-type prostaglandins, leading to an increase in intracellular cAMP levels (Millar & Rozengurt, 1988). cAMP has been shown to be a mitogenic signal for Swiss 3T3 cells, and neuropeptides which bind to receptors coupled to $G_S$ (G-protein subtype that stimulates cAMP synthesis) are mitogenic for these cells (Rozengurt, 1986; Withers *et al*, 1996).

### 1.3.2. Vasopressin

Arginine vasopressin (AVP), also known as antidiuretic hormone (ADH), belongs to the neurohypophysial family of hormones which includes oxytocin. AVP is more traditionally known for its role as a vasoconstrictor hormone acting on vascular smooth muscle cells and its antidiuretic effect via the renal collecting system. In addition to its function in the regulation of body fluid, vascular tone and blood pressure, AVP has mitogenic effects on a number of cell types and has also been shown to possess immunomodulatory properties (Chikanza *et al*, 2000). The human AVP gene is located on chromosome 20 (20p13). The cyclic nonapeptide is synthesised in the hypothalamus as part of a larger pre-cursor protein which is cleaved in the secretory granules to produce vasopressin, vasopressin-associated neurophysin (VP-NP) and vasopressin-associated glycopeptide (VAG) (North, 1997a). Expression of the neuropeptide is largely restricted to the brain but also detected in peripheral organs as a result of systemic secretion.

AVP exerts its biological effects through binding to three vasopressin receptor subtypes: $V_1$ vascular ($V_1R/V_{1A}R$), $V_2$ renal ($V_2R$) and $V_3$ pituitary membrane

($V_3R/V_{1b}R$) receptors which are coupled to distinct G proteins and second messengers (Thibonnier *et al*, 1998). $V_1$ and $V_3$ receptors predominantly couple to $G_q$ proteins to modify phospholipase activity whereas $V_2$ receptors couple to $G_s$ proteins to regulate adenylate cyclases. AVP receptors are expressed in vascular smooth muscle cells, hepatocytes, blood platelets, adrenal cortex, kidneys, the reproductive organs, spleen, adipocytes, brain and various cell-lines (3T3, A10, WRK-1 and A7r5) (Thibonnier *et al*, 1994). The receptors show tissue-specific distribution and each has a predominant biological role: $V_1$ receptors mediate the vascular effects of AVP; $V_2$ receptors mediate renal effects, while $V_3$ receptors are involved in the regulation of ACTH secretion (Chikanza *et al*, 2000). The human $V_{1A}R$ gene is located on chromosome 12 (12q14-q15) and expressed in the liver, platelets, smooth muscle vascular cells and the central nervous system (Birnbaumer, 2000). Stimulation of the $V_{1A}$ receptor promotes cell contraction and proliferation, platelet aggregation, coagulation factor release, and glycogenolysis (Thibonnier *et al*, 1994). $V_{1A}R$ activation also leads to a mitogenic response in vascular smooth muscle cells, 3T3 cells, renal mesangial cells, hepatocytes and adrenal glomerulosa cells (Thibonnier *et al*, 2000).

As mentioned previously, vasopressin is a mitogen for Swiss 3T3 cells in synergistic combination with insulin, serum, EGF and PDGF (Rozengurt & Sinnett-Smith, 1983). The pathways activated by vasopressin have many signalling intermediates in common with those stimulated by bombesin (Rozengurt, 1991). Stimulation of $V_{1A}$ receptors leads to the activation of phospholipases $A_2$, C and D; generation of $IP_3$ and DAG; mobilisation of intracellular calcium; PKC activation; PKD activation; ERK activation; tyrosine phosphorylation of Src family kinases and FAK (Zachary *et al*, 1991b; Thibonnier, 1992; Thibonnier *et al*, 2000; Chiu *et al*, 2002). The mediators of the mitogenic action of $V_{1A}R$ were also found to include the simultaneous and parallel activation of calcium/calmodulin kinase II and phosphatidylinositol 3 kinase (PI3-K) (Thibonnier *et al*, 2000). Thus, the pathways discussed for bombesin-

mediated mitogenesis should also be applicable for vasopressin-mediated mitogenesis.

The importance of the neuropeptides GRP and AVP in mitogenesis in model cell systems has therefore led to the proposal that these neuropeptides may have a regulatory role in the transformation of tumour cells.

## 1.4 Neuropeptide Growth Factors & Lung Cancer

SCLC tumour growth is sustained in part by multiple autocrine and paracrine loops involving calcium mobilising neuropeptides, including bombesin/GRP, bradykinin, cholecystokinin, gastrin, neurotensin and vasopressin (Sethi & Woll, 1995). It appears that SCLC cells can express autocrine growth loops for an overlapping subset of these neuropeptides. Although SCLC cells in culture secrete a variety of these neuropeptides, they only respond mitogenically to those for which the cognate receptor is co-expressed. GRP and vasopressin, the two neuropeptides most relevant to the study here, are discussed below.

### 1.4.1. Bombesin/Gastrin releasing peptide

As mentioned previously, GRP was the first neuropeptide to be detected in SCLC (Wood *et al*, 1981; Moody *et al*, 1981). GRP is found abundantly expressed in the foetal lung but barely detectable, in contrast, in the neuroendocrine cells lining the bronchi of human adult lung (Moody, 1984). These and other studies have suggested that GRP has an important role in normal lung development (Ghatei *et al*, 1983; Wang *et al*, 1996a; Spindel *et al*, 1987; Cuttitta *et al*, 1988; Li *et al*, 1994).

The demonstration that Bombesin/GRP could stimulate the growth of explants of normal human bronchial epithelial cells suggested that GRP could function as a growth factor in the human lung (Willey *et al*, 1984). A study investigating the effect of GRP on the cloning efficiency of human bronchial epithelial (HBE) cells taken from 13 donors showed that the neuropeptide stimulated colony formation in 8/13 cell-lines (Siegfried *et al*, 1997). The elevated expression of GRP receptor subtypes and increased proliferative response found in some of the HBE cell-lines correlated with a history of smoking (al Moustafa *et al*, 1995). The role of GRP as a lung mitogen was further supported through the ability of bombesin to stimulate growth of a HBE cell-line transfected with GRP receptors (Olincy *et al*, 1999). Expression of the three subtypes of bombesin receptor (GRPR, NMBR and BRS-3E) has been demonstrated in HBE cells (DeMichele, 1994). Interestingly, one study found that transformed HBE cells expressed only the GRPR, indicating a possible role for altered regulation of bombesin receptors in lung transformation (Kane *et al*, 1996).

The normal lung responds to changes in pulmonary oxygenation, such as those associated with birth (increased oxygenation) or with chronic obstructive airways disease (decreased oxygenation), by stimulating pulmonary neuroendocrine cells to secrete bombesin/GRP. Increased GRP secretion has been found to be accompanied by pulmonary neuroendocrine hyperplasia (Schuller, 1991). Further to this, it has been suggested that GRP and abnormal oxygenation may play a role in the initiation of smoking-related tumours (Sethi & Woll, 1995). Accordingly, a high incidence of SCLC has been found in smokers with a history of chronic obstructive disease and also the bronchoaleveolar lavages of normal smokers have been found to have elevated levels of GRP compared with non-smokers (Aguayo *et al*, 1990). The serum bombesin levels in schizophrenic smokers, who are often heavy smokers but have a lower incidence of lung cancer, have been found to be reduced (Olincy *et al*, 1999). In a comparison of GRPR expression in the airway cells and tissues of males and females, GRPR expression was found to be significantly higher in women (who have two expressed copies of the X-linked gene) than in men in the absence of

smoking (Shriver *et al*, 2000). GRPR expression was also found to be activated earlier in women in response to tobacco exposure, and could be a contributing factor in the increased incidence of smoking-related lung cancer in women.

Cloning of human GRP mRNA from pulmonary carcinoid tissue showed that the protein is synthesised as a 148 amino acid precursor; a pre-pro-GRP translation product composed of a signal sequence (amino acids 1-27) and a GRP-gene associated peptide (amino acids 31-125) (Spindel *et al*, 1984). Two types of pre-pro-GRP cDNA clones were isolated, indicating the potential existence of at least two forms of pro-GRP differing in the carboxy-terminal GRP-associated peptide.
A marked correlation between pre-pro-GRP expression and the occurrence of bombesin-like immunoreactivity (BLI) has been found in SCLC cells, confirming that the peptides originate in those cells (Sausville *et al*, 1986). Alternative splicing is thought to give rise to the different forms of pre-pro-GRP mRNA which are found in the SCLC cells and suggests that different forms of pro-GRP may be synthesised. In addition to pre-pro-GRP mRNA, pro-bombesin C-terminal peptide and multiple GRP gene associated peptides have also been demonstrated to be present in SCLC tumours (Cuttitta *et al*, 1988; Sunday *et al*, 1991; Sausville *et al*, 1986).

Although plasma GRP is elevated in patients with extensive metastatic SCLC, it was found that GRP itself was not useful as a tumour marker because of low serum levels and its rapid metabolism in the blood (Bork *et al*, 1988). More recently however, it has been proposed that pro-GRP may be useful in this respect. One study showed that pro-GRP levels were elevated in neuroendocrine tumours (Molina *et al*, 2004). High levels of pro-GRP were found in SCLC patients with local and extensive disease in contrast with only 4% of NSCLC cases. Another study indicated that pro-GRP would not be useful in early detection of SCLC since levels were found to be high at an early stage, whereas levels of the more commonly used marker, NSE, tended to increase with disease progression (Sunaga *et al*, 1999). A correlation was found to exist between a positive response to treatment and a

reduction in serum pro-GRP levels, and patients who relapsed had serum pro-GRP levels which were again elevated. In addition, patients whose pro-GRP levels dropped by more than 50% following treatment survived significantly longer than those patients whose pro-GRP levels decreased by less than that. Thus, serum pro-GRP may be useful as a highly specific SCLC marker for treatment monitoring and prognosis. Scintigraphy using a labelled bombesin ligand (99mtc-bombesin) has been used to detect prostate cancer, including pelvic lymph node metastases, and could be applicable to the detection of other endocrine tumours such as SCLC (De Vincentis *et al*, 2004).

To date, deletion of chromosome 18 (the site of the GRP gene) has not been observed in SCLC which suggests that chromosomal abnormalities do not affect the GRP structural gene in this tumour (Virmani *et al*, 1998). Similarly, re-arrangement or amplification of the GRP structural gene has also not been detected. The most consistent and specific chromosomal abnormality found in SCLC cells is deletion of the short arm of chromosome 3, and this has been associated with poor prognosis in limited stage SCLC patients with no pre-operative chemotherapy.

The specific binding of [$^{125}$I-Tyr$^{4}$] bombesin showed that SCLC cells express bombesin receptors, and therefore that GRP could act as an autocrine growth factor for SCLCs (Moody *et al*, 1985; Layton *et al*, 1988). It was demonstrated that the main bombesin receptor subtypes (GRPR, NMBR and BRS-3R) are expressed in an overlapping subset of human lung carcinoma cell-lines (Fathi *et al*, 1996). In one study using RT-PCR, the GRPR, NMBR and BRS-3R were shown to be expressed in 17/20, 11/20 and 5/20 SCLC cell-lines respectively, and could also be detected in NSCLC cell-lines (Toi-Scott *et al*, 1996). In another study using an RNAase protection assay to detect receptor mRNAs, GRPR and NMBR were detected in 3/7 and 7/7 SCLC cell-lines respectively (Yang *et al*, 1998). Analysis of GRPR and NMBR cDNAs from SCLC cell-lines showed there was no structural change in receptor protein or genomic rearrangement associated with expression of these receptors

(Giladi *et al*, 1993). Recently, glucocorticoids have been implicated in the regulation of GRPR expression in SCLC cells. Dexamethasone treatment of a SCLC cell-line led to increased proliferation, which could be blocked by a GRPR antagonist, and was accompanied by significant up-regulation of GRPR expression (Novak *et al*, 2004). It has been shown that SCLC cell-lines, but not NSCLC cell-lines, co-express GRP and GRP receptors and this suggests that autocrine growth loops are likely only to exist in SCLCs (Ocejo-Garcia *et al*, 2001). Pro-GRP mRNA expression has been shown to correlate with elevated serum pro-GRP levels but not with GRPR expression in tumours from SCLC patients (Uchida *et al*, 2002). Thus, it should be noted that not all SCLCs express an autocrine system for GRP.

GRP has been demonstrated to function as a growth factor in SCLC both *in vitro* and *in vivo*. In one study, GRP was shown to enhance DNA synthesis in SCLC cell-lines and in another study, the cloning efficiency of 9/10 SCLC cell-lines was increased 150-fold by GRP (Weber *et al*, 1985; Carney *et al*, 1987). *In vivo*, growth of SCLC xenografts (NCI-H69) increased 77% above control in nude mice given three daily intra-peritoneal injections of bombesin (Alexander *et al*, 1988). The monoclonal antibody to circulating bombesin (2A11) inhibited the cloning efficiency of 2 SCLC cell-lines in serum-free medium and the growth of a SCLC xenograft in nude mice (Cuttitta *et al*, 1985). The same antibody was shown to inhibit the cloning efficiency of SCLC cells in a GRPR-expression dependent manner (Yang *et al*, 1998). Inhibition of SCLC cloning efficiency has also been demonstrated using the specific bombesin antagonist [$Leu^{13}$-psi-($CH_2NH$)-$Leu^{14}$] bombesin (Trepel *et al*, 1988a).

In terms of mitogenic signalling in SCLC, GRP has been shown to stimulate PLC activity and elevate intracellular calcium levels in SCLC cells (Trepel *et al*, 1988b; Heikkila *et al*, 1987). The modulation of PLC activity by non-hydrolysable GTP analogues showed that the GRP receptor expressed in SCLC coupled to a G-protein for intracellular signalling (Sharoni *et al*, 1990). Inositol phosphate production and calcium mobilisation could be inhibited with a specific bombesin antagonist and a

monoclonal antibody against bombesin (Trepel *et al*, 1988b; Heikkila *et al*, 1987). Downregulation of PKC led to inhibition of $IP_3$ production and intracellular calcium release, demonstrating that these GRP-stimulated events occurred in a PKC dependent manner in SCLC cells (Trepel *et al*, 1988a). A role for the kinases ERK and $p70^{s6k}$ in bombesin-stimulated proliferation and colony formation of SCLC cells has also been demonstrated (Seufferlein & Rozengurt, 1996b; Seufferlein & Rozengurt, 1996c). SCLC cell stimulation with bombesin induced expression of the immediate early response genes *c-fos* and *c-jun*, demonstrating that the neuropeptide is able to regulate nuclear expression in SCLC cells (Draoui *et al*, 1995). Tyrosine phosphorylation, including phosphorylation of FAK, is also observed in SCLC in response to bombesin stimulation (Tallett *et al*, 1996b). The calcium mobilising neuropeptides bradykinin, gastrin and neurotensin also stimulated tyrosine kinase activity, indicating that common signalling events could be induced by various neuropeptides. It therefore appears that SCLC growth may be stimulated by the normal intracellular signals evoked by ligand-dependent activation of bombesin receptors. However, to date, therapeutic strategies aimed at specifically antagonising the GRP receptor have not proved fruitful in clinical studies (Chaudhry *et al*, 1999).

### 1.4.2. Vasopressin

Vasopressin growth loops in SCLC have been less intensively studied than the bombesin autocrine system. However, it has become apparent that expression of vasopressin is a very common feature of SCLC cells (Verbeeck *et al*, 1992; North *et al*, 1980; Sorenson *et al*, 1981; Friedmann *et al*, 1994). Immunohistochemical analysis of 24 SCLC cell-lines (using antibodies directed against vasopressin (VP), vasopressin-associated human neurophysin (VP-NP), the bridging region between the hormone and the neurophysin, and vasopressin-associated human glycopeptide (VAG)), showed that all of the cell-lines expressed at least some part of the vasopressin precursor (Friedmann *et al*, 1994). Positive immunoreactivity for all four regions of

the precursor was detected in 67% of cell-lines while in the remaining 33%, only VP-NP and/or VAG was present. Further investigation has revealed that in some SCLC tumours and cultured cells, intact pro-VP can become localised at the cell surface (North *et al*, 1993). This pro-vasopressin product that aberrantly remains attached to the cell membrane may conceivably contribute to autocrine-driven mitogenesis in SCLCs (Friedmann *et al*, 1994). Antibodies recognising this cell surface antigen have been developed as a potential diagnostic and therapeutic tool that targets SCLC tumours *in vivo* (Keegan *et al*, 2002).

Expression of the pre-pro-vasopressin cleavage products, VP and VP-NP, has also been evaluated in non-neuroendocrine tumours. Vasopressin immunoreactivity was not present in any of the tumours examined, although VP-NP immunoreactivity was detected in 1/12 adenocarcinomas and 1/10 squamous cell carcinomas (Friedmann *et al*, 1993). This demonstrated that vasopressin gene expression is restricted to neuroendocrine tumours and this could potentially be exploited in distinguishing SCLC from NSCLC. Another study also demonstrated that vasopressin is expressed in SCLC cell-lines but not in NSCLC cell-lines, HBE cells or transformed HBE cells (Coulson *et al*, 1999c). As mentioned previously, vasopressin is physiologically expressed largely in the hypothalamus and therefore the mechanisms which facilitate de-regulated expression of vasopressin in SCLC are being investigated. Vasopressin expression can be stimulated in SCLC cells through the modulation of VP gene promoter activity by high osmolality as well as non-osmotic factors such as endothelin-3, angiotensin-II and acetylcholine (Kim *et al*, 1996). Studies of the VP promoter have shown that a short region of the promoter drives expression in SCLC but not NSCLC cell-lines (Coulson *et al*, 1999c). It has also been demonstrated that the transcription factor, USF-2 (upstream stimulatory factor) binds within this promoter region to initiate transcription of the vasopressin gene (Coulson *et al*, 2003b). The study showed that in NSCLC cells, USF-2 is endogenously expressed at levels which are insufficient for transcriptional activation of vasopressin expression. SCLC cells have also been found to over-express a novel splice variant of the transcriptional silencer neuron-restrictive

silencer factor (NRSF) which may prevent normal NRSF repressing vasopressin gene expression (Coulson *et al*, 2000).

$V_{1A}$ receptor expression has been detected in 4/4 SCLC cell-lines (Waters *et al*, 2003). In another study, it was demonstrated that 5/5 SCLC cell-lines expressed $V_{1A}$ receptors whereas NSCLC, HBE and transformed HBE cells did not express the receptors (Coulson *et al*, 1999c). It was shown that vasopressin and $V_{1A}$ receptors were co-expressed in all SCLC cell-lines examined, while no such co-expression was observed for NSCLC (Ocejo-Garcia *et al*, 2001). This demonstrates that SCLC cells express an autocrine system for vasopressin. In general, co-expression of neuropeptides and their receptors was found to be restricted to SCLC cells and it was suggested that particular neuropeptide/receptor pairs would be useful as early markers for the detection of lung cancer (Coulson *et al*, 2003a). Both classic and variant SCLC cell-lines have also been shown to co-express vasopressin and $V_{1A}$ receptors (Fay *et al*, 1994). These SCLC cell-lines were also found to express other vasopressin receptor subtypes, which implies that vasopressin may play a more complex role in SCLC growth than has previously been considered (North *et al*, 1998).

Consistent with a role as a growth factor, vasopressin is able to increase the cloning efficiency of SCLC cells (Sethi & Rozengurt, 1991; Bunn *et al*, 1992; Seufferlein & Rozengurt, 1996b). A role for vasopressin in SCLC metastasis has also been suggested since vasopressin is a chemoattractant for SCLC cells (Ruff *et al*, 1985).

Vasopressin is able to stimulate calcium mobilisation in SCLC cells and this can be blocked by vasopressin antagonists (Woll & Rozengurt, 1989a; Woll & Rozengurt, 1990; Bunn *et al*, 1992; Hong *et al*, 1991; Bunn *et al*, 1994). Interestingly, variant SCLC cell-lines do not mobilise intracellular calcium or proliferate in response to vasopressin although functional, structurally normal $V_{1A}$ receptors have been detected on both classical and variant cell lines (Fay *et al*, 1994; North *et al*, 1998).

Whereas vasopressin stimulates calcium mobilisation in classic SCLC cells, $V_{1A}$ receptor activation in variant SCLC results in an increase in inositol phosphate levels without a corresponding rise in intracellular calcium (North *et al*, 1997b). This indicates that discordant vasopressin signalling occurs at a post-receptor level in variant SCLC cells.

In addition to calcium mobilisation, mitogenic vasopressin signalling in SCLC cells has been shown to involve the activation of ERK in a PKC-dependent manner (Seufferlein, 1996b; Pequeux *et al*, 2004). The downstream target of ERK activation is $p90^{rsk}$, which is phosphorylated in response to vasopressin stimulation of SCLC (Pequeux *et al*, 2004). Through the use of a specific vasopressin antagonist as well as inhibitors of various signalling intermediates, the same study showed that vasopressin stimulated ERK activation and $p90^{rsk}$ phosphorylation was dependent upon PLC, PKC, MEK1/2 and calcium signalling. An absolute requirement for PLC, calcium, PKC and ERK1/2 for vasopressin-stimulated DNA synthesis in SCLC cells was also demonstrated. This indicates that the growth of classic SCLCs may be stimulated by the normal intracellular signals evoked by ligand-dependent activation of $V_{1A}$ receptors. Thus, vasopressin receptors provide an attractive target for SCLC therapy.

### 1.4.3. Strategies for Blocking Neuropeptide Growth Factor Action

Since the growth of SCLC can be sustained by autocrine and paracrine growth loops involving calcium mobilising neuropeptides, interruption of these growth loops provides an opportunity for controlling the growth of this cancer. The mitogenic effect of the neuropeptides are elicited through binding to their receptors, therefore preventing the ligand-receptor interaction is the most immediate way of blocking mitogenesis. The majority of work along these lines has been in the development of bombesin antagonists to disrupt bombesin growth loops.

The monoclonal antibody against circulating bombesin (2A11), which had previously been shown to inhibit SCLC growth *in vitro* and *in vivo*, showed no dose-limiting toxicity in a phase I clinical trial (Chaudhry *et al*, 1999). In the phase II trial, one relapsed SCLC patient went into clinical remission (Kelley *et al*, 1997). More recently, a bi-specific molecule targeting cell surface BN/GRPR on SCLC cells and an immune trigger molecule on host immune effector cells has been developed to direct immune effector cells to SCLC cells (Zhou *et al*, 2003). The bispecific molecule consisted of a synthetic BN/GRP antagonist (Antag 2) and a humanized monoclonal antibody for FcgammaRI (H22). In an animal model of human SCLC xenografts, the study showed that targeted immunotherapy in combination with chemotherapy led to greater SCLC killing. However, the cost-effectiveness of antibody therapeutics coupled with relatively low tissue penetration present potential drawbacks for antibody-mediated therapy of SCLC.

A novel approach has been to use antisense (AS) oligodeoxynucleotides (ODNs) directed against gastrin releasing peptide (GRP) receptor mRNA (Langer *et al*, 2002). AS ODNs significantly inhibited the growth of NCI-H345 SCLC cells *in vitro*, but not the growth of cells that lacked an autocrine system for GRP. The AS ODNs down-regulated GRP receptor expression and also caused a reduction in the number of cells mobilising intracellular calcium in response to GRP. It is hoped that AS ODN specificity and effectiveness in human SCLC cell can be increased through further development of this type of strategy.

A more conventional approach has been the development of peptide antagonists of bombesin. A pseudo-peptide analogue of bombesin [$Leu^{13}$-psi($CH_2NH$)$Leu^{14}$] inhibited the growth of SCLC cells both *in vitro* and *in vivo* (Mahmoud *et al*, 1991). Using similar pseudo-nonapeptides, the Schally group introduced D-Trp or Trp analogue (Tpi) substitutions at position 6 to produce a series of more potent bombesin antagonists (Radulovic *et al*, 1991a). Of these, RC-3095 ([D-$Tpi^6$,$Leu^{13}$ psi($CH_2NH$)$Leu^{14}$-bombesin (6-14)]) has been shown to inhibit the growth of GRPR expressing SCLC cells *in vitro* and *in vivo*, but not the growth of non-GRPR expressing NSCLC cells (Pinski *et al*, 1994b). The mechanism of *in vivo* growth

inhibition involves the down-regulation of GRPR and EGFR expression (Koppan *et al*, 1998; Halmos *et al*, 1997). More recently, the antagonist has also been found to have anti-angiogenic effects in various tumour models (Bajo *et al*, 2004; Heuser *et al*, 2005; Stangelberger *et al*, 2005a). RC-3095 delayed the progression of pre-malignant lesions to invasive cancer in a hamster model (Liebow *et al*, 1993). RC-3095 has also demonstrated anti-tumour activity against an extensive range of tumour types which express bombesin receptors (Table 1.5), which highlights the significance of GRP as a cancer growth factor. RC-3095 has recently completed a phase-I trial in patients with advanced solid malignant disease of various types, which demonstrated that the agent was well-tolerated without clinically relevant side effects and that there were signs of anti-tumour effects even at low doses (Schwartsmann G and colleagues, unpublished data). A bombesin antagonist (RC-3094) conjugated to a doxorubicin derivative (AN-201) has recently been developed for targeted chemotherapy. *In vivo* studies indicated that the conjugate inhibited SCLC and prostate cancer growth *in vivo* more effectively and with less toxicity than the cytotoxic agent alone (Kiaris *et al*, 1999a; Stangelberger *et al*, 2005b).

Bradykinin is also an important growth factor for SCLC. The bradykinin receptor is expressed in most of the human lung cancer cell-lines, and bradykinin is one of the most potent neuropeptides that causes intracellular calcium flux in these SCLC and NSCLC cell-lines (Bunn *et al*, 1992). Peptide and non-peptide bradykinin antagonists have been developed which demonstrate anti-cancer activity (Stewart, 2003). In particular, a non-peptide bradykinin antagonist dimer inhibited the growth of SCLC and prostate cancer xenografts in nude mice more potently than standard anti-cancer drugs (Stewart *et al*, 2005). In the same study, it was demonstrated that anti-angiogenesis and anti-matrix metalloprotease activities are components of the anti-cancer effect.

**Table 1.5. Tumour types sensitive to growth inhibition in xenograft models by bombesin antagonist (RC-3095)**

| Tumour Type |
|---|
| Small Cell Lung Cancer |
| Prostate Cancer |
| Renal Adenocarcinoma |
| Breast Cancer |
| Colorectal Cancer |
| Pancreatic Cancer |
| Gastric Cancer |
| Malignant Glioblastoma |
| Ovarian Cancer |

Combination chemotherapy forms the basis of many cancer treatment regimes. Thus, while a single agent may not fully eliminate cancer, a more potent effect can be obtained when used in combination with other drugs, particularly those which target a different aspect of cancer growth. For example, through the combined use of a cytotoxic drug to inhibit growth, an angiogenesis inhibitor to block neovascularisation and a matrix-metalloprotease inhibitor to prevent invasion. The finding that some of these single neuropeptide antagonists also have anti-angiogenic and anti-matrix metalloprotease properties suggests that the combined chemotherapy effect may be attainable through the use of a single compound. However, the heterogeneity of neuropeptide receptor expression and responsiveness in SCLC is a potential hindrance to the therapeutic usefulness of single neuropeptide antagonists. Targeting a single neuropeptide growth loop would therefore be unlikely to inhibit SCLC growth fully and instead, simultaneously blocking the action of multiple neuropeptide growth factors would

be required. For treatment to be fully effective using single neuropeptide antagonists, a cocktail of neuropeptide antagonists would need to be administered according to the neuropeptide receptor profile of the tumour. The ideal alternative would be the development of antagonists which inhibit the action of a broad range of neuropeptide growth factors. Substance-P analogues were the first compounds found to possess broad-spectrum antagonist activity against multiple neuropeptide growth factors.

### *1.4.3.1. Substance-P analogues as novel anti-cancer agents*

Substance-P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met) is a member of the tachykinin family of neuropeptides. A series of substance-P analogues were developed, with various D-amino acid substitutions, as antagonists of substance-P activities. Remarkably, substance-P antagonist A (**SP-A**: [D-Arg$^{1}$, D-Pro$^{2}$,D-Trp$^{7,9}$, Leu$^{11}$]) was found to also antagonise the cellular effects of structurally unrelated bombesin (Jensen *et al*, 1984). In swiss 3T3 cells, SP-A inhibited $^{125}$I-GRP binding and early signalling events and mitogenesis stimulated by bombesin (Zachary & Rozengurt, 1985; Woll & Rozengurt, 1988b). The first indication that substance-P analogues may have broad-spectrum activity came from the finding that SP-A was also able to block the specific binding of $^{3}$H-vasopressin and vasopressin stimulated calcium mobilisation and DNA synthesis (Zachary & Rozengurt, 1986b). This showed that SP-A could interact with receptors for three structurally unrelated neuropeptides, and so further substance-P analogues were investigated.

Antagonist G (**SP-G**: [Arg$^{6}$,D-Trp$^{7,9}$,MePhe$^{8}$]-substance-P (6-11); a truncated substance-P analogue) and antagonist D (**SP-D**: [D-Arg$^{1}$, D-Phe$^{5}$,D-Trp$^{7,9}$, Leu$^{11}$]) inhibited signal transduction and DNA synthesis stimulated by bombesin, GRP, bradykinin and vasopressin (Woll & Rozengurt, 1988c; Woll & Rozengurt, 1990). The analogues were also able to inhibit the binding of these ligands to their receptors. As well as inhibiting neuropeptide binding and calcium mobilisation,

substance-P analogues have been shown to inhibit PLCβ, PKC, ERK and FAK activation by bombesin and vasopressin (Seckl *et al*, 1996a).

In SCLC cells, SP-G inhibited calcium mobilisation induced by bombesin, bradykinin, gastrin, galanin, vasopressin, cholecsytokinin and neurotensin (Woll & Rozengurt, 1990). Various substance-P analogues inhibit SCLC cell growth *in vitro* in liquid and semi-solid media (Bepler *et al*, 1988; Layton *et al*, 1988; Woll & Rozengurt, 1988c; Woll & Rozengurt, 1990; Sethi *et al*, 1992; Bunn *et al*, 1994). SP-D and SP-G are equipotent in their growth inhibitory effects, with half-maximal effect at around 20μM, whereas SP-A is five-fold less potent (Woll & Rozengurt, 1988c). Table 1.6 illustrates the range of potencies with which substance-P analogues inhibit H69 SCLC cell growth. A more potent inhibitor of SCLC cell growth, [D-Arg$^{1}$,D-Trp $^{5,7,9}$,Leu$^{11}$] Substance-P (ICRT5), was identified out of a panel of novel substance-P analogues (Seckl *et al*, 1997). This analogue inhibited proliferation in liquid culture and semisolid media, and also vasopressin- or bradykinin-induced calcium mobilisation and ERK activation.

SP-D inhibited DNA synthesis and cloning efficiency of 6/6 SCLC, 1/1 NSCLC (squamous), 2/2 ovarian and 1/1 squamous cervical carcinoma cell-lines, demonstrating that the growth inhibitory effects were not restricted to effects on SCLC cells alone (Everard *et al*, 1992). Another study assessed the effects of a panel of substance-P analogues and showed that the compounds inhibited the growth of peptide-sensitive SCLC cell lines more efficiently than their inhibition of peptide-insensitive NSCLC or breast cancer cell lines (Bunn *et al*, 1994). Growth inhibition of a range of tumour types (SCLC, NSCLC, ovarian, pancreatic and colorectal carcinoma cell-lines) by SP-G correlated with GRPR expression (Waters *et al*, 2003). Of particular interest was the finding that SP-G was able to inhibit the growth of SCLC, NSCLC and ovarian cell-lines which were resistant to standard chemotherapeutic agents such as etoposide. A comparison of specific bombesin, cholecystokinin or AVP antagonists with a number of broad specificity substance-P analogues showed that the substance-P analogues were more effective at inhibiting SCLC growth *in vitro* (Bunn *et al*, 1994).

**Table 1.6. Substance-P analogue structures and inhibition of H69 SCLC cell growth in liquid culture on day 12 (adapted from Seckl and Rozengurt, 1998)**

| Substance-P analogue structure | % of Growth inhibited by 25μM |
|---|---|
| Arg[1]-Pro[2]-Lys[3]-Pro[4]-Gln[5]-Gln[6]-Phe[7]-Phe[8]-Gly[9]-Leu[10]-Met[11]-$NH_2$ **(Substance-P)** | 0 |
| **Arg[6]-D-Trp[7]-MePhe[8] -D-Trp[9]**-Leu[10]-Met[11]-$NH_2$ **(SP-G)** | 30 |
| **Arg[6]-D-Trp[7]-MePhe[8] -D-Trp[9]**-Leu[10]-**D-Met[11]** -$NH_2$ | 32 |
| **Arg[6]-D-Trp[7]-MePhe[8] -D-Trp[9]-D-Leu[10]**-Met[11] -$NH_2$ | 27 |
| **Arg[6]-D-Trp[7]-MePhe[8] -D-Trp[9]-D-Leu[10]-Val[11]** -$NH_2$ | 19 |
| **Arg[6]-D-Trp[7]-MePhe[8] -D-Trp[9]-D-Leu[10]-Gly[11]**-$NH_2$ | 16 |
| **Arg[6]-D-Trp[7]-MePhe[8] -D-Trp[9]-D-Leu[10]**-Met[11] **-OH** | 13 |
| **D-Pro[2]**-Lys[3]-Pro[4]-**D-Phe[5]**-Gln[6]-**D-Trp[7]**-Phe[8]-**D-Trp[9]**-Leu[10]-Met[11]-$NH_2$ | 61 |
| **D-Pro[2]**-Lys[3]-Pro[4]-**D-Trp[5]**-Gln[6]-**D-Trp[7]**-Phe[8]-**D-Trp[9]**-Leu[10]-Val[11]-$NH_2$ | 36 |
| **D-Pro[2]**-Lys[3]-Pro[4]-**D-Phe[5]**-Gln[6]-**D-Trp[7]**-Phe[8]-**D-Trp[9]**-Leu[10]-Gly[11]-$NH_2$ | 20 |
| **D-Pro[2]**-Lys[3]-Pro[4]-**D-Phe[5]**-Gln[6]-**D-Trp[7]**-Phe[8]-**D-Trp[9]**-Leu[10]-$NH_2$ | 21 |
| **Ac**-Lys[3]-Pro[4]-**D-Phe[5]**-Gln[6]-**D-Trp[7]**-Phe[8]-**D-Trp[9]**-Leu[10]-**Leu[11]**-$NH_2$ | 16 |
| **Ac**-Lys[3]-Pro[4]-**D-Phe[5]**-Gln[6]-**D-Trp[7]**-Phe[8]-**D-Trp[9]**-Leu[10]-**Gly[11]**-$NH_2$ | 0 |
| **Arg[4]-D-Phe[5]**-Gln[6]-**D-Trp[7]**-Phe[8]-**D-Trp[9]**-Leu[10]-**Leu[11]**-$NH_2$ | 39 |
| **Arg[4]-D-Phe[5]**-Gln[6]-**D-Trp[7]**-Phe[8]-**D-Trp[9]**-Leu[10]-**Gly[11]**-$NH_2$ | 42 |
| **D-Arg[1]**-Pro-Lys-Pro-**D-Phe[5]**-Gln-**D-Trp[7]**-Phe-**D-Trp[9]**-Leu-**Leu[11]** **(SP-D)** | 55 |
| **D-Arg[1]**-Pro-Lys-Pro-**D-Trp[5]**-Gln-**D-Trp[7]**-Phe-**D-Trp[9]**-Leu-**Leu[11]** **(ICRT5)** | 92 |

In mouse xenograft model studies, SP-D and SP-G inhibited the growth of WX332 SCLC tumours and the inhibitory effects were maintained beyond the duration of administration (Langdon *et al*, 1992). SP-D and ICRT5 also inhibited H69 SCLC xenografts, and analysis of the biodistribution of SP-D in the mice showed that although there was poor uptake into the tumour xenograft, there was good uptake into the lungs indicating that it may be possible to attain therapeutic concentrations in primary lung tumours (Seckl *et al*, 1997; Jones *et al*, 1997). A phase I clinical trial using SP-G has been completed which evaluated the effects of dose-escalation up to 400mg/m$^2$. Volunteer cancer patients received a 6hr infusion every three weeks for up to 12 cycles. No dose-limiting toxicity was found and plasma concentrations up to 40μM were achieved (Clive *et al*, 2001). This concentration is within the range of $IC_{50}$ values for SCLC growth inhibition derived from *in vitro* studies.

More recently, studies of the most potent substance-P analogue [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$] have been carried out using a well-differentiated pancreatic cancer cell-line which expresses multiple GPCRs for mitogenic agonists and produces pro-angiogenic chemokines (Guha *et al*, 2005). The analogue inhibited multiple neuropeptide-induced calcium mobilisation, DNA synthesis, and anchorage-independent growth *in vitro* and significantly blocked the growth of HPAF-II tumour xenografts in nude mice beyond the treatment period. The novel finding of this study was that the analogue markedly reduced tumour-associated angiogenesis in the HPAF-II xenografts and was also shown to specifically inhibit IL-8/CXCL8-induced angiogenesis in a rat corneal micropocket assay *in vivo*. This demonstrates that substance-P analogues can inhibit tumour growth through a dual mechanism which involves both anti-proliferative and anti-angiogenic properties.

## The mechanisms underlying substance-P analogue induced growth inhibition

Due to the clinical requirement for compounds with broad-spectrum specificity against multiple neuropeptide receptors for the treatment of neuroendocrine

tumours such as SCLC, it has been of great importance to elucidate the mechanisms underlying substance-P analogue induced growth inhibition. The substance-P analogues have broad-spectrum specificity against mitogenic neuropeptides whose receptors couple to $G_q$ proteins to activate a common set of signal transduction pathways (Sethi & Woll, 1995). In accordance with this, substance-P analogues do not inhibit the mitogenic signalling of platelet-derived growth factor (which binds to a RTK to mobilise intracellular calcium), or vasoactive intestinal peptide (which induces cAMP accumulation without calcium mobilisation through a $G_s$-coupled receptor) (Mitchell *et al*, 1995; Seckl *et al*, 1996a). Given the ability of these analogues to inhibit binding and subsequent signalling of multiple, structurally unrelated neuropeptides it was originally believed that the compounds were acting as competitive antagonists of ligand binding. However, several lines of evidence suggested the mechanism was more complex than simple competitive antagonism.

The growth inhibitory effects of the analogues were found to be at least partly mediated through the active induction of apoptosis. SP-G induced apoptosis in SCLC cell-lines at similar concentrations to which it caused growth inhibition (Tallett *et al*, 1996a; MacKinnon *et al*, 1999). The pro-apoptotic and growth inhibitory effects of the analogues could not be reversed by exogenously added neuropeptides (Mitchell *et al*, 1995; MacKinnon *et al*, 1999). In terms of intracellular signalling, SP-D prevented ERK activation at low concentrations of bombesin (1-10nM) whereas at higher concentrations of bombesin (>10nM) the analogue enhanced bombesin-stimulated ERK activation (Mitchell *et al*, 1995). This suggested that the GRP receptor could still be capable of signalling even when bombesin-induced PLC activation was fully blocked by analogue. It was subsequently found that SP-G was able to induce the sustained activation of c-Jun-N-terminal kinase (JNK) in SCLC cells and this activation could not be reversed by high concentrations of neuropeptides (MacKinnon *et al*, 1999). Thus, SP-G was demonstrated to possess neuropeptide independent agonist activity in addition to its previously known antagonist properties. It had previously been demonstrated that JNK activation by GPCRs could be mediated through receptor coupling to $G_{12}$ proteins (Prasad *et al*,

1995). SP-D was also shown to be able to activate JNK and it was found that this JNK activation was dependent on the expression of GRP receptors (Jarpe *et al*, 1998). Jarpe *et al* (1998) suggested that SP-D activated G-proteins of the $G_{12}$ family whilst simultaneously blocking signal transduction via $G_q$-proteins, and proposed the novel pharmacological term 'biased agonism' to describe this behaviour.

Direct evidence for biased agonism was provided through studies of SP-D agonist activity in cells expressing bombesin receptors (Mackinnon *et al*, 2001). SP-D inhibited bombesin binding and bombesin-stimulated calcium mobilisation within the same concentration range which caused sustained JNK and ERK activation. SP-D stimulated JNK activation was blocked by dominant negative inhibition of $G\alpha_{12}$ and ERK activation inhibited by pertussis toxin. In contrast, bombesin stimulated ERK activity was not sensitive to pertussis toxin showing that SP-D facilitated bombesin receptor coupling with $G\alpha_{12}$ and $G_i$ proteins whilst blocking receptor activation of $G_q$ proteins. Thus it was demonstrated that SP-D was capable of differentially modulating the activation of the G-proteins $G\alpha_{12}$, $G_i$ and $G_q$ compared with bombesin. It has been proposed that the biased agonist activity of SP-D, which results in the inhibition of intracellular calcium mobilisation and the prolonged stimulation of ERK and JNK, is central to the growth inhibitory effects of this agent (MacKinnon *et al*, 2001; Waters *et al*, 2003).

## 1.5 Plan of study

As discussed above, substance-P analogues represent a novel therapeutic strategy for the treatment of neuroendocrine tumours such as SCLC. The demonstration that SP-D acts as a biased agonist at bombesin receptors is of great importance as it shows that a ligand can selectively direct intracellular signalling pathways to alter the normal biological outcome of receptor signalling. Targeting multiple mitogenic neuropeptide receptors with a novel pharmacological agent which acts as a

molecular switch converting a normally growth promoting signal into a death signal offers a novel opportunity to inhibit cancer progression. It would therefore be of consequence to the clinical use of substance-P analogues as anti-cancer agents to establish whether their biased agonist activity is restricted to effects at GRP receptors alone. The aim of this study is to determine whether substance-P analogues exhibit biased agonist activity at receptors other than the GRP receptor. The factors which may influence the ability of substance-P analogues to modulate neuropeptide signalling will also be investigated as this could aid in the future development of novel biased agonists. The following specific questions will be addressed:

1) Are substance-P analogues biased agonists of vasopressin receptors as well as GRP receptors? Model cell systems expressing vasopressin or GRP receptors will be established for this purpose. Substance-P analogue activity will be investigated at the levels of receptor binding, calcium mobilisation and ERK activation in order to further investigate the mechanism underlying biased agonism.
2) Does specific expression of GRP and $V_{1A}$ receptors lead to cellular transformation in a model CHO-K1 cell system? Cell growth, migration and adhesion will be assessed in order to evaluate the oncogenic potential of neuropeptide receptors expressed in CHO-K1 epithelial cells. In addition, the chemosensitivity of the model cell systems will be investigated to ascertain whether neuropeptide growth factor signalling could play a role in the acquisition of chemoresistance.
3) Does neuropeptide receptor expression alter the ability of substance-P analogues to signal and inhibit growth? Substance-P analogue-induced growth inhibition will be assessed under both anchorage dependent and independent conditions. This may indicate whether a correlation between biased agonist activity and analogue induced growth inhibition exists. The effect of substance-P analogues on migration will also be

investigated to determine whether they can function as chemoattractants for neuropeptide receptor expressing cells.

4) Does modulation of the stoichiometry of receptor to G-protein alter substance-P analogue directed signalling?

5) What regions of the $V_{1A}$ receptor are necessary for substance-P-analogue induced signalling? Using the $V_{1A}$ receptor as a model, chimeric receptors comprising substitutions from the V2 receptor will be used in an attempt to elucidate whether any particular receptor regions are involved in substance-P analogue directed trafficking of receptor signalling.

# Chapter 2

# Materials and Methods

## 2.1. Materials

The epithelial CHO-K1 cell-line was purchased from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK). The small cell lung cancer cell-line, NCI-H345 SCLC, was purchased from the American Type Culture Collection (ATCC, Rockville, USA). All tissue culture flasks and plates were from Costar unless otherwise stated.

**Table 2.1. List of materials used and sources**

| Product | Description | Source |
|---|---|---|
| Dulbecco's modified eagles medium (DMEM) | Cell culture reagent | Sigma, Poole |
| Foetal Calf Serum (FCS) | Cell culture reagent | |
| Phosphate Buffered Saline (PBS) | Cell culture reagent | Sigma, Poole |
| Hanks Balanced Salt Solution (HBSS) with calcium | Cell culture reagent | Sigma, Poole (H-8264) |
| Hanks Balanced Salt Solution (HBSS) without calcium | Cell culture reagent | Sigma, Poole (H-6648) |
| GIBCO® Non-essential amino acids (NEAA) | Cell culture reagent | Invitrogen, Paisley |
| GIBCO® L-glutamine | Cell culture reagent | Invitrogen, Paisley |
| GIBCO® Penicillin | Antibiotic | Invitrogen, Paisley |
| GIBCO® Streptomycin | Antibiotic | Invitrogen, Paisley |
| GIBCO® G418-sulphate | Antibiotic | Invitrogen, Paisley |
| GIBCO® Trypsin-EDTA | Cell culture reagent | Invitrogen, Paisley |
| Soybean Trypsin Inhibitor | Trypsin Inhibitor | Sigma, Poole |
| LipofectAMINE PLUS | Transfection reagent | Invitrogen, Paisley |
| Bombesin (Bn) | Neuropeptide | Sigma, Poole (B-4272) |
| Arginine$^{8}$-vasopressin (AVP) | Neuropeptide | Sigma, Poole (V-9879) |
| Substance P | Neuropeptide | Sigma, Poole (S-6883) |
| Lysophosphatidic acid | Phospholipid | Sigma, Poole (L-7260) |
| SP-G [D-Arg$^{6}$,D-Trp $^{7,9}$,N$^{me}$Phe$^{8}$]-substance P (6-11) | Substance P analogue | Cancer Research UK, London |

(Table 2.1. continued)

| Product | Description | Source |
|---|---|---|
| SP-D [D-Arg$^{1}$,D-Phe$^{5}$,D-Trp$^{7,9}$,Leu$^{11}$] | Substance P analogue | Cancer Research UK, London |
| Spantide [D-Arg1, D-Trp7,9, Leu11]-Substance P | Substance P analogue | BACHEM, Switzerland |
| Monoclonal antibody against diphosphorylated ERK 1 and 2 | Antibody | Sigma, Poole (M-8579) |
| Anti-mouse IgG HRP conjugate | Antibody | DAKO, UK |
| [β-mercapto-β-β-cyclopentamethylene-propionyl$^{1}$O-Me-Tyr$^{2}$-Arg$^{8}$]-Vasopressin | Vasopressin antagonist (Manning compound) | Sigma, Poole (V-2255) |
| RC3940-II ([Hca$^{6}$,Leu$^{13}\psi$[CH$_2$N]Tac14-Bombesin(6-11)) | Bombesin antagonist (Schally compound) | A.V. Schally, Endocrine, polypeptide and cancer institute, LA, USA |
| Cytochalasin D | Actin cytoskeleton depolymerizer | Calbiochem, Nottingham |
| Wortmannin | P13K inhibitor | Sigma, Poole |
| Staurosporine | PKC inhibitor | Sigma, Poole (S-4400) |
| PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) | Src-family tyrosine kinase inhibitor | Calbiochem, Nottingham |
| FTS (S-*trans,trans*-farnesylthiosalicylic acid) | Ras inhibitor | Calbiochem, Nottingham |
| AG 1478 [4-(3-Chloroanilino)-6,7-dimethoxyquinazoline] | EGF receptor kinase inhibitor | Calbiochem, Nottingham |
| Pertussis Toxin | Endotoxin inhibiting $G_i$, $G_o$ and $G_t$ by ADP-ribosylation | Alexis Biochemicals, Switzerland |
| FURA-2/AM | Fluorescent calcium indicator dye | Calbiochem, Nottingham |
| [$^{125}$I]-argininevasopressin (2000Ci/mmol) (3-[[[$^{125}$I]]*iodotyrosyl*$^{2}$) Vasopressin[Arg$^{8}$] | Iodinated radioligand | Amersham Biosciences, Amersham |
| [$^{125}$I]-GRP (2000Ci/mmol) (3-[$^{125}$I]*iodotyrosyl*$^{15}$) Gastrin-releasing Peptide | Iodinated radioligand | Amersham Biosciences, Amersham |
| [$^{3}$H]-arginine vasopressin (73Ci/mmol) | Tritiated radioligand | New England Nuclear, PerkinElmer, USA |
| Agarose, Low melting point | | Sigma, Poole |
| Seakem® GTG® Agarose | | BioWhittaker Molecular Applications, USA |
| Fibronectin (human plasma) | Plasma glycoprotein | Sigma, Poole (F-0895) |

## 2.2. Cell Culture

CHO-K1 cells were maintained in DMEM supplemented with 10% (v/v) foetal calf serum (FCS, heat-inactivated at 57°C for 1 h), 1% (v/v) non-essential amino acids, 50 U $ml^{-1}$ penicillin, 50 μg $ml^{-1}$ streptomycin and 5 μg $ml^{-1}$ L-glutamine in a humidified atmosphere of 5% $CO_2$:95% air at 37°C. CHO-K1 cells stably transfected with GRPr or V1Ar were maintained in the same DMEM growth media with the addition of 400 μg $ml^{-1}$ G418-sulphate (Promega, WI, USA). For routine passage, cells were detached from the culture flask with trypsin-EDTA, quenched with serum containing media and pelleted at 300g for 4 minutes. Cells were divided into ten new flasks in fresh media. Cells were quiesced in DMEM supplemented with 0.1% (v/v) foetal bovine serum (heat-inactivated at 57°C for 1 hour), 1% (v/v) non-essential amino acids, 50 U $ml^{-1}$ penicillin, 50 μg $ml^{-1}$ streptomycin and 5 μg $ml^{-1}$ L-glutamine when necessary. For experimental purposes, cell-lines were continually cultured only up to ten passages before being replaced with cells of earlier passage number from liquid nitrogen stocks.

Stock NCI-H345 SCLC cells were cultured in RPMI-1640 medium with 25mM HEPES supplemented with 10% (v/v) FCS, 50U $ml^{-1}$ penicillin, 50μg $ml^{-1}$ streptomycin and 5μg $ml^{-1}$ L-glutamine. Cell cultures were allowed to grow to a density of $10^6$ cells $ml^{-1}$ in a humidified atmosphere of 5% carbon dioxide/95% air at 37°C. For experimental purposes, the cells were grown in SITA medium consisting of RPMI-1640 medium containing SIT supplement (30nM selenium, 5μg $ml^{-1}$ insulin, 10μg $ml^{-1}$ transferrin from Sigma) and 0.25% (w/v) bovine serum albumin (BSA).

## 2.3. Plasmid DNA preparation

### 2.3.1 Transformation

A frozen aliquot of transformation competent *Escherichia coli* DH5α (Invitrogen, Paisley) was thawed on ice. Plasmid cDNA (pcDNA) [1-10ng] was added to cells and incubated on ice for 30 minutes. Cells were then heat-shocked at 42°C for 60

seconds and put back on ice for two minutes. Transformed cells were then grown in SOC media (Invitrogen, Paisley) at 37°C with agitation (500rpm) for 60 minutes. The culture was then spread onto agar plates containing 10μg $ml^{-1}$ ampicillin and incubated overnight at 37°C. Individual colonies were picked into LB broth containing ampicillin and grown overnight at 37°C with agitation.

### 2.3.2 DNA purification

Plasmid cDNA was prepared from bacterial cultures using the Endofree® Plasmid Maxi Prep kit (Qiagen, Crawley, UK) according to the manufacturers' instructions. Purified pcDNA was quantified using a Pharmacia Biotech Ultraspec2000 UV spectrophotometer.

Diagnostic restriction digests of purified pcDNA were carried out using appropriate restriction enzymes (Promega). Restriction products were visualised by UV exposure after being resolved on 1-2% agarose gels containing 0.3μg $ml^{-1}$ ethidium bromide.

## 2.4. Transfection

CHO-K1 cells were transfected using LipofectAMINE-PLUS reagents from Invitrogen (MD, USA). 10cm tissue culture dishes or 6 well plates were seeded with $2x10^6$ or $0.5x10^6$ cells respectively and incubated until 60-70% confluent (overnight). Cells were transfected by pre-complexing pcDNA (Table 2.2) with PLUS™ reagent in serum-free DMEM. The pre-complexed pcDNA was combined with LipofectAMINE™ reagent (diluted in serum-free DMEM) and incubated for 15 minutes at room temperature. Meanwhile, the cell monolayers were washed and media replaced with serum-free DMEM before gently adding the LipofectAMINE-pcDNA-PLUS complexes and incubating for 5 hours at 37°C. At the end of this period, serum-levels were increased by the addition of complete medium (10% FCS).

For transient expression, cells were washed the following morning and incubated with fresh complete media. 24 hours post-transfection, cells were washed again and incubated overnight with quiescence media. Quiescent, transiently-transfected cells were then used in experiments the following day (48 hours post-transfection).

For stable clones, the day after transfection the cells were split 1:2 and incubated overnight in fresh complete medium. 24 hours later, 800μg $ml^{-1}$ G418 was added and the media changed every three days until isolated colonies were visible. Cells were trypsinised and diluted in complete medium containing 800μg $ml^{-1}$ G418 to a density of 5 cells $ml^{-1}$ and 200μl was added to each well of a 96 well plate (2 plates per transfection). Cell growth was monitored for two weeks until single colonies were visible in the wells. Individual wells were trypsinised and 12 clones from each transfection were expanded. The clones were screened for the presence of transfected receptors by testing their ability to mobilise intracellular calcium in response to receptor ligands. Positive clones were further expanded and stocks frozen. Stable cell cultures were maintained in the presence of 400 μg $ml^{-1}$ G418.

**Table 2.2. Plasmid cDNA transfected into CHO-K1 cells**

| Transfection | cDNA in pcDNA3.1 vector | μg pcDNA transfected (stably/transiently) | Source |
|---|---|---|---|
| GRP receptor | Full-length human GRP receptor | 10μg per 10cm dish (stably) | J. Battey, Albert Einstein College of Medicine, New York USA |
| $V_{1A}$ receptor | Full-length human $V_{1A}$ receptor | 10μg per 10cm dish (stably) | M. Thibonnier, Case Western Reserve University School of Medicine, Ohio, USA |
| $V_{1A}R_{i2}$ Chimera | Rat $V_{1A}$ receptor with aa152-172 exchanged for human V2 receptor aa140-161 | 10μg per 10cm dish (transiently) | Dr Wess, National Institute of Health, Bethesda, Maryland, USA |
| $V_{1A}$Ri3 Chimera | Rat $V_{1A}$ receptor with aa237-303 exchanged for human V2 receptor aa225-277 | 10μg per 10cm dish (transiently) | Dr Wess, National Institute of Health, Bethesda, Maryland, USA |
| $G\alpha_{i-3}$ | G protein alpha $_{i-3}$ subunit | 6μg per 10cm dish 1μg per well of 6 well plate (transiently) | Guthrie cDNA Resource Centre, Guthrie Research Institute, OK, USA |
| $G\alpha_q$ | G protein alpha $_q$ subunit | 6μg per 10cm dish 1μg per well of 6 well plate (transiently) | Guthrie cDNA Resource Centre, Guthrie Research Institute, OK, USA |
| $G\alpha_{q(Q209L/D277N)}$ | Dominant negative G protein alpha $_q$ subunit with Q209L/D277N double mutations | 6μg per 10cm dish 1μg per well of 6 well plate (transiently) | Guthrie cDNA Resource Centre, Guthrie Research Institute, OK, USA |

## 2.5 Receptor binding

### 2.5.1 Whole cell binding assay

Receptor binding was carried out on confluent and quiescent cultures of vector or neuropeptide receptor transfected CHO-K1 cells grown in 24 well plates. Cell monolayers were washed twice with DMEM containing 1mg $ml^{-1}$ BSA and 225µl of a binding medium containing DMEM, 1mg $ml^{-1}$ BSA and radioligand (1.0 nM GRP containing 2nCi [$^{125}$I]-GRP for GRPR transfected cells or 0.6 nM AVP containing 45nCi [$^{125}$I]-AVP for $V_{1A}R$ transfected cells). 25µl control diluent or increasing concentrations of inhibitors (neuropeptide, Sp-D or Sp-G) were added and plates incubated for 30 minutes at 37°C. Non-specific binding was defined in the presence of 1 µM bombesin or AVP respectively. The reaction was stopped by transferring plates to ice and unbound ligand was removed by washing three times (twice for [$^{125}$I]-AVP) with ice-cold phosphate buffered saline. Plates were left to dry before adding 250µl solubilisation buffer (0.1 M NaOH, 2% $Na_2C0_3$, 1% SDS). Samples were transferred to scintillation vials, scintillation fluid added and left to stand for 1 hour or overnight. Bound ligand was estimated by liquid scintillation counting using a PACKARD scintillation counter. Specific binding in the transfected cells was 60-90% of the total binding. Nonlinear regression analysis of the binding data was used to determine the $IC_{50}$ (concentration of drug displacing 50% specific binding). The binding parameters $K_d$ (dissociation constant) and $B_{max}$ (number of binding sites) were then calculated from competition binding isotherms with unlabelled ligand according to the following equations derived by DeBlasi *et al.* (1989):

$$K_d = IC_{50} - [\text{ligand}]$$

$$B_{max} = \text{Specific Binding} \times IC_{50} / [\text{ligand}]$$

Cells grown in a separate 24 well plate were trypsinised and counted using a coulter counter to enable maximum binding ($B_{max}$) to be adjusted for cell numbers.

The inhibitory constant ($K_i$), of unlabelled analogues was calculated based on the assumption that the labelled and unlabelled ligands have equal affinities for the receptor. The $K_d$ is therefore equal to the inhibitory constant $K_i$, enabling $K_i$ to be derived from the $IC_{50}$ and $K_d$ according to the Cheng-Prusoff equation (Cheng & Prusoff, 1973):

$$K_i = IC_{50} / (1 + ([ligand] / K_d))$$

## 2.5.2 Membrane binding assay

### *2.5.2.1. Membrane preparation*

Membranes were prepared from confluent cell cultures grown in 500cm$^2$ tissue culture plates. Cell monolayers were washed twice with PBS (without calcium) containing 5mM EDTA and cells removed by scraping in 25mls PBS/EDTA solution using a plastic cell lifter. The cell suspension was transferred to a 50ml falcon tube on ice and plates washed twice with 12.5ml PBS/EDTA to harvest remaining cells. Cells were pelleted by centrifugation at 1000g for 10 minutes (0°C) followed by a further wash in 20mls PBS/EDTA solution. Pelleted cells were resuspended in 4ml Hypotonic lysis buffer (10mM Tris (pH 7.4), 5mM EDTA, 5mM EGTA and protease inhibitors) and left to swell for 15 minutes on ice. Cells were homogenised on ice by 3x5sec bursts of a Polytron homogeniser followed by centrifugation at 1700rpm for 10 minutes (4°C) to pellet cell debris and unhomogenised cells. The supernatant (SN1, containing membranes) was retained and the pellet resuspended in 4ml hypotonic lysis buffer was subject to further 3x5sec bursts of homogenisation. Cell debris was pelleted again and the resulting supernatant (SN2) combined with SN1. Membranes were collected from the supernatants by centrifugation at 49000g for 20 minutes at 4°C (Beckman JA20 rotor). The membrane pellet was resuspended in 2ml of 50mM Tris-HCl (pH 7.4) by homogenisation, adjusted to 1mg ml$^{-1}$ following protein assay and aliquots stored at -70°C.

### *2.5.2.2. Binding Assay*

Receptor binding assays were carried out using a 96 well glass fibre filter plate (MultiScreen-FB plates from Millipore) coupled to a vacuum manifold. Filters were pre-wet by adding 100µl wash buffer (50mM Tris (pH 7.4), 3mM $MgCl_2$) to each well and applying vacuum to draw buffer through. Homogenised membranes (100µg) were added to wells containing assay buffer (50mM Tris (pH 7.4), 0.5% BSA, 3mM $MgCl_2$), 1nM [$^3$H]-vasopressin and increasing concentrations of unlabelled vasopressin in a total volume of 200µl. Non-specific binding was determined by the addition of excess unlabelled vasopressin. Plates were incubated at 37°C for 30 minutes and then transferred to the vacuum manifold to filter through binding media. Wells were washed six times with 200µl cold wash buffer. Filters were removed from wells using forceps, transferred to scintillation vials and scintillation fluid added. Vials were vortexed and bound ligand estimated by liquid scintillation counting in a PACKARD scintillation counter. Nonlinear regression analysis of the binding data was used to determine the binding parameters as previously described (section 2.4.1).

## 2.6. Intracellular $[Ca^{2+}]_i$ flux assay

CHO-K1 cells expressing the GRP or $V_{1A}R$ receptor were grown to confluence on 10cm plates and quiesced overnight. Cells were washed with PBS and detached from plates using trypsin, which was then neutralised by the addition of 1 mg $ml^{-1}$ soybean trypsin inhibitor (STI). The cell suspension was washed and $5x10^6$ cells loaded with 2µM of the calcium indicator Fura-2-tetraacetoxymethylester AME (from 1mM FURA-2/AM stock in DMSO) in calcium-free Hank's balanced salt solution (HBSS) in the dark for 30 minutes at 37°C. The cells were pelleted by centrifugation at 300g for four minutes and resuspended in 2ml of HBSS containing 1.26mM $CaCl_2$. After a short recovery period on ice, the FURA-2 loaded cells were transferred to a plastic fluorimeter cuvette (Sigma, C-0793) containing a Teflon-

coated magnetic bar for continuous stirring and placed in the thermostat-regulated sample chamber of a fluorescence spectrophotometer (PerkinElmer LS50B). Fluorescence was monitored in real-time at 37°C following alternate dual wavelength excitation at 340nm and 380nm and FURA-2 fluorescence emission measurement at 510 nm (measured in arbitrary fluorescence units). Test agents were added at 100x[final] followed by the addition of 1% FCS (v/v) as a positive control response. Before terminating each measurement, cells were lysed with 1% (v/v) Triton X-100 in order to saturate all of the calcium indicator (FURA-2 in completely bound state giving maximum fluorescence intensity measurement) and then 20mM EGTA added to chelate all calcium (FURA-2 in unbound form giving minimum fluorescence intensity measurement).

The intracellular $[Ca^{2+}]_i$ was calculated by ratiometric analysis of bound and unbound FURA-2 using 'FLWinLab' software which relates the calcium concentration to the measured fluorescence intensity according to the equation $[Ca^{2+}]_i = \beta \times K\ (R-R_{min})/(R_{max}-R)$: where R is the ratio of the induced sample, $R_{max}$ is the ratio after the addition of 1% triton X-100 and $R_{min}$ is the ratio after $[Ca^{2+}]_i$ chelation with 10 mM EGTA and $\beta$ is the ratio of the denominator (380nm) fluorescence intensities. K is the dissociation constant of Fura-2 for $[Ca^{2+}]_i$, which was 224 nM as determined previously by Grynkiewicz *et al.* (1985). Data points from the intracellular calcium measurements represent the total peak area of the $[Ca^{2+}]_i$ transients elicited.

Antagonist potency (the concentration of antagonist which produces a 2-fold shift in the agonist response curve) was determined from a Schild plot of the (DR-1)log for a series of antagonist concentrations against the [antagonist]log, where the dose ratio (DR)

$$= \frac{IC_{50} \text{ in the presence of antagonist}}{EC_{50} \text{ of control response}}$$

The antagonist potency was obtained from the x-intercept of the line.

## 2.7. ERK activation

### 2.7.1. ERK stimulation and preparation of cell lysates

Quiescent cell cultures in 6 well plates were washed twice with warm HBSS and pre-incubated in a 37°C waterbath with HBSS. Test agents were added (as described in figure legends) at 100x[final] into a final volume of 1ml. At the end of the incubation period, the 6 well-plate was placed on ice and the media aspirated. 0.25 ml ice-cold lysis buffer (20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 2.5mM sodium pyrophosphate, 1% triton X-100, 1mM β-glycerphosphate, 0.5mM dithiothreitol, 1mM sodium orthovanadate, protease inhibitors (Roche, Germany; from 25X stock solution prepared according to manufacturers instructions) and 1mM PMSF was added to each well and cells removed from the plate by scraping with a plastic cell scraper. Cell suspensions were transferred to chilled eppendorfs and then vortexed for 15 minutes at 4°C to lyse cells. Lysates were clarified by centrifugation at 13000rpm for 10 minutes and the protein concentration of retained supernatants determined using Pierce BCA protein assay reagent (Pierce).

Pertussis toxin was activated by adding an equal volume of activation buffer (1% CHAPS, 10mM DTT, 1mM ATP) for 15 minutes at 37°C (Kaslow & Burns, 1992). Cells were pre-treated with Pertussis toxin by overnight incubation with 100ng $ml^{-1}$ toxin in quiescence media. All other inhibitors (e.g. FTS, PP2) were added shortly before ERK stimulation for pre-incubation periods as indicated.

### 2.7.2. Determination of protein concentration in cell lysates

A series of BSA protein standards [0.1-0.5 mg $ml^{-1}$] was prepared in lysis buffer to generate a standard curve from which the protein concentrations of test samples could be derived. Test samples were diluted 1:5 then 200µl of BCA reagent (prepared from 1:50 dilution of reagent B with reagent A) was added to 10µl of standard or test sample in a 96 well plate and incubated at 37°C for 30 minutes.

Absorbance was read at 560nm using a 96 well-plate reader (MRXII, Dynex Technologies). Samples for SDS-PAGE analysis were equilibrated for protein and denatured by heating (5 minutes at 95°C) in 4X SDS-PAGE loading buffer (50mM Tris, 10% glycerol, 2% SDS, 0.1% bromophenol blue and 10% β-mercaptoethanol (pH 7.4)).

### 2.7.3. SDS-PAGE and Western Blotting

Molecular weight markers (Benchmark™ pre-stained protein ladder, Invitrogen) and 15μg lysate/lane were resolved on a 12% SDS-PAGE gel as described by Laemmli (1970). Gels comprised of a stacking gel (0.125M Tris-HCl (pH6.8), 0.1% SDS and 12% acrylamide/bisacrylamide polymerised with ammonium persulphate (APS) solution (50μl of a 25% solution per 10ml gel) and TEMED (10μl per 10ml gel)) and a separating gel (0.375M Tris-HCl (pH 8.8), 0.1% SDS solution and 4.5% acrylamide/bisacrylamide, 25% APS (50μl per 10ml gel) and TEMED (10μl per 10ml gel)). Gels were run at 170V for 80 minutes then transferred to nitrocellulose membrane at 110V for 60 minutes in transfer buffer (210mM Glycine, 24.7mM Tris base and 20% methanol). Nitrocellulose membranes were stained with Ponceau S solution (Sigma) to confirm transfer then blocked for 1 hour at room temperature with 3% BSA in TBS containing 0.05% Tween-20 (TBST). Bands were detected by incubation of blots with primary antibody (Table 2.3) diluted in 3% BSA-TBST for 1 hour at room temperature (or overnight at 4°C). Blots were washed three times with TBST then incubated with a HRP-labelled secondary antibody (Table 2.3) for 1 hour at room temperature (or overnight at 4°C). After washing blots with TBST again, bands were visualized and quantified using enhanced chemifluorescence (ECL Plus™, Amersham Biosciences) and a phosphor imager (Storm™, Molecular Dynamics™, Amersham Biosciences) equipped with ImageQuant software.

Absorbance was read at 560nm using a 96 well-plate reader (MRXII, Dynex Technologies). Samples for SDS-PAGE analysis were equilibrated for protein and denatured by heating (5 min at 95°C) in 4X SDS-PAGE loading buffer (50mM Tris, 10% glycerol, 2% SDS, 0.1% bromophenol blue and 10% β-mercaptoethanol (pH 7.4)).

### 2.7.3. SDS-PAGE and Western Blotting

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**Table 2.3. Antibodies used for western blotting**

| | Primary Antibody | Secondary Antibody |
|---|---|---|
| Phosphorylated ERK 1/2 | Mouse monoclonal antibody against diphosphorylated ERK 1 and 2 (SIGMA)<br><br>[1:1000 dilution] | Anti-mouse IgG HRP conjugate (DAKO)<br><br>[1:2000 dilution] |
| ERK 1/2 | Rabbit polyclonal antibody against ERK 1 (Santa Cruz)<br>Rabbit polyclonal antibody against ERK 2 (Santa Cruz)<br><br>[1:2000 dilution of each] | Anti-rabbit IgG HRP conjugate (DAKO)<br><br>[1:2000 dilution] |
| $G_i$ | Rabbit polyclonal antibody against $G_i$<br><br>[1:1000 dilution] | Anti-rabbit IgG HRP conjugate (DAKO)<br><br>[1:2000 dilution] |
| $G_q$ | Rabbit polyclonal antibody against $G_{q/11}$<br><br>[1:1000 dilution] | Anti-rabbit IgG HRP conjugate (DAKO)<br><br>[1:2000 dilution] |

## 2.8. Growth Assays

### 2.8.1. Liquid growth

Exponentially growing cells were trypsinised and 10cm plates seeded with $5x10^4$ cells in DMEM containing 5% or 1% FCS in the presence or absence of mediators in triplicate. Cells were grown for 1-9 days and cell number determined at various time-points using a Coulter Counter (model Z1, Coulter Electronics).

### 2.8.2. Clonogenic assay

Molten 5% agarose (2.5% (w/v) Seakem® GTG® agarose and 2.5% (w/v) Low melting point agarose) was diluted ten-fold with DMEM containing 1% or 5% FCS to provide a solid base of 0.5% agarose in Ultra Low Attachment 6 well tissue culture plates (Corning® Life Sciences, USA). Viable vector or neuropeptide receptor

transfected CHO-K1 cells suspended in DMEM containing 1% or 5% FCS in the presence or absence of mediators were used to dilute 3% agarose (1.5% (w/v) Seakem® GTG® agarose and 1.5% (w/v) low melting point agarose) with a final cell density of $1x10^4$ cells $ml^{-1}$. 2ml of the 0.3% agarose containing cells was layered over the 0.5% solid base and allowed to set. Plates were wrapped in foil to prevent drying out and incubated at 37°C for 1-10 days. Viable colonies were stained by incubating plates with 4mg $ml^{-1}$ MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) for four hours. Colonies from 10 randomly-selected fields were counted using a microscope with a X4 objective. Cloning efficiency was calculated as the % of original number of seeded cells forming colonies of > 6 cells.

### 2.8.3. Suspension growth

Wild-type and transfected CHO-K1 cells suspended in DMEM containing 1% or 5% FCS in the presence or absence of mediators were seeded at a density of $2x10^4$ cells per well into Ultra low attachment tissue culture plates over a solid base of 0.5% agar. Under these conditions the cells did not adhere. Cells were maintained in culture for up to 9 days and viable cell number determined at various time-points as follows. Cell suspensions were washed twice with PBS and briefly trypsinised to disaggregate cell clusters. Cells were washed and resuspended in 500µl PBS and kept on ice. Cells were counted by FACS after the addition of 10µM TO-PRO3 (Molecular Probes, USA) to distinguish live cells from dead cells.

### 2.8.4. Chemosensitivity assay

Exponentially growing cells were trypsinised and 96 well-plates seeded with $1x10^4$ cells in DMEM containing 10% FCS. After incubation overnight at 37°C, cells were washed and incubated with serum-free media containing increasing concentrations of etoposide with or without 50nM neuropeptide for 48 hours. Cell viability was then determined by incubation with 4mg $ml^{-1}$ MTT at 37°C. After four hours, all media was aspirated and the blue formazan crystals solubilised with 200µl DMSO.

Absorbance was read at 560nm using a 96 well-plate reader (MRXII, Dynex Technologies). Results are expressed as % viability in the absence of neuropeptide.

### 2.8.5. SCLC growth studies

H345 SCLC cells were cultured in SITA medium for 2-3 days then disaggregated into a single cell suspension by two passes through a 19 gauge needle followed by one pass through a 23 gauge needle. Cells were re-suspended in SITA medium at a density of $1x10^5$ cells $ml^{-1}$. Cells were cultured in 24 well plates in the presence of increasing concentrations of test agents in duplicate and total cell number determined after 0, 3, 5, 7, 10, 12 and 14 days. At each time-point, cell clusters were disaggregated as before and the total cell number determined using a Coulter counter.

## 2.9. Adhesion assay

Wells of a 96 well plate were coated with 10μg $ml^{-1}$ human plasma fibronectin in PBS for 1 hour at room temperature (or overnight at 4°C). After coating, the fibronectin solution was aspirated and wells washed twice with PBS. Plates were blocked with 1mg $ml^{-1}$ BSA for 1 hour at room temperature followed by washing twice with PBS. Vector and neuropeptide receptor-transfected CHO-K1 cells (quiesced in DMEM containing 0.1% BSA) were detached from sub-confluent flasks using trypsin and 1mg $ml^{-1}$ soybean trypsin inhibitor (STI) added to the cell suspension. Cells were washed twice then resuspended at a density of $2x10^5$ cells $ml^{-1}$ in DMEM containing 0.1% (w/v) BSA and the appropriate test agent. In the cold room, media only (negative control), $2x10^4$ cells in DMEM-BSA (positive control) or $2x10^4$ cells per treatment were added to wells. Plates were incubated at 37°C for 20 minutes. Each plate was vortexed (3x10s at 800rpm) using a thermomixer and media aspirated from all wells except positive control wells. Wells were washed with PBS, vortexed and washed again before adding 100% methanol to fix cells (2 minutes at room temperature). Cells in the positive control wells were allowed to

adhere fully by incubating the plate for a further hour. The media was then removed, cells washed with PBS and fixed with methanol. Cells were stained at room temperature by adding Diff-Quik® Solution I for 2 minutes. The stain was removed using a multichannel pipette and the plate inverted on a pad of absorbent paper to remove any remaining solution. Diff-Quik® Solution II was then added for 2 minutes and removed as before. Wells were washed by repeatedly flooding the plate in a container of tap water. Plates were allowed to dry before the stain was eluted with 0.5M HCl and absorbance read at 630nm using a 96 well plate reader (MRXII, Dynex Technologies). % Adhesion was calculated relative to the positive control where all cells adhered. Experiments were performed in quadruplicate and repeated five times.

## 2.10. Migration assay

Transwell migration was evaluated using 96 well MultiScreen™-MIC plates (Millipore, USA) composed of a 96 well filter plate (8μm pore size) and a 96 tear-drop well plastic receiver plate housed in a single well tray with lid. Filters were coated overnight (4°C) by placing the filter plate in the plastic receiver plate containing 10μg $ml^{-1}$ fibronectin. The filter plate was washed twice with PBS and allowed to dry. Meanwhile, vector and neuropeptide receptor-transfected CHO-K1 cells (quiesced in DMEM containing 0.1% BSA) were detached from sub-confluent flasks using trypsin and 1mg $ml^{-1}$ STI added to the cell suspension. Cells were washed twice then resuspended at a density of $1x10^6$ cells $ml^{-1}$ in DMEM containing 0.1% (w/v) BSA. DMEM containing 10% FCS (positive control response) or chemotactic agents (neuropeptides or Substance P analogues) diluted in DMEM-BSA were added to the receiver plate (150μl) and the filter plate carefully placed on top. The cell suspension ($7.5x10^4$ cells) was pipetted slowly into the centre of the filter plate wells and the plate incubated for 4 hours at 37°C in a humidified atmosphere. In IGF-stimulated migration experiments, cells were resuspended in DMEM-BSA containing neuropeptide or analogue and 100ng $ml^{-1}$ IGF-1

(Calbiochem) was added to the receiver plate. At the end of the assay, the filter plate was removed from the receiver plate and media aspirated from negative control (no cell input) and positive control (total cell input) wells. Non-migrated cells from all other wells were removed by wiping inside the wells with a cotton bud. Cells remaining on the underside of the filter were then fixed and stained as for the adhesion assay to quantify the number of cells that had migrated. Stain was eluted by placing the filter plate in a receiver plate containing 150µl 0.5M HCl per well. Eluted stain (100µl) was transferred to a 96 well plate and absorbance read colorimetrically at 630nm. The control used to determine unstimulated random migration was DMEM containing 0.1% BSA in lower wells. Experiments were performed in quadruplicate and repeated three times or more.

Data is expressed as a Migration Index:

$$\frac{\text{OD of cells that migrated in response to agonist}}{\text{OD of cells that migrated randomly (DMEM-BSA only)}}$$

## 2.11. Phospholipase C activation

Cells were grown in 6 well-plates until near-confluent and then labelled with *myo*-1,2-[$^3$H]-inositol at a concentration of 1 µCi ml$^{-1}$ overnight in serum- and inositol-free medium. Labelled cells were washed twice with PBS and incubated in HBSS containing 20 mM HEPES, 1.8% glucose, 0.2% BSA and 20 mM LiCl, for 30 minutes at 37°C. Cells were then treated with agonists for 30 minutes at 37°C. Reactions were terminated by removal of assay buffer and addition of ice cold 10 mM formic acid for 60 minutes on ice. Samples were applied to Dowex columns (200-400 mesh, formate form, 0.5g per column) and washed through with 10ml 60mM ammonium formate: 5mM sodium tetraborate. Inositol phosphates were eluted with 5ml 1.2M ammonium formate: 0.1M formic acid. Radioactively labelled IP fractions were

measured by liquid scintillation counting. Columns were regenerated with 2M ammonium formate and washed with distilled $H_20$.

## 2.12. Statistical analysis

Combined data was analysed by one way ANOVA (with Dunnetts post t-test to compare all columns vs. control column where overall $P<0.05$) using Prism® (GraphPad Software Inc, USA).

# Chapter 3

# Effects of Substance-P Analogues on Neuropeptide Signalling

Since SCLC growth can be driven by multiple neuropeptides, there is a need for broad spectrum neuropeptide antagonists to treat this and other neuropeptide-dependent cancers. Substance-P analogues have been previously characterised as such due to their ability to block binding of neuropeptides and calcium mobilisation induced by multiple neuropeptides (Woll & Rozengurt, 1990; Bunn *et al*, 1994; Sethi *et al*, 1992). Rather than just being simple antagonists which inhibit SCLC growth, substance-P analogues were also found to activate JNK and thus proposed to be biased agonists (Jarpe *et al*, 1998). This concept was first verified using a GRP-receptor expressing model cell system (Mackinnon *et al*, 2001). Binding of SP-D to the GRP receptor was shown to induce receptor coupling to selective G-proteins compared to the natural ligand GRP. However it is not known whether the biased agonist properties of the analogues can be similarly regarded as broad spectrum or specific only to its effects on the GRP receptor. The work presented in this chapter was thus initiated to determine whether Substance-P analogues exhibit biased agonist activity at receptors other than GRP receptors.

While 60% of SCLC cells express the GRP receptor, $V_{1A}$ receptor expression has been detected in all SCLC cell-lines tested to date (Ocejo-Garcia *et al*, 2001; Waters *et al*, 2003). It was therefore decided to investigate the effects of the substance-P analogues on the $V_{1A}$ receptor and the GRP receptor. Model cell systems consisting of Chinese Hamster Ovary (CHO-K1) cells (a non-cancerous, non-neuroendocrine epithelial cell-line) stably expressing GRP or $V_{1A}$ receptors were established for this purpose. In order to better understand the substance-P analogues' mechanism of action, the effects of SP-D and SP-G were then studied at distinct steps of the $V_{1A}R$

and GRPR signalling process: neuropeptide growth factor binding, calcium mobilisation and ERK1/2 activation.

## 3.1 Production of model cell systems

### 3.1.1 Stable transfection of CHO-K1 cells with GRPR or $V_{1A}R$

CHO-K1 cells were transfected with cDNA encoding the $V_{1A}$ receptor as described in materials and methods. These cells were cultured in the presence of 800μg $ml^{-1}$ G418 for one week, after which time the cells were trypsinised and transferred into 2 96 well plates at a density of one cell per well. After a further two week period of growth in the presence of 800μg $ml^{-1}$ G418, the resulting clones were trypsinised and transferred to individual wells of a 6 well plate. Once confluent, each sub-clone was then split equally between a $75cm^2$ tissue culture flask and a 100mm tissue culture dish. The ability of neuropeptide agonist to stimulate intracellular calcium mobilisation was then measured using the $Ca^{2+}$-sensitive dye Fura-2AM, as described in materials and methods, in order to confirm the presence of functional receptors. Confluent, quiescent cultures grown in 100mm tissue culture dishes were screened for the ability of 1nM, 10nM and 100nM vasopressin to induce mobilisation of intracellular calcium. Out of 24 clones screened in this manner, six clones were found to respond with varying levels of calcium release. The six cell-lines were expanded and stocks frozen in liquid nitrogen. Clones V3B and V4I were selected for further characterisation and use in experiments.

The same procedure was followed in order to create a cell-line expressing the GRP receptor. 24 clones were screened for the ability of 1nM, 10nM and 100nM bombesin to induce a calcium response. Of these clones, seven responded to stimulation and these cell-lines were expanded and stocks frozen in liquid nitrogen.

Of the seven positive clones, clones G6H and G6A were selected for further characterisation and use in experiments.

At the same time, CHO-K1 cells were transfected with empty vector and a G418-resistant clone isolated for use in control experiments (CHO-vector cell-line).

## 3.1.2 Characterisation of cell-lines expressing GRPR or $V_{1A}R$

### *3.1.2.1 Intracellular calcium mobilisation*

The mobilisation of calcium from intracellular stores leading to a rapid and transient increase in $[Ca^{2+}]_i$ is one of the earliest events stimulated by neuropeptide binding to GPCRs that signal through $G\alpha_q$-mediated activation of phospholipase C (Exton, 1996; Rozengurt, 1998a). Intracellular calcium mobilisation was measured using Fura-2-AM to demonstrate that functional receptors expressed in the selected stable cell-lines could be activated by neuropeptide ligand.

In an initial series of experiments it was found that vector-transfected CHO-K1 cells responded to 10nM bombesin with a small change in intracellular calcium levels (Figure 3.1a). However, in comparison to the calcium response obtained in CHO-G6H cells, the CHO-vector response to 10nM bombesin was relatively insignificant. CHO-G6A cells did not respond to 10nM vasopressin and the response to 10nM bombesin was greater than the response generated in the parental CHO-K1 cell-line. There was no response in CHO-vector cells to 10nM vasopressin (Figure 3.1b). The calcium response to 10nM vasopressin was much greater in CHO-V3B cells than CHO-V4I cells. CHO-V3B cells did not respond to 10nM bombesin, but there was a response generated in CHO-V4I cells which was similar in magnitude to its response to 10nM vasopressin. Therefore, the CHO-V4I cell-line was not used in any further experiments.

In order to fully characterise the calcium mobilising properties of bombesin and vasopressin, the effect of increasing concentrations of neuropeptide on $[Ca^{2+}]_i$ was measured in CHO-G6H, CHO-G6A and CHO-V3B cell-lines. Bombesin transiently increased the level of intracellular calcium in CHO-G6H (Figure 3.2) and CHO-G6A (Figure 3.3) cells in a concentration dependent manner. The concentration of bombesin required to induce half-maximal effects ($EC_{50}$) in the CHO-G6H and CHO-G6A clones was 2.0 ± 0.45 nM and 8.6 ± 3.7 nM respectively (n=5). Vasopressin similarly increased the intracellular calcium in a concentration-dependent manner in CHO-V3B cells (Figure 3.4), with an $EC_{50}$ of 3.1 ± 0.6 nM (n=5).

To confirm that the increase in $[Ca^{2+}]_i$ in CHO-G6H, CHO-G6A and CHO-V3B cells in response to neuropeptides was mediated by specific Gq-coupled receptors, ligand specific antagonists to bombesin and vasopressin were used. The addition of the potent and specific bombesin peptide antagonist RC3940-II ($Hca^{6}$,$Leu^{13}\psi[CH_2N]$Tac14-Bombesin(6-11)) prior to adding agonist inhibited the bombesin-induced increase in $[Ca^{2+}]_i$ causing a rightward parallel shift in both CHO-G6H (Figure 3.5) and CHO-G6A (Figure 3.6) dose response curves. The addition of the selective vasopressin antagonist [β-mercapto-β-β-cyclopentamethylene-propionyl$^{1}$,0-Me-Tyr$^{2}$,Arg$^{8}$]-vasopressin, which binds to the $V_{1A}$ receptor, similarly inhibited the increase in $[Ca^{2+}]_i$ induced by vasopressin in CHO-V3B cells (Figure 3.7). Stimulation of intracellular calcium release by FCS was used as a positive control to ensure that a lack of response to neuropeptide was not due to any abnormality in the mechanism of calcium mobilisation. The $EC_{50}$ values derived from the curves were used to determine the antagonist potency ($pA_2$) of the analogues for calcium flux inhibition, as described in materials and methods. The bombesin antagonist potently inhibited bombesin-induced calcium mobilisation with antagonist potencies ($pA_2$) of 8.5 and 8.1 in CHO-GRP and CHO-G6A cells respectively. The vasopressin antagonist similarly inhibited vasopressin-induced

calcium mobilisation with $pA_2$ of 8.4. The shift in dose response curves is consistent with the activity of competitive antagonists.

The data confirms that the transfected receptors are capable of forming a productive ligand-receptor interaction in the stable cell-lines and that the receptors are effectively able to couple to $G\alpha_q$.

### 3.1.2.2 Receptor binding

The functional expression of stably transfected receptors was verified using [$^{125}$I]-labelled GRP or [$^{125}$I]-labelled AVP. Confluent cultures of cells were incubated with the appropriate radioligand and increasing concentrations of unlabelled neuropeptide (competitor) added. After 30 minutes incubation at 37°C, cells were washed and remaining radioactivity counted after solubilisation of the cells. The resulting competition binding curves show that increasing the concentration of unlabelled ligand causes a decrease in radioligand binding. The affinity of ligand for the receptor ($K_d$) and receptor expression ($B_{max}$) were estimated from binding curves as described in materials and methods.

In CHO-G6H cells, addition of unlabelled bombesin inhibited [$^{125}$I]-GRP binding in a concentration dependent manner (Figure 3.8) with an apparent affinity $K_d$ = 2.55 ± 0.84 nM (n=4) and $B_{max}$ of 5086 ± 489 sites/cell (n=4). Concentration dependent inhibition of [$^{125}$I]-GRP binding by bombesin to CHO-G6A cells (Figure 3.9) occurred with $K_d$ = 0.82 ± 0.26 nM (n=4) and $B_{max}$ of 846 ± 168 sites/cell (n=4). Addition of unlabelled AVP similarly inhibited [$^{125}$I]-AVP binding to CHO-V3B cells in a concentration dependent manner (Figure 3.10). In this cell-line, $V_{1A}$ receptors were expressed at a similar level as in CHO-G6A, with $B_{max}$ = 720 ± 90 sites/cell (n=4) and $K_d$ = 2.98 ± 0.71 nM (n=4). [$^{125}$I]-GRP or [$^{125}$I]-AVP binding to vector transfected

CHO-K1 cells was minimal. Although 10nM bombesin stimulated a calcium response in vector transfected CHO-K1 cells, the binding of [$^{125}$I]-GRP to these cells was not significant enough to allow quantification of receptor. This indicates that endogenous GRP receptors are expressed at very low levels in CHO-K1 cells.

Although in terms of receptor expression, CHO-G6A and CHO-V3B expressed similar levels of receptor, CHO-G6H was selected for use in further experiments. The selection was made on the basis of functional response to neuropeptide, since the magnitude of calcium response generated in the CHO-V3B cell-line was more similar to that produced in CHO-G6H than CHO-G6A cells. Therefore, the CHO-G6H and CHO-V3B cell-lines were used to evaluate neuropeptide signalling and Substance-P analogue activity from this point forward. The GRP and $V_{1A}$ receptor expressing cell-lines are hereafter referred to as CHO-GRP (CHO-G6H) and CHO-$V_{1A}$ (CHO-V3B).

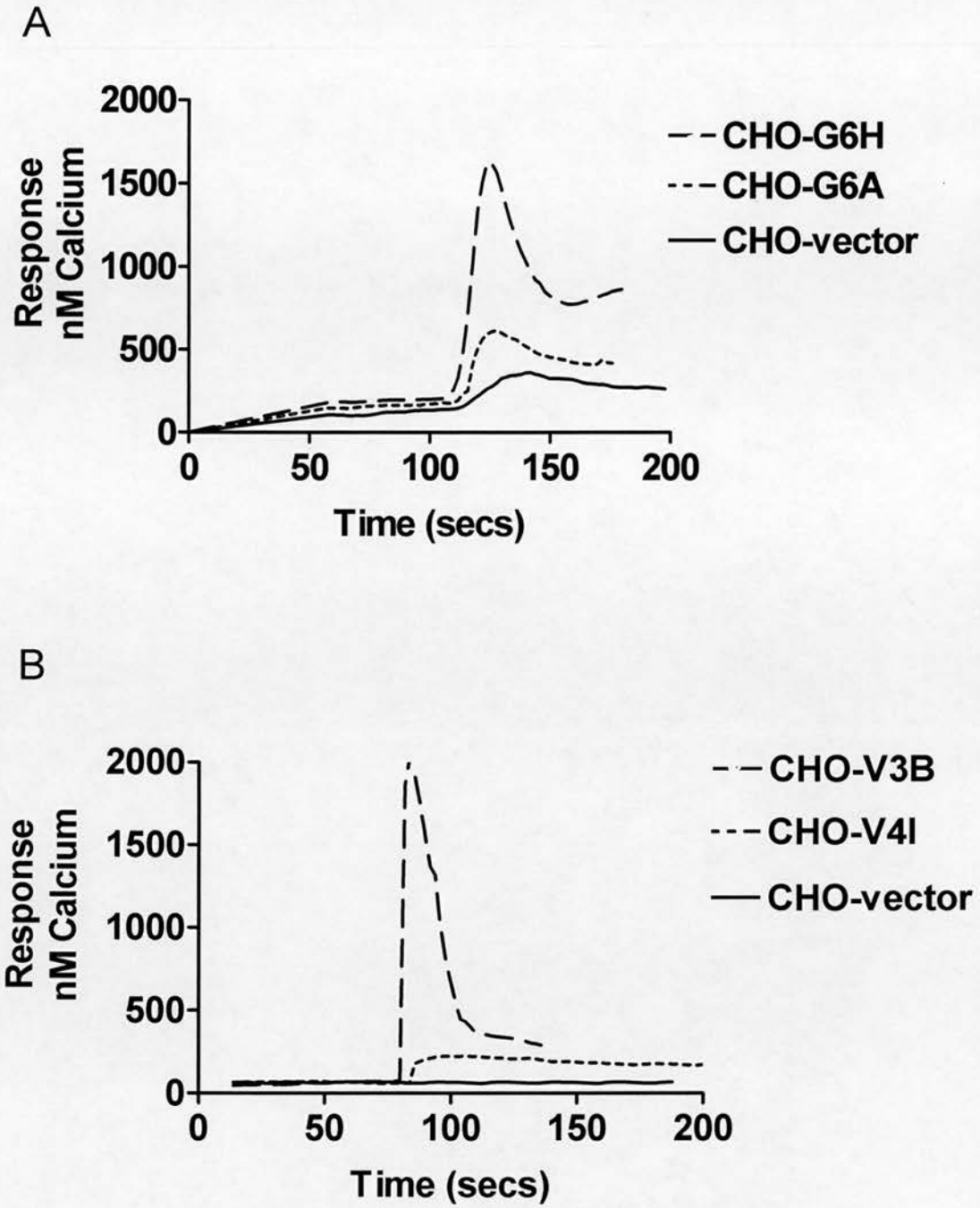


**Fig 3.1. Intracellular calcium mobilisation by neuropeptides.** Measurement of intracellular calcium mobilisation in Fura-2AM loaded cells was carried out as described in materials and methods. **A)** Representative traces of changes in intracellular calcium levels in response to 10nM Bombesin in vector and GRPR-transfected CHO-K1 cells. **B)** Representative traces of changes in intracellular calcium levels in response to 10nM vasopressin in vector and $V_{1A}R$-transfected CHO-K1 cells.

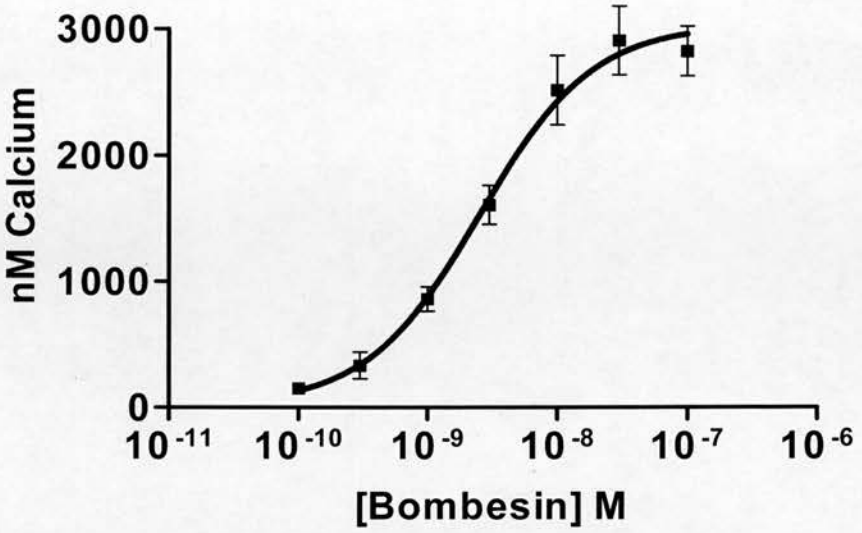


**Figure 3.2. Concentration response curves for calcium mobilisation in CHO-G6H cells.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of bombesin and intracellular calcium release measured by ratiometric fluorimetry as described in materials and methods. Data represents the mean ± s.e.m. of five experiments. $EC_{50}$ = 2.00 ± 0.4 nM

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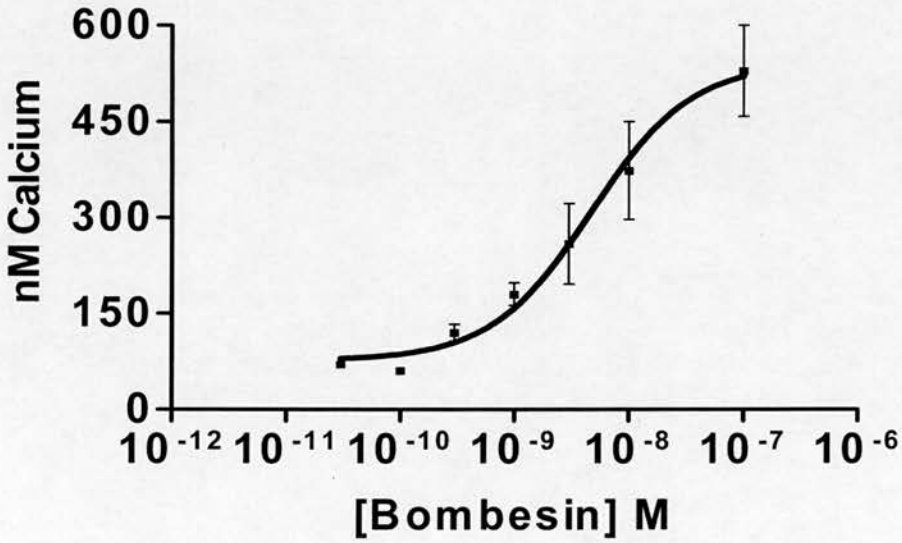



**Figure 3.3. Concentration response curves for calcium mobilisation in CHO-G6A cells.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of bombesin and intracellular calcium release measured by ratiometric fluorimetry as described in materials and methods. Data represents the mean ± s.e.m. of five experiments. $EC_{50}$ = 8.6 ± 3.7 nM

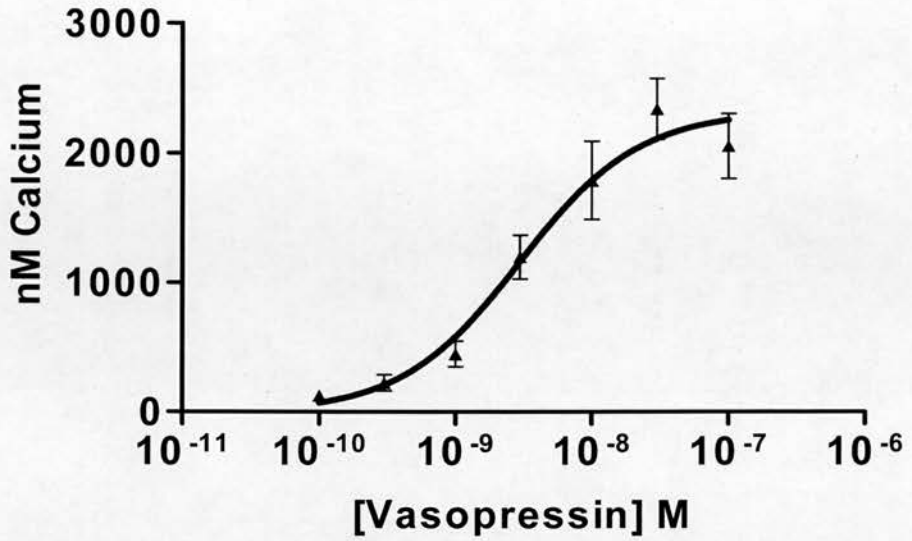



**Figure 3.4. Concentration response curves for calcium mobilisation in CHO-V3B cells.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of vasopressin and intracellular calcium release measured by ratiometric fluorimetry as described in materials and methods. Data represents the mean ± s.e.m. of five experiments. $EC_{50}$ = 3.1 ± 0.6 nM

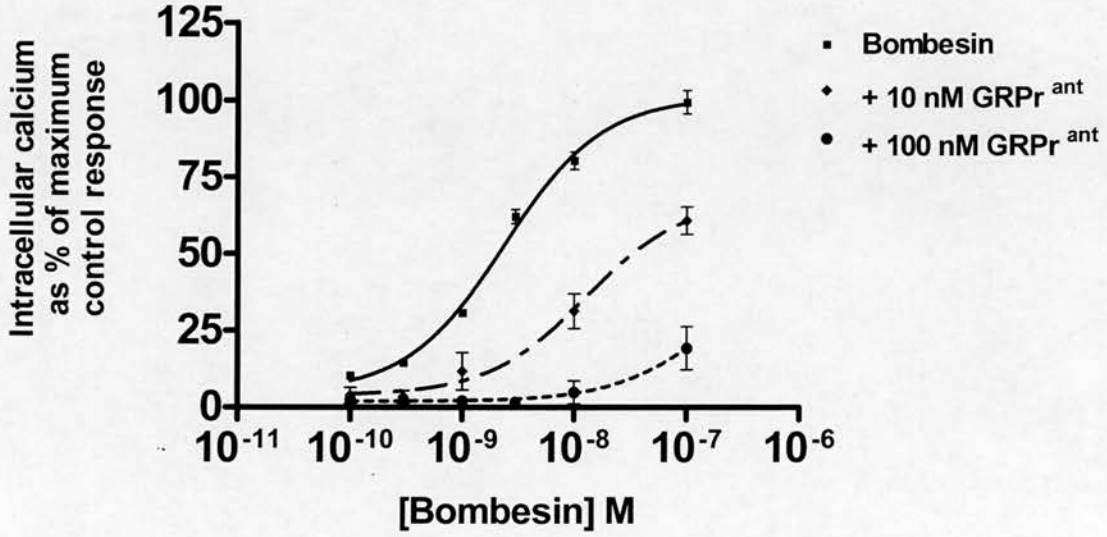



**Figure 3.5. Intracellular calcium mobilisation in CHO-G6H cells is inhibited by GRP receptor antagonist RC-3940II.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of bombesin alone (■) or in the presence of 10nM (♦) or 100nM (●) receptor antagonist. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods and data represents the mean ± s.e.m. of three experiments.

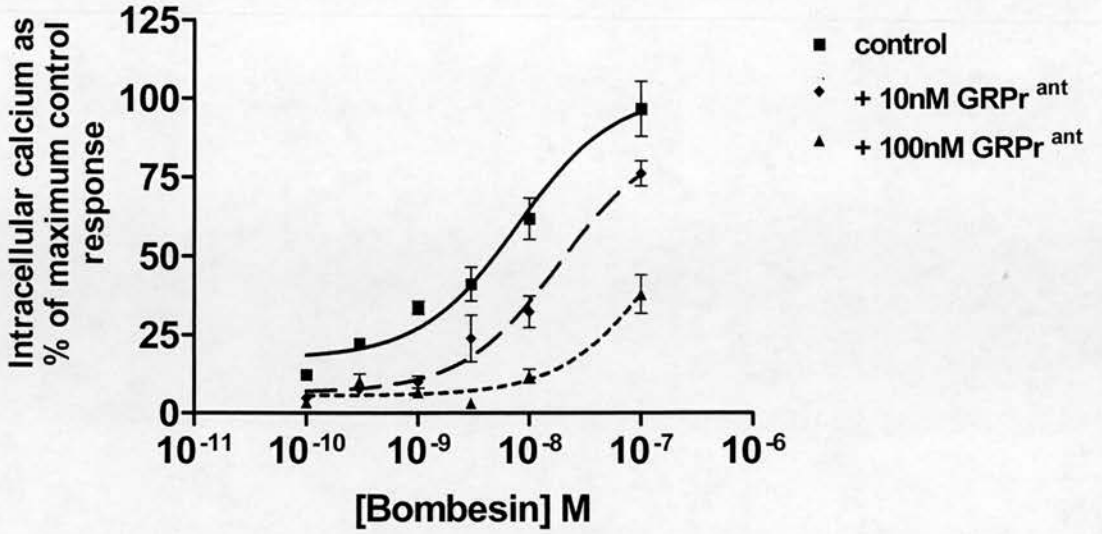



**Figure 3.6. Intracellular calcium mobilisation in CHO-G6A cells is inhibited by GRP receptor antagonist RC-3940II.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of bombesin alone (■) or in the presence of 10nM (♦) or 100nM (●) receptor antagonist. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods and data represents the mean ± s.e.m. of three experiments.

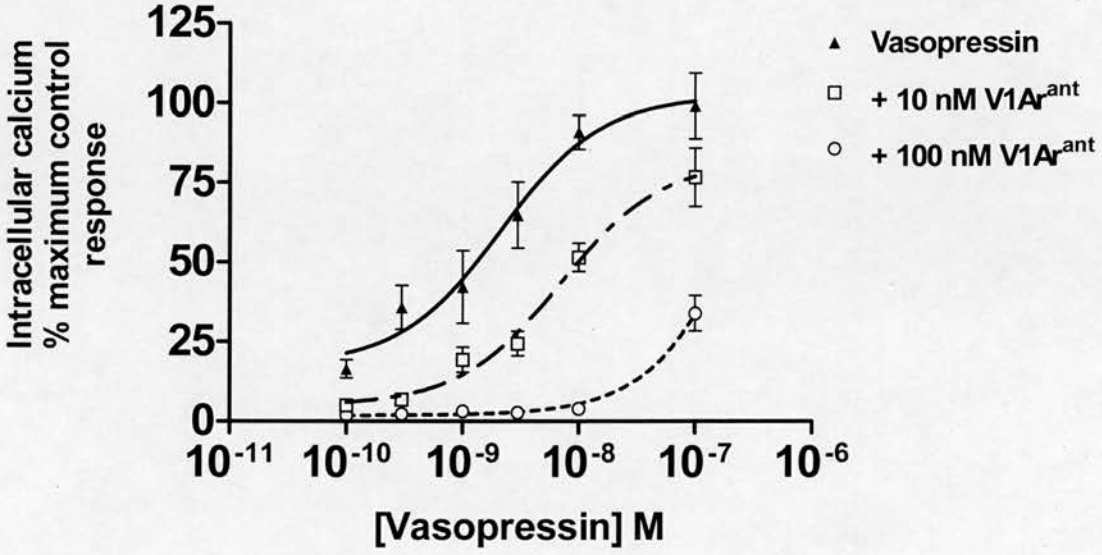



**Figure 3.7. Intracellular calcium mobilisation in CHO-V3B cells is inhibited by $V_{1A}$ receptor antagonist [β-mercapto-β-β-cyclopentamethylene-propionyl1,0-Me-Tyr2,Arg8]-Vasopressin.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of vasopressin alone (▲) or in the presence of 10nM (□) or 100nM (○) receptor antagonist. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods and data represents the mean ± s.e.m. of three experiments.

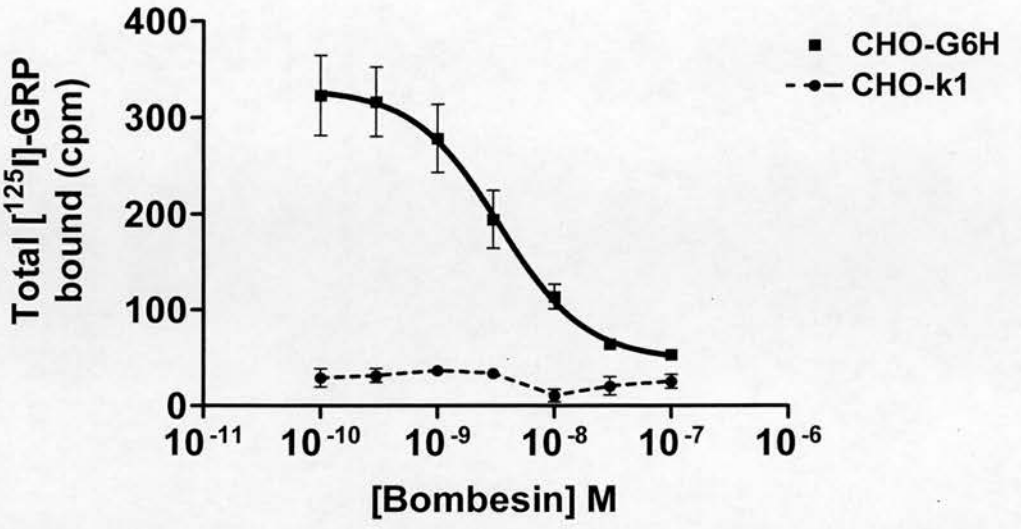



**Figure 3.8. Dose-dependent inhibition of [$^{125}$I]-GRP binding to CHO-G6H cells by bombesin.** Confluent, quiescent cultures of CHO-vector and CHO-G6H cells were incubated with [$^{125}$I]-GRP and various concentrations of unlabelled bombesin added (37°C; 30min). Results are expressed as total radioligand bound (cpm) and represent the mean ± s.e.m. of four experiments performed in triplicate.

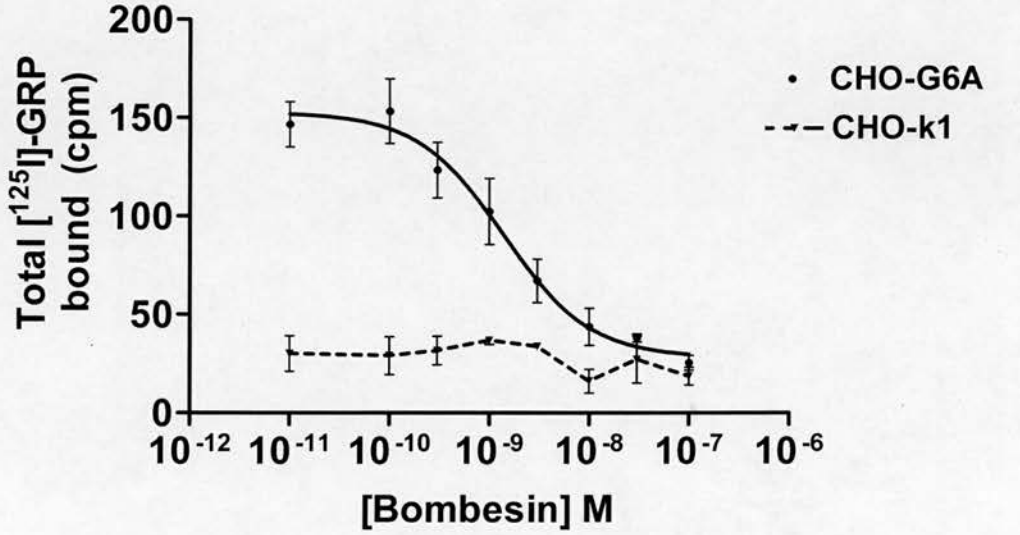



**Figure 3.9. Dose-dependent inhibition of [$^{125}$I]-GRP binding to CHO-G6A cells by bombesin.** Confluent, quiescent cultures of CHO-vector and CHO-G6A cells were incubated with [$^{125}$I]-GRP and various concentrations of unlabelled bombesin added (37°C; 30min). Results are expressed as total radioligand bound (cpm) and represent the mean ± s.e.m. of four experiments performed in triplicate.

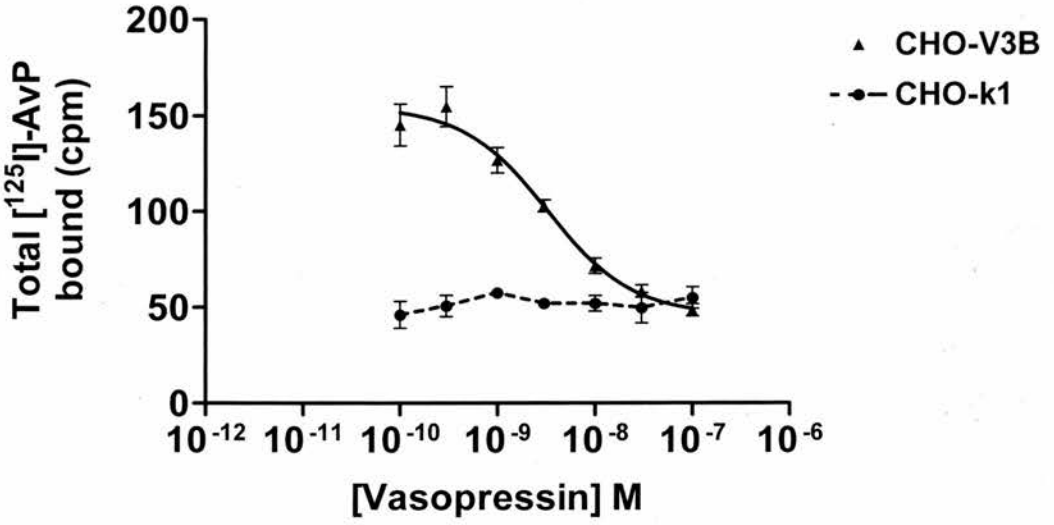



**Figure 3.10. Dose-dependent inhibition of [$^{125}$I]-AVP binding to CHO-V3B cells by vasopressin.** Confluent, quiescent cultures of CHO-vector and CHO-V3B cells were incubated with [$^{125}$I]-AVP and various concentrations of unlabelled vasopressin added (37°C; 30min). Results are expressed as total radioligand bound (cpm) and represent the mean ± s.e.m. of four experiments performed in triplicate.

## 3.2 Effect of Substance-P analogues on calcium response

SP-D and SP-G alone did not stimulate a calcium response in CHO-GRP or CHO-$V_{1A}$ cells. However, the analogues were able to inhibit neuropeptide-induced calcium release in both cell-lines. When neuropeptide-stimulated calcium mobilisation was measured in the absence and presence of 1μM and 10μM SP-D or SP-G there was a rightward parallel shift in concentration response curves in both CHO-GRP (Figure 3.11) and CHO-$V_{1A}$ cells (Figure 3.12). In CHO-GRP cells, SP-D potently inhibited the bombesin induced calcium mobilisation with antagonist potency ($pA_2$) of 7.1. As shown in Figure 3.12b, 1μM SP-G did not significantly inhibit bombesin induced calcium mobilisation although 10μM SP-G did ($pA_2$ 5.46). However, SP-G was a more potent inhibitor of the vasopressin response in CHO-$V_{1A}$ cells (Figure 3.12b). $pA_2$ values for AVP antagonism were 6.22 and 6.72 for SP-G and SP-D respectively. This shows that the Substance-P analogues are effective antagonists of the $G_q$-coupled calcium response.

Spantide is also an analogue of substance-P which only differs from SP-D by the lack of an amino acid substitution at position 5. This analogue (initially referred to as antagonist-B) had previously been shown to lack inhibitory effects on the growth of H345 SCLC cells (Woll & Rozengurt, 1990) so it was anticipated that it would not have any effects on neuropeptide induced calcium release. For comparison, the ability of 30μM SP-D, SP-G and Spantide to inhibit the calcium response to a fixed concentration of neuropeptide was therefore assessed in both cell-lines. At these concentrations, none of the analogues alone stimulated any change in intracellular calcium levels. In CHO-GRP cells, 30μM SP-D, SP-G and Spantide were similarly able to inhibit the calcium response to 1nM bombesin (Figure 3.13). Likewise, all three analogues almost completely inhibited the calcium response to 1nM vasopressin in CHO-V1A cells (Figure 3.14). Therefore, at 30μM, all three analogues are similarly able to inhibit the calcium response induced by 1nM bombesin or

vasopressin. This shows that Spantide is also an effective antagonist of neuropeptide-stimulated calcium release despite its apparent inability to block SCLC cell growth.

## 3.3 Effect of Substance-P analogues on receptor binding

Competition binding experiments were performed using increasing concentrations of SP-D or SP-G to compete with radioligand binding to receptors. Increasing concentrations of analogue caused a decrease in specific binding of radioligand to each cell-line. The resulting competition binding curves were used to determine the affinity ($K_i$) with which the analogue inhibits radioligand binding to each receptor, as described in materials and methods. The substance-P analogues SP-G and SP-D inhibited [$^{125}$I]-GRP binding to CHO-GRP cells (Figure 3.15) with affinities in the micromolar range (SP-G $K_i$ = 19.4 ± 6.3 μM (n=4), SP-D $K_i$ = 0.64 ± 0.05 μM (n=4)). In CHO-$V_{1A}$ cells, both analogues also inhibited $V_{1A}$ receptor binding (Figure 3.16 (SP-G $K_i$ = 3.50 ± 0.82 μM (n=4), SP-D $K_i$ = 8.58 ± 1.47 μM (n=4)). This data shows that SP-G is relatively (6 fold) more selective for the $V_{1A}$ receptor whereas SP-D is GRP receptor selective (13 fold).

In another series of experiments, the effect of a fixed concentration of analogue on radioligand binding was assessed. Cells were incubated with 1μM or 5μM analogue and the appropriate radioligand followed by the addition of increasing concentrations of unlabelled neuropeptide. 1μM and 5μM SP-D caused a significant reduction and complete inhibition of [$^{125}$I]-GRP binding to CHO-GRP cells respectively (Figure 3.17a). In comparison, 1μM and 5μM SP-G had little effect on the ability of unlabelled bombesin to inhibit [$^{125}$I]-GRP binding to receptors (Figure 3.17b). In CHO-$V_{1A}$ cells, 1μM SP-D and SP-G both slightly inhibited [$^{125}$I]-AVP binding whereas binding was reduced to a greater extent similarly by both 5μM SP-D and SP-G (Figure 3.18). Co-incubation with SP-D or SP-G did not significantly

alter the $IC_{50}$ of unlabelled GRP or AVP competing with radioligand for receptor binding.

A direct comparison of Maximum Specific Binding in the presence and absence of analogue in CHO-GRP cells revealed that the reduction in binding in the presence of both 1μM and 5μM SP-D was significant ($P<0.01$, ANOVA) whereas in the presence of SP-G it was not ($P>0.05$, ANOVA). A similar comparison of CHO-$V_{1A}$ data shows that whilst 1μM SP-D and SP-G did not cause a significant change in Maximum Specific Binding ($P>0.05$, ANOVA), the reduction in binding by both 5μM SP-D and SP-G was significant ($P<0.01$, ANOVA). The effective reduction in maximum binding suggests that the analogues act non-competitively to inhibit radioligand binding.

A similar experiment was performed on CHO-GRP cells using 5μM and 25μM Spantide. The presence of 5 μM Spantide caused a significant reduction in [$^{125}$I]-GRP binding to the bombesin receptors while 25 μM Spantide completely abolished specific [$^{125}$I]-GRP binding (Figure 3.19). This shows that Spantide also inhibits bombesin binding to receptor.

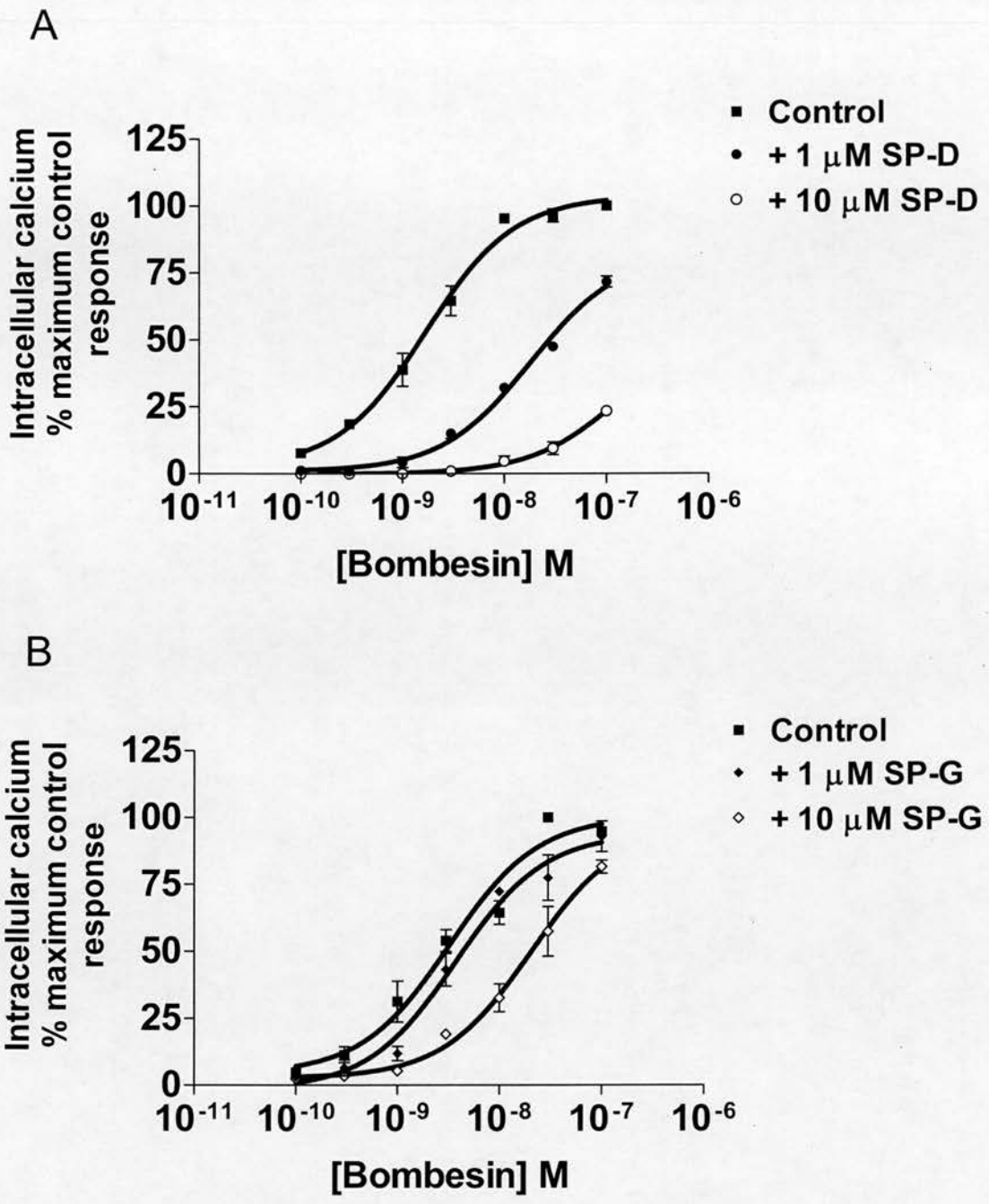



**Figure 3.11. Inhibition of intracellular calcium mobilisation in CHO-GRP by substance-P analogues.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of **A)** Bombesin alone (■) or in the presence of 1μM (•) or 10μM (o) SP-D. **B)** Bombesin alone (■) or in the presence of 1μM (♦) or 10μM (◊) SP-G. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods and data represents the mean ± s.e.m. of three experiments.

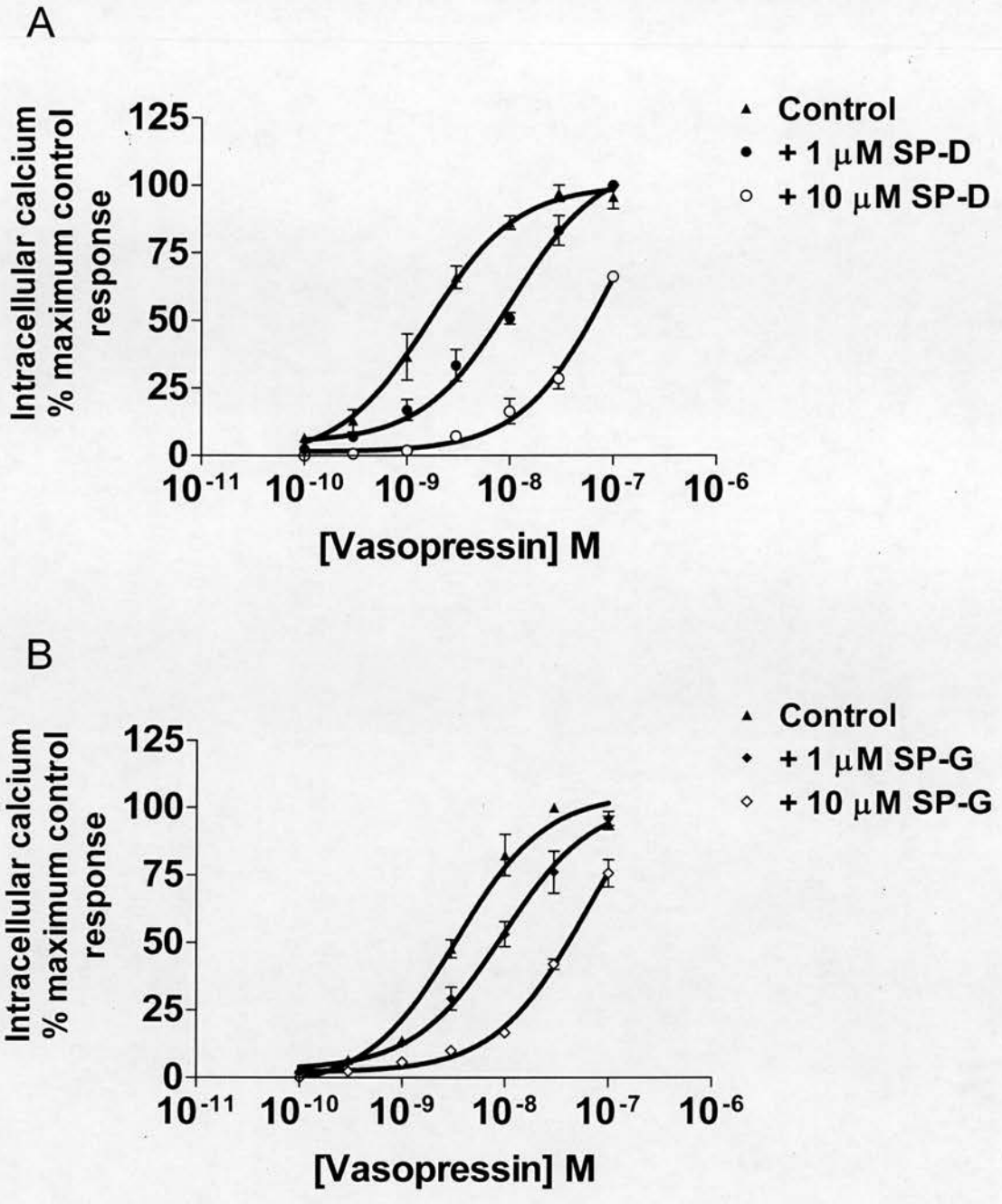



**Figure 3.12. Inhibition of intracellular calcium mobilisation in CHO-$V_{1A}$ by substance-P analogues.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of **A)** Vasopressin alone (▲) or in the presence of 1μM (•) or 10μM (o) SP-D. **B)** Vasopressin alone (▲) or in the presence of 1μM (♦) or 10μM (◊) SP-G. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods and data represents the mean ± s.e.m. of three experiments.

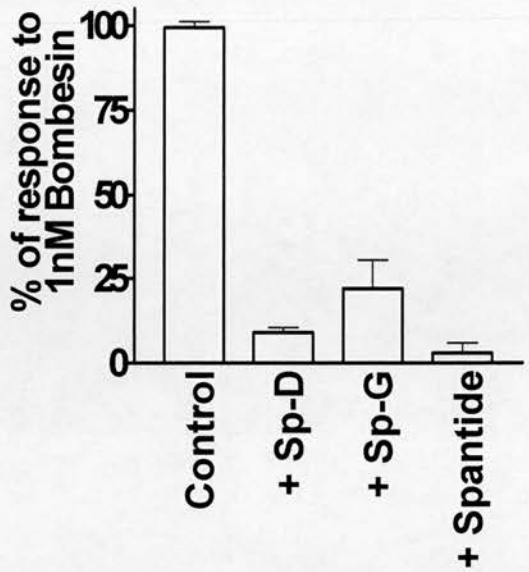



**Figure 3.13. Effect of 30μM analogue on calcium response to 1nM Bombesin in CHO-GRP cells.** Quiescent cells loaded with FURA-2AM were stimulated with 1nM bombesin alone (control) or in the presence of 30μM Spantide, SP-D or SP-G. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods. Data is expressed as % control response and represents the mean ± s.e.m. of three experiments.

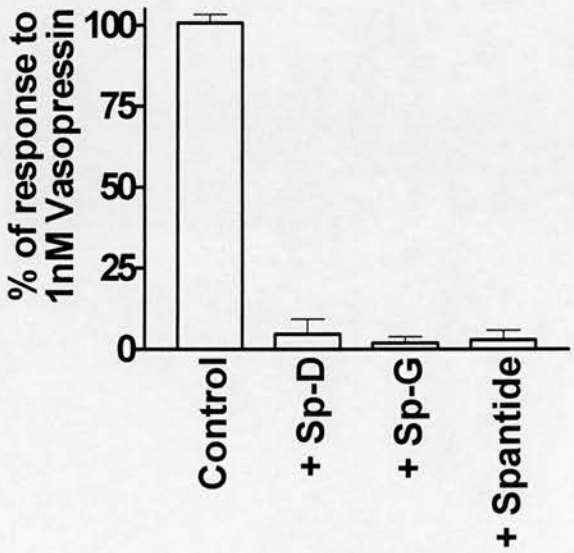



**Figure 3.14. Effect of 30μM analogue on calcium response to 1nM Vasopressin in CHO-$V_{1A}$ cells.** Quiescent cells loaded with FURA-2AM were stimulated with 1nM vasopressin alone (control) or in the presence of 30μM Spantide, SP-D or SP-G. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods. Data is expressed as % control response and represents the mean ± s.e.m. of three experiments.

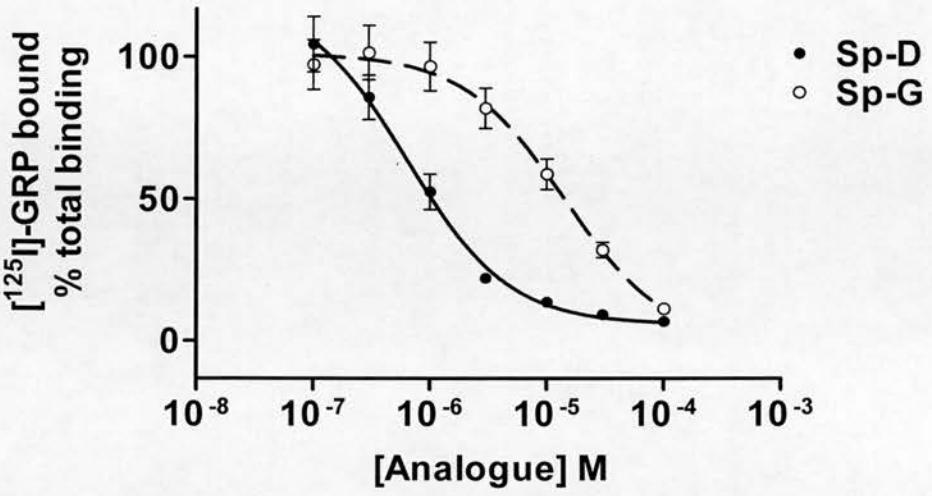



**Figure 3.15. Dose-dependent inhibition of [$^{125}$I]-GRP binding to CHO-GRP cells by substance-P analogues.** Confluent, quiescent cultures of CHO-GRP cells were incubated with [$^{125}$I]-GRP and various concentrations of unlabelled competitor added (37°C; 30min). Results are expressed as % of total radioligand binding and represent the mean ± s.e.m. of four experiments performed in triplicate.

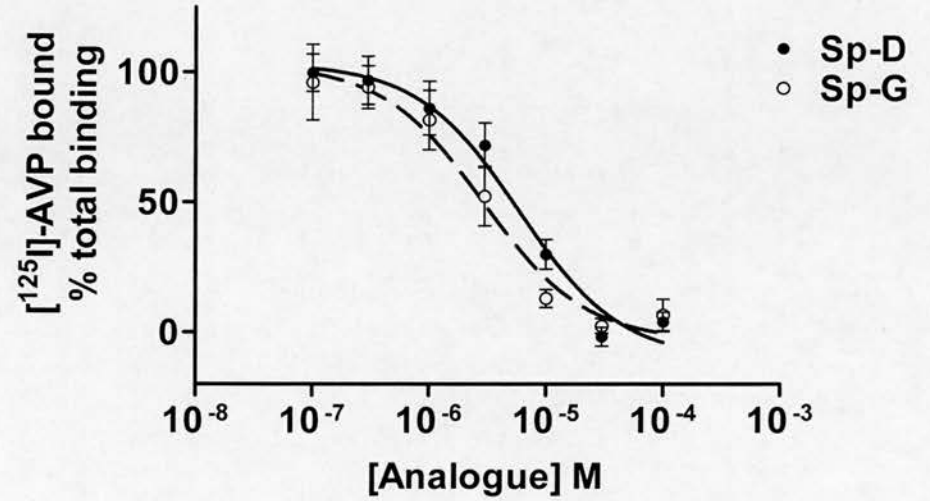



**Figure 3.16. Dose-dependent inhibition of [$^{125}$I]-AVP binding to CHO-$V_{1A}$ cells by substance-P analogues.** Confluent, quiescent cultures of CHO-$V_{1A}$ cells were incubated with [$^{125}$I]-AVP and various concentrations of unlabelled competitor added (37°C; 30min). Results are expressed as % of total radioligand binding and represent the mean ± s.e.m. of four experiments performed in triplicate.

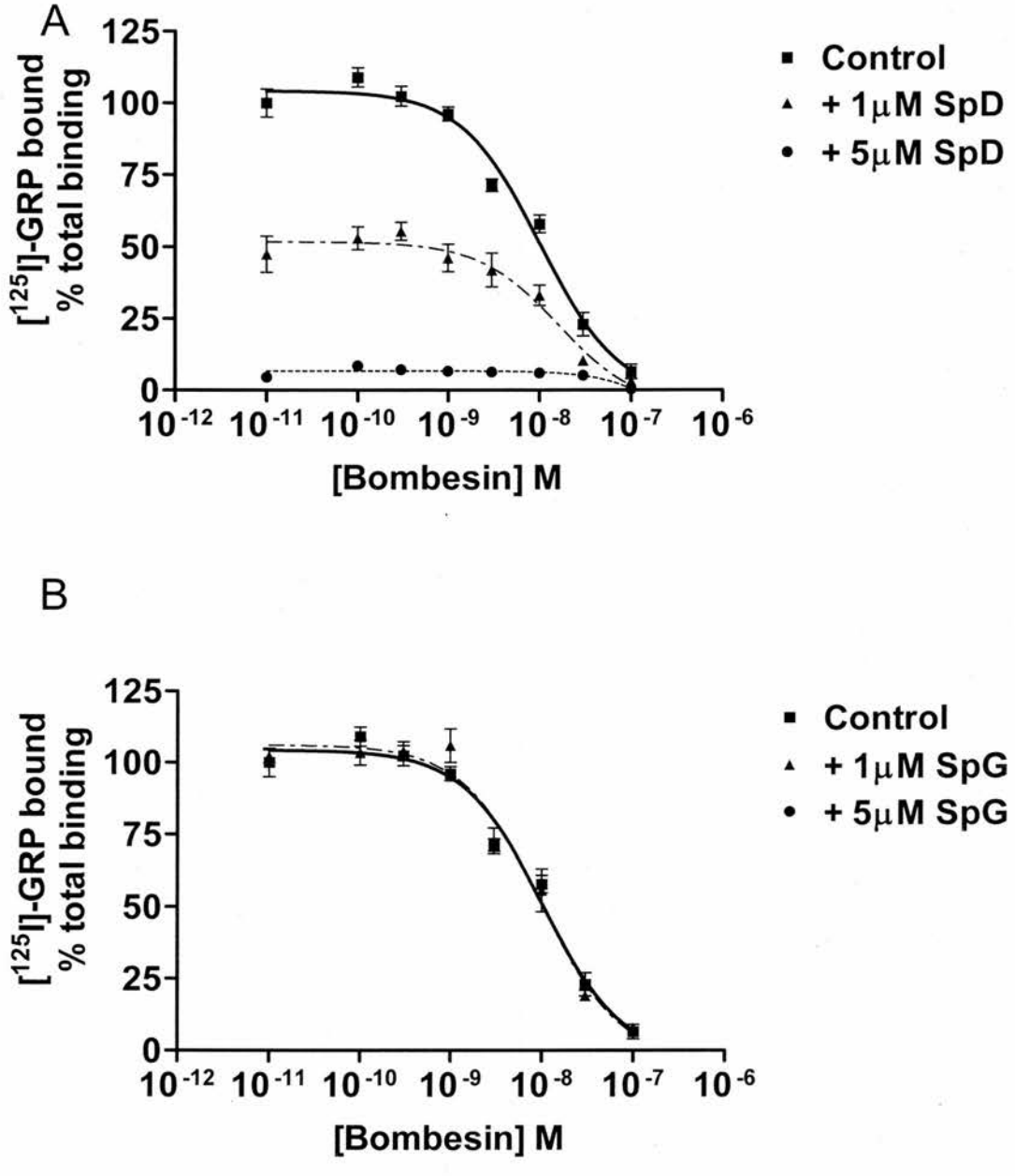



**Figure 3.17. Effect of substance-P analogues on [$^{125}$I]-GRP binding to CHO-GRP cells.** Confluent, quiescent cultures of CHO-GRP cells were incubated with [$^{125}$I]-GRP binding media alone (control) or containing **A)** 1μM or 5μM SP-D. **B)** 1μM or 5μM SP-G, then various concentrations of unlabelled bombesin were added (37°C; 30min). Results are expressed as % of total radioligand binding and represent the mean ± s.e.m. of three experiments performed in triplicate.

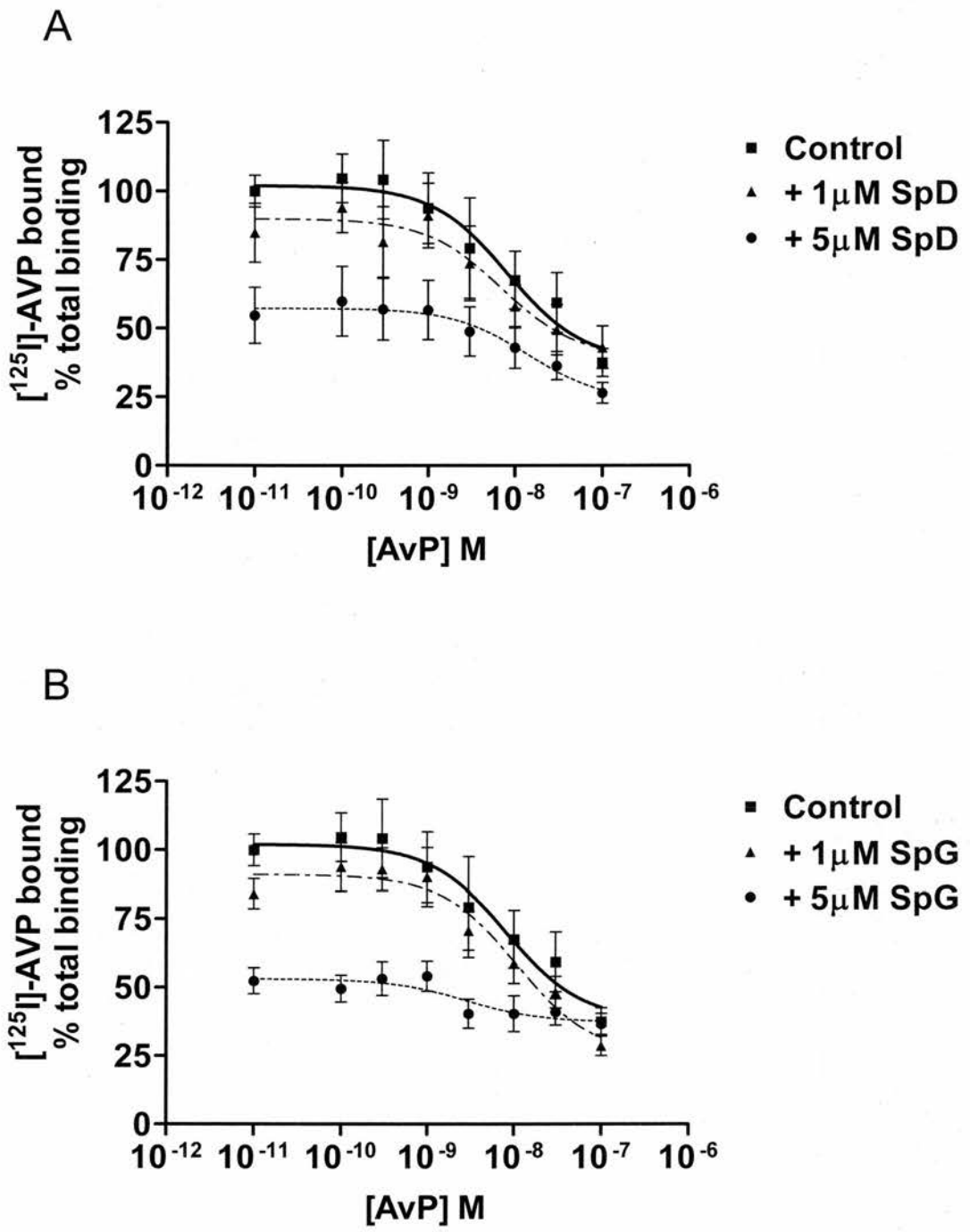



**Figure 3.18. Effect of substance-P analogues on [$^{125}$I]-AVP binding to CHO-$V_{1A}$ cells.** Confluent, quiescent cultures of CHO-$V_{1A}$ cells were incubated with [$^{125}$I]-AVP binding media alone (control) or containing **A)** 1μM or 5μM SP-D. **B)** 1μM or 5μM SP-G, then various concentrations of unlabelled vasopressin were added (37°C; 30min). Results are expressed as % of total radioligand binding and represent the mean ± s.e.m. of three experiments performed in triplicate.

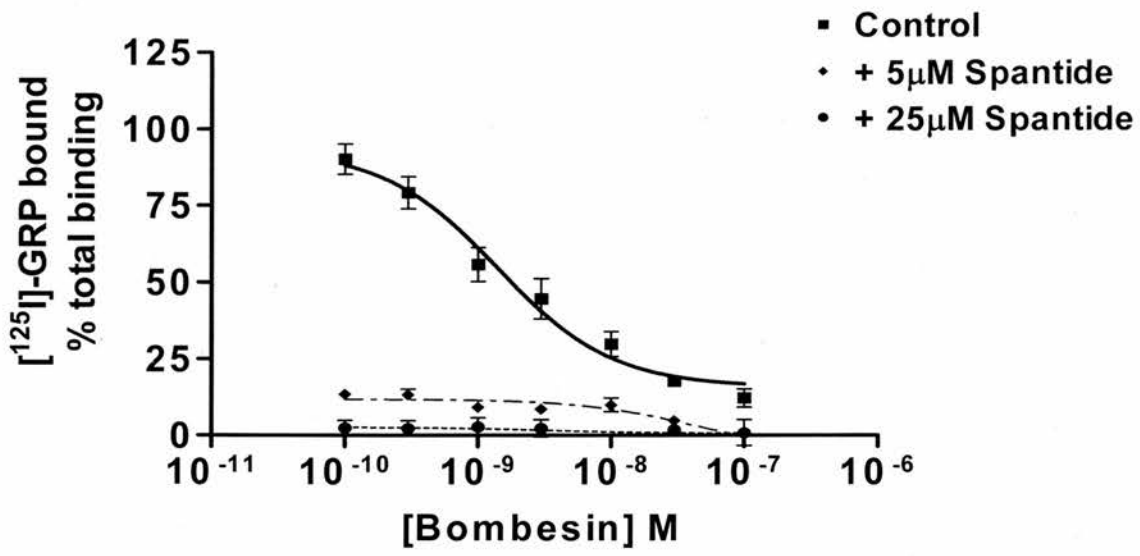



**Figure 3.19. Effect of Spantide on [$^{125}$I]-GRP binding to CHO-GRP cells.** Confluent, quiescent cultures of CHO-GRP cells were incubated with [$^{125}$I]-GRP binding media alone (control) or containing 5μM or 25μM Spantide, then various concentrations of unlabelled bombesin were added (37°C; 30min). Results are expressed as % of total radioligand binding and represent the mean ± s.e.m. of two experiments performed in triplicate.

## 3.4 ERK activation by neuropeptide receptors

Extracellular regulated protein kinases (ERK 1 and 2) are activated by AVP and bombesin in a variety of cell types expressing the cognate receptors (Thibonnier, 1992; Seufferlein, 1996b; Rozengurt, 1998a; Chiu *et al*, 2002). Although the majority of the data suggests a $G_q$- and PKC-dependent mechanism, these observations are thought to be cell type-dependent (Thibonnier, 1992; Seufferlein *et al*, 1996a; Charlesworth & Rozengurt, 1997; Jian *et al*, 1999; Chiu *et al*, 2002). Previous studies have shown that substance-P analogues reversibly inhibit ERK activation by neuropeptides in Swiss 3T3 cells (Mitchell *et al*, 1995; Seckl *et al*, 1996). However, studies in our lab revealed that in rat-1 fibroblasts and in human SCLC cells, in the absence of bombesin, substance-P analogues activated ERK and c-jun kinase (JNK) in a GRP receptor-dependent manner (MacKinnon *et al*, 2001). Having assessed the antagonist properties of the Substance-P analogues by their effects on neuropeptide-induced calcium responses and receptor binding, the following part of this study was undertaken to investigate their agonist properties.

ERK activation was measured in CHO cells expressing GRP or $V_{1A}$ receptors in response to the appropriate neuropeptide or substance-P analogue. Lysates from quiesced cells stimulated with either neuropeptide, SP-D or SP-G were separated on 12% gels and blotted for phospho-ERK1/2 or total ERK2. Activation was estimated by quantification of pERK band intensity ratios using ImageQuant software following chemifluorescent visualisation of immunoblots. Blots were also probed for total ERK2 in order to ensure that any observed increase in ERK phosphorylation was not due to a change in total ERK expression or unequal loading of gels (observed as equivalent ERK2 bands in the absence and presence of treatment).

### 3.4.1 Neuropeptide stimulated ERK activation

Cells were stimulated with increasing concentrations of neuropeptide for 10 minutes and ERK activation measured. In CHO-GRP cells, bombesin increased ERK phosphorylation in a concentration dependent manner. Quantification of 5 separate experiments showed that bombesin produced a maximal 3.5 fold increase in ERK phosphorylation at 1 nM with an $EC_{50}$ of 0.56 nM (Figure 3.20a). AVP also stimulated a dose-dependent increase in ERK phosphorylation in $V_{1A}R$ expressing cells with an $EC_{50}$ of 0.72 nM (n=4) and a maximal 4.2 fold increase stimulated by 1nM AVP (Figure 3.20b). Neither AVP nor GRP stimulated ERK phosphorylation in CHO-vector cells (Figure 3.21).

### 3.4.2 Substance-P analogue stimulated ERK activation

Cells were stimulated with increasing concentrations of SP-D or SP-G for 5 minutes at 37°C and ERK phosphorylation measured. SP-D stimulated ERK in CHO-GRP and CHO-$V_{1A}$ cells in a concentration dependent manner (Figure 3.22), with activation of ERK evident at 3 μM. Maximal stimulation with 50μM Sp-D caused a 2.8 fold (CHO-GRP) and 2 fold (CHO-$V_{1A}$) increases in ERK phosphorylation but this was still less than that observed with neuropeptide. SP-G also stimulated a concentration dependent increase in ERK phosphorylation in both cell-lines (Figure 3.23). SP-G had greater efficacy in $V_{1A}$ receptor expressing cells stimulating a 4.3 fold increase in ERK phosphorylation at 50 μM compared to a 2.1-fold stimulation in CHO-GRP cells. ERK stimulation by SP-D and SP-G was also observed in CHO-vector cells but at a lower level than in receptor expressing cell-lines. These data show that at concentrations which block neuropeptide binding and neuropeptide-stimulated calcium release, SP-D and SP-G are agonists for ERK activation in both GRP and $V_{1A}$ receptor expressing CHO cells.

Interestingly, 30μM Spantide did not activate ERK in CHO-GRP or CHO-$V_{1A}$ cells (Figure 3.24) although the same concentration completely inhibited neuropeptide-stimulated calcium release as well as [$^{125}$I]-GRP binding.

Pre-treatment of CHO-GRP cells with the GRP antagonist RC3940-II (1 μM; 30 min) completely blocked 1 nM bombesin-induced ERK1/2 phosphorylation but not 30 μM SP-D stimulated ERK activation (Figure 3.25). Similarly, the selective $V_{1A}$ receptor antagonist [β-mercapto-β-β-cyclopentamethylene-propionyl$^{1}$,0-Me-Tyr$^{2}$,Arg$^{8}$]-vasopressin (1 μM; 30 min) inhibited 1 nM vasopressin-induced but not 30 μM SP-G stimulated ERK phosphorylation. Antagonists typically bind to the orthosteric ligand binding site of a receptor (Boeynaems & Dumont, 1980) and would thus prevent native ligand binding to the receptor and the subsequent activation of downstream signals. Since the analogues are still able to activate ERK in the presence of antagonist, this suggests that the analogues act at a site on the receptor which is distinct from the neuropeptide binding site. This is in accordance with findings from radioligand binding studies which also suggest that the analogues bind to a site on the receptor which is distinct from the agonist binding site.

### 3.4.3 Kinetics of ERK activation

In order to determine the time-course for ERK phosphorylation by neuropeptide and Substance-P analogues, CHO-$V_{1A}$ cells were treated with 1nM AVP or 30μM SP-G for increasing lengths of time. Both AVP and SP-G stimulated ERK phosphorylation in a time-dependent manner. Neuropeptide-stimulated ERK phosphorylation was rapid, reaching a maximum 4 fold increase within 2 minutes (Figure 3.26). After 30 minutes exposure to AVP, the level of phosphorylation decreased but remained above basal levels for up to 60 minutes post-stimulation. In contrast, SP-G stimulated ERK phosphorylation peaked at 10 minutes, at levels up to 2.5-fold above basal, and was sustained at this level thereafter. The divergent

kinetics of ERK activation indicated that the mechanism by which neuropeptide and Substance-P analogue activate ERK may differ. The work in the following section was subsequently undertaken to investigate the mechanisms by which neuropeptides and Substance-P analogues activate ERK.

## 3.5 Mechanisms of ERK activation

The mechanisms of GRP- and $V_{1A}$-receptor mediated ERK activation are not fully understood although it has been shown in some cell types that activation is mediated by $G_q$-induced PKC activation and subsequent activation of raf. Other GPCRs have been shown to activate ERK in a $G_i$ and ras-dependent mechanism, which may or may not involve the activation of PI3K. There is also a growing body of evidence to implicate receptor tyrosine kinases such as the EGF receptor as being central in the activation of ERK by GPCRs. Inhibitors of various intracellular signalling intermediates were used to ascertain their respective roles in the activation of ERK by neuropeptides and Substance-P analogues in CHO cells.

### 3.5.1. Role of G-proteins of $G_i$ subtype

In order to establish whether $G_i$ proteins play any role in the activation of ERK, cells were treated overnight with 100 ng $ml^{-1}$ pertussis toxin (PTx). The toxin catalyses the ADP-ribosylation of $G_i$ protein α-subunits which results in the uncoupling of $G_i$ from receptors (Kaslow & Burns, 1992). Figure 3.27 shows that in CHO-GRP cells PTx produced a small but non-significant inhibition of bombesin-stimulated ERK activation whereas the responses to 30 μM SP-D and SP-G were completely abolished ($P<0.05$, ANOVA). In CHO-$V_{1A}$ expressing cells the response to AVP was not significantly inhibited by PTx pre-treatment although the response to both SP-G and SP-D (30 μM) was completely blocked ($P<0.05$, ANOVA). In addition, PTx

had no effect on ERK phosphorylation induced by 0.1nM bombesin and vasopressin in CHO-GRP and CHO-$V_{1A}$ cells respectively (data not shown). In both cell types ERK activation by 1 μM LPA (which activates ERK through the $G_i$-coupled LPA receptor) was completely inhibited by PTx treatment (data not shown). This differential sensitivity to pertussis toxin indicates that dual $G_i/G_q$-coupled mechanisms activate the ERK 1/2 cascade via GRP and $V_{1A}$ receptors expressed in CHO cells.

The effect of pertussis toxin on neuropeptide-induced calcium mobilisation and the ability of Substance-P analogues to inhibit this response were also assessed to determine whether $G_i$ proteins were involved. Figure 3.28 shows that PTx did not affect 1 nM bombesin-induced calcium mobilisation or the ability of 30 μM Sp-D to inhibit the calcium response in CHO-GRP cells. Similarly, in CHO-$V_{1A}$ cells calcium mobilisation stimulated by 1 nM vasopressin and the inhibition of this response by 30 μM SP-G was not affected by PTx. This shows that neuropeptide-stimulated calcium mobilisation does not involve receptor coupling to $G_i$ proteins and that the inhibition of calcium release by substance-P analogues is not mediated by $G_i$ proteins.

In the following experiments, CHO-GRP and CHO-$V_{1A}$ cells were stimulated with both SP-D and SP-G. The various inhibitors had the same effects on SP-D and SP-G stimulated ERK activation in each cell-line. Hence, for simplicity only the data for SP-D stimulated ERK phosphorylation is shown for CHO-GRP cells and SP-G stimulated ERK phosphorylation shown for CHO-$V_{1A}$ cells in the following sections.

### 3.5.2. Role of Src kinases and PI-3 kinases

Src-family tyrosine kinases have been implicated in the activation of ERK mediated by both pertussis toxin sensitive and pertussis toxin insensitive G proteins. Src activation via a PKC-and calcium-independent pathway is thought to be specifically mediated by G protein βγ subunits (Igishi *et al*, 1998). The $G_i$ protein βγ subunits are able to associate with and activate phosphotidylinositol-3-kinases (PI-3-K) (Crespo, 1994; Lopez-Ilasaca *et al*, 1997). Following PI-3-kinase activation, Src-family tyrosine kinases phosphorylate adaptor proteins, e.g. Shc, recruiting Grb2-Sos complexes to the membrane, enhancing the GDP-GTP exchange of Ras, and finally linking to the ERK/MAPK cascade (Gutkind, 1998a; Dikic & Blaukat, 1999).

The effect of PP2, which is a selective Src-family kinase inhibitor (Hanke *et al*, 1996), was tested. Cells were pre-treated with 5 μM PP2 for 30 minutes then stimulated with 1 nM neuropeptide or 30μM analogue. In CHO-GRP cells, ERK activation by Sp-D was completely blocked by Src kinase inhibition whilst inhibition of bombesin-induced ERK phosphorylation was incomplete (Figure 3.29). Likewise, SP-G-stimulated ERK phosphorylation was fully inhibited by PP2 treatment in CHO-$V_{1A}$ cells whereas AVP-stimulated ERK phosphorylation was incompletely blocked. This shows that activation of ERK by substance-P analogues and neuropeptides is dependent on Src-family kinases.

The role of PI-3-K was examined in cells pre-treated for 30 minutes with 100 nM Wortmannin (Cross *et al*, 1995). Inhibition of PI3K significantly blocked substance-P analogue- and neuropeptide-stimulated ERK activation in both GRP and $V_{1A}$ receptor expressing cells (Figure 3.30). This shows that substance P analogue and neuropeptide-induced ERK activation involves signalling via PI-3-K dependent pathways.

### 3.5.3. Role of small G-protein Ras

The small GTPase Ras is an upstream regulator of ERK activity and can be activated by both receptor tyrosine kinases and non-receptor tyrosine kinases (Sadoshima & Izumo, 1996). The Ras antagonist FTS (S-*trans-trans*-Farnesylthiosalicyclic acid) dislodges Ras from its membrane-anchoring sites and accelerates degradation with a resultant decrease in total cellular Ras (Haklai *et al*, 1998). Figure 3.31 shows that FTS pre-treatment (50 μM; 30 minutes) inhibited neuropeptide and substance-P analogue-induced ERK activation similarly in both $V_{1A}$ and GRP receptor expressing cells. This shows that the pathways leading to ERK activation by the substance-P analogues and neuropeptides require Ras activation. Basal ERK phosphorylation was also completely inhibited with FTS in both cell-lines indicating that some level of constitutive Ras activity exists in CHO-K1 cells.

### 3.5.4. Role of Signalling Scaffolds

Various studies have indicated that both $G_q$ and $G_i$ coupled receptors are able to induce ERK activation through transactivation of the EGF receptor tyrosine kinase (Daub *et al*, 1997; Gutkind, 2000; Santiskulvong *et al*, 2001). Activated receptor tyrosine kinases (RTKs) can function as scaffolds for the plasma membrane recruitment and assembly of Ras activation complexes, such as the Grb2-mSos guanine nucleotide exchange factor complex (Luttrell *et al*, 1997a; Della Rocca, 1999). So in order to determine whether EGF receptor transactivation was involved in neuropeptide and substance-P analogue induced ERK phosphorylation, cells were pre-treated with the specific inhibitor AG1478 (1μM; 30 minutes) to block EGF receptor kinase activity. In CHO-GRP cells, AG1478 completely abolished analogue-induced ERK1/2 phosphorylation but had no significant effect on bombesin-induced ERK1/2 phosphorylation (Figure 3.32). AG1478 similarly abrogated SP-G-induced but not AVP-stimulated ERK phosphorylation in CHO-

$V_{1A}$ cells. This reveals that EGF receptor transactivation is required for ERK activation by substance-P analogues in CHO cells.

Integrin-based focal adhesions serve as points of cell attachment to the extracellular matrix and of cytoskeletal anchoring to the plasma membrane. They can also function as scaffolds for the GPCR-induced assembly of Ras activation complexes (Luttrell *et al*, 1999b). Disruption of the actin cytoskeleton with cytochalasin D (2μM; 30 minutes) inhibited neuropeptide and analogue stimulated ERK activity in both CHO-GRP and CHO-$V_{1A}$ cells (Figure 3.33). This shows that focal adhesions provide a scaffold upon which signalling complexes assemble for ERK activation following GRP or $V_{1A}$ receptor stimulation.

To summarise, in CHO cells expressing neuropeptide receptors, neuropeptides activate $G_q$ (and to lesser extent $G_i$) leading to ERK phosphorylation through both EGFR, Src and PI3K dependent and independent pathways. In contrast, the substance-P analogues activate $G_i$ leading to ERK activation via EGFR, Src and PI3K dependent pathways only. Ras activation serves as a point of convergence for signals emanating from receptor stimulation with substance-P analogues or neuropeptides. In addition, ERK activation by both analogues and neuropeptides is dependent upon the formation of focal adhesion complexes.

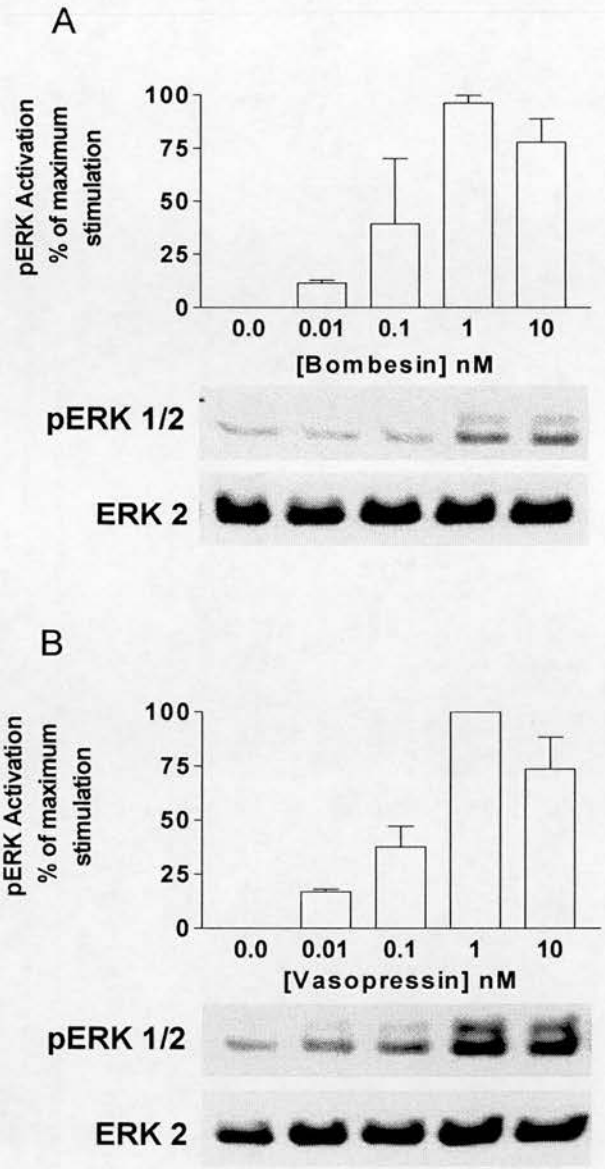



**Figure 3.20. Neuropeptide stimulated ERK phosphorylation in receptor expressing cells.** Confluent, quiescent cultures of A) CHO-GRP and B) CHO-$V_{1A}$ cells were stimulated for 10 min with increasing concentrations of neuropeptide. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal anti-pERK1/2 antibody (upper panel) or polyclonal anti-ERK2 antibody (lower panel). Representative blots are shown. Bar graphs represent the mean band intensity ratios ± s.e.m. of four experiments performed in duplicate. Blots not normalised to total ERK2.

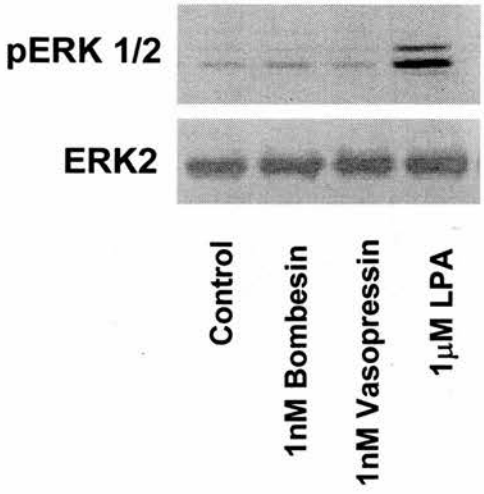



**Figure 3.21. Neuropeptide stimulated ERK phosphorylation in CHO-vector cells.** Confluent, quiescent cultures of CHO-vector cells were stimulated for 10 min with water, 1nM bombesin, 1nM vasopressin or 1μM LPA. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal anti-pERK1/2 antibody (upper panel) or polyclonal anti-ERK2 antibody (lower panel). Representative blots are shown.

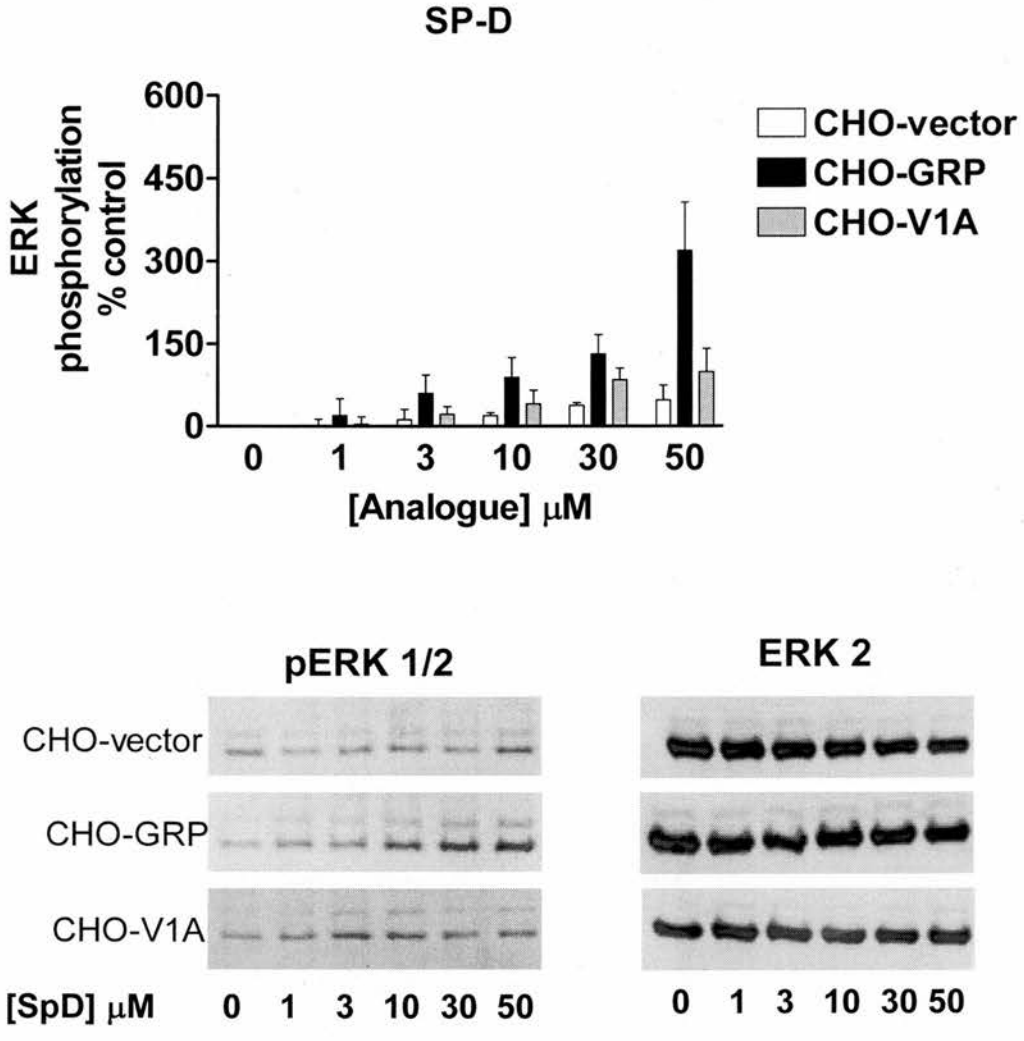



**Figure 3.22. SP-D stimulated ERK phosphorylation.** Confluent, quiescent cultures of CHO-vector, CHO-GRP and CHO-$V_{1A}$ cells were stimulated for 5 min with increasing concentrations of SP-D. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal anti-pERK1/2 antibody (left panel) or polyclonal anti-ERK2 antibody (right panel). Representative blots are shown. Bar graphs represent the mean band intensity ratios ± s.e.m. of four experiments performed in duplicate.

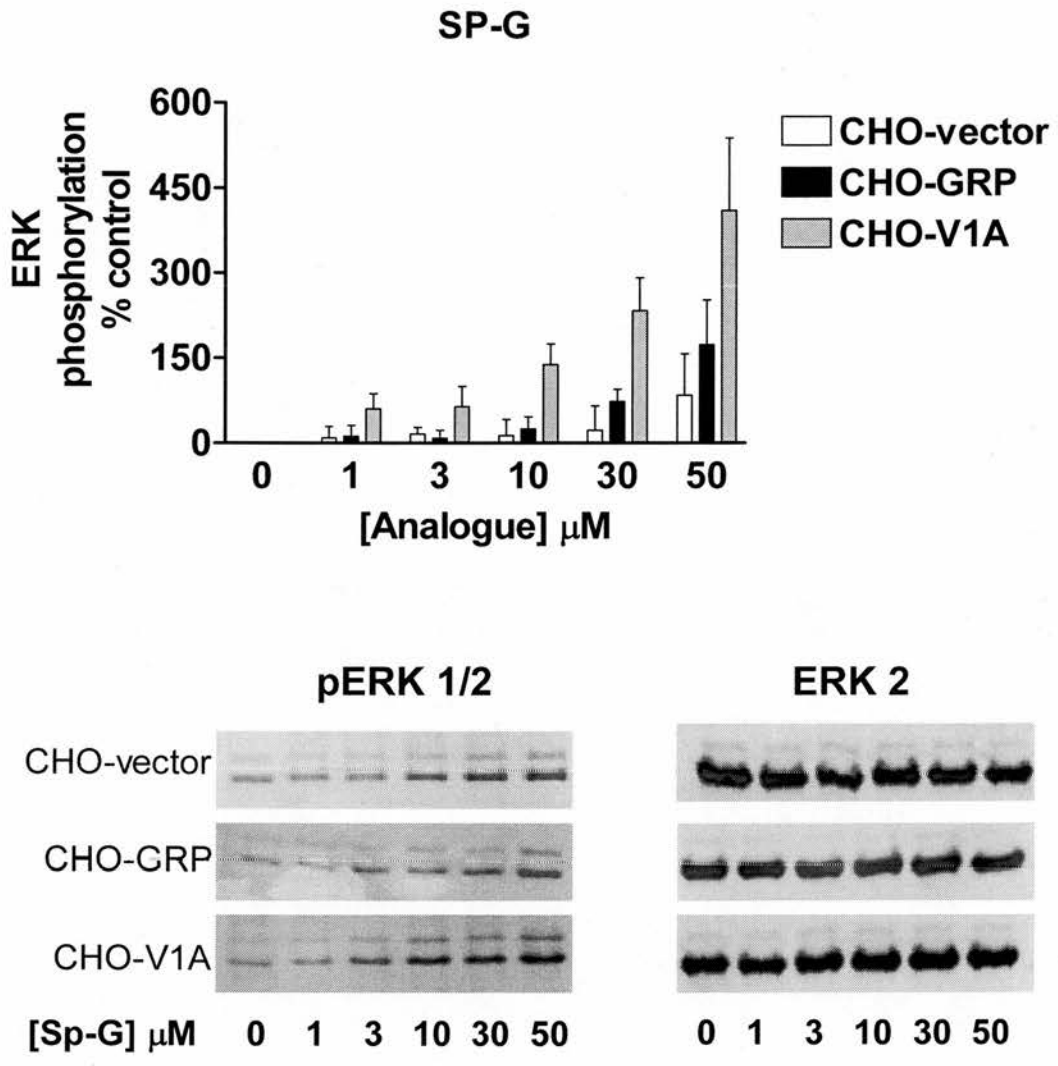



**Figure 3.23. SP-G stimulated ERK phosphorylation.** Confluent, quiescent cultures of CHO-vector, CHO-GRP and CHO-$V_{1A}$ cells were stimulated for 5 min with increasing concentrations of SP-G. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal anti-pERK1/2 antibody (left panel) or polyclonal anti-ERK2 antibody (right panel). Representative blots are shown. Bar graphs represent the mean band intensity ratios ± s.e.m. of four experiments performed in duplicate.

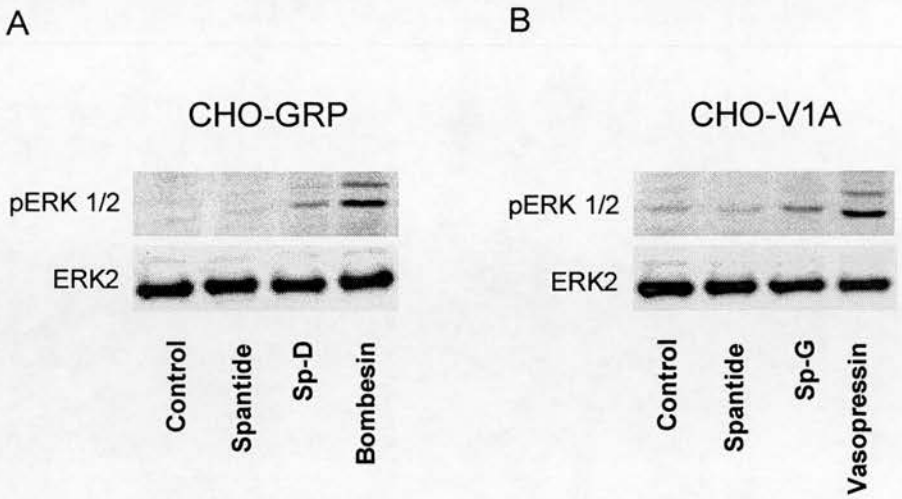



**Figure 3.24. Effect of Spantide on ERK phosphorylation.** Confluent, quiescent cultures of **A)** CHO-GRP: stimulated with $dH_20$ (control), 30μM Spantide, 30μM SP-D or 1μM BN (37°C; 5min). **B)** CHO-$V_{1A}$: stimulated with $dH_20$ (control), 30μM Spantide, 30μM SP-G or 1μM AVP (37°C; 5min). Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal anti-pERK1/2 antibody (upper panel) or polyclonal anti-ERK2 antibody (lower panel). Representative blots are shown. Experiments performed >six times in duplicate.

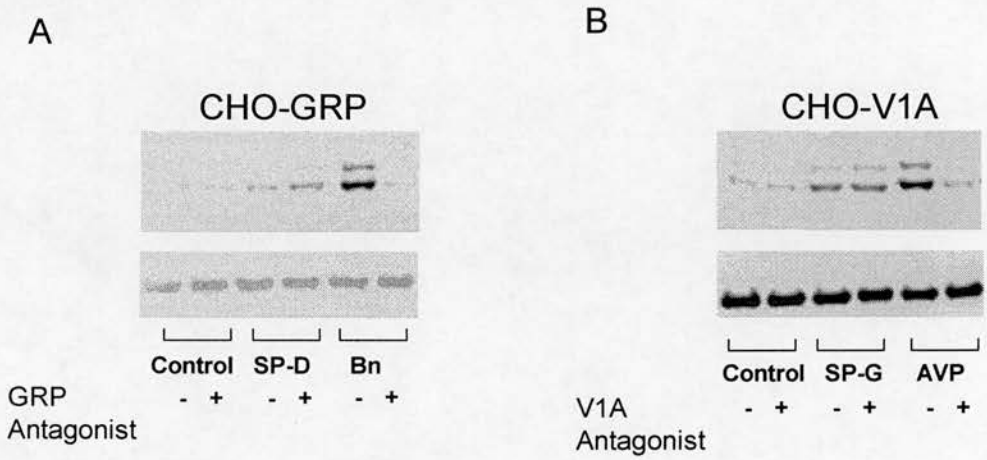



**Figure 3.25. Inhibition of ERK phosphorylation by receptor-antagonists. A)** CHO-GRP stimulated with $dH_20$ (control), 30μM SP-D or 1nM bombesin (37°C; 5min) with or without 1μM RC3940II pre-treatment. **B)** CHO-$V_{1A}$ stimulated with $dH_20$ (control), 30μM SP-G or 1nM vasopressin (37°C; 5min) with or without 1μM [β-mercapto-β-β-cyclopentamethylene-propionyl[1],0-Me-Tyr[2],Arg[8]]-vasopressin pre-treatment. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal anti-pERK1/2 antibody (upper panel) or polyclonal anti-ERK2 antibody (lower panel). Representative blots are shown. Experiments performed three times in duplicate.

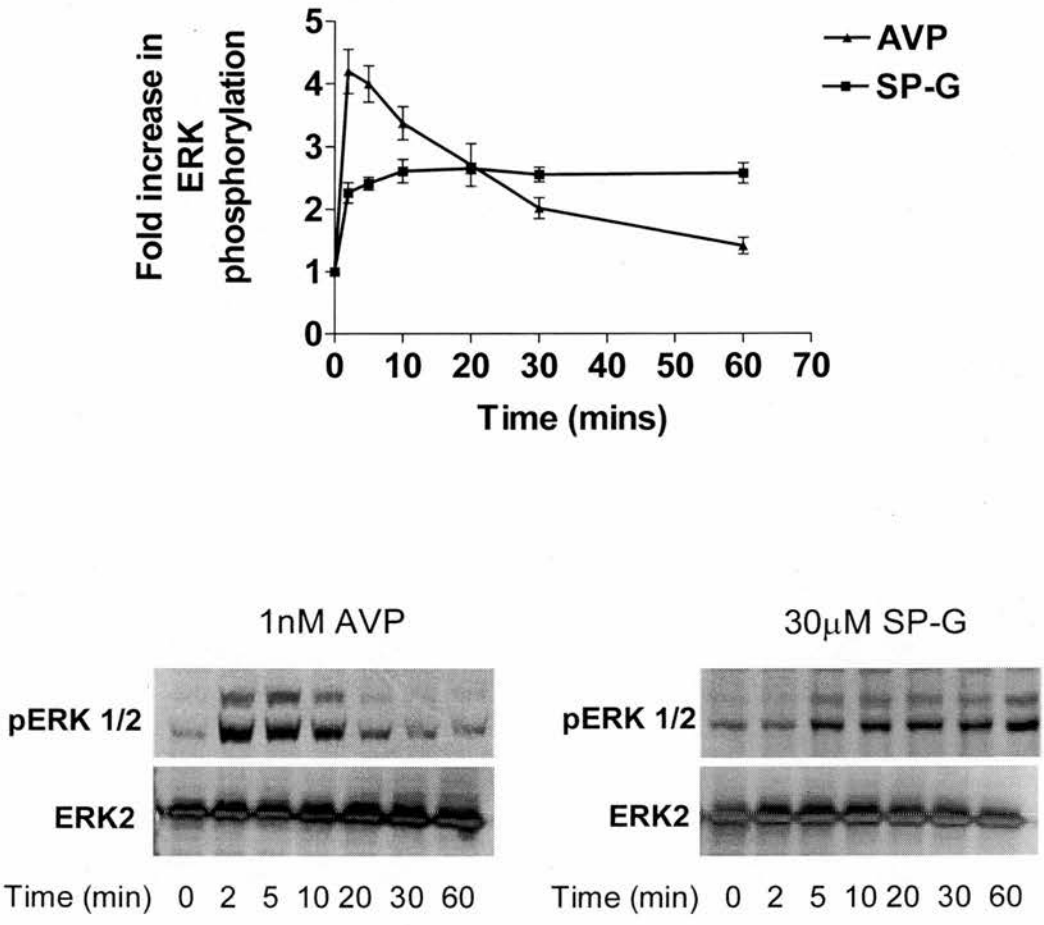



**Figure 3.26. Kinetics of ERK phosphorylation in CHO-$V_{1A}$ cells.** Confluent, quiescent cultures of CHO-$_{V1A}$ cells were stimulated with 1nM AVP or 30μM SP-G for the indicated times at 37°C. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal anti-pERK1/2 antibody (upper panel) or polyclonal anti-ERK2 antibody (lower panel). Representative blots are shown. Time-course plot represents the mean band intensity ratios ± s.e.m. of three experiments performed in duplicate.

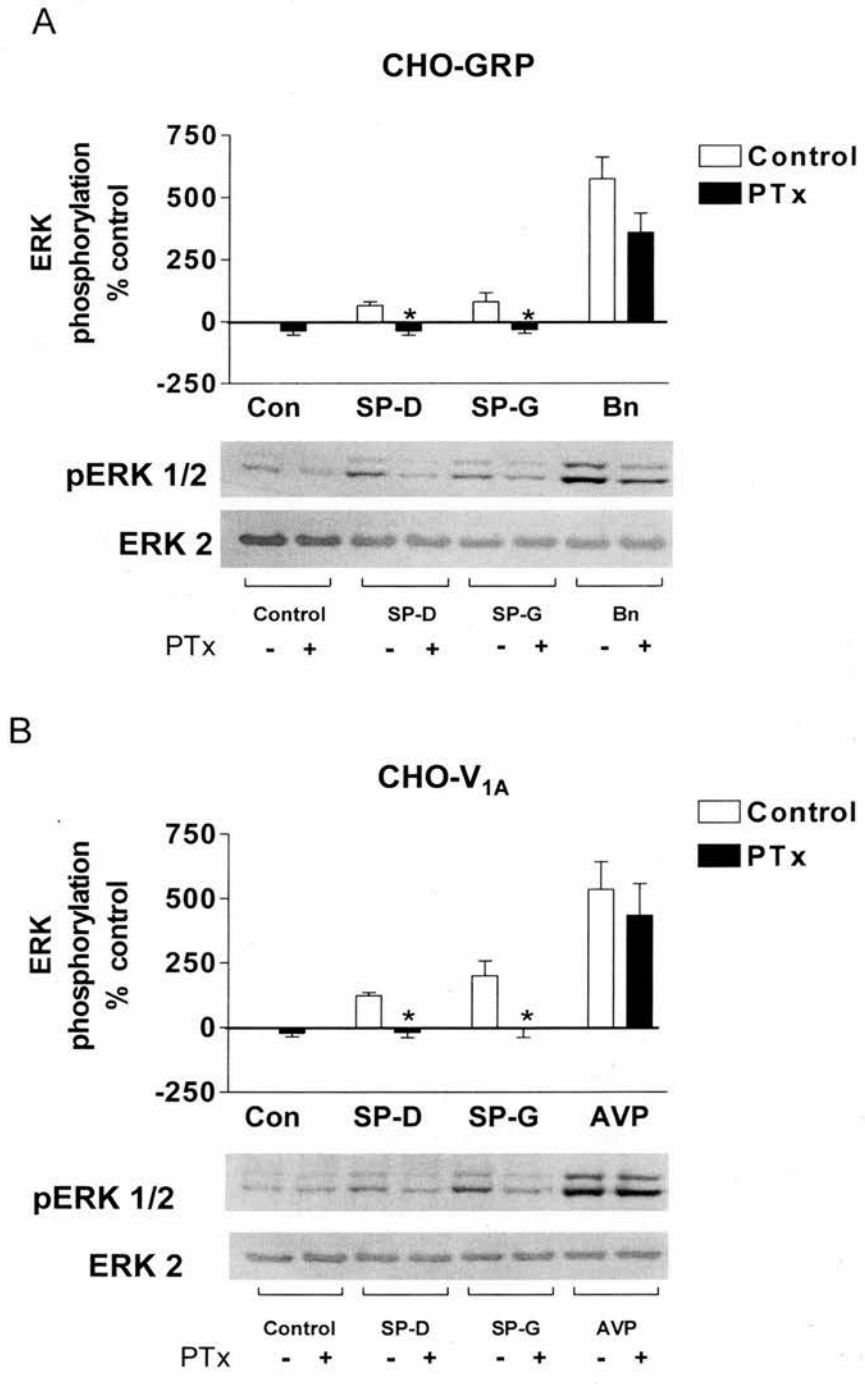



**Figure 3.27. Effect of pertussis toxin on ERK phosphorylation.** Confluent cultures were quiesced overnight in the presence or absence of 100ng ml-1 pertussis toxin (PTx). Cells were stimulated for 5 min with 30μM SP-D or SP-G or 1nM neuropeptide as indicated. Lysates were resolved by SDS-PAGE and western blots probed with monoclonal pERK1/2 antibody (upper panel) or polyclonal ERK2 antibody (lower panel). Representative blots are shown. Bar graphs represent the mean band intensity ratios ± s.e.m. of four separate experiments performed in duplicate (*P<0.05, ANOVA).

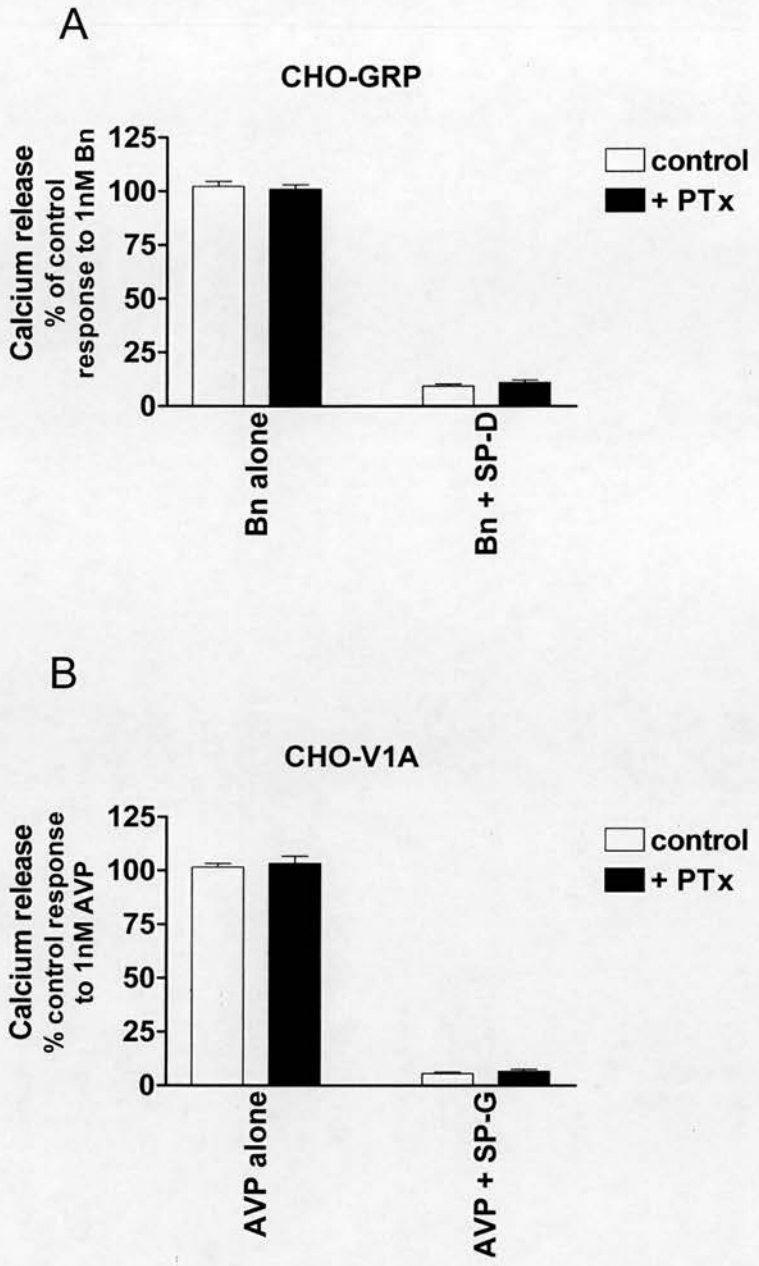



**Figure 3.28. Effect of pertussis toxin on calcium mobilisation. A)** Quiescent CHO-GRP cells loaded with FURA-2AM were stimulated with 1nM bombesin alone (control) or in the presence of 30μM SP-D. **B)** Quiescent CHO-$V_{1A}$ cells loaded with FURA-2AM were stimulated with 1nM AVP alone (control) or in the presence of 30μM SP-G. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods. Data is expressed as % control response and represents the mean ± s.e.m. of three experiments.

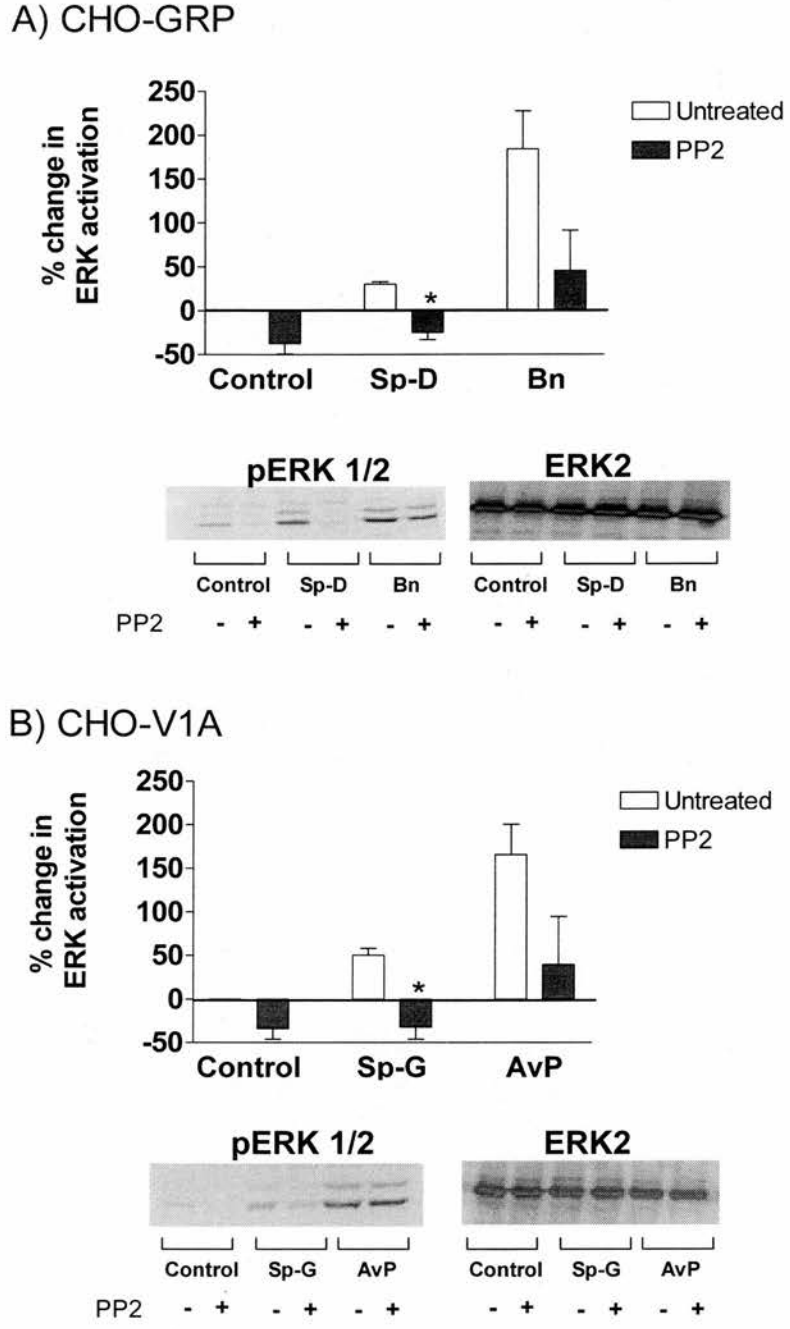



**Figure 3.29. Effect of inhibition of Src-like kinases on ERK phosphorylation.** Confluent, quiescent cultures were pre-treated with PP2 (5μM; 30 min) at 37°C. **A)** CHO-GRP cells stimulated for 5 min with 30μM SP-D or 1nM Bn. **B)** CHO-$V_{1A}$ cells were stimulated for 5 min with 30μM SP-G or 1nM AVP. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal pERK1/2 antibody (left panel) or polyclonal ERK2 antibody (right panel). Representative blots are shown. Bar graphs represent the mean band intensity ratios ± s.e.m. of three separate experiments performed in duplicate (*P<0.05, ANOVA).

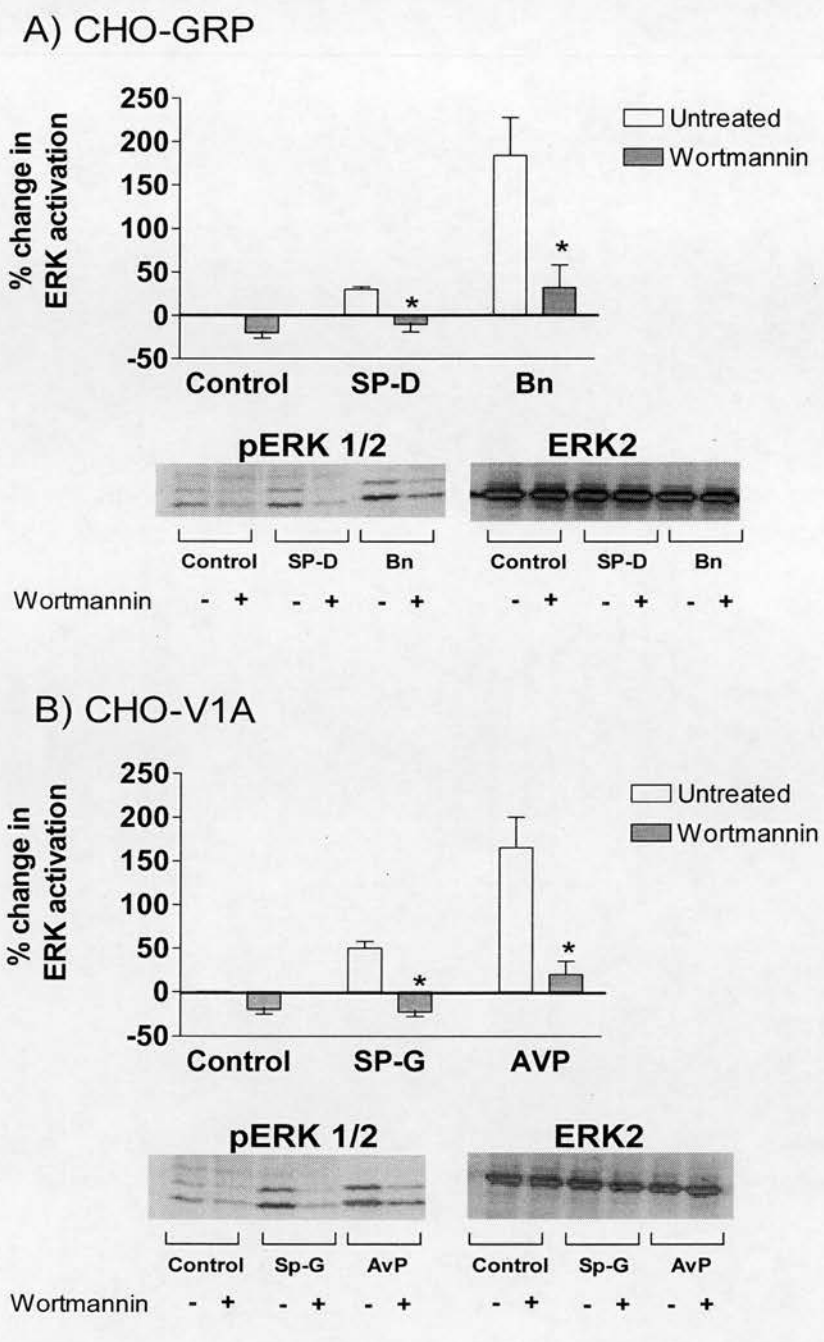



**Figure 3.30. Effect of PI-3-K inhibition on ERK phosphorylation.** Confluent, quiescent cultures were pre-treated with Wortmannin (100nM; 30 min) at 37°C. **A)** CHO-GRP cells stimulated for 5 min with 30μM SP-D or 1nM Bn. **B)** CHO-$V_{1A}$ cells were stimulated for 5 min with 30μM SP-G or 1nM AVP. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal pERK1/2 antibody (left panel) or polyclonal ERK2 antibody (right panel). Representative blots are shown. Bar graphs represent the mean band intensity ratios ± s.e.m. of three separate experiments performed in duplicate (*P<0.05, ANOVA).

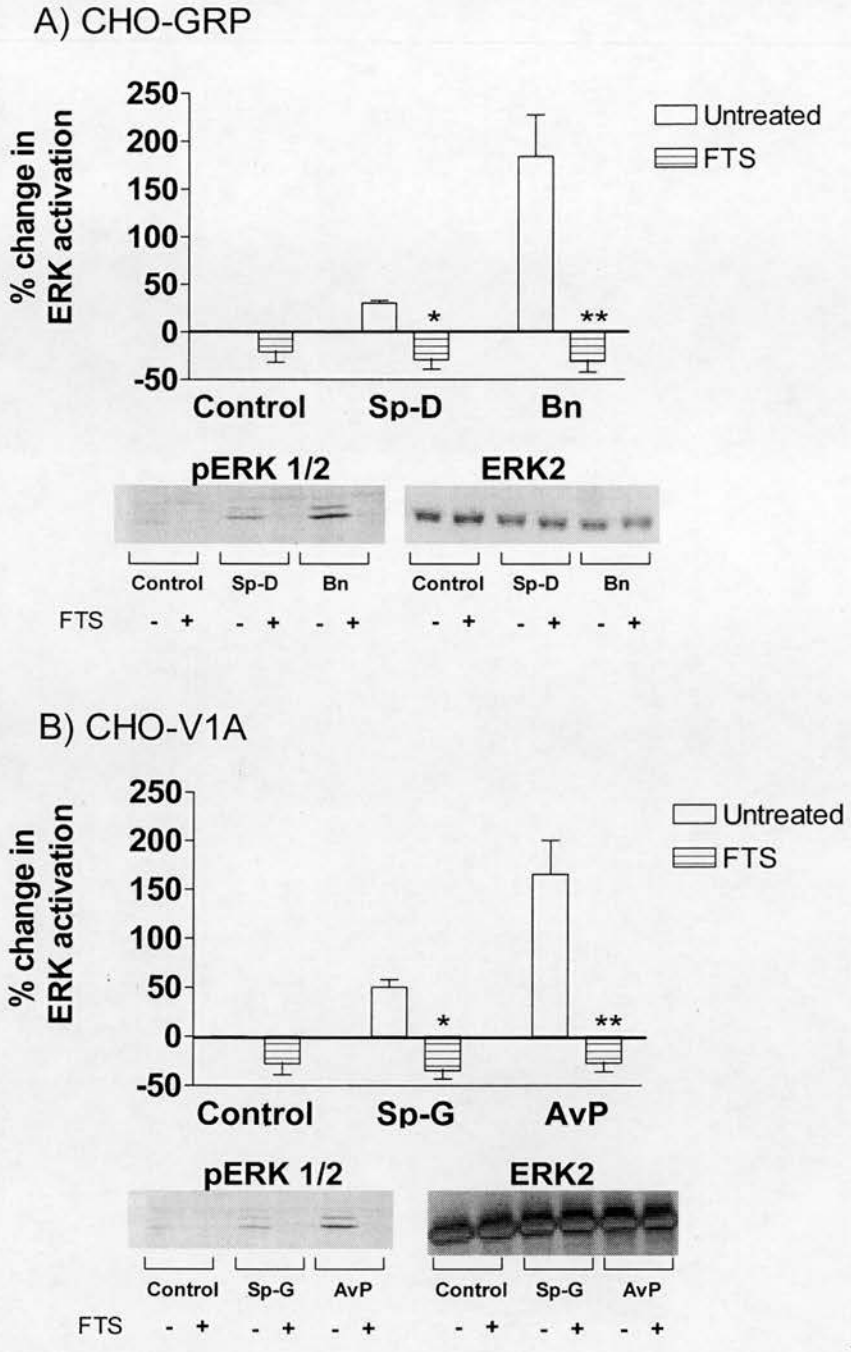



**Figure 3.31. Effect of Ras inhibition on ERK phosphorylation.** Confluent, quiescent cultures were pre-treated with FTS (50μM; 30 min) at 37°C. Cells were then stimulated for 5 min with 30μM analogue or 1nM neuropeptide as indicated. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal pERK1/2 antibody (left panel) or polyclonal ERK2 antibody (right panel). Representative blots are shown. Bar graphs represent the mean band intensity ratios ± s.e.m. of three separate experiments performed in duplicate (*P<0.05, **P<0.01, ANOVA).

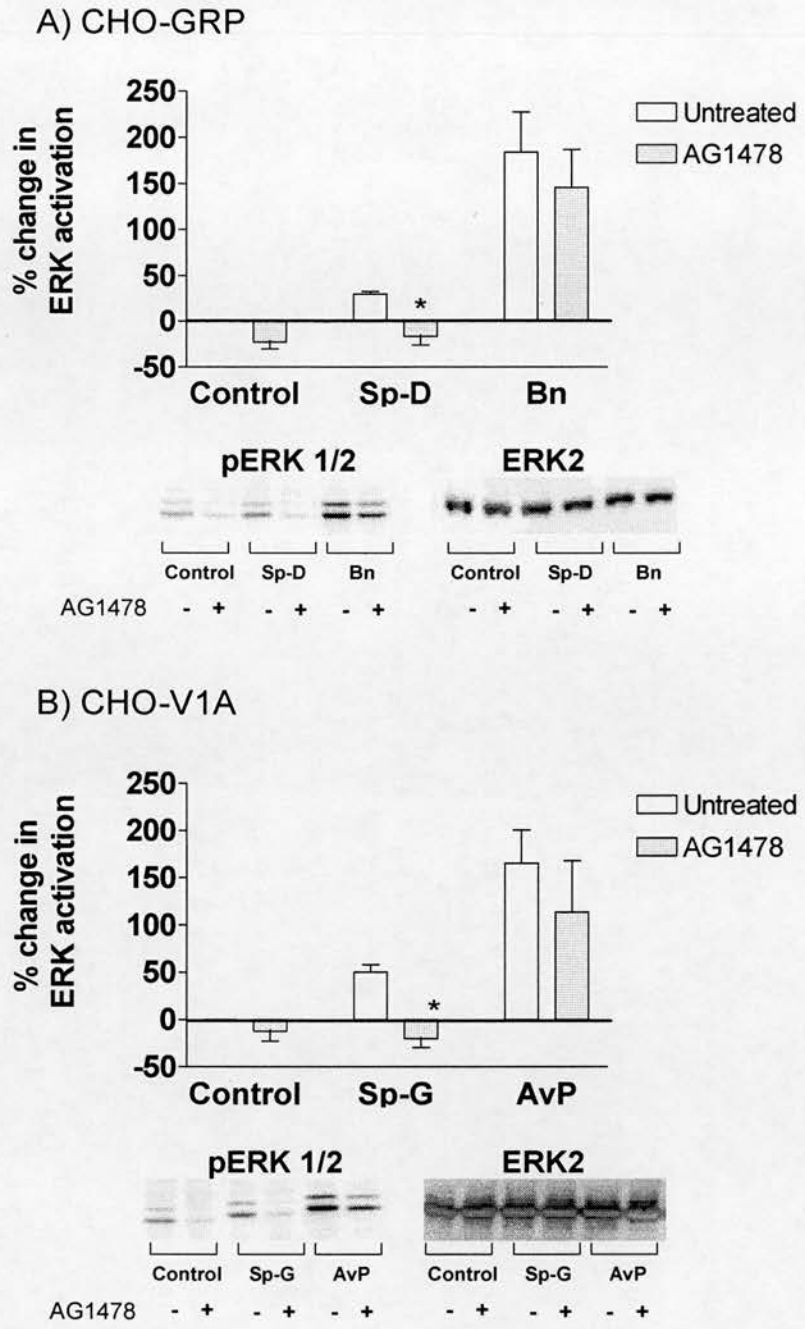



**Figure 3.32. Effect of EGFR tyrosine kinase inhibition on ERK phosphorylation.** Confluent, quiescent cultures were pre-treated with AG1478 (1μM; 30 min) at 37°C. **A)** CHO-GRP cells stimulated for 5 min with 30μM SP-D or 1nM Bn. **B)** CHO-$V_{1A}$ cells were stimulated for 5 min with 30μM SP-G or 1nM AVP. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal pERK1/2 antibody (left panel) or polyclonal ERK2 antibody (right panel). Representative blots are shown. Bar graphs represent the mean band intensity ratios ± s.e.m. of three separate experiments performed in duplicate (*P<0.05, ANOVA).

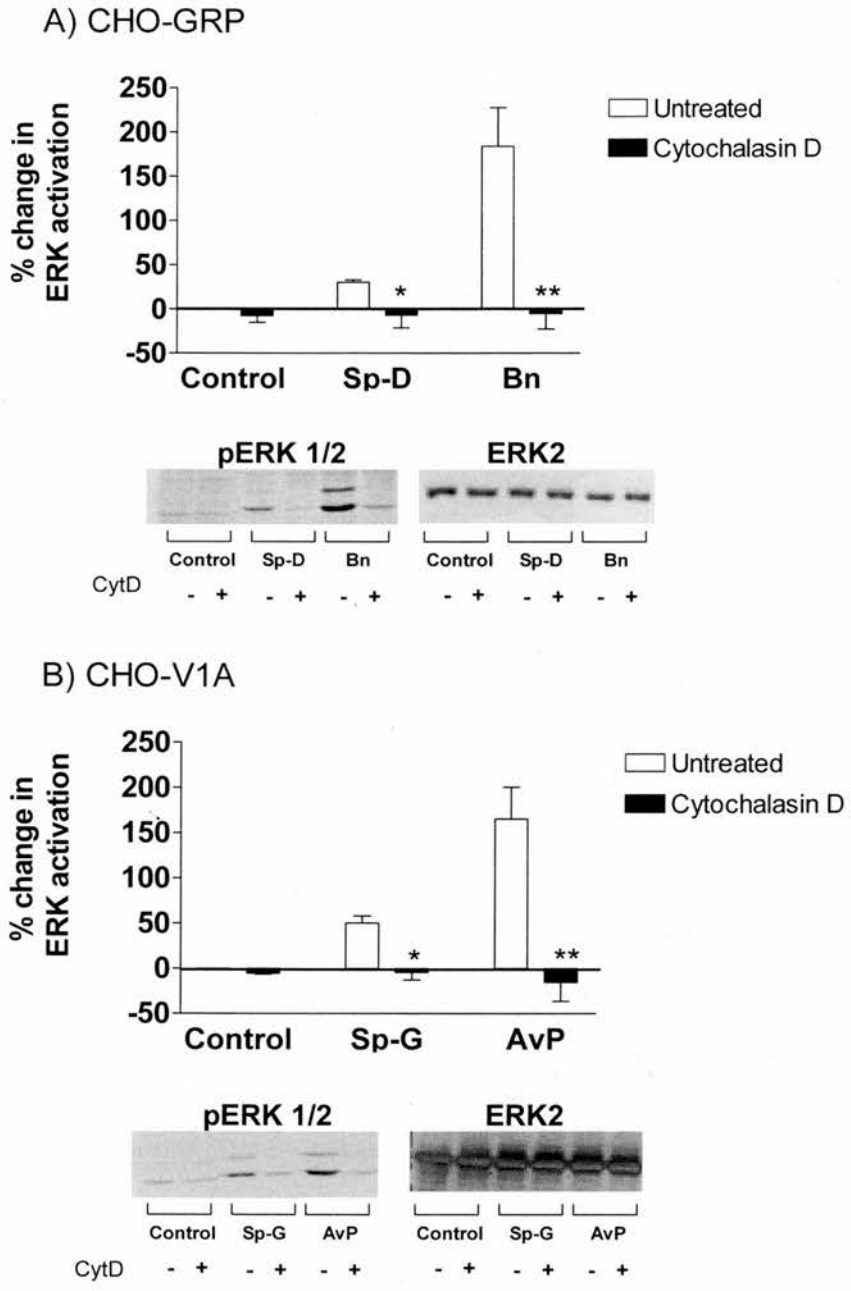



**Figure 3.33. Effect of disrupting actin cytoskeleton on ERK phosphorylation.** Confluent, quiescent cultures were pre-treated with cytochalasin D (2μM; 30 min) at 37°C. **A)** CHO-GRP cells stimulated for 5 min with 30μM SP-D or 1nM Bn. **B)** CHO-$V_{1A}$ cells were stimulated for 5 min with 30μM SP-G or 1nM AVP. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal pERK1/2 antibody (left panel) or polyclonal ERK2 antibody (right panel). Representative blots are shown. Bar graphs represent the mean band intensity ratios ± s.e.m. of three separate experiments performed in duplicate (*P<0.05, ** P<0.01, ANOVA).

## 3.6 Discussion

The question addressed in this chapter was whether substance-P analogues are biased agonists of receptors other than the GRP receptor. Substance-P analogue properties were studied using model cell systems stably expressing the GRPR or $V_{1A}R$. The hamster epithelial CHO-K1 cell-line was used to generate the model cell systems as it is a non-transformed, non-neuroendocrine cell-line. It may also be of relevance to SCLC since this tumour type may be derived from epithelial cells lining the larger airways (Garber *et al*, 2001). The cell-line has an acceptable null background for the transfection of neuropeptide receptors, unlike COS-7 cells which are more amenable to transfection but endogenously express bombesin receptors. CHO-K1 cells have previously been used for the over-expression of various GPCRs and the subsequent investigation of intracellular signalling events. In particular, CHO-K1 cells have been transfected with $V_{1A}$ receptors in order to define the signalling pathways which mediate vasopressin mitogenicity (Thibonnier *et al*, 2000).

Of the cell-lines created, CHO-GRP expressed 5086 ± 489 receptors/cell whereas CHO-$V_{1A}$ expressed 720 ± 90 receptors/cell. The levels of receptor expression are within the range measured in a variety of native and tumour tissues (100-50,000 sites/cell) (Cardona *et al*, 1992; Fay *et al*, 1994; Tahara *et al*, 1998a; Tahara *et al*, 1999; Jensen *et al*, 2001). The affinities of GRP and AVP for their cognate receptors expressed in CHO-GRP and CHO-$V_{1A}$ respectively are similar to previously reported values (Cardona *et al*, 1992; Thibonnier *et al*, 1994; Ferris *et al*, 1997; Tsuda *et al*, 1997; Tahara *et al*, 1998b). Stably expressed GRPR and $V_{1A}R$ effectively mobilised intracellular calcium in response to neuropeptide stimulation, with $EC_{50}$ values reflecting the affinity of each neuropeptide for its receptor. The $EC_{50}$ values for calcium mobilisation were in accordance with previous reports (Wang *et al*, 1996b; Tahara *et al*, 1998b). The pertussis toxin insensitivity of the calcium responses

is also in accord with previous observations that the GRP and $V_{1A}$ receptors couple to a pertussis toxin insensitive G-protein belonging to the $G_q$/11 family (Zachary *et al*, 1987b; Thibonnier *et al*, 1994; Wang *et al*, 1996b; Dyer *et al*, 2005). The receptors also responded to neuropeptide stimulation by activating ERK predominantly via $G_q$-mediated signalling pathways. Neuropeptide stimulated calcium mobilisation and ERK activation in CHO-GRP and CHO-$V_{1A}$ cells was blocked by specific neuropeptide antagonists. This confirmed that the effects of BN and AVP on calcium flux and ERK activity, in CHO-GRP and CHO-$V_{1A}$ cells respectively, were elicited through specific receptors. Receptors expressed in the model cell-systems were thus fully functional and could be regarded as physiologically relevant models.

In vector-transfected cells, BN induced a small calcium response whereas AVP produced no response. This implied that CHO-K1 cells endogenously express GRP receptors. However, endogenous GRP or AVP receptor expression was low, as shown by the lack of significant radio-labelled GRP or AVP binding. In addition, these cells did not activate ERK in response to neuropeptide stimulation indicating that the level of GRP receptor expression is not sufficiently high enough to activate all cell signalling pathways. However, this also suggests that a relatively low number of receptors are adequate for a calcium response to be observed in CHO-K1 cells but not an ERK response.

In order to better understand the substance-P analogues' mechanism of action, their effects were studied at distinct steps of the $V_{1A}$R and GRPR signalling process: neuropeptide growth factor binding, calcium mobilisation and ERK1/2 activation. The analogues inhibited neuropeptide binding and antagonised calcium mobilisation but were agonists for ERK activation by the $V_{1A}$R as well as the GRPR.

The analogues SP-D and SP-G competitively inhibited natural ligand binding to both the GRP and $V_{1A}$ receptors. SP-G exhibited greater affinity for the $V_{1A}$ receptor whereas SP-D had greater affinity for the GRP receptor. The selectivity of SP-G for the $V_{1A}$ receptor is in accordance with previous findings (Seckl *et al*, 1995b). Substance-P analogues are indeed able to inhibit the action of many different neuropeptides but the mechanism underlying this broad-spectrum activity is unclear. A comparative study of specific antagonists and substance-P analogues against bombesin, vasopressin and bradykinin receptors in Swiss 3T3 cells showed that only the substance-P analogues demonstrated cross-inhibition between receptors (Woll & Rozengurt, 1988a). It was therefore proposed that whereas the specific antagonists only interact with the ligand binding site the analogues must be able to recognise a common domain shared by the three receptors.

Consistent with this theory, the analogues caused a reduction in the number of binding sites, suggesting that they bind to a site which is distinct from the orthosteric agonist binding site. In order for the analogues to be able to inhibit the effects of a broad-spectrum of neuropeptides, the analogue binding site is likely to be present within a common structural motif present amongst analogue-sensitive receptors. The variable affinity with which the analogue binds to different analogue-sensitive receptors probably reflects the contribution the respective receptors primary structure makes to the configuration of the analogue binding site. Thus, the data shows that substance-P analogue binding modulates the conformation of the receptor through stabilising a receptor conformation which is unable to bind the natural ligand.

A change in the steepness (Hill factor) of the binding curves is an indicator that the unlabelled ligand is not simply competing for a single class of binding site and can be used to identify co-operativity between binding sites. The steepness of each binding curve (Figures 3.17 and 3.18) was determined using the sigmoidal dose response (variable slope) equation to fit data in Prism, to provide the Hill (slope) factor (data not shown). This showed that there was a reduction in the Hill coefficient of binding curves in the presence of analogue, which indicates that

negative co-operativity may exist between the analogue binding site and neuropeptide binding site on the receptor. In this case, binding of the analogue may distort the neuropeptide binding pocket thus preventing neuropeptide binding. A structural basis for this can be implied from the phenomenon of receptor dimerization. It is becoming apparent that most, if not all GPCRs, form dimers (Rios *et al*, 2001). Indeed, $V_{1A}$ receptors have been shown to constitutively form homodimers during synthesis (Terrillon *et al*, 2003). It has been proposed that the dimerization process can facilitate cross-talk between protomers which may be manifest in the co-operative binding of ligands to these protomers (Durroux, 2005). Models formulated to describe this process suggest that negative co-operativity would result from the conversion of independent binding sites to dependent ones (Durroux, 2005). In accordance with this, the neuropeptide ligand may bind to independent sites (i.e. to monomers) whereas the analogue may bind to dependent sites (i.e. sites dependent on dimer formation). This would also account for the reduction in the number of binding sites in the presence of analogue and could also have implications for signalling.

SP-D and SP-G were effective antagonists of the transient calcium responses induced by neuropeptide stimulation of both $V_{1A}$ and GRP receptors. Previous work has also shown that substance-P analogues inhibit bombesin and vasopressin induced calcium mobilisation in Swiss 3T3 cells (Seckl *et al*, 1996a) and SCLC cell-lines (Bunn *et al*, 1994). In accordance with findings from binding studies, SP-G more potently inhibited the $V_{1A}R$ calcium response. However SP-D, which inhibited GRP binding more potently than AVP binding, inhibited the calcium response to both neuropeptides with similar potency. The inhibition of neuropeptide stimulated calcium release by the analogues was not altered by pertussis toxin pre-treatment. This showed that antagonism of the calcium response did not result from activation of $G_i$ protein-dependent signalling by the analogues. Inhibition of calcium mobilisation is therefore likely to be a consequence of bound analogue preventing the neuropeptide from binding, which is necessary for

activation of the receptor-$G_q$ protein interaction. In Swiss 3T3 cells, SP-D did not inhibit the activation of PLCβ by a constitutively active $G_q$-like protein, indicating that the peptide acts at a point upstream of the activated form of the G-protein alpha subunit (Mitchell *et al*, 1995). Consistent with this finding, the data presented in this chapter indicates that inhibition of neuropeptide stimulated calcium mobilisation is a direct result of the analogue-binding to the receptor in a manner which stabilises a receptor conformation which does not couple to $G_q$ proteins.

In addition to the aforementioned antagonist properties of the analogues against neuropeptide-mediated processes, as shown in this chapter, these compounds were also agonists. Stimulation of the $V_{1A}R$ as well as the GRPR with SP-D and SP-G resulted in ERK activation in the absence of neuropeptides. SP-D and SP-G activated ERK at concentrations which reflected their affinities for the GRPR and $V_{1A}R$. ERK activation by SP-D and SP-G occurred in the absence of calcium mobilisation and was facilitated entirely by receptor coupling with pertussis toxin sensitive $G_i$ proteins. In contrast, neuropeptide-stimulated ERK activation was largely mediated through $G_q$ proteins, with $G_i$-dependent signalling making only a minor contribution and free calcium playing a role as a second messenger. Previous work has shown that ERK can be activated in a calcium- and PKC-independent manner (Faure *et al*, 1994; Mochizuki *et al*, 1999). GPCR coupling with $G_i$ proteins, in addition to $G_q$ proteins, has also been demonstrated previously (Letterio *et al*, 1986; Profrock *et al*, 1992). In particular, GRP receptor coupling to $G_i$ in Swiss 3T3 cells has previously been demonstrated to play a role in bombesin-induced mitogenesis but not in bombesin-stimulated calcium mobilisation (Zachary *et al*, 1987b). In the same cells, $V_{1A}$ receptors have exhibited cell-cycle dependent coupling to $G_i$ proteins for AVP-induced calcium mobilisation (Abel *et al*, 2000). SP-D has also previously been shown to facilitate receptor association with $G_i$ proteins, with SP-D induced calcium mobilisation in human neutrophils being sensitive to pertussis toxin pre-treatment (Jarpe *et al*, 1998). In contrast to neuropeptide stimulated $Ca^{2+}$

mobilisation in fibroblasts, in neutrophils the βγ subunits released upon activation of $G_i$ are thought to mediate stimulation of PLCβ isoforms leading to calcium mobilisation (Wu *et al*, 1993). The G-protein selectivity of substance-P analogues demonstrated in this study is in agreement with previous results where GRP receptor expressing rat 1A cells were stimulated with SP-D (Mackinnon *et al*, 2001).

Evaluation of the kinetics of ERK activation following $V_{1A}$ receptor stimulation with AVP or SP-G showed distinct temporal patterns of activation. Unlike neuropeptide induced ERK activation which was rapid and transient, activation of ERK by SP-G was sustained. GRP stimulation of rat1A cells expressing GRP receptors similarly resulted in rapid and transient ERK activation whereas SP-D stimulated ERK activity was sustained although not delayed (Mackinnon *et al*, 2001). The peak level of ERK phosphorylation induced by SP-G was lower than that obtained following AVP-stimulation. This could be a reflection of the different intracellular pathways employed by SP-G and AVP for ERK activation and suggests that $G_q$-stimulated ERK activity is necessary to attain the maximum levels of ERK phosphorylation stimulated by AVP. The magnitude and duration of ERK signalling is in itself believed to determine cell fate (Qui & Green, 1992; Traverse *et al*, 1992; Cowley *et al*, 1994). The ability of a receptor to produce more sustained activation of MAPK has been shown to determine the ability of a cell to differentiate or proliferate, depending on the cell type (Thibonnier *et al*, 1997). In PC12 cells for instance, sustained ERK activation is associated with ERK translocation to the nucleus and results in neuronal differentiation, whereas transient activation causes cytosolic activation and leads to proliferation (Dikic *et al*, 1994). It would be of interest to determine whether the spatial localisation of ERK is altered following receptor stimulation with analogues compared with neuropeptides as this may contribute to the growth inhibitory response to neuropeptide receptor stimulation with analogues. Following receptor activation, desensitization mechanisms control receptor signalling and thus limit the amplitude and/or duration of the signal transduction cascade (Grady *et al*, 1997). Ligand degradation, GPCR-kinase (GRK)

mediated receptor phosphorylation, β-arrestin binding and receptor internalization can all contribute to this process (Tiberi *et al*, 1996; Claing *et al*, 2002). GRP receptors undergo desensitization upon exposure to bombesin and altered agonist responses have been shown to result from differences in receptor desensitization and down-regulation (Benya *et al*, 1994b). In rat1A fibroblasts expressing GRP receptors, it was found that receptors stimulated with SP-D were less efficiently desensitized than those stimulated with GRP (Mackinnon *et al*, 2001). Therefore, the delayed and sustained ERK activation induced by SP-G in CHO-$V_{1A}$ cells could be attributed to altered receptor desensitization. $Ca^{2+}$/Calmodulin (CaM) dependent pathways have also been implicated in $G_i$ and $G_q$-mediated ERK activation (Della Rocca *et al*, 1997; Della Rocca *et al*, 1999). As mentioned previously, ERK activation by SP-D and SP-G occurs in the absence of calcium mobilisation. A change in the level of ERK1/2-inactivating phosphatases could contribute to sustained ERK phosphorylation induced by SP-G since the expression of at least one of the ERK1/2-inactivating phosphatases, MKP1, is $Ca^{2+}$ dependent (Cook *et al*, 1997). In addition, Bosch *et al* (1998) have shown that CaM was essential for inhibition of sustained ERK1/2 activation following stimulation of cultured fibroblasts with growth factors.

Data presented in this chapter demonstrates that stimulation of ERK activity by bombesin and AVP is dependent upon the formation of focal adhesion complexes. In contrast, analogue stimulated ERK activation requires both EGFR and focal adhesions to function as scaffolds for the assembly of ERK activation complexes. Dissection of the molecular mechanisms underlying neuropeptide receptor stimulated ERK activity showed that activation of ERK by bombesin and AVP is predominantly independent of EGFR transactivation but requires Src and PI3K. In contrast, the substance-P analogues stimulate ERK via EGFR, Src and PI3K dependent pathways only. Nonetheless, Ras activation serves as a point of convergence for signals transduced by either substance-P analogue or neuropeptide stimulated GRP and V1A receptors.

Previous work has demonstrated that stimulation of GRP receptors in Swiss 3T3 cells leads to inhibition of [$^{125}$I]-EGF binding to its cellular receptor, via a process mediated by protein kinase C (Zachary & Rozengurt, 1985; Zachary & Rozengurt, 1986a). MAPK activation by bombesin occurs in a PKC-dependent manner in Swiss 3T3 cells (Seufferlein *et al*, 1996a) and a PKC-independent manner in rat1A cells (Charlesworth & Rozengurt, 1997). The activation of ERK following $V_{1A}$ receptor stimulation in rat vascular smooth muscle cells and 3Y1 fibroblasts has been shown to occur via both PKC dependent and independent pathways (Granot *et al*, 1993; Nishioka *et al*, 1995). In addition, PI3K activation has been demonstrated to be a component of the PKC-independent pathway (Nishioka *et al*, 1995). PI-3-K activation has also been implicated in GRP stimulated ERK activity (Bocker & Verspohl, 2001). In accordance with this, data presented in this chapter showed that ERK activation following neuropeptide stimulation of transfected receptors in CHO-K1 cells was partially dependent on PI-3-K. Conversely, analogue stimulated ERK activity was fully dependent upon PI-3-K activity. Gβγ-subunits directly activate PI-3-Kγ as well as the more widely distributed PI-3-Kβ (Schwindinger & Robishaw, 2001). PI-3-kinases can be involved in ERK signalling both upstream and downstream of EGFR transactivation. PI-3-K may induce Src-mediated EGFR transactivation (upstream role) and recruitment of Ras activation complexes. In addition, direct binding of the catalytic subunit of PI-3-K to activated Ras can lead to further stimulation of PI-3-K activity (downstream role) following GPCR-induced activation of PI-3-K (Cantley, 2002). While the role of PKC in neuropeptide and analogue stimulated ERK activity in the CHO model systems was not determined in this study, it is nevertheless conceivable that the EGFR independent pathway leading to ERK stimulation by bombesin and AVP represents a PKC-dependent pathway.

A requirement for transactivation of growth factor receptors for ERK activation has previously been demonstrated for some G-protein-coupled receptors (Della Rocca *et al*, 1999). Stimulation of the $G_i$-coupled receptor for LPA has been shown to induce ERK activation through a Gβγ subunit mediated Ras dependent pathway (Crespo *et*

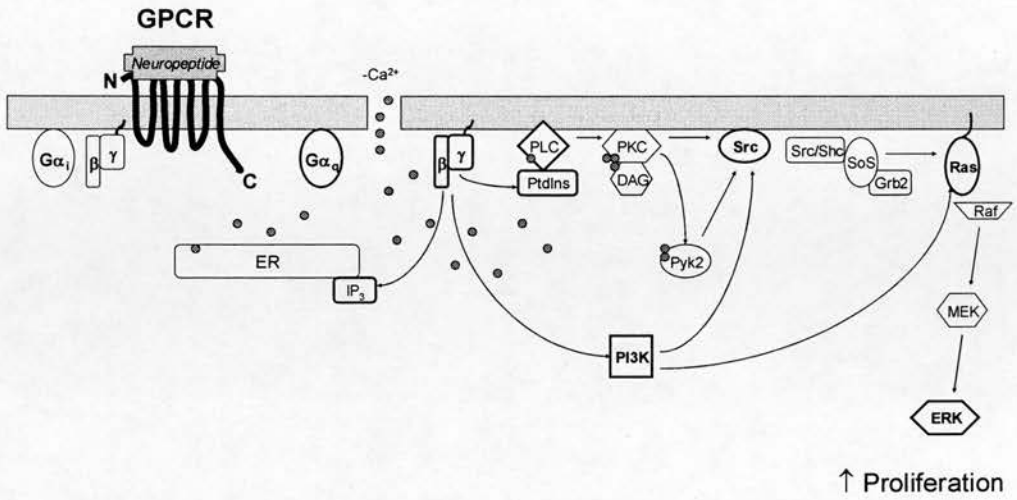
*al*, 1994; van Biesen *et al*, 1995). Although the intermediates between βγ and Ras have not been defined clearly, tyrosine kinases including Src and activated EGFR have been implicated (Pierce *et al*, 2001b). This would be in accordance with the findings presented here that $G_i$-mediated ERK activation by the substance-P analogues involves EGFR activation and Src kinases. The Src kinases may play a role upstream of EGFR transactivation and/or downstream. Transactivation of EGF receptors can result from activation of Src-like kinases following GPCR stimulation (Luttrell *et al*, 1997b; Zhang *et al*, 2004). Src kinases have been shown to facilitate cleavage of membrane bound EGF-like ligands by membrane bound metalloproteases leading to EGFR activation in a classic autocrine manner (Prenzel *et al*, 2001; Zhang, 2004). Alternatively, analogue signalling in the absence of calcium mobilisation may account for EGFR transactivation since it has been suggested that CaM inhibits the RTK transactivation process. $Ca^{2+}$/CaM can modulate Src activity and directly or indirectly affect Ras activity or signalling elements downstream of Ras (Belcheva & Coscia, 2002). Inhibition of CaM has been demonstrated to stimulate cleavage of several membrane proteins in CHO and human epithelial cells, including EGFR binding ligands, and this process is PKC independent (Bosch *et al*, 1998). The endocytic pathway has also been implicated in EGFR transactivation following GPCR activation whereby a complex is formed within clathrin coated pits which includes EGFR, β-arrestin and Src (Maudsley *et al*, 2000). In such cases, β-arrestin functions as an adaptor which recruits Src to mediate EGFR phosphorylation (Miller *et al*, 2000). Either way, activated EGFR recruits Grb2-Sos complexes directly or via Src to activate Ras. Thus, analogue signalling may utilise Src kinases for EGFR activation (EGFR upstream role) and Ras activation (EGFR downstream role) in a number of ways.

Non-receptor tyrosine kinases are also implicated in EGFR independent pathways leading to ERK stimulation. $G_q$-coupled receptors can use Src kinases to activate Ras in a manner similar to that described for RTK activation of Ras (Sadoshima & Izumo, 1996). Src-mediated phosphorylation of the adaptor protein Shc recruits Grb2-Sos complexes to the plasma membrane facilitating Ras activation leading to

ERK activation (Gutkind, 1998a; Dikic & Blaukat, 1999). Alternatively, neuropeptide stimulated ERK activity may involve activation of the $Ca^{2+}$-dependent focal adhesion kinase, Pyk2 (Belcheva & Coscia, 2002). Stimulation of the $G_q$-coupled bradykinin receptor induced Pyk2 activation, Src recruitment, Src mediated Pyk2 phosphorylation, and binding of Grb2 (Dikic *et al*, 1996). In addition, the dependence of GPCR-mediated ERK1/2 activation on intact focal adhesions has been found to correlate with Pyk2 expression in some cell types (Della Rocca *et al*, 1999). Thus, neuropeptide signalling to ERK probably utilises Src to mediate Ras activation.

It thus appears that activation of ERK by neuropeptides occurs simultaneously through parallel signalling pathways, whereby the predominant ($G_q$) pathway in CHO-K1 cells is likely to be mediated by PKC and is EGFR independent, whereas PI3K, EGFR and Src activation contribute to the minor ($G_i$) pathway. In contrast, substance-P analogues activate only a subset of the downstream signals available to the neuropeptide receptor ($G_i$-mediated). Figure 3.34 illustrates the multiple signalling pathways which may putatively link activated GRP and $V_{1A}$ receptors to ERK activation in transfected CHO-K1 cells, in accordance with the 'multi-track' model for GPCR signalling to the ERK pathway as proposed by Wetzker and Bohmer (2003).

A) Neuropeptide stimulated pathways




B) Substance-P analogue stimulated pathways

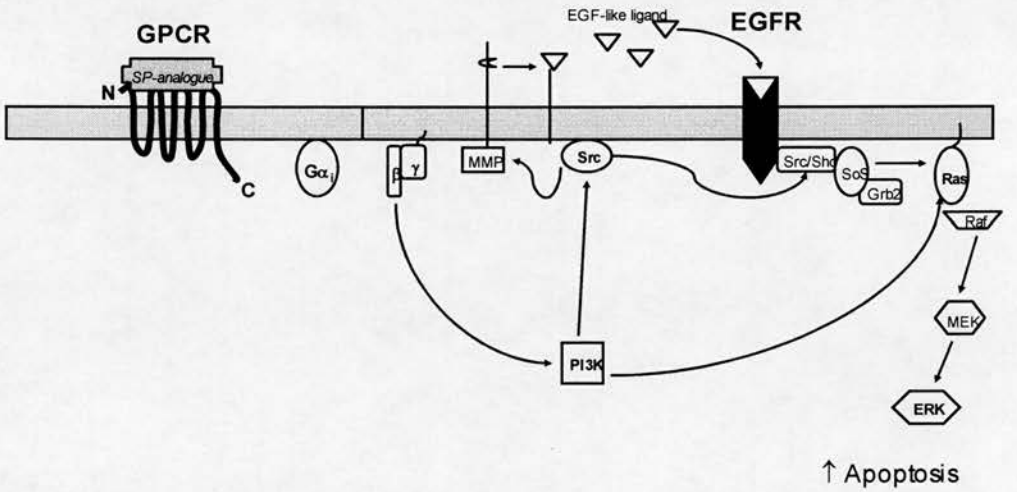



**Figure 3.34. Multi-track signalling pathways leading to ERK activation following neuropeptide receptor stimulation in receptor expressing CHO-K1 cells.** A) ERK activation by bombesin or vasopressin. B) ERK activation by Substance-P analogues SP-D and SP-G. Intermediates identified as being effectors of ERK activity in the present study are highlighted in bold. Signalling components are shaped according to their function as follows: G proteins as vertical ellipses; non-receptor tyrosine kinases as horizontal ellipses; Ser-Thr kinases as larger hexagons; $Ca^{2+}$ as circles. $IP_3$=inositol triphosphate; MMP=matrix metalloproteases; PtdIns= phosphatidylinositide. (Figure adapted from Belcheva *et al*, 2002).

The analogues, particularly SP-G, also induced ERK activation in CHO-vector cells. However, ERK activation in receptor-expressing cells was greater than that observed in vector-transfected CHO-K1 showing that this effect is at least partly receptor-mediated. Analogue induced ERK activation in CHO-vector cells may have been via stimulation of the endogenously expressed GRPR present at low levels or other endogenously expressed analogue sensitive neuropeptide receptors. As well as binding to neuropeptide receptors, SP-D has also been shown to bind to non-neuropeptide receptor members of the same GPCR subfamily (e.g. IL-8 chemoattractant receptor) (Jarpe *et al*, 1998). Sphingosine-1-phosphate induced intracellular calcium mobilisation in CHO-K1 cells (data not shown) therefore the cells probably express receptors for this chemoattractant. Therefore, it is conceivable that the substance-P analogues may have stimulated ERK activation via such receptors endogenously expressed in CHO-vector cells.

Alternatively, analogue stimulated ERK activity in CHO-vector cells could have been due to direct G-protein activation by the analogues. Some substance-P analogues have been shown to inhibit the interaction between receptor and G-protein by directly binding to the G-protein (Mukai *et al*, 1992). In H69 SCLC membranes, SP-G was found to stimulate G-protein activity and this activity was not blocked by a selective $V_{1A}$ receptor antagonist (MacKinnon *et al*, 1999). It was therefore suggested that SP-G may directly modulate G-protein activity as well as interact with neuropeptide receptors.

However, analogue binding to a site distinct from the agonist/antagonist binding site (as the radioligand binding studies suggest) could also account for the failure of the $V_{1A}$ receptor antagonist to block SP-G stimulated G-protein activity. Data presented in this chapter similarly showed that analogue induced ERK activation was not blocked by selective receptor antagonists whereas neuropeptide stimulated ERK was. This adds support to the indication that the analogues bind to a site distinct from the orthosteric agonist binding site to mediate their effects. Nonetheless, it is not possible at this point to conclude whether the distinct analogue binding site is associated with direct interaction with G-proteins or not.

As mentioned previously however (page 136), ERK activation by the analogues is enhanced in receptor-expressing cells and thus cannot be attributed to direct G-protein stimulation alone.

The ability of substance-P analogues to stimulate G-protein activity directly is based upon the amphiphilic nature of the peptides. Amphiphilic peptides such as mastoparan, bradykinin, substance-P and some analogues of substance-P are able to directly promote G-protein activation (Mousli *et al*, 1990b). It has been suggested that mastoparan and substance-P mimic agonist-bound receptors through direct interaction with the C-terminus of G protein alpha subunits to stimulate G-protein activity (Mousli *et al*, 1990a). The N-terminal portion of substance-P is believed to be responsible for such receptor-independent effects whereas the C-terminal region is thought to mediate receptor-dependent effects via interaction with specific neurokinin receptors (Landry *et al*, 1990). Data presented in this chapter showed that SP-G, SP-D and Spantide all inhibit radioligand binding and neuropeptide induced calcium flux while only SP-G and SP-D activate ERK. ERK activation by SP-D and SP-G via the GRP and $V_{1A}$ receptors occurs at concentrations that completely inhibit neuropeptide binding to receptors. Spantide used at a concentration which completely inhibits neuropeptide binding to the GRP receptor does not activate ERK. This illustrates that the process of inhibiting $G_q$ mediated signalling can be dissociated from $G_i$ coupling for ERK activation. A structure-function analysis of substance-P analogues indicates that the analogues could have similar properties to substance-P to achieve such effects. In such a case, the C-terminal region of the analogues would be responsible for receptor mediated effects such as inhibition of radioligand binding and neuropeptide induced calcium mobilisation whereas the N-terminal region would mediate receptor independent effects such as G-protein stimulation for ERK activation. Further to this, it is only the N-terminal region which differs between SP-D [D-Arg$^1$,D-Phe$^5$,D-Trp$^{7,9}$,Leu$^{11}$] and Spantide [D-Arg$^1$,D-Trp$^{7,9}$,Leu$^{11}$], with Spantide lacking an amino acid substitution at position 5 of the peptide. Whereas this residue is polar uncharged in Spantide, in SP-D it is substituted with a residue which has an aromatic side chain. Thus substituting a

hydrophilic moiety for a hydrophobic one at this position appears to be critical for agonist activity, as well growth inhibitory capacity. The bulky hydrophobic side chain may participate in hydrophobic interactions with the receptor to elicit steric effects on the conformation of the receptor, facilitating efficient receptor coupling with differential G-proteins (e.g. $G_i$ for ERK activation). Alternatively, hydrophobic interactions may occur directly with the G-protein for activation. Another substance-P analogue, ICRT5 [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$], which was identified as being the most potent inhibitor of SCLC growth (Seckl *et al*, 1997), has an even bulkier aromatic side chain at this position. This may enable it to be even more effective in stabilising a G protein–selective receptor conformation and/or stimulating G-protein activity and thus account for its superior potency. Also in accordance with this hypothesis, deletion of the C-terminal Leu of SP-D to yield [D-Arg$^1$,D-Phe$^5$,D-Trp$^{7,9}$]SP1-10 resulted in a selective loss of inhibitory activity of this analogue against bombesin- but not vasopressin-stimulated DNA synthesis, Ca2+ mobilization, and MAP kinase activation (Seckl *et al*, 1996a). The data presented in this chapter showed that SP-G which has Met$^{11}$ at the C-terminus is selective for the $V_{1A}$ receptor. Since SP-D without Leu$^{11}$ is selective for the $V_{1A}$ receptor (Seckl *et al*, 1996a), this suggests that the penultimate amino acid of SP-G and SP-D contributes to $V_{1A}$ receptor selectivity whereas the final amino acid dictates GRP receptor selectivity. Since Spantide lacks agonist activity, it appears to act no differently than a classical antagonist other than that it binds to a site distinct from the orthosteric agonist binding site. It could thus be the case that the C-terminal region confers antagonist properties upon the peptides whereas the N-terminal portion dictates agonist properties, with a biased agonist possessing both of these properties. Further structure-function analyses of various substance-P analogues should be carried out to verify this hypothesis.

To summarise, the question addressed in this chapter was whether substance-P analogues are biased agonists of receptors other than the GRP receptor and the mechanism underlying such activity was also investigated. As well as verifying that

SP-D is indeed a biased agonist for the GRP receptor, this study has shown that SP-G is also a biased agonist for this receptor. Importantly, these analogues are also biased agonists of $V_{1A}$ receptors. It could thus be advocated that the growth inhibiting substance-P analogues are broad spectrum biased agonists. The substance-P analogues modulate neuropeptide receptor signalling by stabilising a receptor conformation (possibly dimers) which favours coupling to $G_i$ rather than $G_q$ proteins and the subsequent activation of only a subset of possible downstream signals. The inapt signalling transduced through the normally mitogenic receptor may contribute to the growth inhibitory effects of these analogues and is the subject of study in the following chapter.

# Chapter 4

# Effect of Neuropeptide Receptor Expression and Substance-P Analogues on Growth

The agonist-dependent transformation of cells through ectopic expression of wild-type GPCRs (e.g 5-$HT_{2C}$ receptors) demonstrates that GPCRs have oncogenic potential (Julius *et al*, 1989; Gutkind *et al*, 1991). SCLC-secreted neuropeptides can contribute to tumourigenesis in a similar manner through chronic stimulation of GPCRs in their role as cancer growth factors. GPCRs can also be constitutively active in a manner dependent on the cellular environment in which the GPCR is ectopically expressed and has been observed for the GRP receptor over-expressed in a non-malignant human colon epithelial cell-line (Ferris *et al*, 1997). The work in this chapter was carried out to determine whether expression of GRP and $V_{1A}$ receptors can transform CHO-K1 epithelial cells. The characteristics of the wild-type CHO-K1 cells were compared with neuropeptide receptor expressing cells in terms of growth, migration and adhesion. The chemosensitivity of the model cell systems was also investigated to ascertain whether neuropeptide growth factor activity plays a role in the acquisition of chemoresistance.

Substance P analogues can inhibit the growth of various tumour types and this has been shown to correlate with GRP receptor expression (Waters *et al*, 2003). Since the $V_{1A}$ receptor has been found to be expressed on all SCLC cell-lines tested to date, it is important to determine whether cells expressing $V_{1A}$ receptors are sensitive to growth inhibition by substance P analogues. The work presented in this chapter was therefore performed to investigate the effect of SP-D and SP-G on growth of the model cell systems. The ability of the analogues to modulate the motility of neuropeptide receptor expressing cells was also investigated.

## 4.1. Sensitivity of SCLC cells to Substance-P analogues

A preliminary experiment was performed to verify the ability of the selected analogues, SP-D and SP-G, to inhibit the growth of small cell lung cancer cells. The proliferation of H345 SCLC cells was measured in the absence and presence of various concentrations of substance-P analogues. Cell numbers were determined by coulter counter at various time-points over a 12-14 day period. Data from cells in exponential growth phase (day 12 counts) were used to determine the $IC_{50}$ values (concentration of drug required to cause 50% inhibition of control growth) for each analogue.

The resulting time course in liquid culture revealed that both analogues inhibited H345 growth in a dose-dependent manner. Concentrations of SP-D and SP-G at or above 30μM reduced the number of cells present over time compared with untreated cells. The $IC_{50}$ for inhibition of H345 proliferation by SP-D was 14 ± 3 μM (n=2, Figure 4.1). SP-G inhibited H345 proliferation less potently with an $IC_{50}$ = 27.2 ± 1.6 μM (n=2, Figure 4.2). In contrast, spantide did not inhibit H345 proliferation (Figure 4.3). This shows that H345 SCLC cells are sensitive to growth inhibition by both SP-D and SP-G but not spantide.

## 4.2 Cell growth in liquid culture

### 4.2.1. Effect of neuropeptide receptor expression on cell growth and morphology in liquid culture

Through the course of routine tissue culture, it became apparent that whereas the parental CHO-K1 cells had regular, elongated shapes, CHO-GRP (Figure 4.4) and CHO-$V_{1A}$ (Figure 4.5) cells appeared more rounded. The neuropeptide receptor expressing cells also exhibited increased membrane ruffling and possessed more granules. This shows that increased neuropeptide receptor expression has an impact on cell morphology.

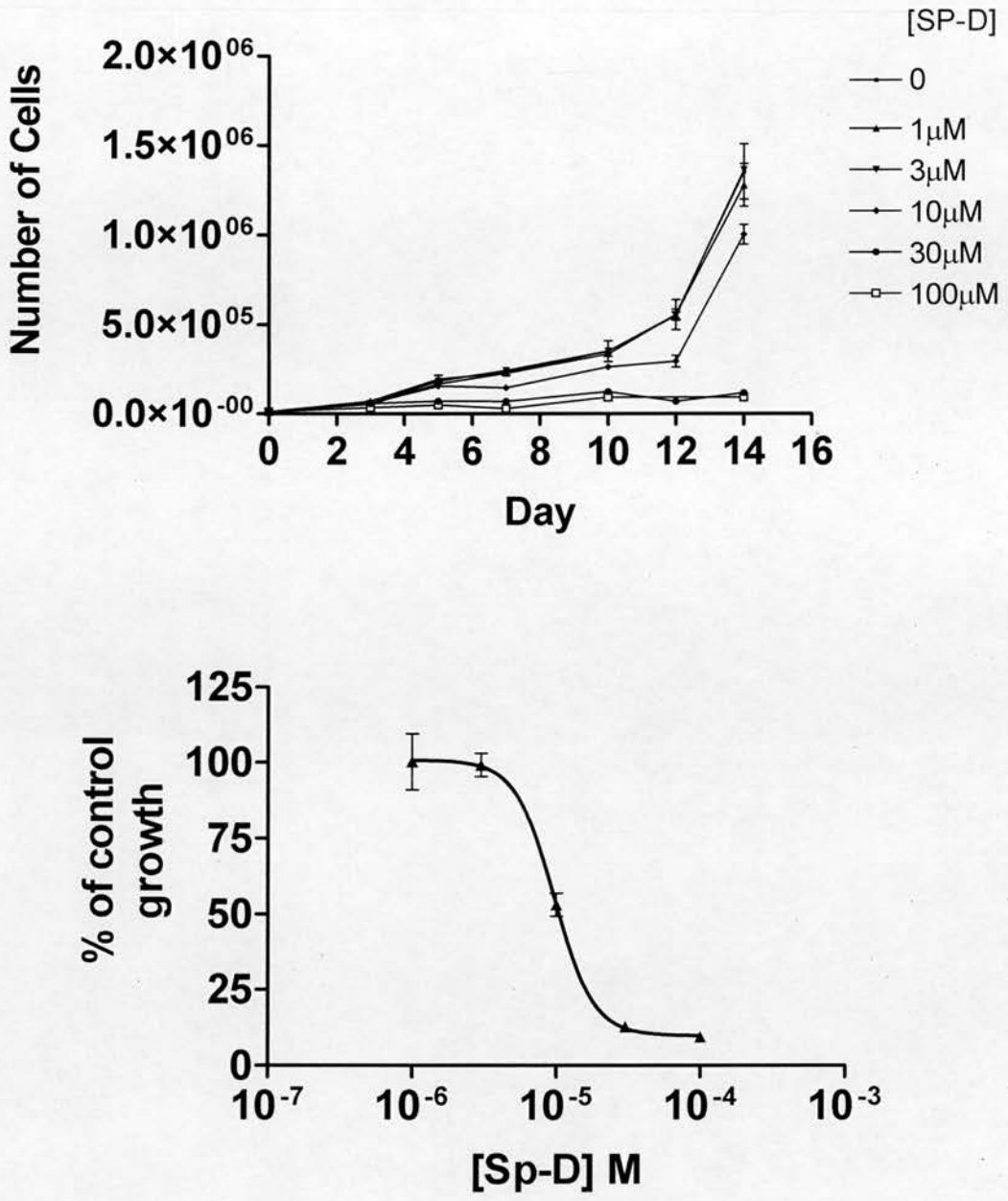



**Figure 4.1. Growth inhibition of H345 cells by SP-D.** A) H345 SCLC cells were incubated with increasing concentrations of SP-D. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean ± s.e.m. of triplicate samples. B) $IC_{50}$ determination for SP-D using day 12 counts from two independent experiments to plot curve. Curve fitting was carried out using non-regression analysis of data in Prism. Mean $IC_{50} = 14 \pm 3$ μM

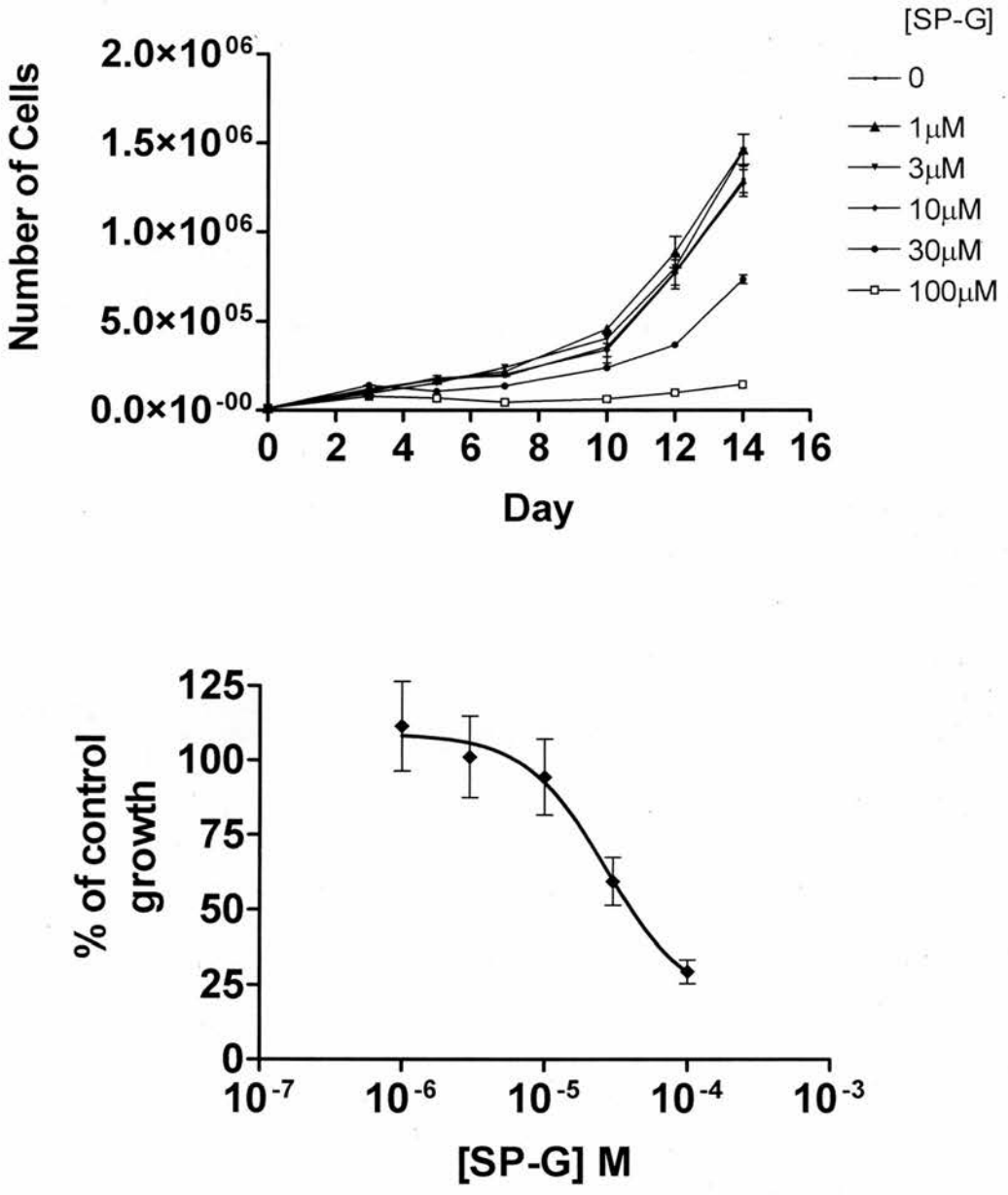



**Figure 4.2. Growth inhibition of H345 cells by SP-G.** A) H345 SCLC cells were incubated with increasing concentrations of SP-G. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean ± s.e.m. of triplicate samples. B) $IC_{50}$ determination for SP-G using day 12 counts from two independent experiments to plot curve. Curve fitting was carried out using non-regression analysis of data in Prism. Mean $IC_{50} = 27.2 \pm 1.6$ μM

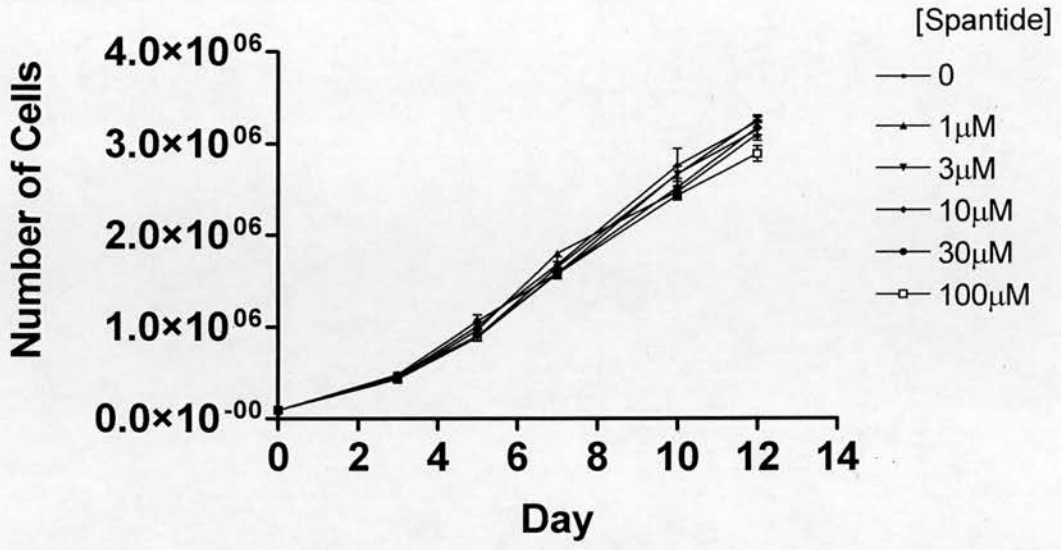



**Figure 4.3. Growth inhibition of H345 cells by Spantide.** H345 SCLC cells were incubated with increasing concentrations of Spantide. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean ± s.e.m. of triplicate samples.

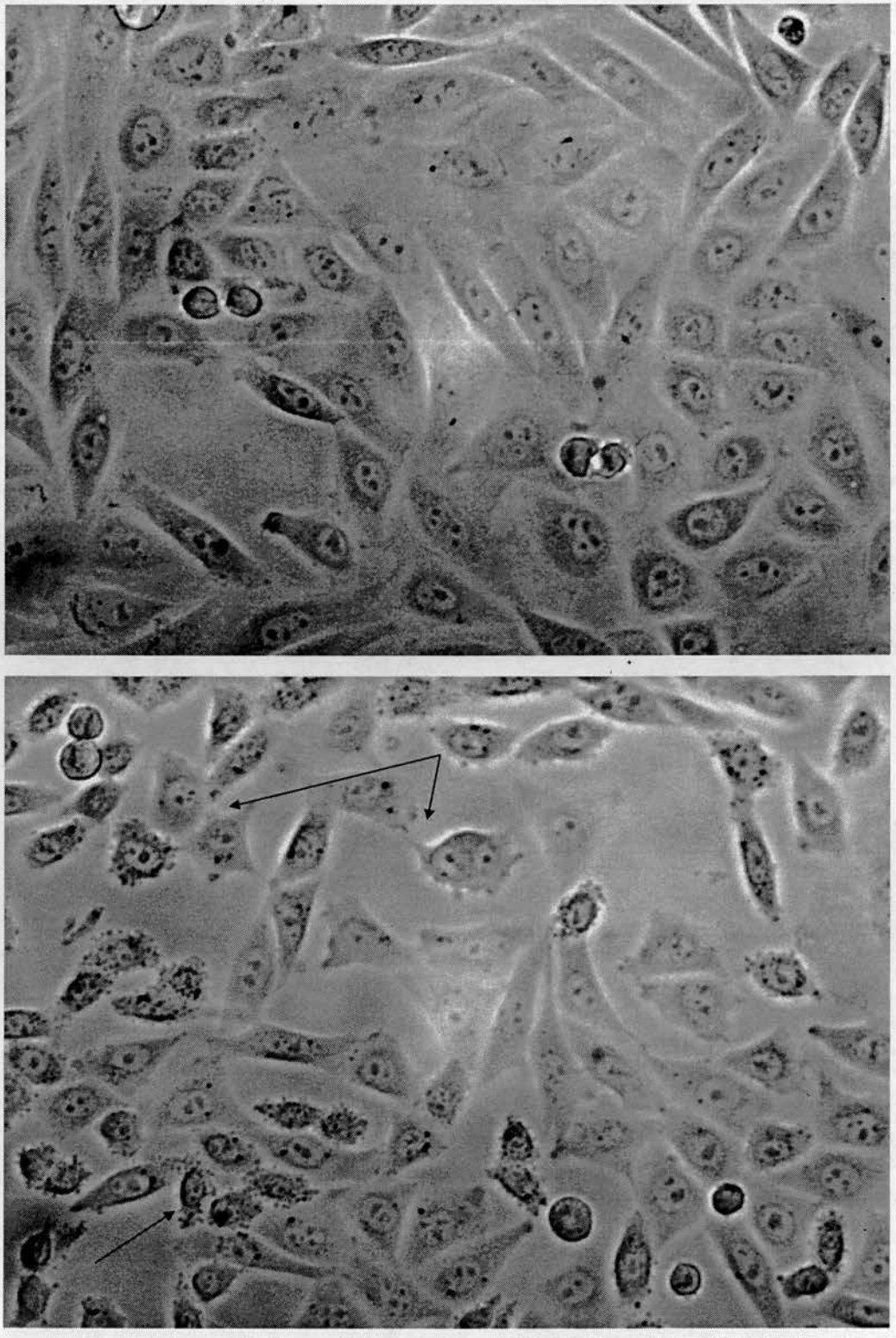


**Figure 4.4. Morphology of sub-confluent vector and GRPR transfected CHO-K1 cells.** Top: Vector transfected CHO-K1 cells have regular elongated shapes. Bottom: CHO-GRP cells are more rounded and numerous ruffled edges and granules are more apparent (arrows). 32X magnification DIC images.

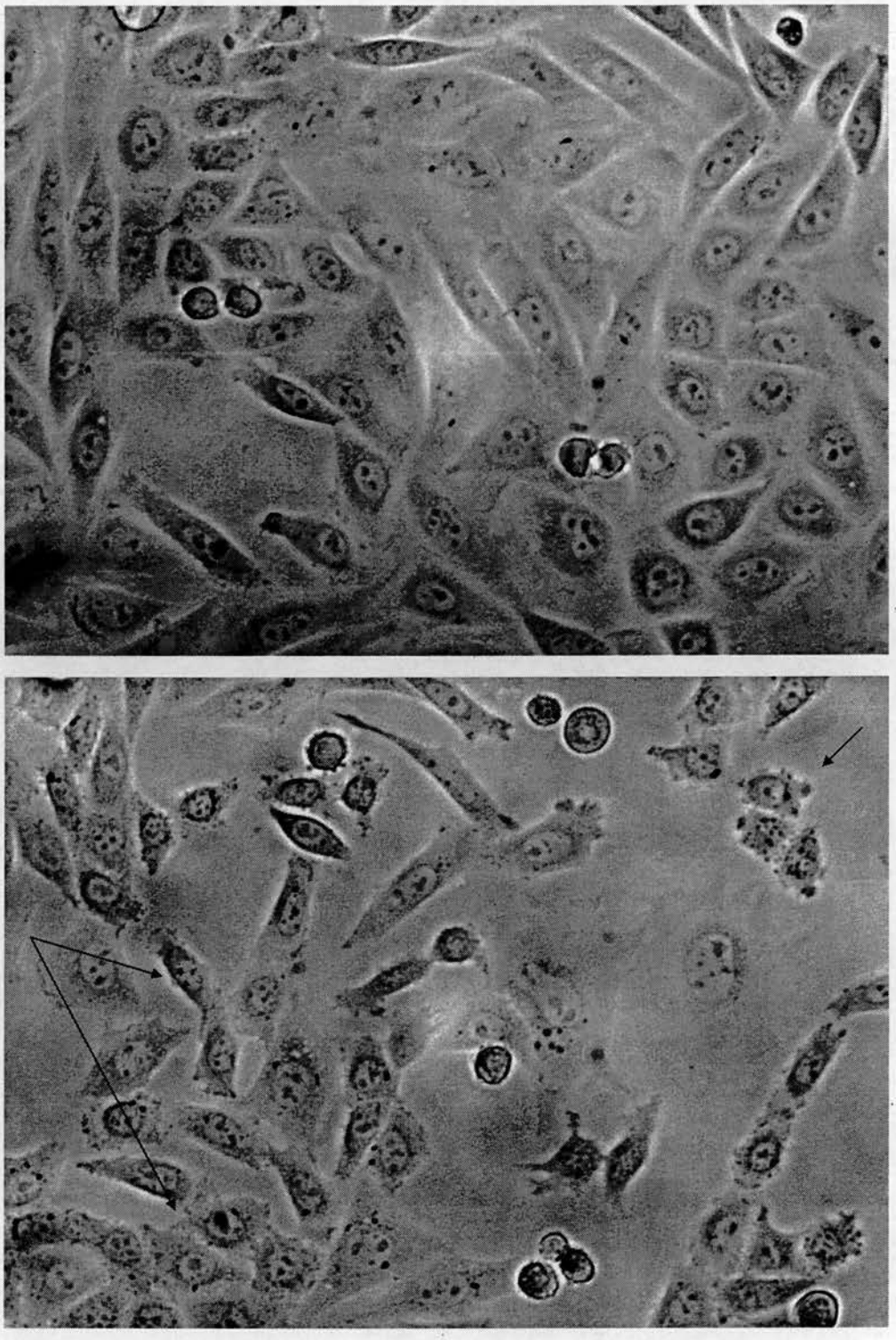


**Figure 4.5. Morphology of sub-confluent vector and $V_{1A}R$ transfected CHO-K1 cells.** Top: Vector transfected CHO-K1 cells have regular elongated shapes. Bottom: CHO-$V_{1A}$ cells are more rounded and numerous ruffled edges and granules are more apparent (arrows). 32X magnification DIC images.

The growth characteristics of the CHO-GRP and CHO-$V_{1A}$ cells also appeared to differ from the parental CHO-K1 cells. An investigation into the growth of these cells in normal liquid culture showed that in the presence of 5% FCS, the CHO-GRP and CHO-$V_{1A}$ cells initially proliferated at a similar rate to untransfected CHO-K1 cells. However, differences in the growth rates became apparent by the fourth day after seeding (Figure 4.6). Six days post-seeding, CHO-GRP cells had reached a density of 4.5 x $10^6$ cells per 100 mm dish. At confluence, the cells did not appear to be contact inhibited and exhibited the rounded morphology. Cells over grew one another and significant numbers of cells were observed to be detached and growing in clusters (Figure 4.7). Trypan blue exclusion revealed that the detached cells were >95% viable. A similar though less pronounced effect was observed with the CHO-$V_{1A}$ cells, which reached a density of 3.1 x $10^6$ cells per 100 mm dish. The parental cell line exhibited normal contact inhibition, had flat epithelial morphology and did not show significant detachment. The endpoint density of these cells was 2.1 x $10^6$ cell per 100 mm dish. These observations suggest that transfection of GRP and $V_{1A}$ receptors cause CHO-K1 cells to lose contact inhibition and adopt a more transformed phenotype.

### 4.2.2. Effect of substance-P analogues on growth in liquid culture

The effect of substance-P analogues on CHO-vector, CHO-GRP and CHO-$V_{1A}$ cell growth in normal liquid culture was determined by counting cells after a 5 day incubation with media containing 5% FCS in the presence or absence of 30μM SP-D or SP-G. Figure 4.8 shows that SP-D inhibited CHO-GRP and CHO-$V_{1A}$ cell growth ($P<0.01$, $n=2$) but not the growth of CHO-vector cells. Similarly, SP-G did not inhibit the growth of CHO-vector cells whereas CHO-GRP ($P<0.01$, $n=2$) and CHO-

$V_{1A}$ ($P<0.01$, n=2) cell growth was inhibited. This shows that neuropeptide receptor expression confers sensitivity to substance-P analogue induced growth inhibition.

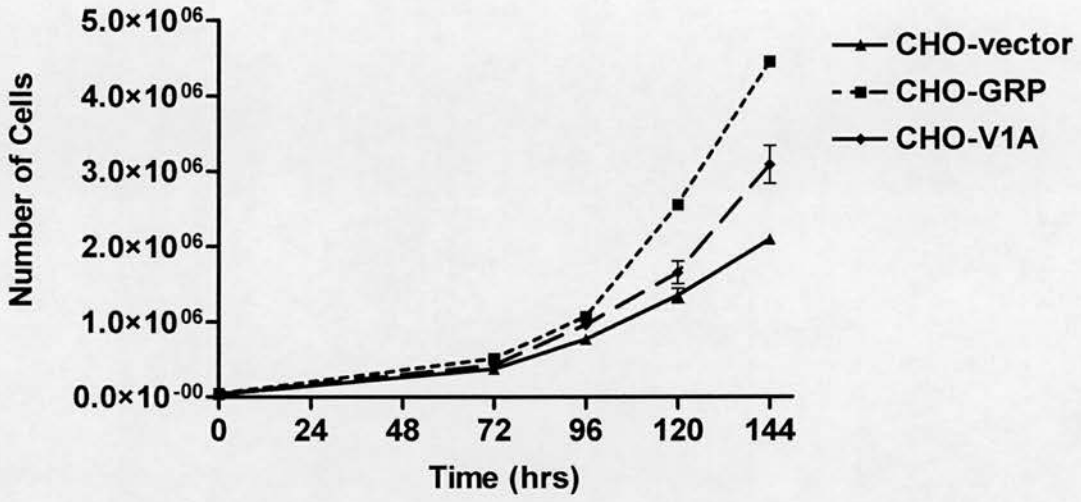



**Figure 4.6. Effect of neuropeptide receptor expression on cell growth.** CHO-K1 cells expressing vector, the GRP or $V_{1A}$ receptor were plated at a density of $1x10^4$ cells/100 mm tissue culture dish in 5% FCS and incubated at 37°C. Cells were harvested at various times and counted. Results represent the mean ± s.e.m. of 3 experiments performed in duplicate.

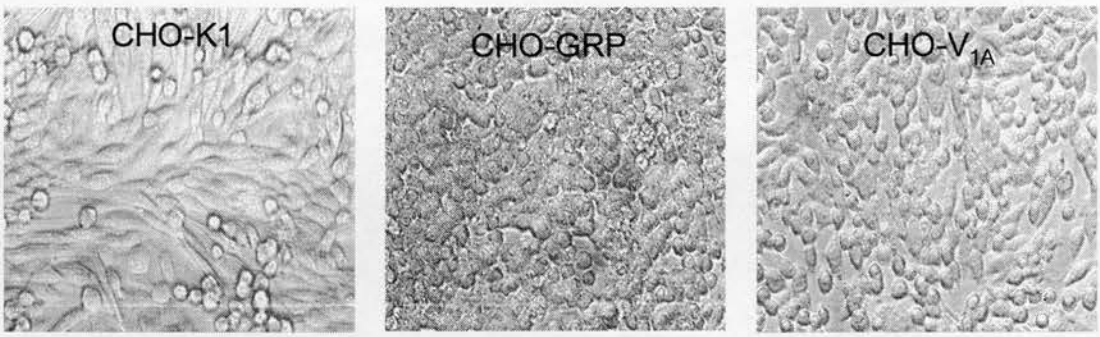



**Figure 4.7. Cell morphology of vector and neuropeptide receptor transfected CHO-K1 cells at confluence.** The rounded cobblestone features and higher cell density are evident in neuropeptide receptor expressing cells (CHO-GRP- middle panel; CHO-$V_{1A}$- right panel) compared to the elongated contact inhibited monolayer exhibited by vector transfected controls (left panel).

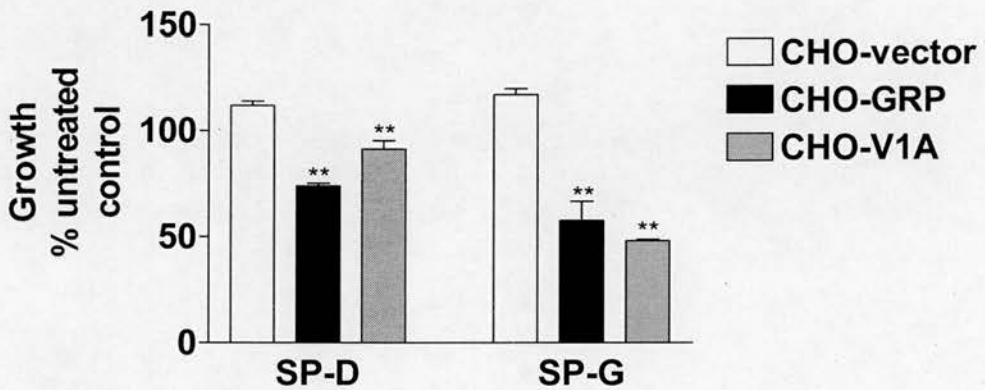



**Figure 4.8. Effect of Substance-P analogues on liquid growth.** Vector and neuropeptide receptor expressing CHO-K1 cells were seeded at a density of $1x10^4$ cells/100 mm tissue culture dish in 5% FCS media alone (control) or with 30μM SP-D or SP-G. Cells were harvested following 5 days incubation at 37°C and counted using a coulter counter. Data is expressed as % of cell numbers in untreated control and represents the mean ± s.e.m. of 2 experiments performed in duplicate (** $P<0.01$; ANOVA).

## 4.3 Cell growth in semi-solid media

### 4.3.1. Effect of neuropeptide receptor expression on growth in semi-solid media

Growth in semi-solid media is widely regarded as being a hallmark of the transformed phenotype. The effect of neuropeptide receptor expression on growth in semi-solid media containing 1% or 5% FCS was examined over an 11 day period. Cells were trypsinised and suspended in 0.3% agarose in DMEM ($1 \times 10^4$ cells $ml^{-1}$) over a 0.5% layer of agarose in DMEM. Clusters of cells (>6 cells) were visualised by MTT staining and counted to enable the cloning efficiency of the cells to be determined. Figure 4.9 shows that following 9 days of growth in 5% FCS, the cloning efficiency was higher in the GRP expressing cells (14.1 ± 1.5%, $P<0.05$, ANOVA) and the $V_{1A}$ receptor expressing cells (8.5 ± 1.0%, $P<0.05$, ANOVA) than the vector control cells (5.5 ± 0.02%). Colony formation was also evident in low serum, although a statistically significant difference in cloning efficiency did not occur at every time-point. Nevertheless, a trend of increased cloning efficiency was exhibited, particularly by the CHO-GRP cell line. The results suggest that neuropeptide receptor expression confers anchorage-independent growth capability upon CHO-K1 cells.

### 4.3.2. Effect of substance-P analogues on growth in semi-solid media

In a separate series of experiments the effects of SP-D and SP-G on cell growth in semi-solid media were examined. During an 8 day incubation with 30μM SP-D or SP-G, both analogues significantly reduced colony formation by CHO-GRP and CHO-$V_{1A}$ cells, but had no effect on the cloning efficiency of vector control CHO-

K1 cells (Figure 4.10). This shows that neuropeptide receptor expression confers sensitivity to substance-P analogue induced inhibition of clonogenicity.

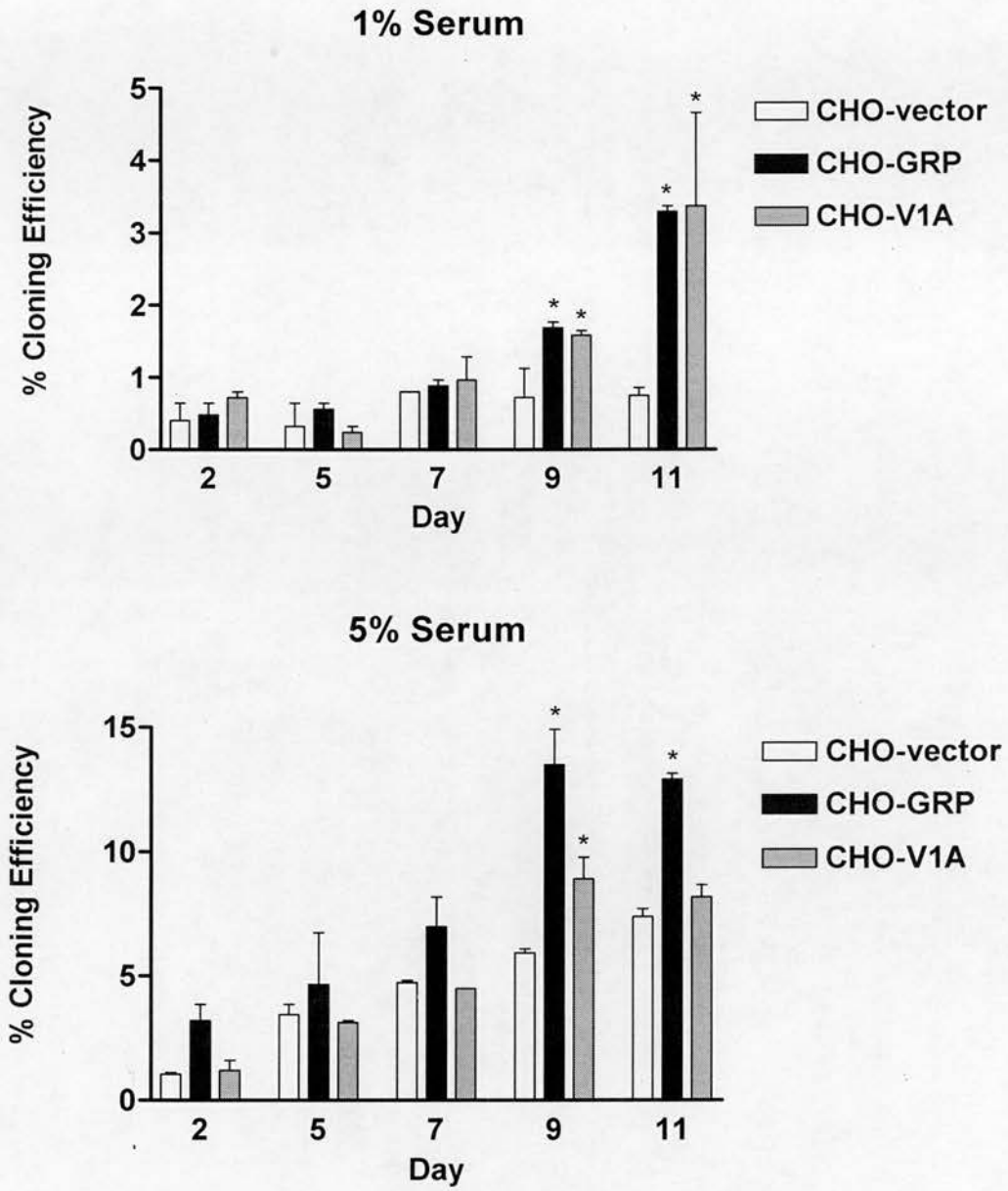



**Figure 4.9. Effect of neuropeptide receptor expression on growth in semi-solid media.** Sub-confluent CHO-vector, CHO-GRP or CHO-$V_{1A}$ cells were trypsinised and suspended (1 x $10^4$ cells $ml^{-1}$) in 0.3% agarose in DMEM containing 1% FCS (top) or 5% FCS (bottom) over a 0.5% layer of agarose in DMEM. At various time-points, cells were stained with MTT and colonies (>6 cells) counted at X10 magnification. Bar graph represents the mean ± s.e.m. of three experiments performed in duplicate ( *P<0.05, ANOVA).

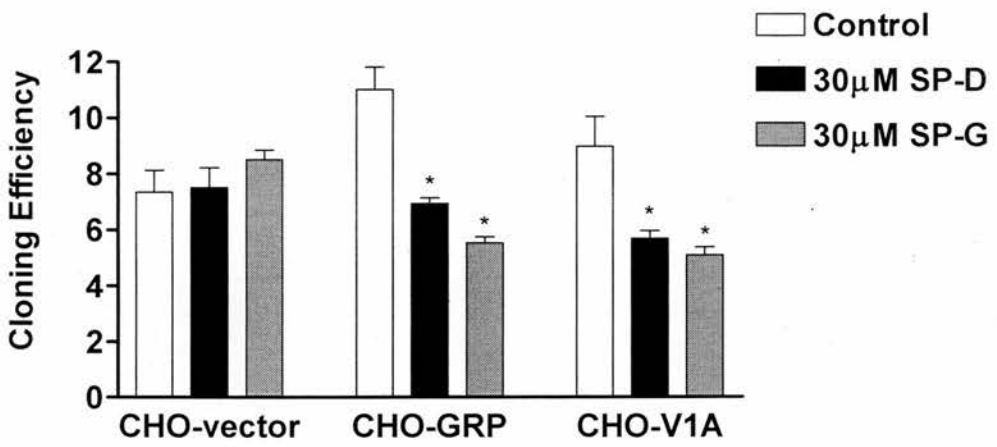



**Figure 4.10. Effect of Substance-P analogues on growth in semi-solid media.** Sub-confluent CHO-vector, CHO-GRP or CHO-$V_{1A}$ cells were trypsinised and suspended (1 x $10^4$ cells $ml^{-1}$) in 0.3% agarose in 5% FCS-DMEM in the absence (open bar) or presence of 30μM SP-D (black bar) or SP-G (grey bar) over a 0.5% layer of agarose in DMEM. After an 8 day incubation at 37°C, cells were stained with MTT and colonies (>6 cells) counted at X10 magnification. Bar graph represents the mean ± s.e.m. of three experiments performed in duplicate ( *P<0.05, ANOVA).

## 4.4 Cell growth in suspension

### 4.4.1. Effect of neuropeptide receptor expression on growth in suspension

An alternative method for evaluating the transformed phenotype is through an aggregation assay where cells are prevented from adhering to solid substrata and instead grow as aggregates in suspension. The effect of receptor expression on growth in suspension was examined. Stably transfected CHO-K1 cells were suspended in DMEM containing 1% or 5% FCS and seeded at a density of $2x10^4$ cells per well into ultra low attachment tissue culture plates over a solid base of 0.5% agar. Cells were maintained in culture for up to 9 days and viable cell number determined at various time-points by TO-PRO3 exclusion. There was no significant difference between the ability of these cell-lines to grow as aggregates in media containing 1% FCS (Figure 4.11). However, by the fifth day of growth in suspension in media containing 5% FCS, a small difference in growth was observed in receptor expressing cell-lines relative to CHO-vector cells. This provides further evidence to suggest that neuropeptide receptor expression leads to CHO-K1 cell transformation. In addition, the morphology of the cell aggregates also differed between vector and receptor transfected cells growing in 5% FCS. Figure 4.12 shows that whereas CHO-vector cells formed large, compact aggregates when grown in suspension, receptor expressing cells grew in numerous small clusters.

### 4.4.2. Effect of substance-P analogues on growth in suspension

The effect of SP-G on cell growth in suspension was examined by treating cells with increasing concentrations of SP-G. After 7 days, cell clusters were disaggregated and viable cells counted. Figure 4.13 shows that SP-G inhibited CHO-GRP and CHO-

$V_{1A}$ cell growth and a slight effect on the growth of CHO-vector cells. This shows that neuropeptide receptor expression confers sensitivity to substance-P analogue-induced growth inhibition.

## 4.5 Effect of neuropeptide receptor expression on chemosensitivity

Since neuropeptide receptor expression led to cell transformation, it was of interest to determine whether receptor expression had any impact on sensitivity to chemotherapeutic drugs. This was assessed through treating vector and receptor transfected cells with etoposide in the absence or presence of neuropeptides and measuring cell growth by MTT accumulation in viable cells. As shown in Figure 4.14, after a 48h treatment with etoposide in serum-free media, a dose-dependent inhibition of proliferation was observed in all cell types ($IC_{50}$ = 12.4 ± 3.1, 8.1 ± 2.3 and 14.2 ± 4.0 μg $ml^{-1}$ in CHO-vector, CHO-GRP and CHO-$V_{1A}$ cells respectively). Co-incubation of CHO-GRP and CHO-$V_{1A}$ cells with 50 nM bombesin or AVP respectively, produced a small but significant protection from etoposide which was not observed when both neuropeptides were added to CHO-vector cells ($IC_{50}$ = 13.0 and 26.9 μg $ml^{-1}$ in control and AVP treated CHO-$V_{1A}$ cells respectively; and 6.30 and 12.7 μg $ml^{-1}$ in control and bombesin treated CHO-GRP cells respectively). At 40 μg $ml^{-1}$ etoposide, vasopressin treated CHO-$V_{1A}$ cells accumulated 92% more MTT than control cells ($P<0.01$). In GRPR expressing cells, bombesin treatment caused a 52% increase in MTT accumulation compared to untreated cells ($P<0.01$). These results suggest that neuropeptide receptor stimulation may contribute to an increase in chemoresistance.

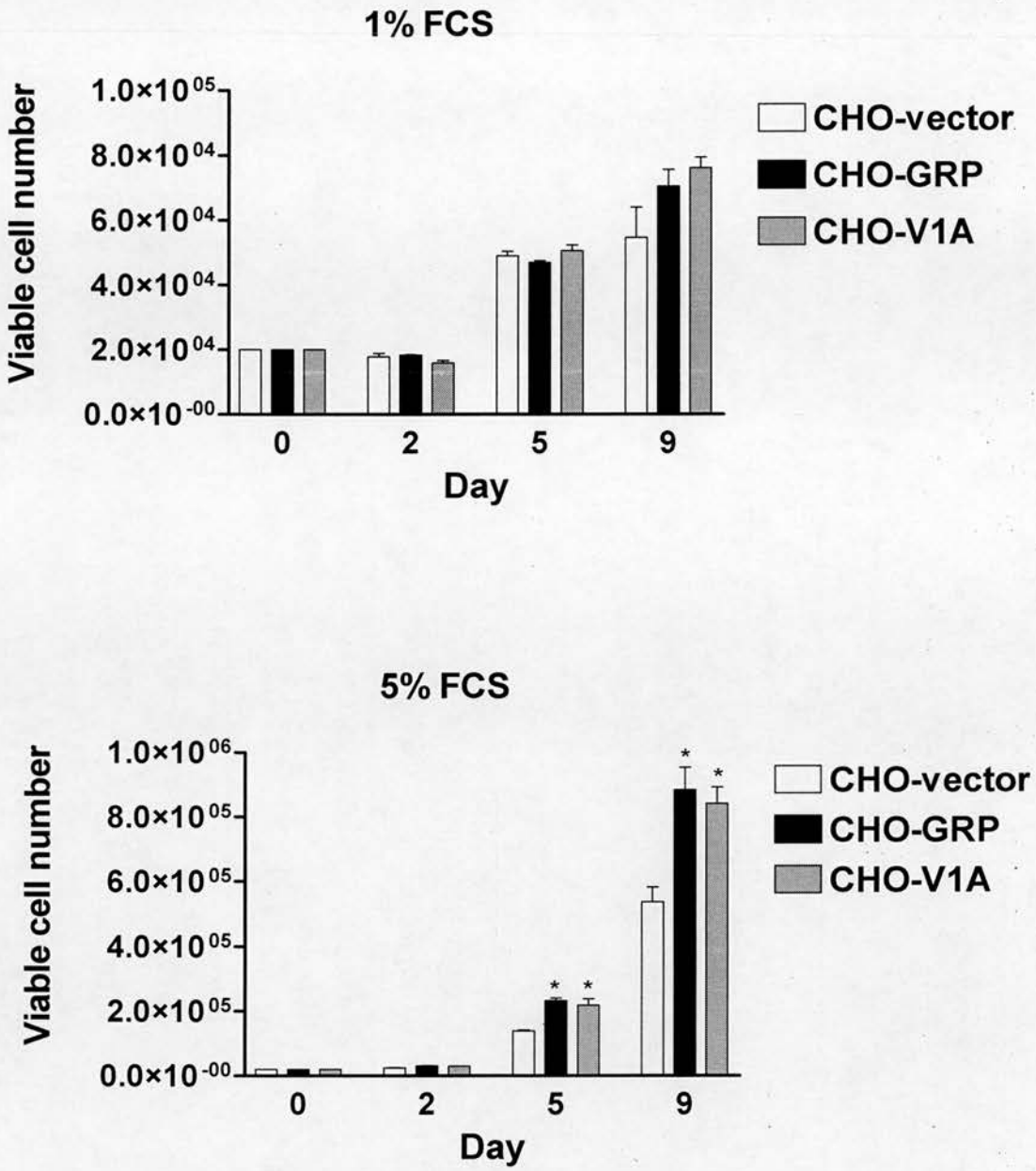



**Figure 4.11. Effect of neuropeptide receptor expression on growth in suspension.** CHO-vector, CHO-GRP and CHO-$V_{1A}$ cells were seeded (2 x$10^4$ cells in media containing 1% FCS (top) or 5% FCS (bottom)) into low adhesion tissue culture plates over a base layer of 0.5% agarose in DMEM. Cells were incubated at 37°C for various time intervals. At each time-point, cells were briefly trypsinised to disaggregate clusters and viable cells counted by TO-PRO3 exclusion using FACS. Bar graphs represent the mean ± s.e.m. of two experiments performed in duplicate (*P<0.05, ANOVA).

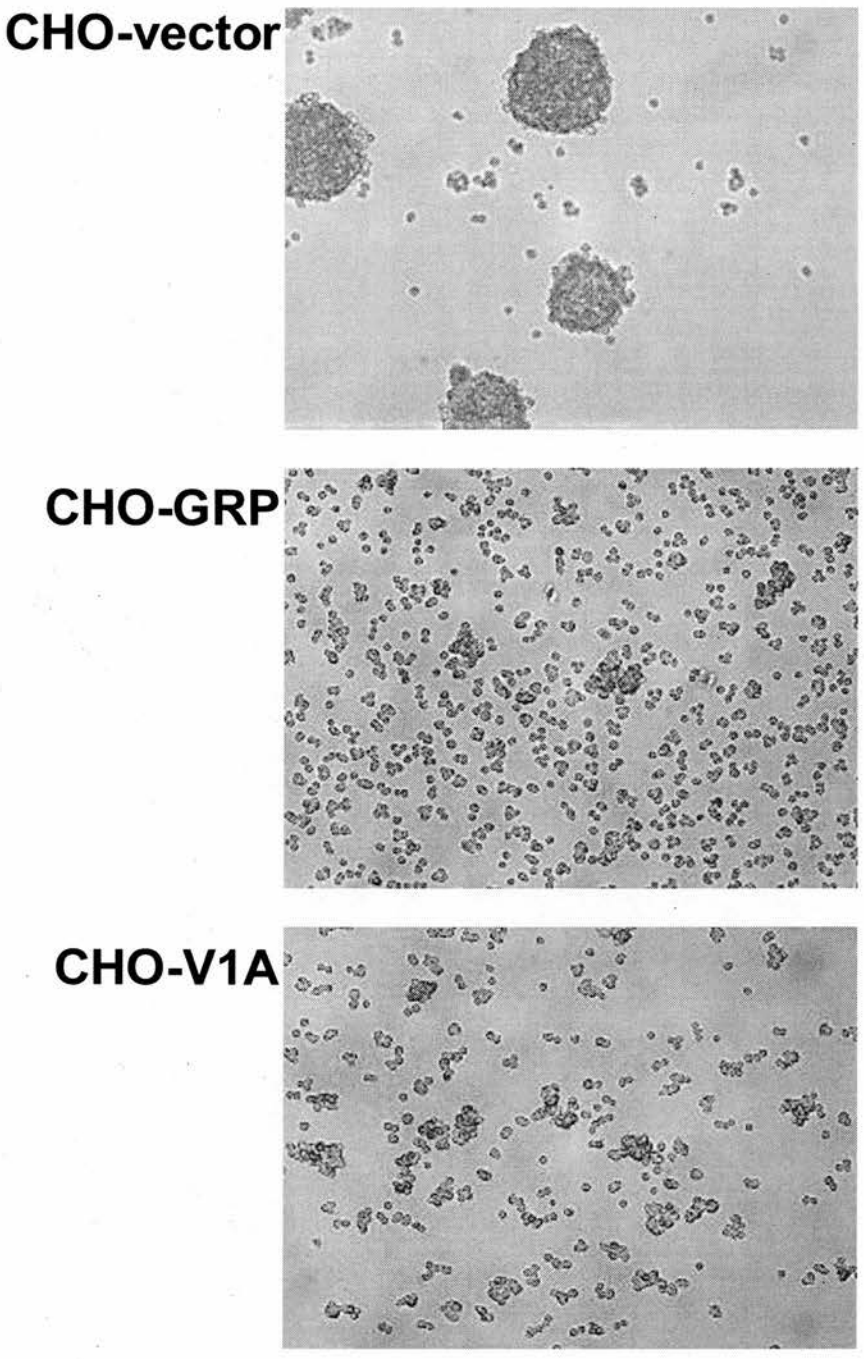



**Figure 4.12. Morphology of cell aggregates.** Morphology of CHO-vector, CHO-GRP and CHO-$V_{1A}$ cells after 9 days growth in suspension in media containing 5% FCS. CHO-vector cells grow as large, compact aggregates whereas neuropeptide receptor expressing cells form smaller, but more numerous clusters. Representative images are shown (x10 magnification)

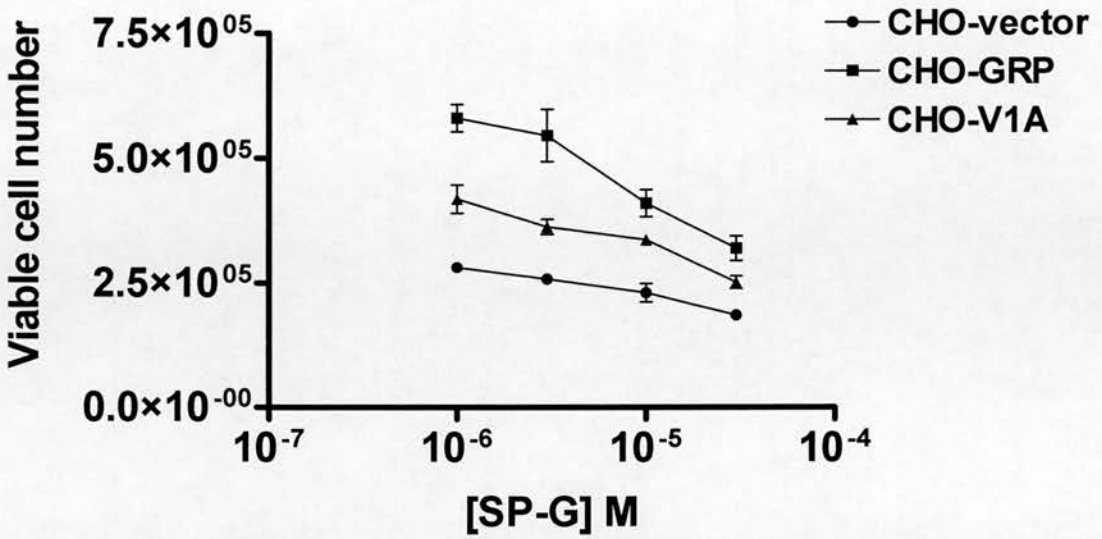



**Figure 4.13. Effect of SP-G on growth in suspension.** CHO-vector, CHO-GRP and CHO-$V_{1A}$ cells were seeded (2 x$10^4$ cells in media containing 5% FCS) into low adhesion tissue culture plates over a base layer of 0.5% agarose in DMEM with increasing concentrations of SP-G. Cells were incubated at 37°C for 7 days then briefly trypsinised to disaggregate clusters. Viable cells were counted by TO-PRO3 exclusion using FACS. Data represents the mean ± s.e.m. of two experiments performed in duplicate.

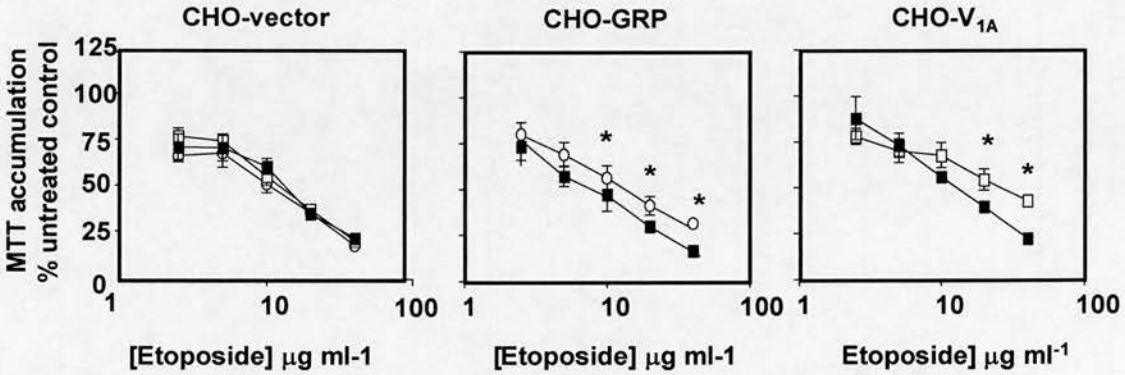



**Figure 4.14. Effect of neuropeptide on chemosensitivity.** CHO-vector (left), CHO-GRP (middle) and CHO-$V_{1A}$ (right) cells were plated at a density of 1 x$10^4$ cells per well of a 96 well tissue culture plate in 10% FCS-DMEM and incubated overnight at 37°C. Cells were then incubated in serum-free media containing etoposide as indicated and in the absence (filled squares) or presence of 50nM GRP (open circles), 50nM AVP (open squares) or both neuropeptides (CHO-vector cells) for 48 hrs at 37°C. Cell viability was assessed by MTT staining. Results are expressed as % viability in the absence of neuropeptide and represents mean ± s.e.m. of four independent experiments (* $P<0.05$ = significantly different from control cells, ANOVA).

## 4.6 Effect of neuropeptide receptor expression on cell adhesion and migration

A combination of events contribute to the invasive process in cancer, with initial changes in adhesive properties allowing transformed cells to migrate, gain access to the circulation and form metastatic colonies (Aprikian *et al*, 1997). Various neuropeptides have been shown to stimulate monocyte and human tumour cell chemotaxis (Ruff *et al*, 1985; Cuttitta *et al*, 1985; Festuccia *et al*, 1998). In addition, monocyte chemotaxis towards SP-D has been demonstrated to occur via bombesin receptors suggesting that the biased agonist activity of these analogues may play a role in normal and tumour cell migration. The work described in this section was carried out in order to investigate the migratory and adhesive properties of the neuropeptide receptor expression-transformed cells.

### 4.6.1. Modulation of cell migration by neuropeptide receptor expression

Chemotactic migration of vector and neuropeptide transfected cells through fibronectin coated filters (8μm pore) was measured in a 96 well format following four hours incubation at 37°C. Cells suspended in 0.1% BSA were added to the upper chamber and chemoattractants added to the lower chamber of the transwell migration plates. In experiments assessing chemotaxis towards FCS (lower chamber), it was found that CHO-GRP and CHO-$V_{1A}$ cells migrated more than CHO-vector cells (Figure 4.15). Similarly, receptor expressing cells migrated more than CHO-vector cells towards IGF-1 (Figure 4.16). However, the magnitude of the chemotactic response to substance-P analogues was similar in all three cell-lines (Figure 4.17). Together these results show that expression of neuropeptide receptors enhances CHO-K1 cell migration generally to growth factors but is not agonist-specific.

### 4.6.2. Modulation of cell adhesion by neuropeptide receptor expression

Cell interaction with extracellular matrix (ECM) proteins influences a tumour cell's ability to form metastases at distant sites. CHO-vector, CHO-GRP and CHO-$V_{1A}$ cell adhesion to the ECM component fibronectin (Fn) was measured by incubating cells (suspended in 0.1% BSA) for 20 min at 37°C in Fn-coated 96 well tissue culture plates. CHO-GRP and CHO-$V_{1A}$ cell adhesion to Fn was reduced (50 ± 9% and 55 ± 13% respectively) compared with the adhesiveness of CHO-vector (69 ± 5%) cells (Figure 4.18). The basal level of adhesion of each cell-line to Fn-coated plates was not significantly altered by incubation with neuropeptides, substance-P analogues or IGF-1 (data not shown). This suggests that neuropeptide receptor expression reduces the cells' overall ability to interact with fibronectin.

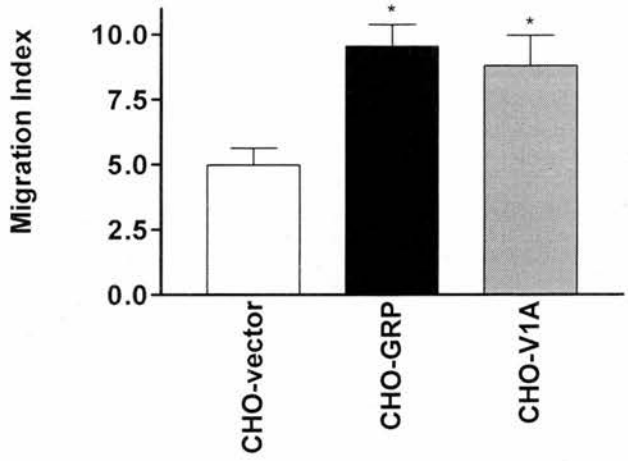



**Figure 4.15. Effect of neuropeptide receptor expression on chemotactic response to FCS.** Sub-confluent CHO-vector, CHO-GRP and CHO-$_{V1A}$ cells were resuspended in DMEM containing 0.1% (w/v) BSA and $1x10^5$ cells added to the upper chamber of a 96 transwell migration plate with fibronectin (10μg $ml^{-1}$) coated filters. FCS was added to the lower chamber and migration allowed to proceed for 4hrs at 37°C. Migration was evaluated as described in materials and methods. Bar graphs represent the OD of agonist-induced migration relative to OD of BSA-induced migration and are the mean ± s.e.m of at least two experiments performed in quadruplicate (*significantly different from vector control cells $P<0.05$, ANOVA).

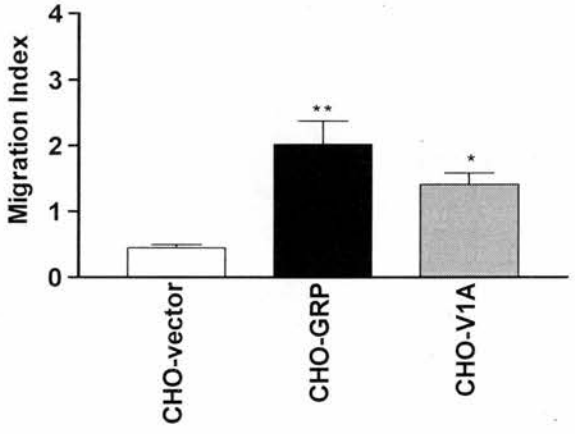



**Figure 4.16. Effect of neuropeptide receptor expression on chemotactic response to IGF-1.** Sub-confluent CHO-vector, CHO-GRP and CHO-$_{V1A}$ cells were resuspended in DMEM containing 0.1% (w/v) BSA and $1x10^5$ cells added to the upper chamber of a 96 transwell migration plate with fibronectin coated filters (10μg $ml^{-1}$). IGF-1 [100ng $ml^{-1}$] was added to the lower chamber and migration allowed to proceed for 4hrs at 37°C. Migration was evaluated as described in materials and methods. Data represents the OD of agonist-induced migration relative to OD of BSA-induced migration and is the mean ± s.e.m of at least two experiments performed in quadruplicate (*$P<0.05$, **$P<0.01$, significantly different from vector control cells, ANOVA).

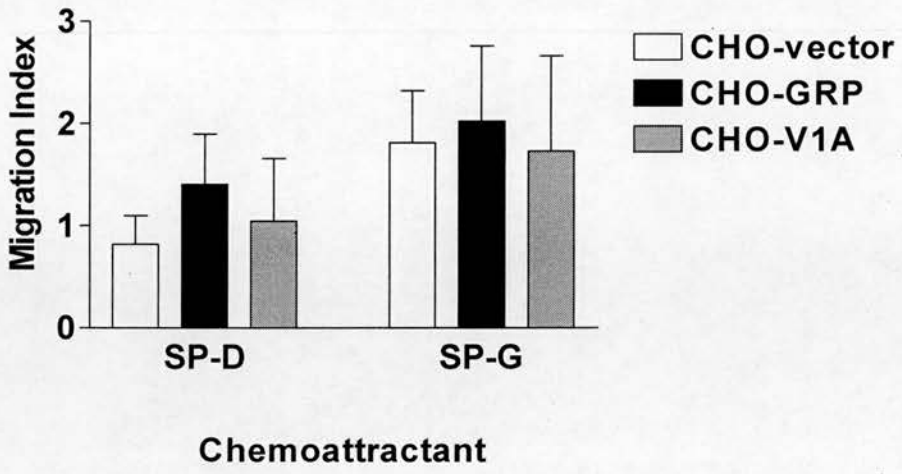



**Figure 4.17. Effect of neuropeptide receptor expression on chemotactic response to substance-P analogues.** Sub-confluent CHO-vector (A), CHO-GRP (B) and CHO-$V_{1A}$ (C) cells were resuspended in DMEM containing 0.1% (w/v) BSA and $1x10^5$ cells added to the upper chamber of a 96 transwell migration plate with fibronectin (10μg $ml^{-1}$) coated filters. 30μM SP-D or SP-G was added to the lower chamber and migration allowed to proceed for 4hrs at 37°C. Migration was evaluated as described in materials and methods. Data represents the OD of agonist-induced migration relative to OD of BSA-induced migration and is the mean ± s.e.m of at least two experiments performed in quadruplicate.

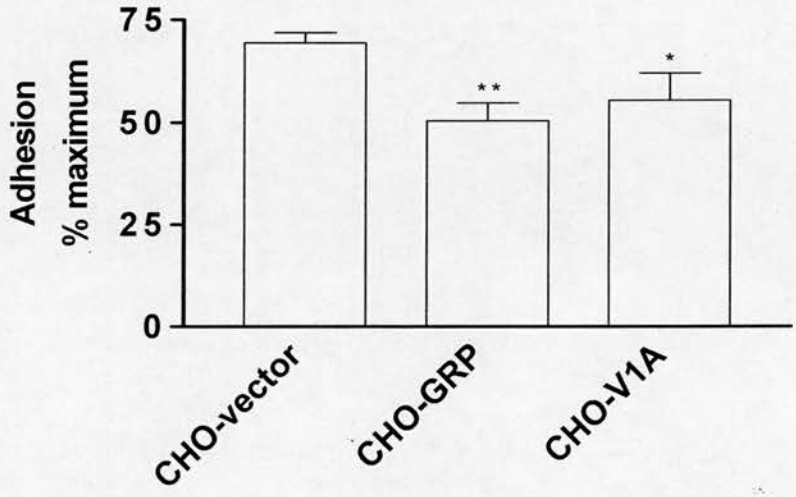



**Figure 4.18. Effect of neuropeptide receptor expression on cell adhesion.** Sub-confluent CHO-vector, CHO-GRP and CHO-$V_{1A}$ cells were resuspended in DMEM containing 0.1% (w/v) BSA. $2x10^4$ cells were added to fibronectin-coated (10μg $ml^{-1}$) wells of a 96 well tissue culture plate and allowed to adhere for 20 min at 37°C. Adhesion was evaluated as described in materials and methods. Results represent the % adhesion relative to control where all cells adhered and are the mean ± s.e.m of at five experiments performed in quadruplicate (*P<0.05, **P<0.01, significantly different from vector control cells, ANOVA).

## 4.7 Discussion

The oncogenic nature of GPCRs was first highlighted through screening analyses for novel oncogenes whose expression caused the cellular transformation of NIH3T3 mouse fibroblasts. The first GPCR identified to possess transforming properties in this manner was the product of the *mas* oncogene (Young *et al*, 1986). It has since been discovered that chronic stimulation or mutational activation of mitogenic receptors can also lead to oncogenic transformation (Whitehead *et al*, 2001). The effect of GRP and $V_{1A}$ receptor expression, the two most common neuropeptide receptors present on SCLC, on growth and transformation of the epithelial CHO-K1 cell-line was examined. Data presented in this chapter shows that expression of these receptors transforms CHO-K1 cells and also leads to increased sensitivity to substance-P analogues. Ectopic expression of neuropeptide receptors in epithelial CHO-K1 cells led to morphological transformation of the cells, increased saturation density in monolayer culture and an anchorage-independent phenotype. The study also found that expression of neuropeptide receptors had an impact on the adhesive and migratory properties of CHO-K1 cells.

The morphological and behavioural characteristics of transformed cells are distinct from those of counterpart normal cells. The acquisition of anchorage-independent growth is an indicator of the transformed status of a cell population and is commonly demonstrated through colony formation assays in semi-solid media (MacPherson & Montagnier, 1964; DiPaolo *et al*, 1969; DiPaolo *et al*, 1972). Although *in vivo* tumourigenicity provides verification for cell transformation, it has been demonstrated that colony formation in soft-agar does not necessarily indicate tumourigenic potential (Steuer *et al*, 1977). In contrast, the ability of various clonogenic cell types to survive in suspension in aggregate form has been shown to correlate with tumourigenicity (Steuer *et al*, 1977). Using fibroblast and epithelial cell types from various species (including humans) it was demonstrated that only those cells which were tumourigenic were able to survive and grow in aggregate

form. Thus, growth in suspension can be used as an alternative assessment of cell transformation.

Data presented in this chapter showed that neuropeptide receptor expressing cells proliferated more than vector-transfected control cells when cultured as monolayers. In addition, although vector-transfected CHO-K1 cells were able to form colonies in soft agar and grow as multicellular aggregates in suspension, these capabilities were enhanced in cells expressing neuropeptide receptors. Under normal circumstances, detachment of non-transformed epithelial cells from their substratum induces a specialised form of apoptosis called anoikis (Wei *et al*, 2001). The cell-type dependent phenomenon is thought to constitute a protective mechanism which operates to prevent growth of displaced epithelial and endothelial cells in inappropriate environments (Bretland *et al*, 2001). In the present study, only around 5% of the cell population was non-viable following 9 days growth in suspension (data not shown) which indicates resistance to detachment induced apoptosis. The establishment of epithelial cell-lines can be hindered by sensitivity to anoikis, a factor which has been found to affect the development of non-transformed colonic epithelial cell-lines but not colonic tumour cell-lines (Bretland *et al*, 2001). Therefore, through the initial process of establishing the CHO-K1 cell-line, a degree of anoikis-resistance may have been acquired which could account for the ability of this cell-line to survive detachment from the ECM and grow under anchorage independent conditions at all.

Since cell growth in suspension occurs in the absence of cell-ECM interaction, growth as multicellular aggregates has also provided a means of assessing the involvement of intercellular adhesion (Kantak & Kramer, 1998). Data presented in this chapter showed that when grown in suspension, neuropeptide receptor expressing cells formed morphologically different aggregates to vector transfected CHO-K1 cells which could be attributed to altered intercellular adhesion. In accordance with the findings of the present study, alteration of cultured epithelial cell morphology by another neuropeptide receptor (gastrin/cholecystokinin-2

receptor) has been demonstrated recently and the receptors found to modulate intercellular adhesion and cell differentiation *in vivo* (Bierkamp *et al*, 2004). Cadherins mediate intercellular adhesion as components of adhesion junctions and play an important role in epithelial morphogenesis (Vizirianakis *et al*, 2002). Human breast cancer cells transfected with a dominant-negative form of E-cadherin fail to compact and remain as loosely associated cells (similar here to neuropeptide receptor expressing cells) whereas wildtype cells form multicellular aggregates that tightly compact into spheroids (similar here to vector transfected CHO-K1 cells) (Vizirianakis *et al*, 2002). Also, just as the dominant-negative E-cadherin transfected breast cancer cells had an increased proliferation rate; neuropeptide receptor-expressing cells similarly demonstrated increased growth in suspension. Metastatic fibroblasts have also been shown to form morphologically different aggregates, with normal cells forming compact aggregates and transformed cells forming loose aggregates (Matsuyoshi *et al*, 1992). As a consequence of neuropeptide receptor expression in cells, it is conceivable that fluctuations in E-cadherin mediated cell adhesion could arise due to signals transduced by the receptors. This has previously been demonstrated for other GPCRs such as the $M_3$ muscarinic acetylcholine receptor (mAChR) in SCLC (Williams *et al*, 1993), and led to the hypothesis that neoplastic transformation induced by activation of this neurotransmitter receptor or other GPCRs may involve receptor mediated changes in adhesion molecule activity. The degree of altered morphology observed in the present study may correlate with neuropeptide receptor expression levels since CHO-$V_{1A}$ cells, which express less receptors than CHO-GRP, form aggregates which are more CHO-like in appearance or intermediate between CHO-vector and CHO-GRP in appearance. This suggests that a correlation between neuropeptide receptor expression and modulation of intercellular adhesion may also exist.

In addition to increased proliferation, neuropeptide receptor expressing cells were not subject to density-dependent inhibition of growth. This provides further evidence that the neuropeptide-receptor expressing cells are able to over-ride

growth inhibitory signals, consistent with a transformed phenotype. Glover *et al* (2005) maintain that GRP acts as a morphogen when it and its receptor (GRP-R) are aberrantly upregulated in colon cancer, primarily modulating processes which contribute to the assumption or maintenance of tumour differentiation. Studies using a colon cancer cell-line showed that when cells were plated at subconfluent densities, GRP/GRPR were rapidly upregulated followed by their progressive decrease as confluence was achieved (Glover *et al*, 2005). Relative to this finding, constitutive receptor expression in GRP or $V_{1A}$ receptor expressing cell-lines may enable the cells to continue growth even after confluence has been achieved.

CHO-GRP and CHO-$V_{1A}$ cells also exhibited a rounded morphology and increased membrane ruffling compared with CHO-vector cells, which could be taken as a primary indication that increased Rho/Rac activity exists in the neuropeptide receptor expressing cells. Small GTPases of the Rho family, primarily Rac, cdc42 and Rho, regulate actin organisation and have been identified as key regulators of epithelial architecture, as well as of cell migration (Sugimoto *et al*, 2003). Activation of Rho GTPases is mediated by G protein alpha 12 and alpha 13 subunits (Buhl *et al*, 1995), and both bombesin and vasopressin have been shown to stimulate Rho-dependent responses through receptor coupling to $G_{12}/G_{13}$ proteins (Gohla *et al*, 1999; Yuan *et al*, 2001). Rho mediates LPA- and bombesin-induced formation of focal adhesions and actin stress fibres in Swiss 3T3 cells, whilst Rac is required for the PDGF-, insulin- and bombesin-stimulated actin polymerisation at the plasma membrane that results in membrane ruffling (Nobes *et al*, 1995). In addition, Rho GTPase activity has been shown to contribute to cell rounding, transformation and hypertrophic growth (Morissette *et al*, 2000; Sah *et al*, 2000). Studies of the signalling and transforming properties of numerous GPCR oncoproteins have also shown that abnormal growth regulation is promoted through Rho GTPase activation (Whitehead *et al*, 2001). It could therefore be informative to determine whether Rho family GTPases play a role in the changes in morphology and growth effected by neuropeptide receptor expression in CHO-K1 cells.

As mentioned previously, as well as having a greater propensity to form non-adherent clusters in suspension, cells expressing neuropeptide receptors also formed colonies in semi-solid agarose with greater efficiency than vector-transfected control cells. Since colony growth was also evident in low serum, these findings suggest that expression of these receptors allows for anchorage- and serum-independent growth consistent with transformation. Moreover it suggests that these receptors may be constitutively active, as wild type receptor expression alone was sufficient to increase transformation in the absence of exogenously added neuropeptide. Alternatively, the effect of neuropeptide receptor expression on CHO-K1 cell growth could be attributed to the existence of an autocrine growth loop.

Constitutive receptor activity has been observed at low and high GPCR expression levels, in native and recombinant systems, and has been reported for GPCRs irrespective of G-protein coupling specificity (Seifert & Wenzel-Seifert, 2002). In accordance with the findings here, Ferris *et al* (1997) also found that expression of GRPR in a non-malignant human colon epithelial cell-line led to increased proliferation. However, since the GRPR does not cause proliferation of all cells in which it is expressed (Benya *et al*, 1994b), the tissue milieu is thought to influence the ability of a GPCR to act as an oncogene (Ferris *et al*, 1997). In addition, the intrinsic molecular properties of the GPCR studied can also contribute to basal GPCR activity. Differences in GPCR expression level, GPCR desensitization, G-protein complement and GPCR/G-protein stoichiometry can all contribute to variations in constitutive activity observed in different systems (Seifert & Wenzel-Seifert, 2002). This could explain why GRP receptors expressed in NCM460 cells, a non-malignant human colon epithelial cell line are constitutively active (Ferris *et al*, 1997) but are not in other cell types (Benya *et al*, 1994a). A physiological requirement for constitutive GPCR activity is likely to exist. For example, it is thought that constitutive neurotransmitter GPCR activity may be necessary to support basal neuronal activity (Seifert & Wenzel-Seifert, 2002).

The activation of multiple cell-specific signal transduction pathways following GRP or AVP receptor stimulation controls cell proliferation through the induction of immediate early gene expression (Rozengurt, 1991; Schulze-Lohoff *et al*, 1993; Benya *et al*, 1994b). The immediate-early gene products c-*fos* and c-*jun* form heterodimers that stimulate expression of genes containing AP1 activation elements (Schulze-Lohoff *et al*, 1993; Hipskind *et al*, 1994; Beno *et al*, 1995). Since the GRP gene has a 5'-upstream AP1 binding site (Kim *et al*, 2000), GRP expression can potentially be stimulated through GRP receptor activation (Bajo *et al*, 2002) which would result in the formation of an autocrine growth loop. Analysis of the AVP gene promoter region has identified the presence of AP-1, AP-2, E-box and neuron-restrictive silencer element (NRSE) binding sites which are involved in the regulation of AVP expression (Grace *et al*, 1999; Coulson *et al*, 1999b; Coulson *et al*, 1999a). Selective vasopressin gene expression in SCLC but not normal lung or NSCLC has been attributed to differential activity of the transcriptional regulator Upstream Stimulatory Factor (USF) in the lung tissues (Coulson *et al*, 2003b). In principle, neuropeptide receptor activity could thus stimulate transcription factors which result in neuropeptide gene expression and the subsequent formation of an autocrine growth loop. Whether the neuropeptide receptor expressing cells expressed the associated neuropeptide ligand was not determined in this study. However, numerous granules were observed to be present in the cytoplasm of neuropeptide receptor expressing cells and less apparent in vector-transfected CHO-K1 cells. It is quite possible that these dense granules, reminiscent of the intra-cytoplasmic vesicles which characterise SCLC cells, were storage vesicles for synthesised neuropeptide. If this were the case, it would indicate regulated rather than constitutive secretion of the neuropeptide since there is no storage of peptides in cells with constitutive secretion (Rehfeld, 1998). Had these cell-lines been assessed for neuropeptide secretion (e.g. by immunofluorescent staining of cells using antibodies against bombesin or vasopressin), the existence of an autocrine growth loop could have been determined. This would provide a biological basis for receptor activity and increased growth in the absence of exogenously added ligand in the

CHO cell systems. It is important to note that although receptor expressing cells demonstrated increased growth in the absence of exogenously added ligand, this does not demean the findings presented in chapter 3 which resulted from stimulation of cells with neuropeptide. Together these findings show that although neuropeptide receptors expressed in CHO-K1 cells may be constitutively active, they are still able to respond to stimulation with neuropeptide with subsequent changes in intracellular calcium levels and ERK activity.

Whatever the precise basis for increased neuropeptide receptor activity, the present study demonstrates that $V_{1A}$ receptors, as well as GRP receptors, are oncogenic when expressed in epithelial cells. As discussed earlier, while GRP receptor expression has been shown to transform some cell-types, to my knowledge, this has not previously been demonstrated directly for $V_{1A}$ receptors. This finding may be of particular relevance to lung cancer biology since all SCLC cell-lines have been shown to express an AVP autocrine system (Coulson *et al*, 1999; Waters *et al*, 2003).

The actual mechanisms governing the survival advantage gained in neuropeptide receptor expressing cells are poorly understood. During colon cancer progression, increased expression of GRP receptors leads to tumour cell differentiation, increased motility and adhesion to extracellular matrix via an increased activation of focal adhesion kinase (FAK) (Glover *et al*, 2004). As discussed in chapter 3 (page 132), transactivation of EGF receptors can occur through GPCR stimulation. The significance of GPCR/EGFR cross-talk in SCLC growth is demonstrated through the finding that the Bn/GRP antagonist RC-3095 inhibits SCLC growth through a mechanism which involves downregulation of both GRP and EGF receptors (Halmos & Schally, 1997; Koppan *et al*, 1998). IGF-1 stimulation of COS-7 cells also results in EGFR transactivation (Roudabush *et al*, 2000) which is of significance since the IGF-I/IGF-IR autocrine loop plays a prominent role in SCLC growth (Warshamana-Greene *et al*, 2004). Bombesin antagonists in combination with GHRH antagonists have been shown to down regulate IGF-1, IGF-II, IGF receptors, GRP

and EGF receptors in SCLC and prostate cancer xenografts (Plonowski *et al*, 2000; Kanashiro *et al*, 2003). Bombesin antagonists also inhibit the expression of bFGF, IGF-II and vEGF-A in human experimental breast cancers (Bajo *et al*, 2004), therefore heterologous regulation of RTKs by bombesin is not restricted to effects on the EGFR alone. Further to this, it is conceivable that similar transmodulation of other tyrosine kinase growth factor receptors by neuropeptide receptors may facilitate regulation of signalling pathways that control survival. Transactivation of the IGF-1R by GRP or $V_{1A}$ receptors may particularly contribute to anchorage-independent growth as the receptor mediates signalling through both proliferative and anti-apoptotic pathways (Roudabush *et al*, 2000).

Dysregulated growth is believed to constitute an early event in the transformation process, with alterations in adhesive, migratory and invasive properties contributing to the subsequent development of metastatic potential. It has been hypothesised that increased cell motility (migration) is necessary for cells to become metastatic since metastatic tumour cells generally migrate at a faster rate than normal cells or non-metastatic tumour cells (Parsons *et al*, 2002). It has previously been postulated that high bombesin/GRP receptor mRNA levels correlate with more invasive characteristics of human colon tumours (Saurin *et al*, 1999). In the same way that bombesin/GRP receptor expression may contribute to the cellular events that are critical for invasion/migration of colorectal carcinoma cells (Saurin *et al*, 2002), a similar role for GRP and $V_{1A}$ receptor expression in CHO-K1 cells may exist. Data presented in this chapter showed that neuropeptide receptor expressing cells displayed greater chemotactic migration towards FCS and IGF-1 than vector transfected control cells in transwell migration assays. Previous reports have shown that GRP receptors can activate insulin-like growth factor-1 receptors (IGF-IR) in prostate cancer cells (Sumitomo *et al*, 2001) and that human chorionic gonadotrophin (hCG) upregulates IGF-1 expression in ovarian cancer cells (Kuroda *et al*, 1998). Transmodulation of IGF or other growth factor receptors by

neuropeptide receptors may therefore underlie the enhanced chemotactic response of neuropeptide receptor expressing cells towards growth factors.

Monocyte chemotaxis towards SP-D has previously been demonstrated to occur via bombesin receptors suggesting that the biased agonist activity of these analogues may play a role in normal and tumour cell migration (Djanani *et al*, 2003). In the present study however, cell-lines transfected with vector or neuropeptide receptors displayed similar chemotactic responses to SP-D or SP-G suggesting that the effects were not neuropeptide receptor-mediated. Previous work has shown that SP-D stimulates migration of human neutrophils, which express various chemoattractant receptors (including two IL-8 receptor subtypes) as well as the substance-P receptor (NK-1) (Jarpe *et al*, 1998). Although the identity of the receptor involved in the chemotactic response of neutrophils to SP-D was not determined, it was proposed that the IL-8 receptor may mediate this effect since SP-D binds to this receptor (Jarpe *et al*, 1998). As mentioned in chapter 3 (page 136), sphingosine-1-phosphate induced intracellular calcium mobilisation in CHO-K1 cells indicating the cells express receptors for this chemoattractant. Sphingosine-1-phosphate performs a variety of roles, including the regulation of cell migration, mediated through members of the EDG (endothelial differentiation gene) family of GPCRs (Okamoto *et al*, 2000). Therefore, it is conceivable that the substance-P analogues may have stimulated a chemotactic response via such receptors endogenously expressed in CHO-K1 cells.

Neuropeptide receptor expressing cells failed to demonstrate a chemotactic response to the cognate neuropeptide (data not shown) and this could be attributed to the receptor's being constitutively active. In this case, it is likely that a chemotactic response was not elicited at the concentrations of neuropeptide tested (1nM and 10nM; data not shown) because the receptors are already active. It is important to note that the interpretation of data from the migration assay will differ from that of the growth assays since the former is measuring the response to the chemotactic gradient presented to cells whereas the latter is measuring basal levels of growth in the absence of exogenously added neuropeptide.

Since cell movement is controlled by a complex coordinated series of rearrangements of the actin cytoskeleton and changes in adhesion, the intracellular regulators of cytoskeletal organisation and cellular adhesion strength are likely to be crucial determinants of cell motility (Fincham & Frame, 1998). Cell adhesion is thought to occur through a 3-step process: 1) attachment, 2) spreading and 3) focal adhesion and stress fiber formation (Murphy-Ullrich, 2001). The second step is considered to be an intermediate state between that of weak contact and strong adhesion and is believed to be the level of cellular adhesive strength at which migration occurs (Schwarzbauer, 1997). In accordance with this, data presented in this chapter showed that more migratory neuropeptide receptor expressing cells were less adherent than vector transfected CHO-K1 cells on the ECM component fibronectin. The level of adhesiveness was not significantly altered by neuropeptides, substance-P analogues or IGF-1. This suggests that neuropeptide receptor expression influences cellular adhesiveness overall, consequently enabling cell migration to be increased in response to particular chemotactic gradients. In order to do this, neuropeptide receptors may influence intracellular signalling pathways which regulate cell-cell and cell-ECM interactions. In this respect, the focal adhesion components FAK and paxillin have recently been implicated in facilitating cross-talk between cell-cell and cell-ECM adhesions (Schaller, 2004). The findings of abnormal intercellular adhesions, together with reduced cell-ECM interactions in neuropeptide receptor expressing cells could be relevant to the ability of cells to detach from tumours and metastasise. Thus neuropeptide receptor expression may make a greater contribution to tumour development and progression than has previously been recognized.

Sensitivity to AVP and GRP has previously been shown to increase during the progression to chemoresistance in a set of SCLC cell lines (Waters *et al*, 2003). This would be analogous to the development of autocrine and paracrine growth loops in tumour development *in vivo*, with tumour cells becoming more dependent on neuropeptide growth factors for survival and subsequently developing

chemoresistance. Likewise, data presented in this chapter showed that stimulation of neuropeptide receptor expressing cells with neuropeptides led to a small but significant increase in resistance to the cytotoxic effects of etoposide. Although it is presently unclear how this change in sensitivity relates to the development of chemoresistance *in vivo*, this finding highlights an important area for future investigation. However, these findings do suggest that the increase in neuropeptide receptor expression that occurs as cells become more differentiated may contribute to the development of chemoresistance. Since the acquisition of chemoresistance by SCLC cells underlies the extremely poor prognosis associated with this form of lung cancer, a greater understanding of the mechanism underlying chemoresistance of neuroendocrine tumours may lead to the identification of novel treatment strategies. A possible mechanism leading to the development of chemoresistance in neuroendocrine cells may involve transactivation of IGF-1 receptors by neuropeptide receptors since the stimulation of growth by IGF-1 has been shown to enhance SCLC resistance to the apoptotic effect of chemotherapy (Krystal *et al*, 2002).

Data presented in this chapter showed that SP-D and SP-G inhibited H345 SCLC growth, providing confirmation that the substance-P analogues selected for investigation do indeed inhibit the growth of SCLC cells. The finding that these two analogues, but not spantide, inhibit H345 growth is in accordance with previous results (Woll & Rozengurt, 1990). During the phase I clinical trial of SP-G, plasma levels up to 40μM were obtained in the absence of dose limiting toxicity (Clive *et al*, 2001). The $IC_{50}$ values for both SP-D and SP-G obtained in this study are well within this limit. Substance-P analogues effectively inhibit the growth of chemoresistant tumour cells and can increase the sensitivity of such cells to etoposide-mediated cell death (Waters *et al*, 2003). In addition, the sensitivity of diverse tumour types to SP-D correlates with GRP receptor expression (Waters *et al*, 2003). In accordance with this, data presented in this chapter similarly shows that GRP receptor expressing CHO-K1 cells are more sensitive to growth inhibition by substance-P analogues. In addition, expression of $V_{1A}$ receptors in CHO-K1 cells

confers sensitivity to substance-P analogue induced growth inhibition. This was demonstrated through the inhibition of anchorage dependent and independent growth by substance-P analogues in receptor-transfected but not control cells. Substance-P analogues have previously been shown to inhibit anchorage independent growth of SCLC, ovarian, cervical and pancreatic cancer cells (Bepler *et al*, 1988; Everard *et al*, 1992; Guha *et al*, 2005). Also consistent with the findings here, SP-D has previously been shown to have more potent antiproliferative effects on tumour cells than normal cells (Everard *et al*, 1992). Since anti-cancer drugs should preferably exert minimal side-effects, this suggests that substance-P analogues may be ideal candidates for specifically targeting transformed neuropeptide-dependent cells with minimal effects on normal cells.

Bunn *et al* (1994) previously demonstrated that substance-P analogues were more effective in inhibiting SCLC growth than specific bombesin and vasopressin antagonists. As demonstrated in chapter 3, the substance-P analogues functioned as biased agonists of the GRP and $V_{1A}$ receptor in CHO-GRP and CHO-$V_{1A}$ cell-lines respectively. The substance-P analogues modulated GRPR and $V_{1A}R$ signalling, causing blockade of $G_q$-mediated $Ca^{2+}$ release while inducing $G_i$-mediated ERK activation via a pertussis toxin-sensitive pathway. Data presented in the current chapter shows that these biased agonist properties selectively result in anti-proliferative effects in neuropeptide receptor expressing cells. These findings support the concept that the biased agonist properties of substance-P analogues are central to the growth inhibitory effects of these compounds. Maudsley *et al* (2004) have similarly demonstrated that the anti-proliferative effects of Gonadotrophin-releasing hormones (GnRH) and GnRH analogues in reproductive tumour cells correlate with the ligand-selective activation of a $G\alpha_i$–coupled form of the type I GnRH receptor. They found that structurally related antagonistic GnRH analogues displayed divergent anti-proliferative efficacies but equal efficacies in inhibiting GnRH-induced $G\alpha_q$-based signalling, indicating that the growth inhibitory effects were determined by the ability of the ligands to stabilise/induce a $G_i$-coupled state

of the GnRH receptor. Spantide does not inhibit SCLC growth although it is able to inhibit GRP binding and block $G_q$-based signalling (as demonstrated in chapter 3). Thus, enhanced anti-proliferative activity can be related to the ability of substance-P analogues to activate a $G_i$-coupled state of neuropeptide receptors. This should have implications for the future identification of drugs which can effectively target SCLC and other neuropeptide-dependent tumours.

In summary, introduction of neuropeptide receptors into CHO-K1 cells led to phenotypic transformation of the cells, suggesting a role for neuropeptide receptors in tumour development and progression. In particular, neuropeptide receptor expression conferred an advantage upon the cells growing under both anchorage dependent and independent conditions. Neuropeptide receptor expression altered cell-cell and cell-ECM interaction and led to changes in cell motility. All of these effects were observed in the absence of exogenously added neuropeptides suggesting that these receptors may be constitutively active when expressed in CHO-K1 cells. It was also found that neuropeptide receptor stimulation may contribute to an increase in chemoresistance. Neuropeptide receptor expression conferred sensitivity to the growth inhibitory effects of these agents since $V_{1A}$ and GRP receptor expressing cells were more sensitive to substance-P analogue induced growth inhibition than vector-transfected CHO-K1 cells. Moreover, the anti-proliferative effects of the analogues in neuropeptide receptor expressing cells correlated with biased agonist properties in those cell-lines. The study demonstrates that the therapeutic use of these compounds need not be restricted to tumours expressing GRP receptors only, and could be extended from SCLC therapy to targeting various transformed cell types with neuropeptide dependence. In addition, these biased agonist compounds may be particularly effective in targeting well-differentiated tumours that have developed resistance to chemotherapy.

# Chapter 5

# The underlying basis for Substance-P analogue biased agonism of Vasopressin receptors

Biased agonism is the ability of a ligand to interact with a receptor and differentially regulate each of the multiple signalling pathways coupled to a receptor. It is also known as 'functional selectivity', 'stimulus trafficking' and 'Agonist Directed Trafficking of Receptor Stimulus' (ADTRS) (Clarke, 2005). The mechanism underlying this behaviour is thought to be dependent on the ability of ligands to promote unique, ligand-selective receptor conformations that regulate signal transduction pathways with distinct efficacies. Receptor number and receptor:G protein stoichiometry as well the specific identity of the G-proteins activated are factors which can influence the efficacy with which a compound activates different signalling cascades (Cussac *et al*, 2002). Alteration of the receptor/G-protein stoichiometry has been shown to change the coupling specificity of various GPCRs. Agonist-directed trafficking at human $\alpha_{2A}$-adrenoreceptors was found to be dependent on the level of receptor expression and the presence of receptor reserve (Brink *et al*, 2000). Low level expression of the $V_{1b}$ receptor in CHO cells resulted in receptor coupling to G-proteins of the $G_q$ family whereas at higher expression levels, $G_i$- and $G_s$- coupling was also observed (Thibonnier *et al*, 1997). A similar study using the $G_s$-coupled $V_2$ receptor revealed that the receptors coupled to $G_q$-proteins when expressed in high numbers (Zhu *et al*, 1994). The first part of this study (sections 5.1 and 5.2) was conducted in an attempt to evaluate whether the receptor:G protein stoichiometry influenced the biased agonist activity of substance-P analogues on neuropeptide receptors. The receptor:G protein stoichiometry was altered in two ways: by changing receptor density while maintaining endogenous

G-protein expression levels (section 5.1) and through over expression of specific G-protein sub-units at a fixed receptor density (section 5.2).

## 5.1. Effect of altering receptor numbers on SP-G activity

### 5.1.1. Generation of cells with varying $V_{1A}$ receptor densities

Cell-lines stably expressing different numbers of $V_{1A}$ receptor were generated through transfecting CHO-K1 cells with 10μg, 20μg or 30μg $V_{1A}R$ cDNA as described in materials and methods. Individual clones were obtained through selection and screening in a manner analogous to that used to establish CHO-GRP and CHO-$V_{1A}$ cell-lines (as described in section 3.1). Receptor expression in seven clones (positive for AVP calcium response) was assessed by measuring [$^3$H]-AVP binding to cell membranes in order to identify three clones with varying levels of receptor expression (Figure 5.1). Of the seven clones, 30b, 20i and 10d were chosen to represent a high ($V_{1A}R$-high), medium ($V_{1A}R$-med) and low ($V_{1A}R$-low) expressing cell line respectively. Competitive binding experiments with [$^3$H]-AVP using membrane preparations from these cells were carried out in order to define the binding parameters $B_{max}$ and $K_d$ for each cell-line, as described in materials and methods. $V_{1A}$ receptors were expressed with a $B_{max}$ of 383, 265 and 111 fmoles $mg^{-1}$ protein in $V_{1A}R$-high, $V_{1A}R$-med and $V_{1A}R$-low cell-lines respectively. [$^3$H]-AVP had similar affinity for receptors expressed in all three cell-lines (Table 5.1). This showed that the differences in receptor number did not alter the affinity of vasopressin for its receptor.

### 5.1.2. Intracellular calcium mobilisation

Intracellular calcium mobilisation was measured in the three cell-lines using Fura-2-AM in order to first assess whether level of receptor expression had any affect on this response. AVP stimulated a concentration-dependent increase in intracellular calcium levels in all three cell-lines with $EC_{50}$ values of 3.1 ± 0.6 nM, 3.7 ± 0.84 nM and 6.3 ± 0.84 nM in $V_{1A}$R-high, $V_{1A}$R-med and $V_{1A}$R-low cell-lines respectively (Figure 5.2). While the efficacy of the AVP response increased with increasing receptor numbers, the $EC_{50}$ was not significantly different between the high and medium expressing cells and reflected the affinity ($K_d$) of AVP for the $V_{1A}$ receptor expressed in these cell-lines in receptor binding experiments (Table 5.1). This suggests that there is little receptor reserve for stimulation of a calcium response by AVP.

The effect of SP-G on intracellular calcium mobilisation was assessed in the three cell-lines expressing different $V_{1A}$ receptor numbers. Neither 1μM nor 10μM SP-G alone stimulated a calcium response in any of the cell-lines. This showed that, irrespective of receptor numbers, the analogue had zero efficacy for coupling $V_{1A}$ receptors with $G_q$ proteins for PLC activation and subsequent calcium mobilisation. However, measurement of AVP-stimulated calcium mobilisation in the presence of 1μM SP-G resulted in a rightward parallel shift in the concentration response curve in $V_{1A}$R-High (Figure 5.3) and $V_{1A}$R-Med (Figure 5.4) but not $V_{1A}$R-Low (Figure 5.5) cells. In addition, 10μM SP-G resulted in a rightward parallel shift in the concentration response curves in all three cell-lines. The antagonist potency for SP-G was lowest in the $V_{1A}$R-Low cells ($pA_2$ 5.66) but similar in $V_{1A}$R-Med ($pA_2$ 6.42) and $V_{1A}$R-High ($pA_2$ 6.27) cells. This shows that SP-G maintains antagonism of the $G_q$-coupled calcium response regardless of $V_{1A}$ receptor numbers.

### 5.1.3. ERK activation

ERK activation was measured in $V_{1A}$R-High, $V_{1A}$R-Med and $V_{1A}$R-Low cells in response to AVP or SP-G. Lysates from quiesced cells stimulated for 10 minutes with either AVP or SP-G were separated on 12% gels and blotted for phospho-ERK1/2 or total ERK2. Activation was estimated by quantification of pERK band intensity ratios using ImageQuant software following chemifluorescent visualisation of immunoblots. Blots were also probed for total ERK2 in order to ensure that any observed increase in ERK phosphorylation was not due to a change in total ERK expression or unequal loading of gels (observed as equivalent ERK2 bands in the absence and presence of treatment).

$V_{1A}$R-High, $V_{1A}$R-Med and $V_{1A}$R-Low cells were stimulated with increasing concentrations of AVP and ERK activation measured. AVP stimulated a concentration dependent increase in ERK phosphorylation in all three cell-lines (Figure 5.6). A relatively small response was obtained in $V_{1A}$R-Low cells, with only a maximal 1.5 fold increase in ERK activity stimulated by 100nM AVP. However, the magnitude of the response increased with increasing receptor numbers. Although it was not possible to determine the $EC_{50}$ for the response in $V_{1A}$R-Low cells, similar $EC_{50}$ values were obtained in $V_{1A}$R-Med and $V_{1A}$R-High cells (3.18nM and 2.84nM respectively). The maximal stimulation observed with AVP increased with receptor expression, showing 3 and 4 fold stimulation with 100 nM AVP in the medium and high expressing cells respectively. Stimulation with increasing concentrations of SP-G resulted in a concentration dependent increase in ERK activation in $V_{1A}$R-High, $V_{1A}$R-Med and $V_{1A}$R-Low cells (Figure 5.7). In contrast to AVP, maximal activation by SP-G (50μM) was not achieved but there was no difference in the magnitude of ERK activation between medium and high $V_{1A}$ receptor expressing cells and the dose-response curves were super imposable. Together this data shows that while the efficacy of AVP for ERK activation continues to increase with increasing receptor numbers, the efficacy of SP-G does

not. The difference in efficacy between AVP and SP-G for ERK activation in $V_{1A}$ receptor expressing cells further indicates that the two agonists activate ERK by different mechanisms.

As shown in chapter 3 (page 107), ERK activation by AVP is mediated predominantly through coupling to $G_q$-proteins whereas SP-G stimulated ERK activation is entirely dependent on $G_i$–protein coupling. $V_{1A}$R-High, $V_{1A}$R-Med and $V_{1A}$R-Low cells were pre-treated overnight with 100ng $ml^{-1}$ pertussis toxin (PTx) then stimulated with 30μM SP-G or 1nM AVP in order to assess whether changes in receptor number influence G-protein coupling specificity for this response. SP-G stimulated ERK activity was inhibited by PTx in all three cell-lines whereas AVP stimulated ERK activity was not affected (Figure 5.8). This shows that SP-G retains G-protein selectivity for ERK activation despite changes in receptor numbers.

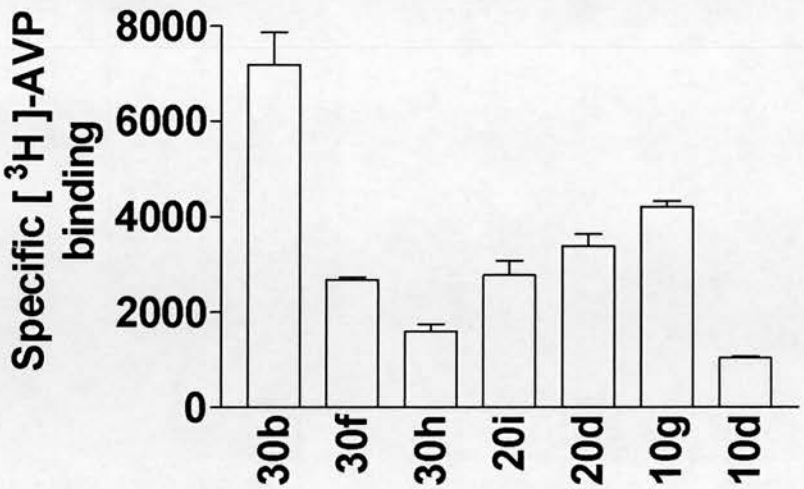



**Figure 5.1. Specific binding of [$^3$H]-AVP to clones isolated from CHO-K1 cells stably transfected with $V_{1A}$ receptors.** Cell membranes were incubated with 1nM [$^3$H]-AVP in the presence (non-specific binding) and absence (total binding) of an excess of unlabelled vasopressin (37°C; 30min). Results are expressed as specific radioligand binding and represent the mean ± s.e.m. of a single experiment performed in triplicate. Clones 30b, 30b, 20i and 10d were chosen to represent a high ($V_{1A}$R-high), medium ($V_{1A}$R-med) and low ($V_{1A}$R-low) expressing cell line respectively.

**Table 5.1. $V_{1A}$ receptor expression in stably transfected CHO-K1 cells**

| **[$^3$H]-AVP binding** | $B_{max}$ (fmoles mg$^{-1}$ protein) | $K_d$ (nM) |
|---|---|---|
| **$V_{1A}$R-high** | 382 ± 74 | 3.27 ± 1.57 |
| **$V_{1A}$R-med** | 265 ± 22 | 1.5 ± 0.77 |
| **$V_{1A}$R-low** | 111 ± 5 | 1.13 ± 0.29 |

Cell membranes from clones of CHO-K1 cells stably expressing different numbers of $V_{1A}$ receptors were incubated with 1nM [$^3$H]-AVP and increasing concentrations of unlabelled vasopressin (37°C; 30min). The binding parameters $B_{max}$ and $K_d$ were calculated from the competition binding isotherms as described in materials and methods. The results represent the mean ± s.e.m. of five independent experiments performed in quadruplicate.

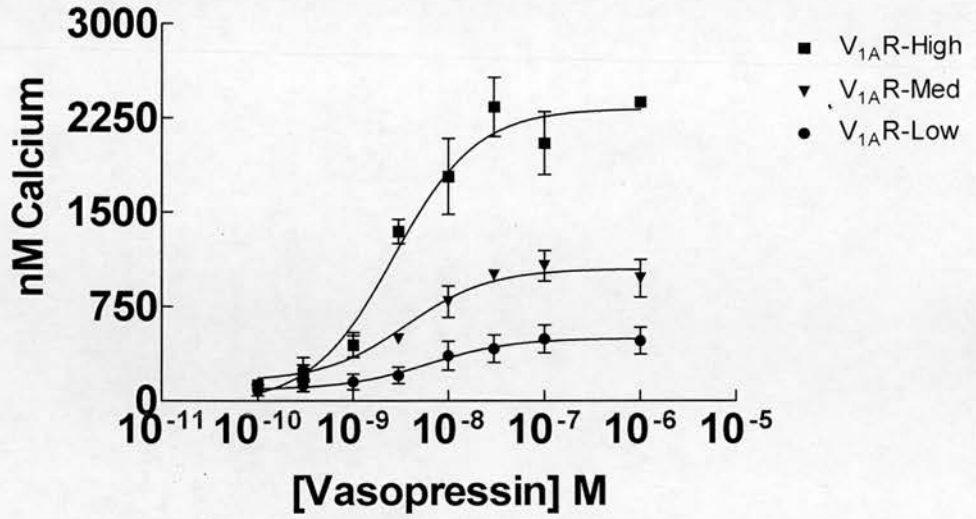



**Figure 5.2. Concentration response curves for calcium mobilisation in CHO-K1 cells expressing different $V_{1A}$ receptor numbers.** Quiescent $V_{1A}$R-high, $V_{1A}$R-med and $V_{1A}$R-low cells loaded with FURA-2AM were stimulated with increasing concentrations of vasopressin and intracellular calcium release measured by ratiometric fluorimetry as described in materials and methods. Data represents the mean ± s.e.m. of four experiments.

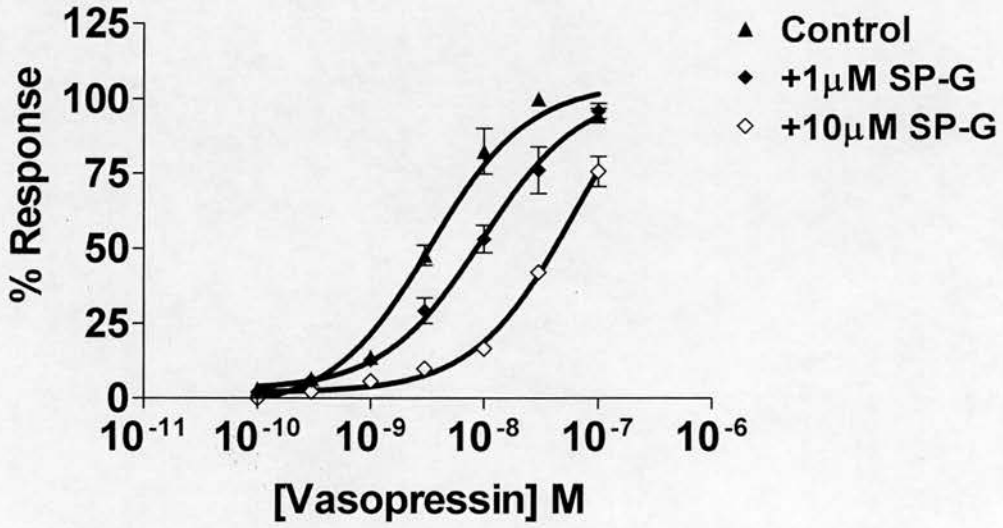



**Figure 5.3. Inhibition of intracellular calcium mobilisation in $V_{1A}$R-high by SP-G.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of vasopressin alone (▲) or in the presence of 1μM (♦) or 10μM (◊) SP-G. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods and data represents the mean ± s.e.m. of three experiments.

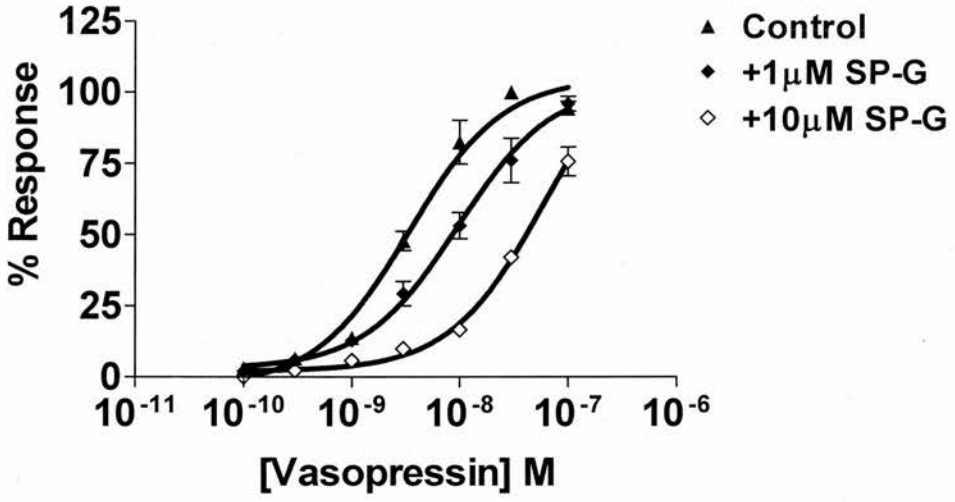



**Figure 5.4. Inhibition of intracellular calcium mobilisation in $V_{1A}$R-med by SP-G.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of vasopressin alone (▲) or in the presence of 1μM (♦) or 10μM (◊) SP-G. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods and data represents the mean ± s.e.m. of three experiments.

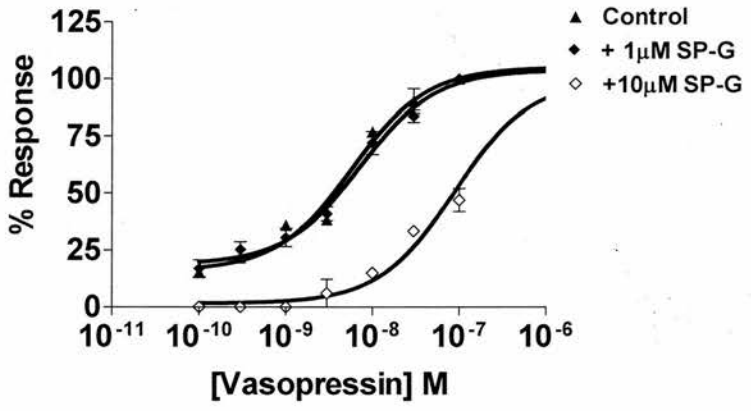



**Figure 5.5. Inhibition of intracellular calcium mobilisation in $V_{1A}$R-low by SP-G.** Quiescent cells loaded with FURA-2AM were stimulated with increasing concentrations of vasopressin alone (▲) or in the presence of 1μM (♦) or 10μM (◊) SP-G. Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods and data represents the mean ± s.e.m. of three experiments.

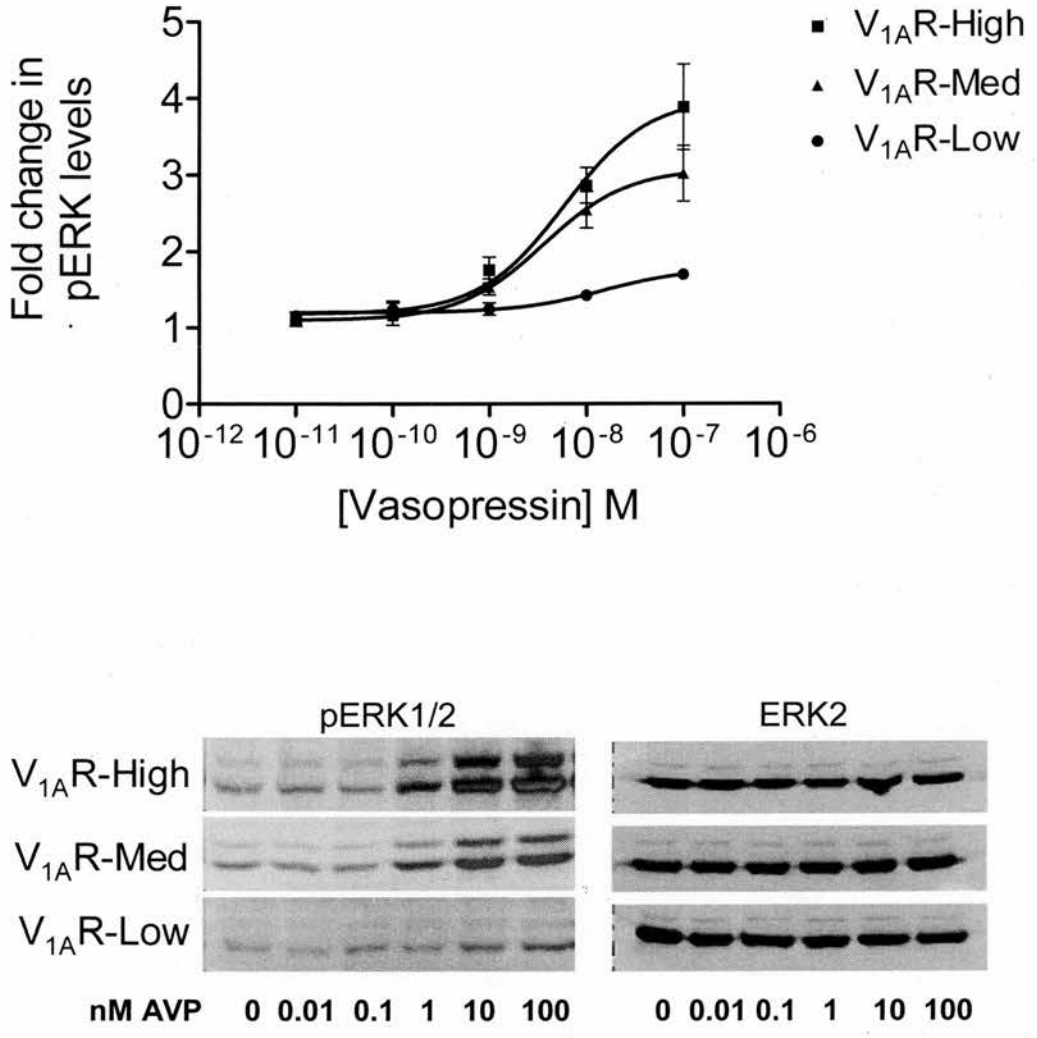



**Figure 5.6. Vasopressin stimulated ERK phosphorylation in $V_{1A}$ receptor expressing cells.** Confluent, quiescent cultures of $V_{1A}$R-high, $V_{1A}$R-med and $V_{1A}$R-low cells were stimulated for 10 min with increasing concentrations of neuropeptide. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal anti-pERK1/2 antibody (left panel) or polyclonal anti-ERK2 antibody (right panel). Representative blots are shown. Graphs represent the mean band intensity ratios ± s.e.m. of four experiments performed in duplicate.

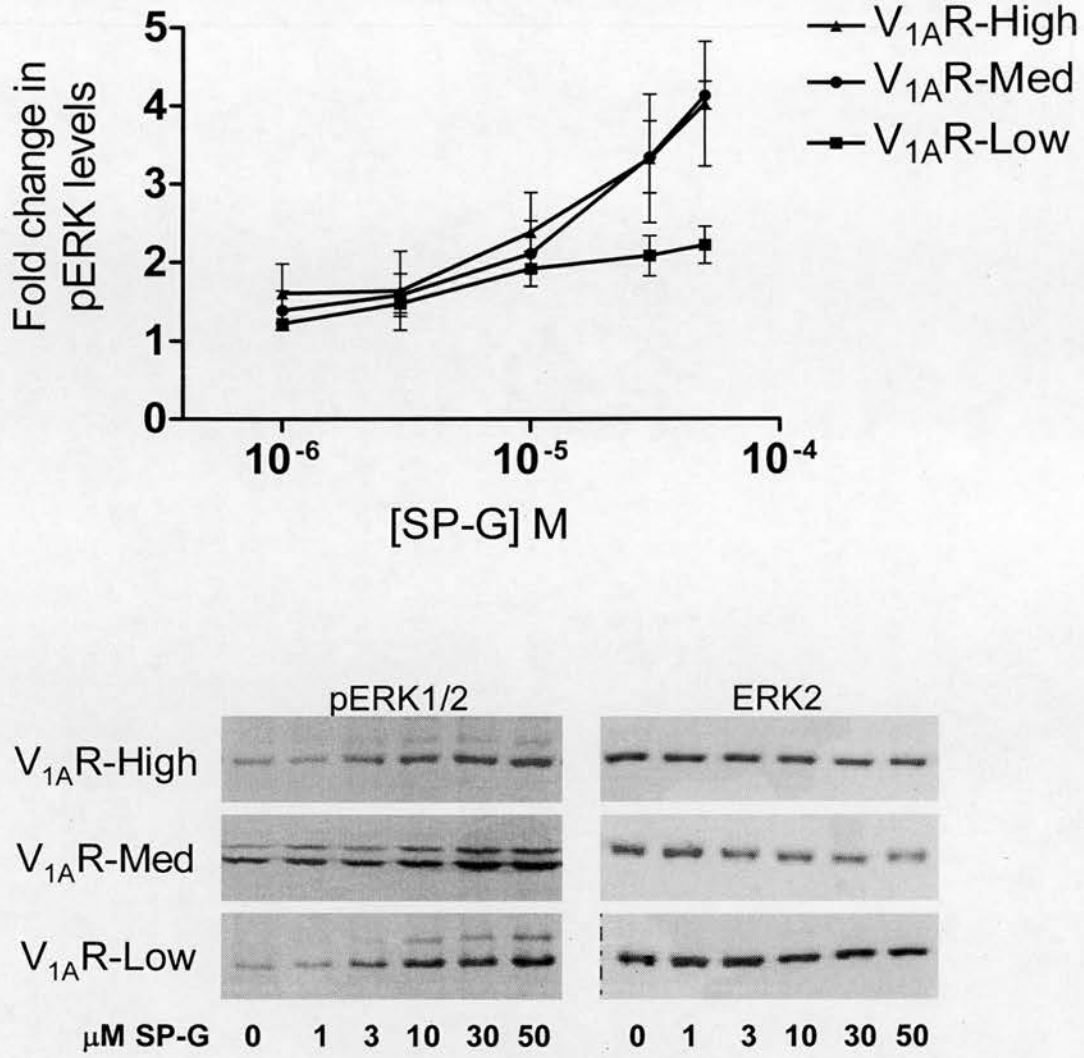



**Figure 5.7. SP-G stimulated ERK phosphorylation in $V_{1A}$ receptor expressing cells.** Confluent, quiescent cultures of $V_{1A}$R-high, $V_{1A}$R-med and $V_{1A}$R-low cells were stimulated for 10 min with increasing concentrations of SP-G. Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal anti-pERK1/2 antibody (left panel) or polyclonal anti-ERK2 antibody (right panel). Representative blots are shown. Graphs represent the mean band intensity ratios ± s.e.m. of four experiments performed in duplicate.

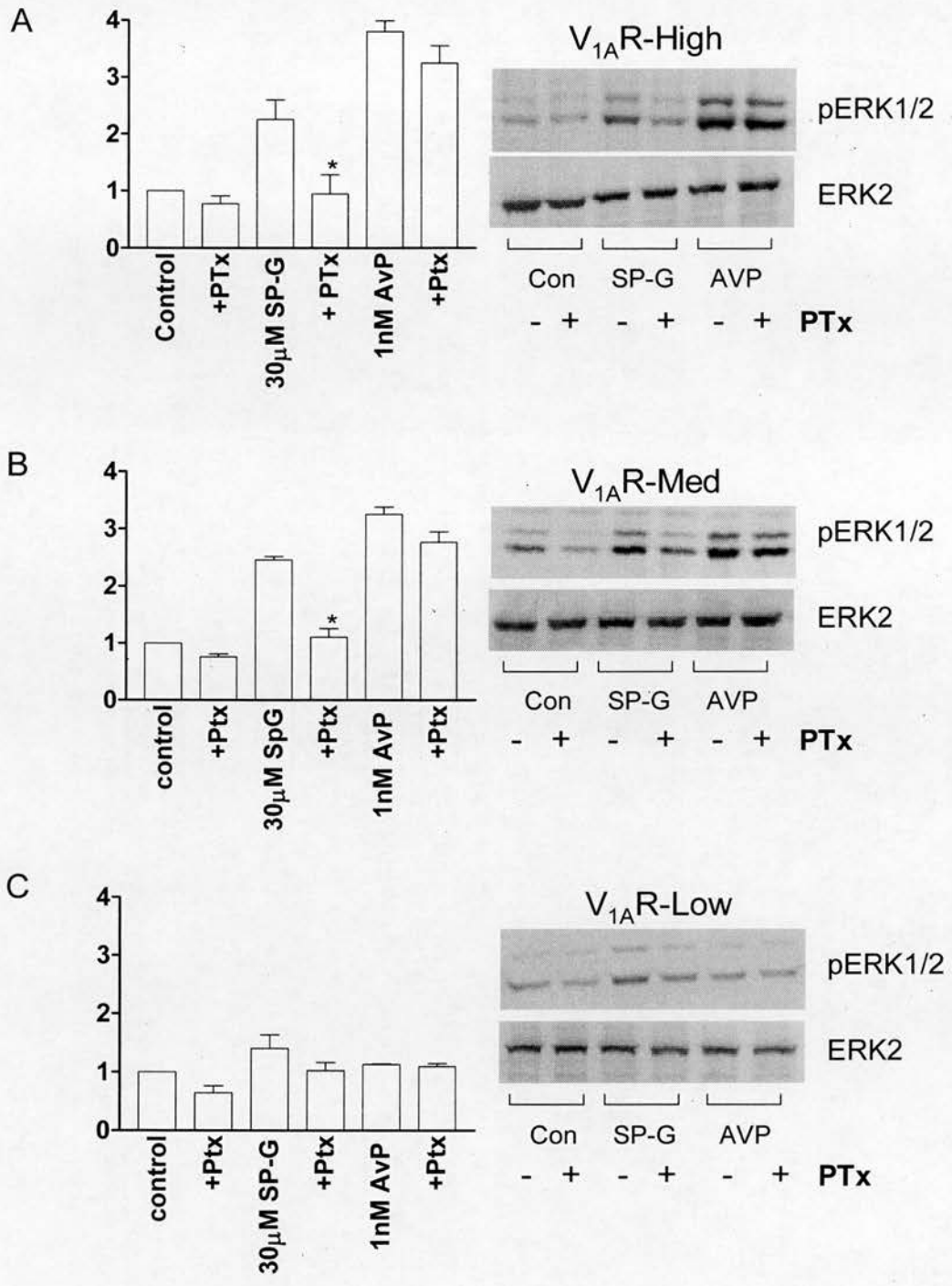



**Figure 5.8. Effect of pertussis toxin on ERK phosphorylation.** Quiescent cultures of A) $V_{1A}$R-high, B) $V_{1A}$R-med and C) $V_{1A}$R-low cells were quiesced overnight in the presence or absence of 100ng ml-1 pertussis toxin (PTx). Cells were stimulated for 10 min with 30μM SP-G or 1nM AVP. Lysates were resolved by SDS-PAGE and western blots probed with monoclonal pERK1/2 antibody (top) or polyclonal ERK2 antibody (bottom). Representative blots are shown. Bar graphs represent the mean band intensity ratios ± s.e.m. of 3 separate experiments performed in duplicate (*P<0.05, ANOVA).

## 5.2. Effect of altering G-protein levels on SP-G activity

CHO-$V_{1A}$ cells were transiently transfected with G-protein alpha subunits of wild-type $G\alpha_{i-3}$ and $G\alpha_q$ or dominant-negative $G\alpha_q^{(Q209L/D277N)}$ sub-types, as described in materials and methods. The effects of G-protein over-expression on SP-G activity in the pathways leading to calcium mobilisation (via stimulation of PLC) or ERK activation were subsequently examined. Unfortunately, G-protein over-expression was not consistently obtained throughout this series of experiments making it difficult to draw proper conclusions from this study (data not shown for over-expression of $G\alpha_i$ subunits). In particular, the dominant-negative $G\alpha_q$ subunit often appeared to be expressed at similar levels as wild-type $G\alpha_q$. Over-expression of the dominant-negative $G\alpha_q$ would have been expected to inhibit AVP-stimulated calcium mobilisation since this is a $G_q$-coupled response. The fact that this effect was not observed during this study is probably because the dominant-negative $G\alpha_q$ subunit was never expressed at sufficiently high levels to suppress wild-type $G\alpha_q$ activity. In contrast, over-expression of the wild-type $G\alpha_q$ subunit, at a level two-fold greater than basal (as determined by western blotting; data not shown), was obtained more frequently. Successful over-expression of $G\alpha_q$ resulted in an increase in the amplitude of AVP-stimulated calcium release, confirming that the $V_{1A}$ receptor coupled to a $G_q$-like protein for PLC activation (Figure 5.9a). The ability of SP-G to inhibit AVP-induced calcium mobilisation was not altered by $G\alpha_q$ over-expression (Figure 5.9b).

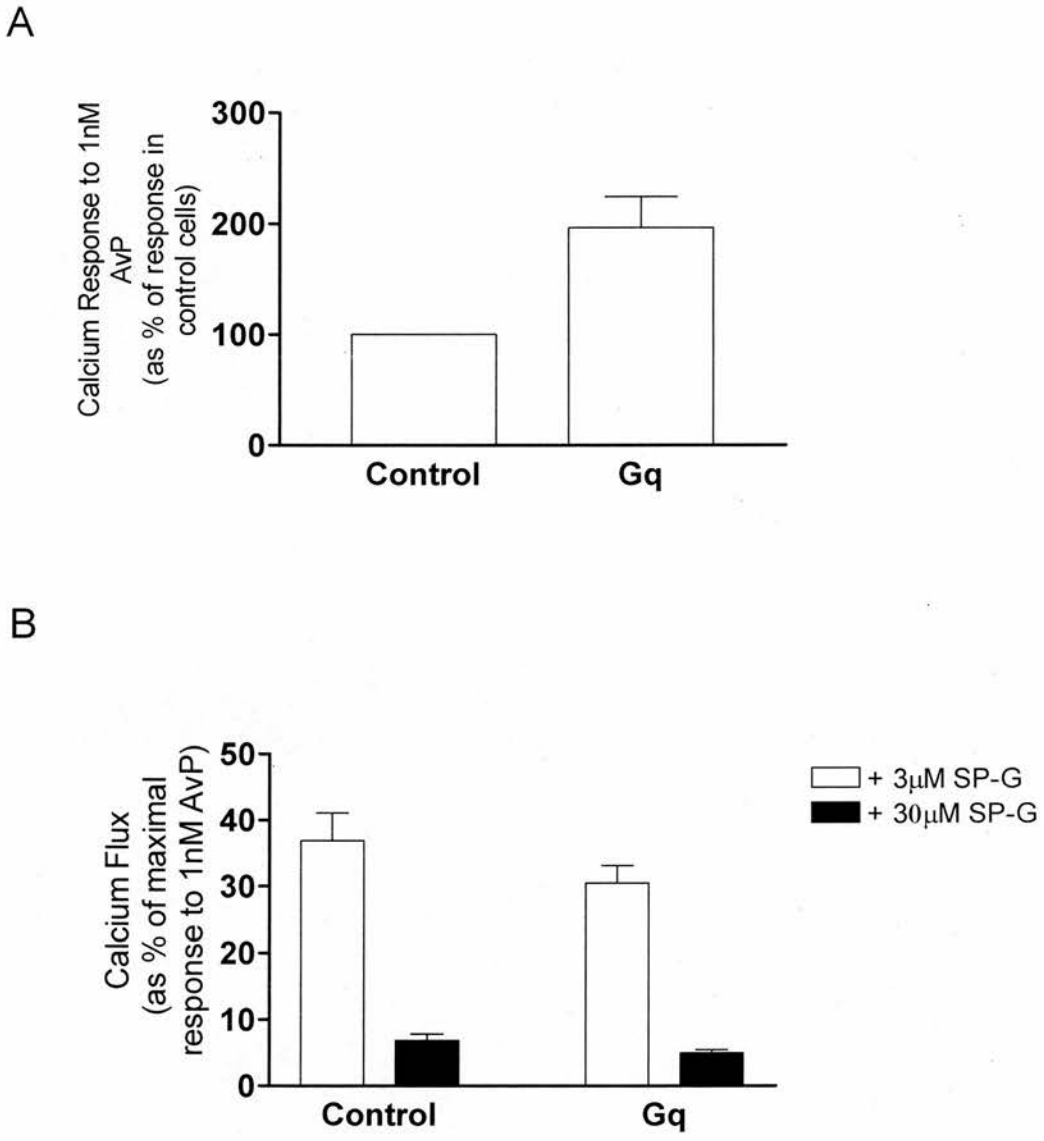



**Figure 5.9. Effect of $G\alpha_q$ over-expression on calcium mobilisation.** Quiescent CHO-$V_{1A}$ cells (control) or CHO-$V_{1A}$ cells transiently transfected with $G\alpha_q$ were loaded with FURA-2AM. A) Cells were stimulated with 1nM AVP (bar graph represents % of response to 1nM AVP in control cells). B) Cells were stimulated with 1nM AVP in the presence of 3μM or 30μM SP-G (bar graph represents the % of maximum response to 1nM AVP in respective cells). Intracellular calcium release was measured by ratiometric fluorimetry as described in materials and methods and data represents the mean ± s.e.m. of three experiments.

## 5.3. Biased agonism of chimeric $V_{1A}$ receptors

The data presented in chapter 3 demonstrated that the substance-P analogues activate ERK exclusively through activation of $G_i$-proteins. Using a linear $V_{1A}$ receptor antagonist, it has been demonstrated that in the absence of ligand the $V_{1A}$ receptor is pre-associated with G-protein subtypes $G\alpha_{q/11}$, $G\alpha_{i3}$ and $G\alpha_s$ in rat liver membranes (Strakova *et al*, 1997). Experimental data reported in the literature suggest that heterotrimeric G-proteins interact with parts of the activated receptor at the transmembrane helix-intracellular loop interface (Sgourakis *et al*, 2005). While the second intracellular loop of the $V_{1A}$ receptor has been shown to be essential for receptor coupling to $G_q$ proteins (Liu & Wess, 1996), at present it is unknown which intracellular region of the receptor is involved in coupling to $G_i$ proteins.

Chimeric $V_{1A}$ receptors, where the second ($V_{1A}i2$) or third ($V_{1A}i3$) intracellular loops have been replaced with the corresponding sequence of the $V_2$ ($G_s$-coupled) receptor, were used to determine whether the analogues directly affect the intracellular G-protein coupling domains of the receptor to induce $G_i$-mediated ERK activation. CHO-K1 cells were transiently transfected with wild-type $V_{1A}$ or chimeric $V_{1A}i2$ or $V_{1A}i3$ receptors (as described in materials and methods) and a preliminary study of the effects on receptor signalling carried out.

Binding studies using [$^3$H]-AVP on membranes prepared from $V_{1A}R$, $V_{1A}i2R$ or $V_{1A}i3R$ cells showed that AVP had similar affinity for the $V_{1A}$ and V1i2 receptors ($K_d$ = 5.71 ± 2.54 nM and 2.64 ± 0.65 nM respectively). AVP had slightly greater affinity for the V1i3 receptor than for the wild-type receptor ($K_d$= 1.14 ± 0.57 nM). Competitive binding experiments using SP-G as unlabelled competitor similarly showed that the analogue had similar affinity for the $V_{1A}$ and $V_{1A}i2$ receptors ($K_i$ = 4.2 ± 1.3μM and 3.9 ± 1.1μM respectively) and slightly greater affinity for the $V_{1A}i3$

receptor ($K_i$= 1.5 ± 0.6μM). This shows that the chimeric receptors retain the ability to bind both AVP and SP-G.

Inositol triphosphate production was measured in the three cell-lines in order to examine the effect of replacing the intracellular loops on this response to AVP. AVP, at concentrations up to 100nM, was unable to increase the generation of inositol triphosphate in $V_{1A}$i2 receptor expressing cells (Figure 5.10). In contrast, a 4-fold increase in inositol phosphates was observed in $V_{1A}$i3R cells which was comparable to the activation observed in wild-type receptor expressing cells ($EC_{50}$ for AVP was 0.89 ± 0.10 nM and 1.04 ± 0.12 nM in $V_{1A}$ and $V_{1A}$i3 receptor expressing cells respectively). This suggests that the second intracellular loop is essential for $V_{1A}$ receptor activation of PLC.

Preliminary measurements of intracellular calcium mobilisation in response to AVP in the three cell-lines were made using FURA-2-AM. Stimulation with 10nM AVP resulted in an increase in intracellular calcium levels in $V_{1A}$R and $V_{1A}$i3R expressing cells whereas cells expressing the $V_{1A}$i2 chimera gave only a weak response (Figure 5.11). At concentrations of 3, 10 and 30μM SP-G was still able to inhibit the $V_{1A}$i3R calcium response to 1nM AVP, but less effectively than in $V_{1A}$R expressing cells (Figure 5.12). This data similarly indicates that the $G_q$-coupled calcium response is dependent upon the second intracellular loop of the $V_{1A}$ receptor and also shows that changing the third intracellular loop does not prevent SP-G from inhibiting this event.

The efficacy of AVP and SP-G in stimulating ERK activation via the chimeric receptors was compared with wild-type $V_{1A}$ receptor stimulation. CHO-K1 cells transiently expressing $V_{1A}$, $V_{1A}$i2 or $V_{1A}$i3 receptors were stimulated for 5 minutes with increasing concentrations of AVP or SP-G and western blot analysis of lysates performed. Preliminary experiments showed that AVP stimulation of the

$V_{1A}$i3 chimera resulted in more potent ERK activation than that observed following wild-type receptor stimulation (Figure 5.13). In contrast, SP-G had lower efficacy for ERK activation in these cells; producing significant stimulation only at 30μM. AVP was less effective at stimulating ERK activation via the chimeric $V_{1A}$i2 receptor than the wild-type receptor. However, the efficacy of SP-G for ERK phosphorylation was not altered by the $V_{1A}$i2 chimera. This data shows that the second and third intracellular loop of the $V_{1A}$ receptor is required for effective ERK activation by AVP and SP-G respectively.

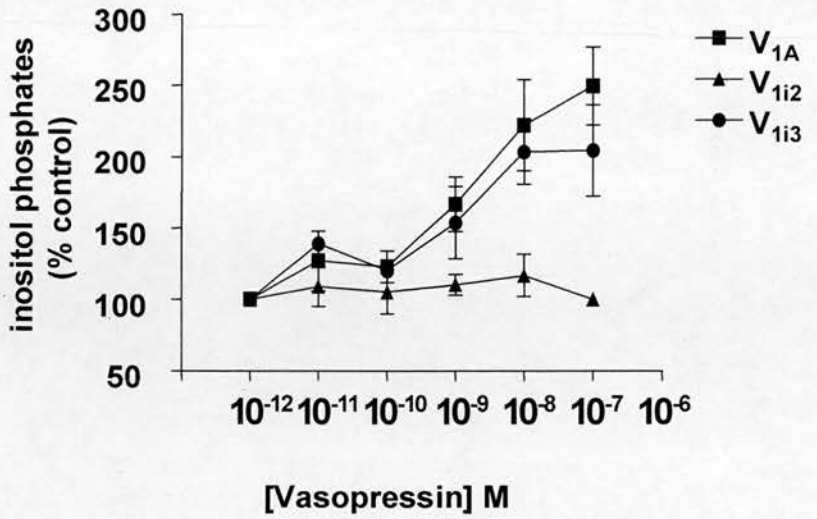



**Figure 5.10. PLC activation in chimeric receptor expressing cells.** CHO-K1 cells transiently transfected with $V_{1A}R$, $V_{1A}$ i2R or $V_{1A}$ i3R were incubated overnight in serum- and inositol-free medium containing 1μCi $ml^{-1}$ myo-[2-$^3$H]-inositol. Cells were washed prior to stimulation with the indicated concentrations of AVP (30 min; 37°C). [$^3$H]-inositol phosphate production was measured as described in materials and methods. Results are expressed as % of unstimulated cells and represent the mean ± s.e.m. of three independent experiments.

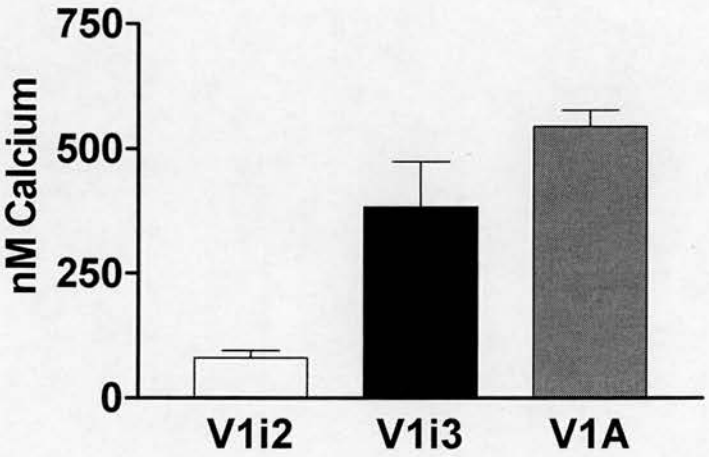



**Figure 5.11. Intracellular calcium mobilisation in chimeric receptor expressing cells.** Quiescent CHO-K1 cells transiently transfected with $V_{1A}R$, $V_{1A}$i2R or $V_{1A}$i3R were loaded with FURA-2AM. Cells were stimulated with 10nM AVP and intracellular calcium release measured by ratiometric fluorimetry as described in materials and methods. Data represents the mean ± s.e.m. of two experiments.

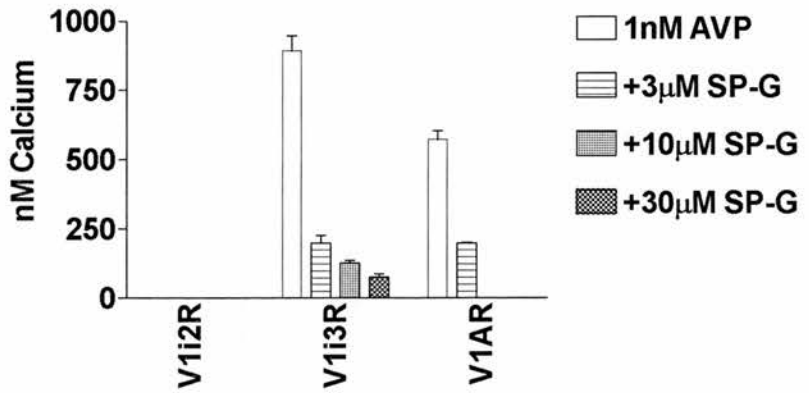



**Figure 5.12. Inhibition of intracellular calcium mobilisation in chimeric receptor expressing cells by SP-G.** Quiescent CHO-K1 cells transiently transfected with $V_{1A}R$, $V_{1A}i2R$ or $V_{1A}i3R$ were loaded with FURA-2AM. Cells were stimulated with 1nM AVP in the absence or presence of 3μM, 10μM or 30μM SP-G and intracellular calcium release measured by ratiometric fluorimetry as described in materials and methods. Data represents the mean ± s.e.m. of two experiments.

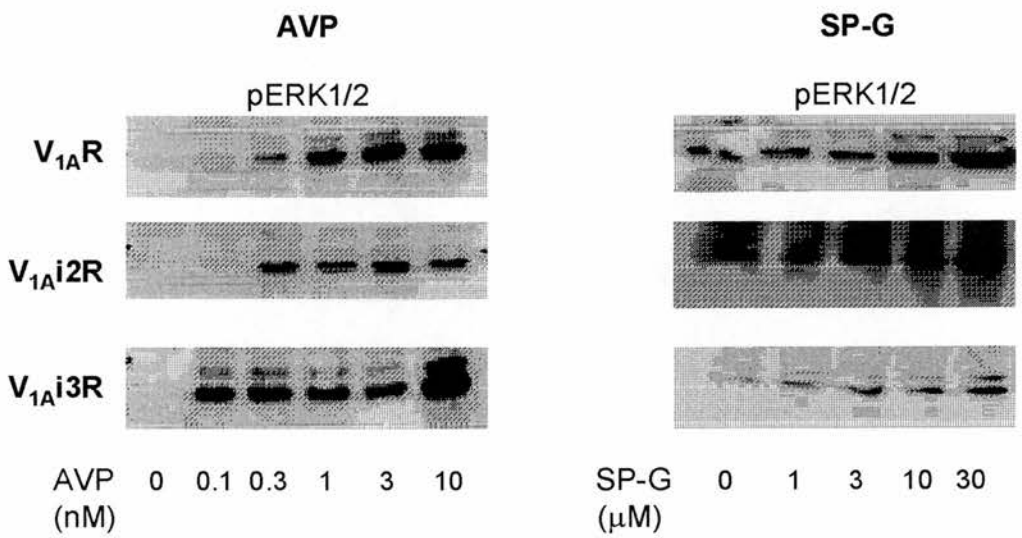



**Figure 5.13. ERK activation in chimeric receptor expressing cells.** Confluent, quiescent cultures of CHO-K1 cells transiently transfected with $V_{1A}R$, $V_{1A}i2R$ or $V_{1A}i3R$ were stimulated for 5 min with increasing concentrations of AVP (left panel) or SP-G (right panel). Aliquots of cell lysate were resolved by SDS-PAGE and western blots probed with monoclonal anti-pERK1/2 antibody. Representative blots are shown.

## 5.4 Discussion

The data presented in chapter 3 showed that neuropeptides induced receptor coupling with $G_q$ proteins for PLC and ERK activation. In contrast, the substance-P analogues inhibited neuropeptide-stimulated calcium release (event downstream of PLC activation) and exclusively activated ERK through receptor coupling to $G_i$ proteins. In this chapter, an attempt was made to investigate the factors which control the biased agonist activity of the substance-P analogues.

Alteration of the receptor/G-protein stoichiometry has been shown to change the coupling specificity of various GPCRs. Low level expression of the $V_{1b}$ receptor in CHO cells resulted in receptor coupling to G-proteins of the $G_q$ family whereas at higher expression levels, $G_i$- and $G_s$- coupling were also observed (Thibonnier *et al*, 1997). A similar study using the $G_s$-coupled $V_2$ receptor revealed that the receptors coupled to $G_q$-proteins when expressed in high numbers (Zhu *et al*, 1994). Alteration of the $V_{1A}$ receptor expression level in CHO-K1 cells therefore may potentially have resulted in changes in the G-protein selectivity of SP-G and/or AVP. The data presented in this chapter showed that increasing $V_{1A}$ receptor number did not alter the affinity of AVP or its potency for calcium mobilisation. Tsuda et al (1997) similarly found that increasing the GRP receptor number had no effect on the affinity of GRP for receptor in binding studies and did not alter the potency ($EC_{50}$) for activating PLC. Similarly here, the efficacy of AVP-induced calcium mobilisation (a PLC-coupled response) increased with increasing receptor numbers, as did the efficacy of AVP-induced ERK activation. In contrast, the efficacy of ERK activation by SP-G was not altered between medium and high expressing cells, suggesting that this response may be limited. Limitations could be imposed upon $G_i$–induced ERK activation through low availability of $G_i$ proteins at

the expression level, restraint/ regulation of $G_i$–proteins by cytoplasmic proteins or sequestration of $G_i$–proteins by other receptors.

An extended ternary complex model in which multiple receptors couple to a single G protein has been used to predict limitations of agonist, receptor and G-protein interaction which were experimentally investigated using neuroblastoma-glioma cells that express alpha 2b-adrenergic, m4 muscarinic, and delta-opiate receptors which all utilise $G_i$ proteins as a transducer (Graeser & Neubig, 1993). Theoretical simulations of the simple ternary complex model predicted that agonist binding with differential affinity only occurred when G-protein was limiting, whereas measurements showed that a significant excess of G-protein over receptor existed in the neuroblastoma-glioma cell system. A second prediction of this model was that binding of an agonist at one receptor would produce competition for G-protein used by another receptor; if the G-protein pool were limiting and freely mobile, this would result in an unlabeled agonist at one receptor decreasing binding of a radiolabeled agonist to another receptor. Experimentally, the $G_i$-protein was made limiting by a partial pertussis toxin treatment, but no cross-talk was observed between the three $G_i$–coupled receptors in the neuroblastoma-glioma cell-line. It was therefore suggested that instead limitations are probably imparted by regulation of G-protein mobility in the membrane through 1) attachment to structural elements, such as the cytoskeleton, 2) sequestration in lipid pools, or 3) organisation into slowly exchanging supramolecular complexes.

Since the efficacy of AVP for calcium mobilisation and ERK activation increased with increasing receptor density without a change in potency, this indicates that there is little receptor reserve for these responses. The ratio of $K_i/EC_{50}$ for a particular G-protein mediated response can also be used as an indicator of the magnitude of receptor reserve for the response (Brink *et al*, 2000). A large $K_i/EC_{50}$ ratio suggests a large receptor reserve and vice versa. In this study, the potency for calcium mobilisation and ERK activation by AVP was equivalent to the affinity of

AVP for the $V_{1A}$ receptor. This reflects small $K_i$/EC50 ratios which further suggest that there is a small receptor reserve for these responses to AVP. Conversely, since the efficacy of SP-G for ERK activation did not change between medium and high expressing cells, this suggests that there is a higher receptor reserve for SP-G induced ERK activation. The different effect altering receptor density has on the efficacy of AVP and SP-G for ERK activation via the $V_{1A}$ receptor further indicates that the two ligands have distinct mechanisms of action.

Fitzgerald *et al* (1999) showed that by increasing h5-$HT_{2C}$ receptor numbers, the partial agonist LSD became a full agonist in HEK 293 cells due to the existence of a receptor reserve for PLC activation. Pre-treatment with pertussis toxin showed that SP-G maintained $G_i$–coupling for ERK activation regardless of $V_{1A}$ receptor number. Therefore, although there may have been a large receptor reserve for SP-G induced ERK activation, altering $V_{1A}$ receptor numbers did not enable SP-G to induce $G_q$-mediated activation of ERK. Similarly, changes in receptor density did not permit SP-G to couple $V_{1A}$ receptors with $G_q$ proteins for PLC activation and subsequent calcium mobilisation. In fact, SP-G maintained antagonism of the $G_q$-coupled calcium response regardless of $V_{1A}$ receptor density. Together, the data shows that SP-G retains biased agonist activity across the range of receptor densities tested. It should be mentioned that a potential limitation of this study may be that the fold differences in receptor numbers between the high, medium and low expressing cells were relatively small. There was a 3.45 fold difference in receptor numbers between low and high expressing cells and a 2.4 fold difference between low and medium expressing cells. Greater fold differences in receptor numbers between the cell-lines may have provided more robust conclusions as it is possible that at extremely high receptor densities SP-G may have behaved differently. Nevertheless, the findings of this part of the study suggest that endogenous G-protein expression may instead be limiting analogue activity.

Alteration of G-protein expression may have clarified this premise, had the various G-protein subunits of interest been sufficiently over-expressed. Only $G\alpha_q$ over-expression was successfully obtained in CHO-$V_{1A}$ cells and resulted in an increase in the efficacy of AVP for calcium mobilisation and ERK activation. This confirmed that the $V_{1A}$ receptor coupled to a $G_q$-like protein to stimulate these responses. $G\alpha_q$ over-expression did not enable SP-G to stimulate a calcium response and the analogue maintained antagonism of the AVP-induced calcium response. The latter finding provides further support for the hypothesis that inhibition of neuropeptide-stimulated calcium mobilisation by substance-P analogues does not involve $G_q$-proteins and is instead dependent upon stabilisation of a receptor conformation which does not couple to $G_q$ proteins for PLC activation.

The data presented in chapter 3 indicated that the substance-P analogues bind to a site on the neuropeptide receptors which is distinct from the neuropeptides' binding site (page 127). This is corroborated by the finding that the alternate set of receptor chimeras in which the $V_2$ receptor intracellular loops were replaced by those from the $V_{1A}$ receptor shows that although these receptors are still able to bind AVP with high affinity, they are no longer able to bind substance-P analogues (M. Seckl, unpublished observations). Substance-P analogues must therefore interact at site distinct from the ligand recognition site. This differential interaction may enable the analogues to stabilise a receptor conformation which differs from that induced by neuropeptide allowing it to couple more effectively to $G_i$ proteins. Many GPCRs are able to couple to G proteins from more than one family (Gudermann, 1997). G-protein coupling is proposed to involve the C-terminal end of the G protein $\alpha$-subunit binding in a pocket constituted by the intracellular loops of the various GPCR families (Tsuda *et al*, 1997). Previous studies have shown that in addition to activating $G_i$-protein coupled responses, the $\alpha_{2A}$-AR is capable of stimulating adenylyl cyclase through activation of $G_s$ proteins, albeit less effectively (Eason *et al*, 1992). Chimeric analysis of the intracellular loops of this receptor showed that the i2,

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N-terminal i3 and C-terminal i3 regions were required for $G_s$-coupling (Eason *et al*, 1995). In contrast, the N-terminal i3 or C-terminal i3 region were independently capable of supporting $G_i$ coupling and activation (Eason & Liggett, 1996). Previous studies have shown that the second intracellular loop of the $V_{1A}$ receptor is necessary for $G_q$-coupling and subsequent PLC activation (Liu & Wess, 1996; Erlenbach & Wess, 1998). It has been suggested that the differential involvement of the intracellular domains in coupling to G proteins might represent the molecular basis for agonist-selective responses through G protein-coupled receptors (Skrzydelski *et al*, 2003). Preliminary experiments using chimeric $V_{1A}$ receptors, where the second ($V_{1A}$i2) or third ($V_{1A}$i3) intracellular loops were replaced with the homologous region of the $V_2$ ($G_s$-coupled) receptor, were carried out to determine whether SP-G required the intracellular G-protein coupling domains of the receptor to elicit biased agonist activity.

Binding studies showed that the chimeric receptors retained the ability to bind both AVP and SP-G. However, the $V_{1A}$i3 receptor had slightly greater affinity for AVP than the wild-type receptor did. It has been suggested that the i3 loop may exert indirect conformational effects on the configuration of the AVP binding site (Liu & Wess, 1996). The finding that the affinity of SP-G for the $V_{1A}$i3 receptor was also slightly increased indicates that some degree of overlap between the analogue and neuropeptide binding sites may exist.

Data presented in this chapter showed that the second intracellular loop was essential for $V_{1A}$ receptor activation of PLC, which subsequently leads to intracellular calcium mobilisation. Preliminary calcium flux assays similarly indicated that the $G_q$-coupled calcium response required the second intracellular loop of the $V_{1A}$ receptor, and exchanging the third intracellular loop of the receptor did not abolish SP-G antagonism of this event. The observation that SP-G inhibited

the $V_{1A}$i3R calcium response less effectively than the $V_{1A}$R calcium response may be related to the increased affinity the receptor has for AVP. Binding of SP-G to the $V_{1A}$R may alter the conformation of the i3 loop to facilitate $G_i$-protein coupling and simultaneously prevent the i3 loop from contributing to the configuration of the neuropeptide binding site, thus reducing AVP binding and $G_q$-coupling. Increased affinity of AVP for the $V_{1A}$i3R may thus sustain the contribution of the i3 loop to the configuration of the neuropeptide binding site, making it more difficult for SP-G to distort the binding pocket.

AVP was less effective at stimulating ERK activation via the chimeric $V_{1A}$i2 receptor than the wild-type receptor. However, the efficacy of SP-G for ERK phosphorylation was not altered by the $V_{1A}$i2 chimera. Given that substitution of the second intracellular region of the $V_{1A}$ receptor did not affect SP-G-induced ERK activation coupled with the finding that SP-G increases ERK via $G_i$ suggests that the i2 region may not be involved in $V_{1A}$ receptor coupling to $G_i$ although it is crucial for receptor binding to $G_q$. The increased affinity of AVP for the $V_{1A}$i3R correlated with more potent ERK activation, whereas SP-G also had greater affinity for $V_{1A}$i3R but was less able to activate ERK. This suggests that the i3 domain may be involved in $V_{1A}$ receptor coupling to $G_i$ and is required for $G_i$–mediated ERK activation by SP-G. This lends further credibility to the existence of agonist selective states, as different agonists would be expected to alter receptor conformation so as to expose different G-protein interacting sequences leading to selective activation of downstream signaling events.

Maudsley *et al* (2004) have similarly demonstrated the existence of agonist selective receptor states using GnRH I, GnRH II and GnRH analogues. Their studies showed that these ligands are able to selectively inhibit the growth of peripheral reproductive tumours and this ability correlates with ligand-selective activation of a $G\alpha_i$–coupled form of the type I GnRH receptor. The human gonadotropin-releasing

hormone (GnRH) type I receptor binds GnRH I (with greater affinity than it binds GnRH II) and couples to $G_q$ proteins in pituitary cells to mediate activation of PLC and calcium mobilisation, culminating in the release of pituitary gonadotrophin hormones. Structurally related antagonistic GnRH analogues displayed divergent anti-proliferative efficacies but demonstrated equal efficacies in inhibiting GnRH-induced $G\alpha_q$-based signalling and this was proposed to be a consequence of their relative abilities to stabilise an active form of the receptor that is capable of coupling productively to $G\alpha_i$ (GnRH ligand-induced selective signalling).

Whereas GnRH I is more potent in the activation of $G_q$ proteins in the gonadotrope, GnRH II is more potent in the stimulation of apoptosis and anti-proliferative effects through activating $G_i$–protein mediated signalling. This ligand-induced selective signalling is proposed to be a consequence of the binding of GnRH I and II selectively stabilising different receptor-active conformations (Lu *et al*, 2005). The molecular basis for this hypothesis was provided through Ala mutagenesis of highly conserved GPCR TM residues in the human GnRH type I receptor, which resulted in an increase in GnRH II binding with little effect on GnRH I binding (Lu *et al*, 2005). Of the three amino acids which differ between the ligands, $Tyr^8$ of GnRH II was important for the increased affinity of the receptor mutants for GnRH II. It was suggested that mutagenesis created a high affinity GnRH II binding site through disruption of particular sets of receptor-stabilising intramolecular interactions which facilitated intermolecular interactions between $Tyr^8$ and receptor contact residues. They interpreted that GnRH ligand binding to the human GnRH receptor breaks intramolecular constraint networks that stabilise the receptor in inactive conformations, resulting in the ligand-specific generation of new sets of inter- and intra-molecular contacts stabilising the receptor in particular active conformations which dictate downstream signalling selectivity. The manner of ligand binding can thus affect downstream signalling from a receptor.

The fact that ERK activation was observed at all in response to SP-G stimulation of the $V_{1A}$i3R may have been due to direct G-protein activation as discussed previously (chapter 3). However, previous studies using the $V_{1A}$i3R chimera have shown that AVP is able to induce $G_s$-coupling to this receptor, resulting in the generation of cAMP (Liu & Wess, 1996). This shows that agonist-induced exposure of the G-protein coupling sequences present in this particular ($V_2$R) i3 loop facilitates receptor coupling to the cognate G-protein. In light of this, it is entirely conceivable that SP-G selects a receptor conformation which exposes G-protein coupling sequences present within the third intracellular loop and facilitates $G_s$-coupling to the $V_{1A}$i3R for ERK activation. The pertussis toxin insensitivity in such a case would confirm this possibility. Indeed, preliminary results have shown that SP-G stimulated ERK activation via the $V_{1A}$i3R is not PTx sensitive (data not shown). However, if SP-G stimulated ERK activation via the $V_{1A}$i3R is PTx sensitive, this could still provide evidence for targeted effects at the G-protein coupling domains. A BBXXB motif (in which B represents a basic residue and X a non-basic residue) located in the C-terminal portion of the third intracellular loop is proposed to be involved in $G_i$–protein activation via the $\alpha_{2A}$-AR (Ikezu *et al*, 1992; Wade *et al*, 1996). Basic residues present within this motif have been shown to contribute to the activation of $G_i$ but are not required for receptor-G protein coupling (Wade *et al*, 1999). This motif is present in the C-terminal region of both the $V_{1A}$ and $V_2$ receptor i3 loops. Thus it is also conceivable that SP-G is able to activate $G_i$ (albeit less effectively) via the normally $G_s$-coupled i3 loop of the V2 receptor due to direct conformational effects on this particular region. Either way, the preliminary study discussed here suggests that the analogue does exert direct conformational effects at the G-protein coupling domains of the $V_{1A}$ receptor to induce pathway selective signalling.

To summarise, the data presented in this chapter showed that altering the density of $V_{1A}$ receptors did not enable SP-G to activate downstream signalling in a manner analogous to AVP stimulation of the same receptors. Similarly, increasing $G\alpha_q$-protein levels did not result in SP-G inducing $G_q$-coupling of the $V_{1A}$ receptor and stimulating a calcium response as the endogenous receptor agonist does. These findings, together with chimeric receptor studies confirm that SP-G functions as a dual efficacy ligand which promotes an agonist state of the $V_{1A}$ receptor that couples to $G_i$ leading to activation of ERK but which blocks AVP-induced activation of PLC and subsequent release of intracellular calcium. The molecular basis for this activity appears to involve a region of the receptor out with the second intracellular loop which is involved in receptor coupling to $G_q$. This study provides a tentative basis for the hypothesis that the substance-P analogues induce specific activation states of the receptor and selectively activate downstream signalling through subsequent effects at the G-protein coupling domains of the receptor.

# Chapter 6

# General Discussion and

# Suggestions for Future Work

The idea of drugs inducing selective activation states has been widely hypothesised and 'biased agonists' or dual efficacy ligands have been described for 5HT, $\alpha_{2A}$, δ-opiate and $\beta_2$ receptors (Brink *et al*, 2000; Cussac *et al*, 2002; Martin *et al*, 2002; Azzi *et al*, 2003). Theoretically this activity has important applications for future drug discovery, but has yet to be proven to be clinically relevant in any pathological condition. In endocrine cancers such as SCLC, mitogenic neuropeptide receptors such as the $V_{1A}$ receptor and the GRP receptor activate downstream signals which control proliferation and differentiation but may also activate death signals leading to JNK activation and apoptosis. A biased agonist agent which could block the proliferative effects of $Ca^{2+}$ mobilising mitogenic neuropeptides whilst stimulating receptor-dependent apoptosis would be highly advantageous in the management of tumour growth. In this respect, the broad spectrum neuropeptide receptor antagonists substance-P analogues, which inhibit the growth of tumour cells, have previously been found to be biased agonists of the GRP receptor.

This project intended to determine whether substance-P analogues exhibit 'biased agonist' activity at receptors other than the bombesin/GRP receptors and investigate factors which influence their ability to modulate neuropeptide signalling. Towards this end, model cell systems comprising of CHO-K1 cells expressing GRP or $V_{1A}$ receptors were created. It was found that neuropeptide receptor expression altered the usual characteristics of the epithelial cell line. The impact of neuropeptide receptor expression on CHO-K1 cell growth and motility was therefore studied in order to reveal the extent of change induced. In addition, the sensitivity of the

model cell systems to substance-P analogue induced growth inhibition was also determined in order to establish a link between the mechanistic basis of analogue activity and the biological consequences of such activity. Although it was found that expression of neuropeptide receptors led to cellular transformation, the sensitivity of cells to substance-P analogue-induced growth inhibition was also increased. As well as being dependent on neuropeptide receptor expression, the anti-proliferative effects of the substance-P analogues correlated with their biased agonist effects upon the neuropeptide receptors.

The novel findings from this study are that 1) GRP and $V_{1A}$ receptor expression leads to the development of a transformed phenotype in CHO-K1 cells. 2) Receptor expressing cells show some increased resistance to the chemotherapeutic agent etoposide. 3) Expression of $V_{1A}$ receptors, as well as GRP receptors, confers sensitivity to substance-P analogue induced growth inhibition. 4) Substance-P analogues act as biased agonists at $V_{1A}$ receptors, as well as GRP receptors. 5) Like SP-D, the analogue SP-G also has biased agonist properties against neuropeptide receptors. 5) This pharmacological activity is crucial for the anti-proliferative effects of these agents and may be of particular benefit in more differentiated cancers that have developed resistance to chemotherapy. 6) The molecular basis for this activity involves a region of the receptor out with the second intracellular loop which is involved in receptor coupling to $G_q$. 7) The substance-P analogues bind to a site which is distinct from the neuropeptide binding site.

This study demonstrated that GRP or $V_{1A}$ receptor expression transformed CHO-K1 cells since these cells exhibited various hallmarks of cancer. The self-sufficiency in growth these cells exhibited was thought to be responsible for increased growth of these cells under anchorage dependent and independent conditions. This may have been a consequence of constitutive neuropeptide receptor activity in this cell-type since these observations were made in the absence of exogenously added

neuropeptides. However, investigation into the existence of an autocrine growth loop should be carried out as this may provide a biological basis for the constitutive receptor activity. Neuropeptide receptor induced transformation is likely to be of relevance to the development and progression of neuropeptide dependent tumours. Bombesin has been implicated in the progression of prostate cancer to androgen independence by virtue of synergistic effects which promote androgen receptor mediated signalling (Dai *et al*, 2002). Cross-talk between neuropeptide receptors and receptor tyrosine kinases may facilitate optimal mitogenic signal pathway activation; therefore the involvement of various RTKs in neuropeptide receptor induced transformation should be investigated further. In particular, it would be of interest to see whether transactivation of the IGF-1R by neuropeptide receptors contributes to the ability of the transformed cells to evade detachment induced apoptosis (anoikis) and promotes anchorage independent growth since this RTK is able to mediate anti-apoptotic signalling. Constitutive FAK activity has been shown to confer anchorage-independence (Frisch *et al*, 1996) and may be responsible for the anchorage independent growth capabilities of neuropeptide receptor expressing CHO-K1 cells. A comparison of FAK activity in vector and neuropeptide receptor transfected cells would determine whether this kinase plays a role in the anchorage-independent growth of these cells. In the present study it was shown that neuropeptide receptor expression alone resulted in increased migration and decreased adhesion to fibronectin. It should be pointed out that the static adhesion assay used in this study primarily investigates cell attachment and that *in vivo*, because of the presence of shear stress, cell detachment (which is not necessarily a biologically equivalent process) would play an important part in governing the metastatic potential of a tumour cell. A recent study by Glover *et al* (1994) showed that phosphorylation of Tyr 397 of FAK was essential for GRPs effects on adhesion and migration and suggested that the GRP acts as a morphogen which causes increased adhesion and decreased deformability; effects which would result in increased tumour differentiation and decreased metastasis in colon cancer. As in the present study addition of GRP to the cells produced no further effect on adhesion or

migration. It was suggested therefore that the GRP receptor in colon cancer cells was activated fully by an autocrine mechanism. This present study however has shown that expression of GRP and V1A receptors in hamster epithelial cells leads to decreased adhesion to fibronectin and increased motility which would fit with a more metastatic phenotype. Overall, investigating the mechanism underlying neuropeptide receptor induced transformation using these model cell systems may contribute to a greater understanding of the role of neuropeptide receptors in the development and progression of neuropeptide-dependent tumours. This may lead to the identification of novel targets for anti-cancer therapy.

The mechanism underlying neuropeptide-induced chemoresistance should be investigated further as this may reveal novel targets for treating neuroendocrine tumours which have developed chemoresistance. Inhibitors of P13K and Akt could be used to determine whether P13K-Akt signalling is involved in this process as this pathway is known to promote survival under apoptotic stress.

A correlation was previously found to exist between GRP receptor expression and SP-G induced growth inhibition of a panel of tumour cell-lines (Waters *et al*, 2003). In addition, SP-D had previously been shown to be a biased agonist against GRP receptors but it remained unclear whether the growth inhibitory effects could be attributed to such activity at these receptors alone. This thesis has illustrated that SP-D and SP-G act as biased agonists at the $V_{1A}$ receptor as well as the GRP receptor and that such activity has growth inhibitory consequences irrespective of which receptor is stimulated. Thus it can be postulated that discordant signalling stimulated by biased agonist activity is central to the growth inhibitory properties of this group of analogues. It would be of further interest to determine whether the analogues are able to synergistically inhibit the growth of cells expressing different analogue-sensitive neuropeptide receptors (as SCLC cells do). This could be examined using the GRP or $V_{1A}$ receptor model cells transfected with other neuropeptide receptors. This would indicate whether further biased agonists of

neuropeptide receptors need essentially be designed to similarly have broad-spectrum activity for maximum effect.

The finding that $V_{1A}$ or GRP receptor expression in CHO-K1 cells leads to increased sensitivity to substance-P analogue induced growth inhibition suggests that screening biopsy samples for expression of GRP and $V_{1A}$ receptors could be useful in predicting clinical responsiveness to treatment with substance-P analogues. In addition, since the substance-P analogues exhibit selectivity towards different neuropeptide receptors there may be potential for tailoring treatment to a specific tumour phenotype. For example, SP-G may be more beneficial in $V_{1A}$ receptor expressing tumours while SP-D may be better for tumours which express both GRP and $V_{1A}$ receptors. Given that SP-D and SP-G inhibited the growth of neuropeptide receptor transformed cells and not normal CHO-K1 cells, this suggests that these anti-cancer drugs should have minimal side-effects. In the phase I clinical trial of SP-G, patient plasma levels of SP-G were achieved which were equivalent to the $IC_{50}$ values of *in vitro* pre-clinical studies, with side-effects (e.g. facial flushing) being generally well tolerated (Clive *et al*, 2001). Thus, these analogues may be clinically effective against a broad spectrum of neuropeptide-dependent tumours.

The $V_{1A}$ receptor expressing cell-line, which expressed a five fold lower density of receptors than the GRP receptor expressing cell-line, was still susceptible to growth inhibition by substance-P analogues indicating that sensitivity to the compounds may not be limited by a critical receptor density. A possible molecular basis for this was provided through experiments which were carried out in an attempt to understand what facilitates substance-P analogue biased agonism. The ratio of receptors to G-proteins was altered and the subsequent effects on analogue activity examined. The preliminary data obtained showed that for the activation of ERK there appears to be a greater receptor reserve available to SP-G for coupling receptors to $G_i$ proteins than exists for AVP to couple receptors to $G_q$ proteins. This

indicates that the analogues only need to activate a small proportion of the available receptors for $G_i$–mediated ERK stimulation, so the analogues could well be effective against tumours regardless of their level of receptor expression. SP-G also maintained coupling to $G_i$–proteins for ERK activation following $G_q$ protein over-expression which similarly suggests that these analogues may be able to maintain activity irrespective of which G-protein is in abundance in different tumours. However, these findings need to be confirmed through further studies, preferably using cell-lines expressing greater fold differences in receptor numbers and also with a greater level of G-protein over-expression of both $G_q$ and $G_i$ proteins. Since conventional transfection of the CHO-K1 cells with bacterial vectors encoding wild type and dominant negative G-protein $\alpha$-subunits was disappointing it would be interesting to increase expression using an adenoviral or retroviral system and to inhibit expression using $G\alpha$ minigenes or siRNA. It may also be informative to examine the role of $G\beta\gamma$ subunits in the transduction of biased agonist signalling through expression of the carboxy-terminal domain of G protein-coupled receptor kinase 2 (GRK2-ct), which is a $G\beta\gamma$-sequestering polypeptide.

Investigations into the mechanism of action of the substance-P analogues showed that SP-D and SP-G inhibited neuropeptide binding and antagonised calcium mobilisation but were agonists for pertussis-toxin sensitive ERK activation by $V_{1A}R$ as well as the GRPR. The anti-proliferative effects of the analogues on CHO-K1 cells were dependent on the expression of GRP or $V_{1A}$ receptors. The biased agonist activity thus correlates with anti-proliferative effects. To further strengthen this hypothesis, spantide, despite having only a single amino acid substitution difference from SP-D, did not act as a biased agonist at GRP or $V_{1A}$ receptors, but did bind to GRP receptors with high affinity and blocked calcium responses. Moreover, spantide did not inhibit the growth of the H345 SCLC cell-line. This would confirm that uncoupling of receptors from $G_q$ proteins is not sufficient to

induce growth inhibition, and $G_i$-protein-mediated signalling (i.e. through biased agonism) is required for the anti-proliferative effects of the analogues.

In CHO-K1 cells expressing neuropeptide receptors, the analogues modulated neuropeptide receptor signalling so that ERK activation was mediated through enhanced coupling to $G_i$ proteins, in comparison with the largely $G_q$ mediated ERK activation induced by the natural ligand. This presumably shifts the balance of intracellular signalling and it would be of interest to investigate further what impact the substance-P analogue induced differences in duration and manner of intracellular signalling (e.g. ERK or JNK activity) have on biological outcome. Previous work in our laboratory has shown that ERK activity at least partially contributes to the pro-apoptotic effects of SP-D on GRPR expressing fibroblasts and H69 SCLC cells. A mechanism by which cells are able to detect differences in the duration of intracellular signalling has been revealed which involves the immediate early gene product *c-Fos* behaving as a sensor for ERK1/2 signal duration (Murphy *et al*, 2002). Sustained ERK signalling leads to stable *c-Fos* phosphorylation and exposes an ERK docking site, facilitating additional phosphorylation and thus *c-Fos* mediated signalling. Putative ERK binding sites have also been identified in other immediate early gene products, including *c-Jun* which is a target of activated JNK (Murphy *et al*, 2002). Sustained ERK signalling can therefore potentially influence JNK activity. Substance-P analogues stimulate JNK activity in SCLC cells which is sustained over time (MacKinnon *et al*, 1999) and has been shown to occur through receptor coupling to $G_{12}$ proteins in GRPR expressing fibroblasts (Mackinnon *et al*, 2001). A number of attempts were made to evaluate JNK activation and apoptosis in response to substance-P analogues using the model cell systems but were unsuccessful. This aspect of the study should be re-visited and if accomplished, the possibility that the sustained pattern of substance-P analogue stimulated ERK activity contributes to the induction of the apoptotic pathway investigated. Substance-P analogue induced JNK activation in SCLC cells in the presence and absence of ERK inhibition could also be measured. Since the intracellular localisation of activated ERK has been shown to have an impact on cell fate, confocal

imaging could be used to determine whether the subcellular localisation of substance-P analogue and neuropeptide stimulated ERK differs. Growth inhibition may involve modulation of receptor trafficking since it has previously been demonstrated that SP-D does not induce GRP receptor desensitization to the same extent as bombesin (Mackinnon *et al*, 2001). Arrestins have traditionally been implicated as mediators of GPCR desensitization and internalisation but have recently been shown to play an important role in the suppression of GPCR-mediated apoptosis (Beekman *et al*, 1998). Therefore it may be informative to investigate whether the substance-P analogues interfere with β-arrestin function in order to transduce an anti-proliferative signal through neuropeptide receptor stimulation instead of a mitogenic one. Such investigation of the consequences of biased agonist induced signalling events may facilitate a better understanding of how dysregulated signalling can influence cell fate; consideration of which may be useful in the design of pathway selective drugs.

Also along the line of investigating the apoptotic effects of the analogues, the contribution that $G_i$–mediated signalling makes to this process could also be investigated to gain a better understanding of the mechanism underlying growth inhibition through biased agonism. Lack of Substance-P analogue induced growth inhibition following $G_i$–protein inactivation using Pertussis Toxin or siRNA to decrease expression of $G\alpha_i$, would confirm a requirement for $G_i$–mediated signalling in the anti-proliferative effects of these compounds.

The finding that the substance-P analogues were capable of stimulating ERK activity in vector-transfected CHO-K1 cells suggested that the compounds may have receptor-independent effects on cells. However, it has previously been demonstrated that SP-G induced apoptosis and JNK activation in SCLC cells is dependent on the generation of reactive oxygen species (ROS) (MacKinnon *et al*, 1999). $G\alpha_{i/o}$ proteins are targets of ROS, resulting in $G_i$–protein activation and the subsequent activation of ERK (Nishida *et al*, 2000). Therefore, it is possible that the generation of ROS by substance-P analogues could account for the activation of ERK

observed in CHO-vector cells. It is promising that despite stimulating ERK activity in CHO-vector cells, the analogues did not inhibit the growth of these cells. This maintains that substance-P analogue induced growth inhibition is a consequence of receptor-mediated effects, i.e. biased agonism.

A body of evidence has accumulated which supports the concept that GPCRs exist as dimers and studies have shown that a number of receptor properties are altered as a consequence (Rios *et al*, 2001). Although the physiological relevance of GPCR dimerisation is unclear, the agonist-induced dimerisation of bradykinin receptors has been demonstrated to have consequences on receptor mediated signalling (AbdAlla *et al*, 1999). Alterations in ligand properties when used in dimeric form rather than monomeric form suggest that receptor dimerisation may be involved in the mechanism of action of such a molecule (Carrithers & Lerner, 1996). In accordance with this, receptor dimerisation may underlie the anti-proliferative effects of the bradykinin antagonist dimer (CU201), which functions as a biased agonist, in contrast with the non-growth inhibiting monomer which functions as an antagonist (Chan *et al*, 2002a). $V_{1A}$ receptors have been demonstrated to form constitutive homodimers during the biosynthetic process (Terrillon *et al*, 2003) and homodimerisation of bombesin receptors has been suggested previously (Carrithers & Lerner, 1996). Therefore, receptor dimerisation may similarly play a role in the biased agonism of substance-P analogues. A greater understanding of the roles of receptor dimerisation will undoubtedly influence the direction of future drug design.

The work presented here suggests that substance-P analogues are able to promote a neuropeptide receptor conformation which couples more efficiently to $G_i$ proteins for ERK activation but is not favourable for calcium mobilisation. Consistent with the ability to recognise and bind a range of unrelated receptors to antagonise the effects of various neuropeptides, this study also showed that substance-P analogues bind to a site on the receptor which is distinct from the neuropeptide binding site in

order to do this. Using chimeric $V_{1A}$ receptors, preliminary studies into the basis of the subsequent generation of $G_i$–mediated signalling suggested that SP-G exerts direct conformational effects on the G-protein coupling domains of the $V_{1A}$ receptor to induce pathway selective signalling. These findings demonstrate that targeting the G-protein coupling domains of GPCRs to induce pathway selective signalling can be an effective way of influencing cell fate. This work also shows that biased agonists can be useful tools for dissecting receptor mediated signal transduction. However, further work should be carried out to determine the precise mechanism underlying the effects of the substance-P analogues on the G-protein coupling domains.

Since low potency and a short half-life are detrimental to the further development of these substance-P analogues, designing new drugs to supersede these compounds is likely to be a beneficial endeavour. Given that the substance-P analogues are dual efficacy ligands, high throughput screening of compounds (which bind mitogenic neuropeptide receptors) for the ability to activate $G_i$ or $G_{12}$ proteins (i.e. activate ERK or JNK with increased efficacy) but not $G_q$ proteins (i.e. block calcium mobilisation) could be used to identify molecules with similar properties. Lead compounds could then be optimised to have greater stability (increased effectiveness) and/or generate non-peptide ligands (reduced cost of synthesis). Due to the existence of an extensive network of autocrine and paracrine growth loops sustaining SCLC growth, novel biased agonists for SCLC treatment developed in the future should ideally incorporate activity against a range of neuropeptide receptors. In order to facilitate this, future work should focus on exactly how substance-P analogues are able to act upon different receptors whose ligands are structurally unrelated. Molecular modelling of the interaction between neuropeptide receptors and substance-P analogues may be informative in this respect. Although neuropeptide receptor structures have not yet been resolved due to difficulties in crystallisation, it may be possible to use the Rhodopsin GPCR

structure as a framework for modelling studies in the meantime. Mutant receptors could be used to identify the GPCR region(s) which contribute to the analogue binding site. Sequence analysis of receptors may subsequently be used to determine whether the site is conserved amongst analogue sensitive receptors. Rational drug strategies are traditionally aimed at identifying high potency drugs to keep the dosage of drug as low as possible and thus minimise side effects. However, it may be that the low affinity with which substance-P analogues bind to the receptors determines their ability to interact with a broad-spectrum of neuropeptide receptors.

This thesis has shown that substance-P analogues, previously characterised as broad-spectrum neuropeptide antagonists, can also be regarded as broad-spectrum neuropeptide biased agonists. It could be anticipated that substance-P analogues which have previously been demonstrated to be antagonists of the mitogenic effects of other neuropeptides are also likely to be biased agonists of their receptors. This widens the range of tumour types which could be sensitive to the growth inhibitory effects of these compounds from those which only express GRP receptors to potentially any tumour possessing growth loops comprising of at least one substance-P analogue sensitive neuropeptide receptor. Biased agonism is important for the antiproliferative and apoptogenic effects of substance-P analogues and this type of pharmacological activity may have clinical relevance in the treatment of neuropeptide-dependent tumours. Finally data from this work shows that SCLC tumours which are resistant following chemotherapy may show increased sensitivity to substance-P analogues. This has far reaching implications for therapy as currently there is no effective therapy for SCLC which has relapsed. *In vivo* studies focussing on the effectiveness of conventional chemotherapy on SCLC cell xenografts manipulated *in vitro* to express varying levels of neuropeptide receptor would go some way to answering this question. Conversely, the effectiveness of substance-P analogue therapy on chemosensitive and chemoresistant tumour growth could be studied.

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1. MacKinnon, A.C., U. Tufail-Hanif, C.D. Lucas, D. Jodrell, C. Haslett, and T. Sethi. 2005. Expression of $V_{1A}$ and GRP receptors leads to cellular transformation and increased sensitivity to substance-P analogue-induced growth inhibition. *Br. J Cancer* 92:522-531.

2. Waters, C.M., A.C. M acKinnon, J. Cumming s, U. Tufail-Hanif, D. Jodrel l, C. Haslett, and T. Sethi. 2003. Increased gastrin-releasing peptide (GRP) receptor expression in tumour cells confers sensitivity to [Arg$^{6}$,D-Trp$^{7,9}$,NmePhe$^{8}$]-substance P (6-11)-induced growth inhibition. *Br. J Cancer* 88:1808-1816.

3. MacKinnon, A.C., U. Tufail-Hanif, D. Jodrell, M. Seckl, C. Haslett, and T. Sethi. Agonist directed trafficking of $V_{1A}$ receptor signalling by substance-P analogues. (Ready for submission)

**British Journal of Cancer (2005) 92,** 522–531
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# Expression of $V_{1A}$ and GRP receptors leads to cellular transformation and increased sensitivity to substance-P analogue-induced growth inhibition

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Small-cell lung cancer (SCLC) is a particularly aggressive cancer, which metastasises early. Despite initial sensitivity to radio- and chemo-therapy, it invariably relapses, so that the 2-year survival remains less than 5%. Neuropeptides particularly arginine vasopressin (AVP) and gastrin-releasing peptide (GRP) act as autocrine and paracrine growth factors and the expression of these and their receptors are a hallmark of the disease. Substance-P analogues including [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]-substance-P (SP-D) and [Arg[6],D-Trp[7,9],$N^{me}$Phe[8]]-substance-P (6–11) (SP-G) inhibit the growth of SCLC cells by modulating neuropeptide signalling. We show that GRP and $V_{1A}$ receptors expression leads to the development of a transformed phenotype. Addition of neuropeptide provides some protection from etoposide-induced cytotoxicity. Receptor expression also leads to an increased sensitivity to substance-P analogue-induced growth inhibition. We show that SP-D and SP-G act as biased agonists at GRP and $V_{1A}$ receptors causing blockade of $G_q$-mediated $Ca^{2+}$ release while directing signalling to activate ERK via a pertussis toxin-sensitive pathway. This is the first description of biased agonism at $V_{1A}$ receptors. This unique pharmacology governs the antiproliferative properties of these agents and highlights their potential therapeutic potential for the treatment of SCLC and particularly in tumours, which have developed resistance to chemotherapy.
*British Journal of Cancer* (2005) **92,** 522–531. doi:10.1038/sj.bjc.6602366 www.bjcancer.com
Published online 1 February 2005
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**Keywords:** SCLC; vasopressin; GRP; transformation; SP-D; SP-G

Lung cancer is the most common fatal malignancy in the developed world. Small-cell lung cancer (SCLC), which constitutes 25% of the total, is a particularly aggressive form of lung cancer. It metastasises early and over 90% of patients have widespread metastasis at presentation precluding curative surgery. Despite initial sensitivity to radio- and chemo-therapy, SCLC almost invariably relapses, so that the 2-year survival remains less than 5% (Smyth *et al*, 1986). Small-cell lung cancer is a paradigm for neuropeptide-driven tumourigenesis. Small-cell lung cancer cells proliferate in response to a range of neuropeptide growth factors and in many cases these neuropeptides are involved in autocrine and paracrine growth loops, which fuel unrestrained proliferation (Moody *et al*, 1981; Sethi and Rozengurt, 1991; Sethi *et al*, 1992; North, 2000). This is thought to be part of a special process of oncogenic transformation called Selective Tumour gene Expression of Peptides essential for Survival (STEPS) (North, 2000). The expression of neuropeptides and their receptors in tumour cells may render them sensitive to pharmacological therapeutic intervention and may be of diagnostic importance for the early detection of lung cancer and for the selection of appropriate treatment. Much of the recent studies in SCLC have focused on the roles of the specific mitogens gastrin-releasing peptide (GRP, the mammalian homologue of bombesin) and arginine vasopressin (AVP).

Gastrin-releasing peptide receptors are frequently aberrantly expressed in human neuroendocrine tumours of the breast, prostate, lung and colon (Carroll *et al*, 1999; Jensen *et al*, 2001) where they have both mitogenic and morphogenic roles and confer a survival advantage in proliferating cancer cells. It has been suggested that the detection of these markers in the peripheral blood of patients may be useful as early markers of SCLC. Scintigraphy with the labelled peptide ligand 99mTc-bombesin/GRP has been shown to detect prostate cancer and invasion of pelvic lymph nodes and should be applicable to other endocrine tumours particularly SCLC (De Vincentis *et al*, 2004). Monoclonal antibodies have been developed against circulating bombesin and one such antibody 2A11 has been shown to inhibit the growth of SCLC *in vitro* and as xenografts in nude mice (Chaudhry *et al*, 1999); however, it has limited efficacy in human trials.

The presence of AVP and $V_{1A}$ receptors on SCLC tumours has been extensively studied and the potential presence of an autocrine growth loop has been established. Small-cell lung cancer patients frequently display symptoms of inappropriate antidiuretic hormone secretion such as hyponatremia and urinary hyperosmolality (Johnson *et al*, 1997). Independent studies have shown the expression of $V_{1A}$ receptors in five out of five SCLC lines and

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Revised 18 November 2004; accepted 7 December 2004; published online 1 February 2005

zero out of four non-small-cell lung cancer (NSCLC) lines (Ocejo-Garcia *et al*, 2001) while we showed expression of $V_{1A}$ receptors in four out of four SCLC lines (Waters *et al*, 2003). Expression of $V_{1A}$ receptors and vasopressin is the most useful diagnostic tool for differentiating SCLC from NSCLC and other cancers (Coulson *et al*, 2003). Therapy that targets $V_{1A}$ receptors may be of potential benefit for SCLC. Small-cell lung cancer cells can also express the AVP gene as provasopressin, which remains attached to the cell membrane and conceivably contributes to the autocrine-driven mitogenesis (Friedmann *et al*, 1994). Antibodies recognising this cell surface antigen have been developed as a potential diagnostic and therapeutic tool that targets SCLC tumours *in vivo* (Keegan *et al*, 2002).

However, the main focus of research in this drug development strategy has been in the development of broad-spectrum neuropeptide antagonists. Synthetic analogues of substance-P for example, [Arg$^6$,D-Trp$^{7,9}$,$N^{me}$Phe$^8$]-substance-P (6–11) (SP-G) were initially identified as antagonists of substance-P-mediated cellular effects and were subsequently found to also antagonise the cellular effects of bombesin (Jensen *et al*, 1984). SP-G and its analogue SP-D inhibit calcium mobilisation stimulated by bombesin and vasopressin in SCLC cells (Woll and Rozengurt, 1988; Langdon *et al*, 1992; Sethi *et al*, 1992). They also inhibit mitogenesis by the same neuropeptides in both Swiss 3T3 cells and SCLC cells (Woll and Rozengurt, 1988; Sethi *et al*, 1992; Seckl *et al*, 1995) In addition to the *in vitro* growth inhibitory effects of substance-P analogues, these compounds inhibit the growth of tumours in xenograft models in nude mice (Langdon *et al*, 1992) and are more effective than specific neuropeptide receptor antagonists. SP-G has recently completed a phase I clinical trial where it shows minimal toxicity at therapeutic plasma concentrations (Clive *et al*, 2001). However, its mechanism of action is still being investigated.

Our previous work has shown that SP-G and SP-D do not act as simple competitive antagonists of GRP receptors but rather act as biased agonists, inhibiting GRP-stimulated PLC activation via $G_q$ while directly stimulating JNK and ERK via $G_{12}$ and $G_i$, respectively (Tallet *et al*, 1996; Jarpe *et al*, 1998; MacKinnon *et al*, 1999, 2001) The prolonged stimulation of JNK and ERK coupled with an inhibition of intracellular $Ca^{2+}$ is fundamental for the antiproliferative and proapoptotic effects of these agents (MacKinnon *et al*, 2001, Waters *et al*, 2003). It is not known whether these compounds interact with vasopressin receptors in the same fashion.

It was the objective of the present study to examine the effect of GRPr and $V_{1A}$r expression, the two most common neuropeptide receptors present on SCLC, on growth and transformation of the epithelial cell line, CHO-K1. We show that expression of these receptors leads to an increase in basal and anchorage-independent growth in low serum, and an increased sensitivity to substance-P analogues. As well as directing GRP receptor signalling, substance-P analogues also act as biased agonists at $V_{1A}$ vasopressin receptors. These combined effects will be useful for SCLC therapy and particularly in targeting well-differentiated tumours, which have developed resistance to chemotherapy.

## METHODS

### Materials

CHO-K1 cells were purchased for the European Cell Culture Collection; Dulbecco's modified Eagle's medium (DMEM), GRP, bombesin and monoclonal antibody to diphosphorylated ERK 1 and 2 (M 8159) were from Sigma (Poole, UK); ([D-Arg$^1$,D-Phe$^5$,D-Trp$^{7,9}$,Leu$^{11}$]-substance-P (SP-D) and [D-Arg$^6$,D-Trp$^{7,9}$,$N^{me}$Phe$^8$]-substance-P (6–11) (SP-G) were synthesised by Cancer Research UK (London, UK). The human $V_{1A}$ receptor construct in pcDNA3.1 was a kind gift from M Thibonnier (Case Western Reserve University School of Medicine, OH, US). The human GRP receptor in pcDNA3.1 was from J Battey (Albert Einstein College of Medicine, NY, USA). [$^{125}$I]-Arginine vasopressin (2000 Ci mmol$^{-1}$) and [$^{125}$I]-GRP were from Amersham International (Amersham, UK).

### Cell culture and transfection

CHO-K1 cells were maintained in DMEM supplemented with 10% (v v$^{-1}$) foetal bovine serum (heat-inactivated at 57°C for 1 h) 50 U ml$^{-1}$ penicillin, 50 $\mu$g ml$^{-1}$ streptomycin and 5 $\mu$g ml$^{-1}$ L-glutamine in a humidified atmosphere of 5% $CO_2$:95% air at 37°C. CHO-K1 cells were transfected with full-length human GRP receptor or human $V_{1A}$ receptor using lipofectamine plus (Invitrogen) as per the manufacturer's instructions. Stable cell cultures were maintained in the presence of 400 $\mu$g ml$^{-1}$ G418-sulphate.

### Receptor binding

Receptor binding was carried out in confluent and quiescent cultures of CHO-K1-$V_{1A}$r and GRPr cells in a binding medium containing DMEM, 1 mg ml$^{-1}$ bovine serum albumin and radioligand (1.0 nM GRP containing 2nCi [$^{125}$I]-GRP or 0.6 nM AVP containing 45 nCi [$^{125}$I]-AVP. Incubations were carried out at 37°C for 30 min in the presence of inhibitors as indicated. Nonspecific binding was defined in the presence of 1 $\mu$M GRP or AVP, respectively. The reaction was stopped on ice and unbound ligand was removed by washing ×3 with ice-cold phosphate-buffered saline. After solubilisation in 0.1 M NaOH, 2% $Na_2C0_3$ containing 1% SDS, bound ligand was estimated by liquid scintillation counting. The binding parameters $K_d$ and $B_{max}$ were calculated from competition binding isotherms with unlabelled ligand (DeBlasi *et al*, 1989). The $IC_{50}$ (concentration of drug displacing 50% specific binding) was converted to the inhibitory constant ($K_i$), where $K_i = IC_{50}/(1 + [\text{ligand}]/K_d)$ (Cheng and Prusoff, 1973).

### Growth assays

*Liquid growth* Exponentially growing CHO-K1 cells were trypsinised and suspended in DMEM with 5% FCS at a density of $5 \times 10^4$ cells per plate in the presence or absence of mediators in triplicate. Cells were grown for 1–9 days and cell number determined using a Coulter Counter (model Z1, Coulter).

*MTT assay* In some assays, MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) formazan production (Sigma) was used to measure proliferation as per the manufacturer's instructions.

*Clonogenic assay* CHO-K1 ($2x10^4$) viable cells were mixed with DMEM containing 0.3% agarose in the presence or absence of mediators and layered over a solid base of 0.5% agarose in DMEM in 35 mm plastic dishes. The cultures were incubated at 37°C for 1–10 days, and then stained with 1 mg ml$^{-1}$ nitro-blue tetrazolium (NBT, Sigma) overnight at 37°C. Colonies from 10 separate fields were counted using a microscope with a ×4 objective. Cloning efficiency is calculated as the percentage of original number of seeded cells forming colonies of >6 cells.

*Aggregation assay* CHO-K1 cells were suspended in DMEM in the presence of 5% FCS and seeded into low adhesion tissue culture plates on top of a layer (1 ml) of 0.5% agar. Under these conditions, the cells did not adhere. Cells were maintained in culture for 7 days briefly trypsinised to disaggregate clusters and viable cells counted.

### Determination of intracellular $Ca^{2+}$ concentration

CHO-K1 cells expressing the GRP or $V_{1A}$ receptor were grown to confluence on 10 cm plates and quiesced overnight in DMEM

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containing 0.1%. FCS. Cells were trypsinised and loaded with fura-2-tetraacetoxymethylester AME (FURA-2-AM, 1 $\mu$M) in calcium-free Hank's balanced salt solution for 10 min at 37°C. The cells were pelleted and resuspended in 2 ml of Hank's balanced salt solution containing 1.8 mM $CaCl_2$. Fluorescence was recorded in a fluorescence spectrophotometer (Perkin Elmer). Alternate dual wavelength excitation at 380 and 410 nm allowed ratiometric analysis of bound and unbound FURA-2AM when measured at 505 nm. $[Ca^{2+}]$ was calculated according to the equation $[Ca^{2+}] = K(F-F_{min})/(F_{max}-F)$, where $F$ is the ratio of the unknown sample, $F_{max}$ is the ratio after the addition of 0.1% Triton X-100 and $F_{min}$ is the ratio after $Ca^{2+}$ chelation with 10 mM EGTA. $K$ is the dissociation constant for Fura-2, which is 224 nM.

### Western blotting

Quiescent cell cultures in six-well plates were treated as described in figure legends and lysed at 4°C in 0.25 ml lysis buffer containing; 25 mM HEPES pH 7.4, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Triton X-100, 20 mM $\beta$-glycerphosphate, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate and protease inhibitors (Boehringer Mannheim, Sussex, UK; prepared as per the manufacturer's instructions). Lysates were clarified by centrifugation, equilibrated for protein using BCA protein assay reagent (Perbio Science, Cheshire, UK) and denatured by boiling (5 min) in SDS–PAGE loading buffer. In all, 20 $\mu$l lysate/lane was resolved on 12% SDS–PAGE gels and electroblotted onto nitrocellulose membranes. Membranes were blocked in 3% bovine serum albumin in PBS containing 0.05% Tween-20. $ERK_{1/2}$ phosphorylation was determined using 1 : 1000 dilution of the primary antibody followed by the appropriate HRP-labelled goat IgG (DAKO, UK) diluted 1 : 5000. Bands were visualized using enhanced chemiluminescence (ECL plus, Amersham) and quantified using a phosphor imager (Storm).

## RESULTS

### Receptor binding

To assess receptor expression, stable cultures of CHO-K1-GRP and CHO-K1-$V_{1A}$ cells were incubated with the appropriate radioligand, and receptor number and affinity were measured from competition binding curves with unlabelled ligand. Figure 1 shows that GRP receptors were expressed with $K_d = 2.55 \pm 0.84$ nM and $B_{max}$ of $1151 \pm 326$ sites/cell ($n = 4$). $V_{1A}$ receptors were expressed at a slightly lower level with a $B_{max} = 450 \pm 80$ sites/cell and $K_d = 2.98 \pm 0.71$ nM ($n = 4$, Figure 1B). The substance-P analogues SP-G and SP-D inhibited [$^{125}$I]-GRP binding with affinities in the micromolar range (SP-G $K_i = 19.4 \pm 6.3$ $\mu$M ($n = 4$), SP-D $K_i = 0.64 \pm 0.05$ $\mu$M ($n = 4$, Figure 1)). Both analogues also inhibited $V_{1A}$ receptor binding ((SP-G $K_i = 3.50 \pm 0.82$ $\mu$M ($n = 4$), SP-D $K_i = 8.58 \pm 1.47$ $\mu$M ($n = 4$)). This data show that SP-G is relatively (six-fold) more selective for the $V_{1A}$ receptor whereas SP-D is GRP receptor selective (13-fold).

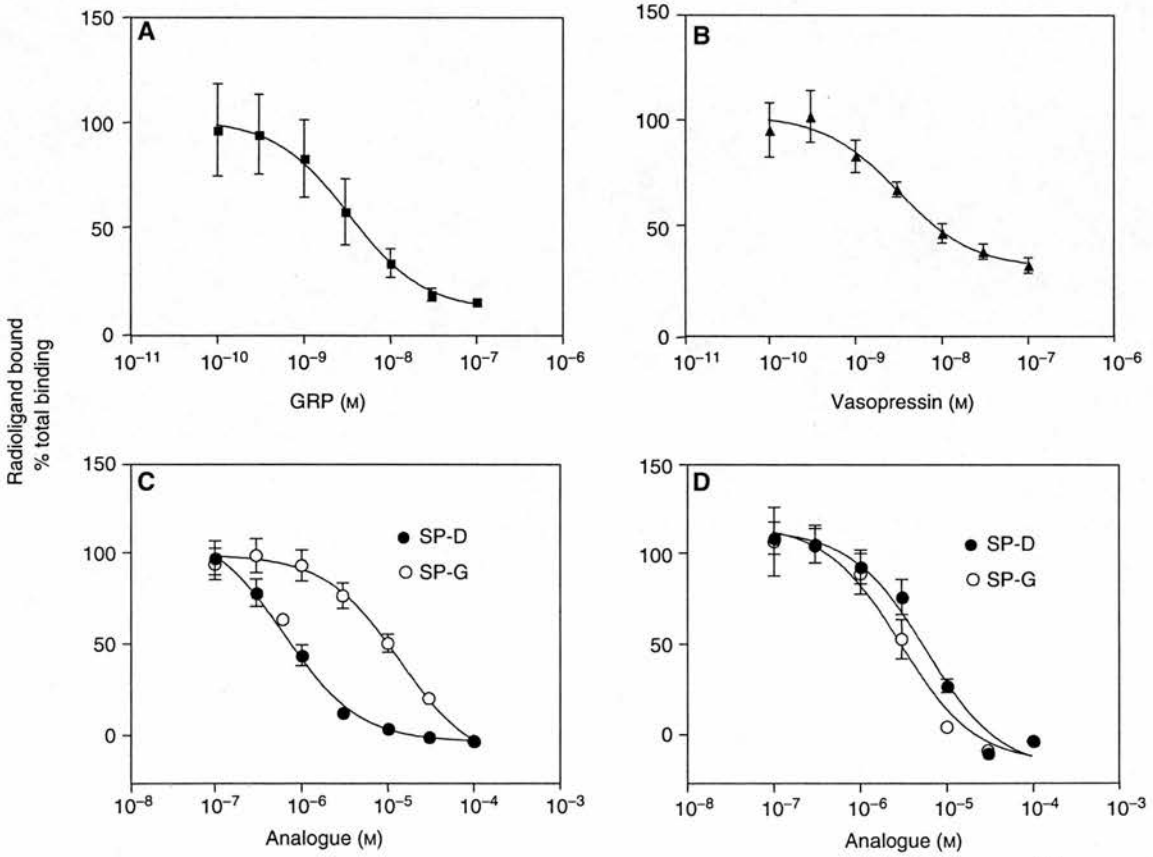


**Figure 1** Receptor binding. Gastrin-releasing peptide (**A**, **C**) or $V_{1A}$ (**B**, **D**) receptor expressing CHO cells were incubated with [$^{125}$I]-GRP or [$^{125}$I]-AVP for 30 min at 37°C as described in Methods in the presence of various concentrations of unlabelled GRP (**A**), unlabelled AVP (**B**), SP-D or SP-G (**C**, **D**). Results are expressed as % total binding and represent the mean ± s.e.m. of four experiments performed in triplicate.


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### Effects of $V_{1A}$ and GRP receptor expression on cell growth in liquid culture and semisolid media

Figure 2A shows that in liquid culture, in the presence of 5% FCS, the GRP and the $V_{1A}$ expressing cells proliferated at a similar rate to untransfected CHOs, but on day 6 after seeding, the GRP cells had reached a density of $4.5 \times 10^6$ cells per 100 mm dish. The cells did not appear to be contact inhibited and exhibited a rounded morphology. Cells over grew one another and significant numbers of cells were observed to be detached and growing in clusters (Figure 2B). Trypan blue exclusion revealed that the cells were >95% viable. A similar, though less pronounced, effect was observed with the $V_{1A}$-transfected cells, which reached a density of $3.1 \times 10^6$ cells per 100 mm dish. The parental cell line exhibited normal contact inhibition and had flat fibroblast morphology and did not show significant detachment. The end point density of these cells was $2.1 \times 10^6$ cells per 100 mm dish. These observations suggested that transfection of GRP and $V_{1A}$ receptors caused CHO-K1 cells to lose contact inhibition and adopt a more transformed phenotype. These findings led us to examine their growth in semisolid media, which is widely regarded as being a hallmark of the transformed phenotype. Cells were trypsinised and suspended in 0.3% agarose in DMEM ($1 \times 10^4$cells ml$^{-1}$) layered over a 0.5% layer of agarose in DMEM. Figure 2C shows that at day 9 after seeding, clusters of cells (>6 cells) were visible with MTT staining. The cloning efficiency was significantly higher in the GRP-expressing cells ($14.1 \pm 1.5\%$, $P<0.05$, ANOVA) and the $V_{1A}$ receptor-expressing cells ($8.5 \pm 1.0\%$, $P<0.05$, ANOVA) than the vector control cells in the presence of 5% FCS ($5.5 \pm 0.02\%$). In a separate series of experiments, the effects of SP-D and SP-G were examined (Figure 3A). At 30 $\mu$M, both analogues significantly reduced colony formation in the GRP and $V_{1A}$ receptor expressing cells but had no effect on the cloning efficiency of vector control CHO-K1 cells. The effect of receptor expression was also examined in an aggregation assay where cells are prevented from adhering and form aggregates in suspension. After 6 days, cells are briefly trypsinised and viable cells counted. These experiments showed that expression of the GRPr and $V_{1A}$r increased anchorage-independent growth compared to wild-type cells or vector control cells. Similarly SP-G and SP-D were able to inhibit cell growth in receptor expressing cells (Figure 3B). Together, these data suggest that expression of the GRP and $V_{1A}$ receptors induce a more transformed phenotype in CHO cells and induce sensitivity to substance-P analogue-induced growth inhibition.

### Chemosensitivity

The response to etoposide in control and receptor-transfected cells was measured by MTT accumulation. Figure 4 shows that after 48 h in culture in the absence of serum, etoposide produced a dose-dependent inhibition of proliferation in all cell types ($IC_{50} = 12.4 \pm 3.1$, $8.1 \pm 2.3$ and $14.2 \pm 4.0$ $\mu$g ml$^{-1}$ in CHO-WT, CHO-GRPr and CHO-$V_{1A}$r cells, respectively). Incubation with 50 nM of either bombesin or AVP added to CHO-GRPr or CHO-$V_{1A}$r produced a small but significant protection from etoposide, which was not observed when both neuropeptides were added to wild-type cells ($IC_{50} = 13.0$ and 26.9 $\mu$g ml$^{-1}$ in control and AVP-treated $V_{1A}$r-expressing cells, respectively; and 6.30 and 12.7 $\mu$g ml$^{-1}$ in bombesin-treated GRPr-expressing cells, respectively). At 40 $\mu$g ml$^{-1}$ etoposide, vasopressin-treated $V_{1A}$r-expressing cells accumulated 92% more MTT than control cells

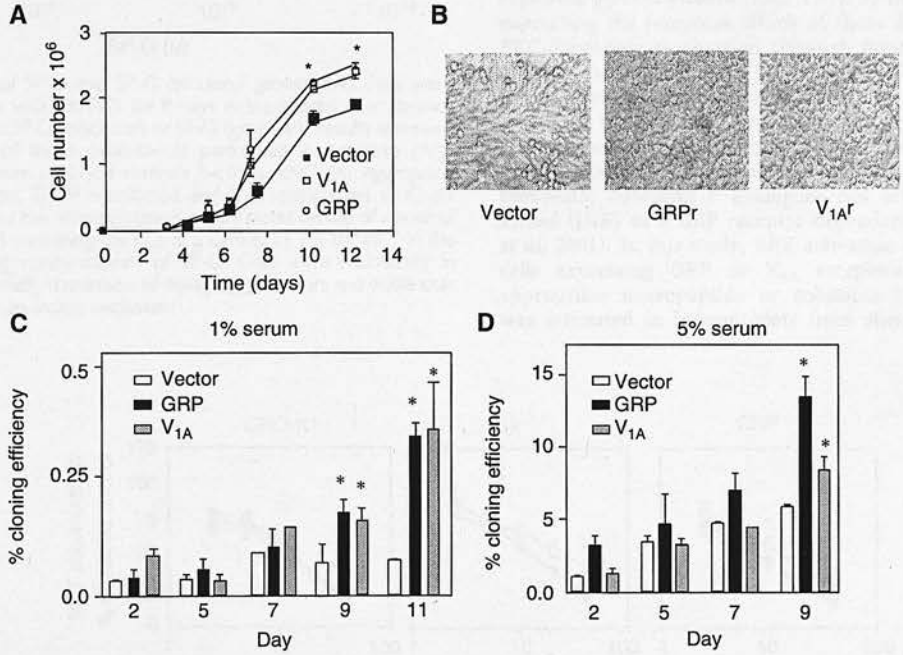


**Figure 2** Effect of neuropeptide receptor expression on cell growth *liquid growth* (**A**) CHO-K1 cells expressing vector, the GRP or $V_{1A}$ receptor were plated at a density of $1e^4$ cells/100 mm tissue culture dish and incubated at 37°C. Cells were harvested at various times and counted. Results represent the mean + s.e.m. of three experiments performed in duplicate (*significantly different from vector control cells $P<0.05$, ANOVA). (**B**) Representative images showing morphological features of transfected cells are shown. The rounded cobblestone features and higher cell density are evident in neuropeptide receptor expressing cells compared to the elongated contact inhibited monolayer exhibited by vector transfected controls. *Clonal growth* vector (open bars), GRP receptor (black bars) and $V_{1A}$ receptor (grey bars) transfected cells were plated at $1e^4$ cells well$^{-1}$ in 0.3% agar in DMEM containing 1% (**C**) or 5% (**D**) FCS. At various time points, cells were stained with MTT and colonies counted at ×10 magnification. Results represent the mean ± s.e.m. of three experiments performed in duplicate (*significantly different from vector control cells $P<0.05$, ANOVA).

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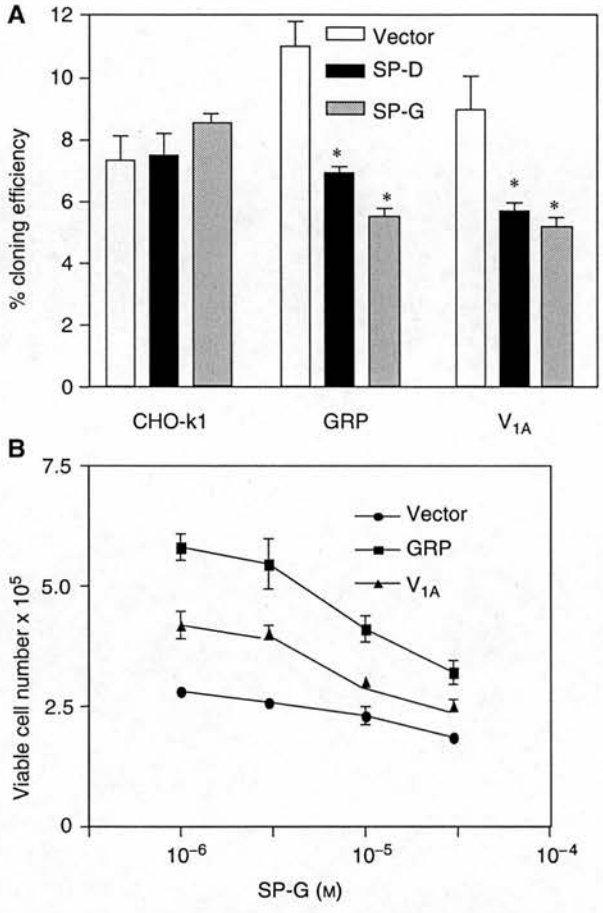


**Figure 3** Effect of SP-D and SP-G on clonal growth. (**A**) Cells were grown in 0.3% agar with 1% FCS for 8 days in the presence or absence (open bar) of 30 $\mu$M SP-D (black bar) or SP-G (grey bar). Results represent the mean $\pm$ s.e.m. of three experiments performed in duplicate (*significantly different from wild-type controls $P<0.05$, ANOVA). *Aggregation assay.* (**B**) Wild-type, GRPR-transfected and $V_{1A}$R-transfected CHO-K1 cells were plated into low adhesion tissue culture plates on top of a layer of 0.5% agar in DMEM containing 5% FCS at a density of $5 \times 10^4$ ml$^{-1}$ in the presence of varying concentrations of SP-G. Cells were maintained in culture for 7 days, briefly trypsinised to dissagregate clusters and viable cells counted by propidium iodide exclusion.

($P<0.01$). In GRPr-expressing cells, bombesin treatment caused an increase of 52% MTT accumulation compared to untreated cells ($P<0.01$). These results suggest that neuropeptide receptor stimulation may contribute to an increase in chemoresistance.

## Intracellular $[Ca^{2+}]_i$

The mobilization of calcium from intracellular stores is one of the earliest events triggered by neuropeptide receptor activation of $G_{\alpha q}$ leading to PLC activation and subsequent generation of $IP_3$. In untransfected CHO-K1 cells, neither GRP nor AVP produced a significant change in $[Ca^{2+}]_i$ (data not shown). In the CHO-GRP cells, GRP produced a concentration-dependent increase in $[Ca^{2+}]_i$ ($EC_{50} = 2.00 \pm 0.4$ nM, $n=5$, Figure 5A). SP-D and SP-G produced no change in $[Ca^{2+}]_i$ on their own but inhibited GRP-induced $[Ca^{2+}]_i$ elevation. Figure 5A and B show that SP-D and SP-G inhibited GRP-induced $[Ca^{2+}]_i$ with resultant $pA_2$ values of 7.21 and 5.72 for SP-D and SP-G respectively. In $V_{1A}$-expressing cells, AVP increased calcium mobilisation with an $EC_{50} = 3.1 \pm 0.60$ nM ($n=4$, Figure 5C and D). As in the GRP-expressing cells, SP-D and SP-G did not mobilise intracellular calcium directly but inhibited the response to AVP (Figure 5C and D). SP-G was more efficacious in inhibiting the vasopressin response than the GRP response. $PA_2$ values for AVP antagonism were 7.23 and 6.53 for SP-D and SP-G, respectively. Together, these data suggest that when stably transfected into CHO cells the GRP and the $V_{1A}$ receptor effectively couple to $G_{\alpha q}$ to increase intracellular calcium and that the substance-P analogues are effective antagonists of this response.

## ERK activation

AVP and bombesin have been shown to activate extracellular regulated protein kinases (ERK 1 and 2) in a variety of cell types expressing the receptors. Much of these data suggest a $G_q$- and PKC-dependent mechanism although these observations may be cell type-dependent (Della Rocca *et al*, 1999; Sinnett-Smith *et al*, 2000). Previous studies have shown that substance-P analogues reversibly inhibit ERK activation by neuropeptides in Swiss 3T3 cells; however, our studies suggest that in rat-1 fibroblasts and in human small-cell lung carcinoma cells (SCLC), in the absence of bombesin, substance-P analogues can activate ERK and c-jun kinase (JNK) in a GRP receptor-dependent manner (MacKinnon *et al*, 2001). In this study, ERK activation was measured in CHO cells expressing GRP or $V_{1A}$ receptors in response to the appropriate neuropeptide or substance-P analogue. Activation was estimated in immunoblots from stimulated lysates using a

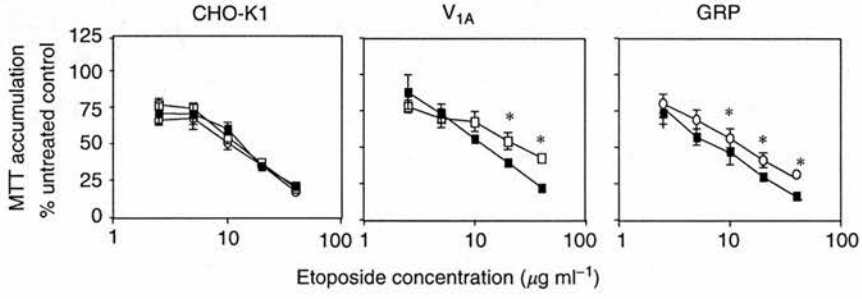


**Figure 4** Effect of neuropeptide on chemosensitivity. Wild-type CHO-K1 cells (left) and cells expressing the $V_{1A}$ (middle) or GRP (right) receptor were plated at a density of $1e^4$ cells per well of a 96-well tissue culture plate in DMEM with 10% FCS and incubated overnight 37°C. Cells were then incubated in serum-free media containing etoposide as indicated and in the absence (filled squares) or presence of either 50 nM AVP (open squares) or 50 nM bombesin (open circles) or both neuropeptides (wild-type cells) for 48 h at 37°C. Cell viability was assessed by MTT staining. Results are expressed as % viability in the absence of neuropeptide and are mean + s.e.m. of four independent experiments (*significantly different from untreated etoposide control, $P<0.05$ ANOVA).


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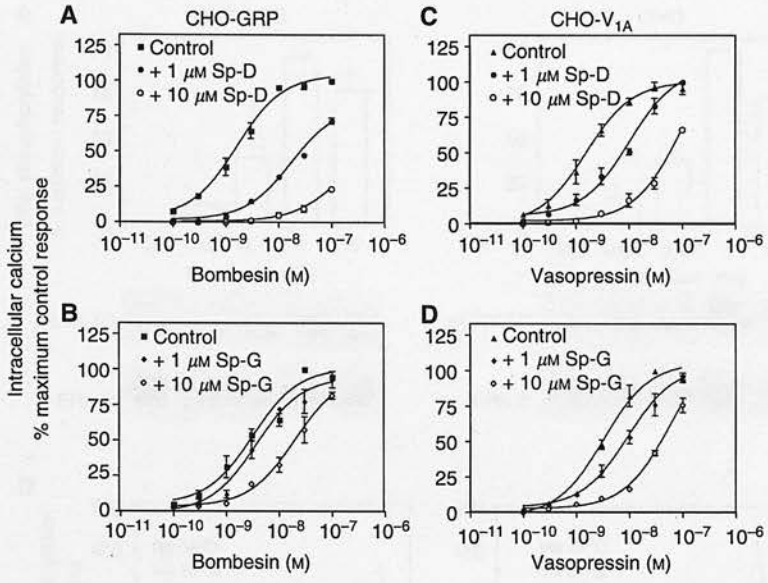


**Figure 5** Intracellular calcium release. Quiescent GRP (**A**, **B**) or $V_{1A}$ (**C**, **D**) receptor expressing CHO cells were incubated with FURA-2AM for 15 min at 37°C and ratiometric fluorescence monitored at 37°C. Concentration–response curves to bombesin were carried out in control cells and in cells pretreated for 2 min with 1 or 10 μM SP-D (**A**) or SP-G (**B**). Concentration–response curves to vasopressin were carried out in control cells and in cells treated with 1 or 10 μM SP-D (**C**) or SP-G (**D**). Results are expressed as % maximum control response to neuropeptide and represent the mean ± s.e.m. of four experiments.

phosphorylation state-specific antibody. Lysates from quiesced cells stimulated for 10 min with either neuropeptide or SP-D or SP-G were separated on 12% gels and blotted for pERK2 or total ERK1/2. Figure 6A shows that bombesin stimulated ERK phosphorylation in GRP-expressing CHO cells. Quantification of five separate experiments showed that bombesin produced a maximal 3.5-fold increase in ERK phosphorylation at 1 nM with an $EC_{50}$ of 0.56 nM. In $V_{1A}$-expressing cells, AVP produced an increase in ERK phosphorylation of 4.2-fold with an $EC_{50}$ of 0.72 nM, which correlated with its affinity for $V_{1A}$ receptors measured in binding studies and for the stimulation of intracellular calcium. Neither AVP nor GRP produced any stimulation of ERK phosphorylation in untransfected CHO cells (data not shown).

SP-D and SP-G stimulated ERK activation in GRP receptor expressing CHO cells (Figure 6B). Activation of ERK was evident at 3 μM for SP-D, but the maximal stimulation was less than that observed with bombesin (2.8-fold for SP-D and 2.1-fold for SP-G at 50 μM). The substance-P analogues also stimulated ERK in $V_{1A}$-expressing cells. However, in these cells SP-G was more efficacious than SP-D giving a 4.3-fold stimulation at 50 μM compared to a 2-fold stimulation by the same concentration of SP-D. The observed increase in ERK phosphorylation was not due to a change in total ERK expression, as ERK1/2 immunoreactivity did not change with drug treatment. These data show that at concentrations, which block neuropeptide-stimulated calcium release, substance-P analogues are agonists for ERK activation in GRP and $V_{1A}$ expressing CHO cells.

### Mechanisms of ERK phosphorylation

The mechanisms of GRP- and $V_{1A}$receptor-mediated ERK activation are not fully understood, although it has been shown in some cell types that activation is mediated by $G_q$-induced PKC activation and subsequent activation of raf. Other GPCRs have been shown to activate ERK in a $G_i$ and ras-dependent mechanism, which may or may not involve activation of PI3 K (Della Rocca *et al*, 1999). To examine the possible recruitment of $G_i$, CHO cells were incubated overnight in quiescent media containing 100 ng ml$^{-1}$ pertussis toxin. Figure 7 shows that in GRP receptor expressing cells, PTX produced a small but nonsignificant inhibition of bombesin-stimulated ERK activation whereas the response to 10 μM SP-D was completely abolished ($P<0.01$, ANOVA). In $V_{1A}$-expressing cells, the response to AVP was not inhibited by PTX pretreatment but the response to SP-G (20 μM) was completely inhibited ($P<0.01$, ANOVA). In both cell types, ERK activation by 1 μM LPA was completely inhibited by PTX treatment (data not shown). This differential sensitivity to pertussis toxin indicates that dual $G_i/G_q$-coupled mechanisms activate the ERK 1/2 cascade via GRP and $V_{1A}$ receptors expressed in CHO cells.

## DISCUSSION

This study utilised a model CHO cell system for neuropeptide receptor expression as these cells have an acceptable null background for these receptors. The results from this work shows that (1) GRP and $V_{1A}$ receptor expression leads to the development of a transformed phenotype in CHO-K1 cells. (2) Receptor expressing cells showed some increased resistance to the chemotherapeutic agent etoposide and increased sensitivity to the substance-P analogues, SP-D and SP-G. (3) SP-D and SP-G act as biased agonists at GRP and $V_{1A}$ receptors. This is the first demonstration of biased agonism at vasopressin receptors. (4) This pharmacological activity is crucial for the antiproliferative effect of these agents, which may be of particular benefit in more differentiated cancers that have developed resistance to chemotherapy.

Introduction of the neuropeptide receptors into CHO cells lead to an increase in proliferation and an increase in the ability of cells to grow as colonies in semisolid medium. The cells also showed a greater propensity to grow as nonadherent clusters. Colony growth was also evident in low serum suggesting that expression of these receptors allows for anchorage- and serum-independent growth consistent with transformation. Moreover, it suggests that these



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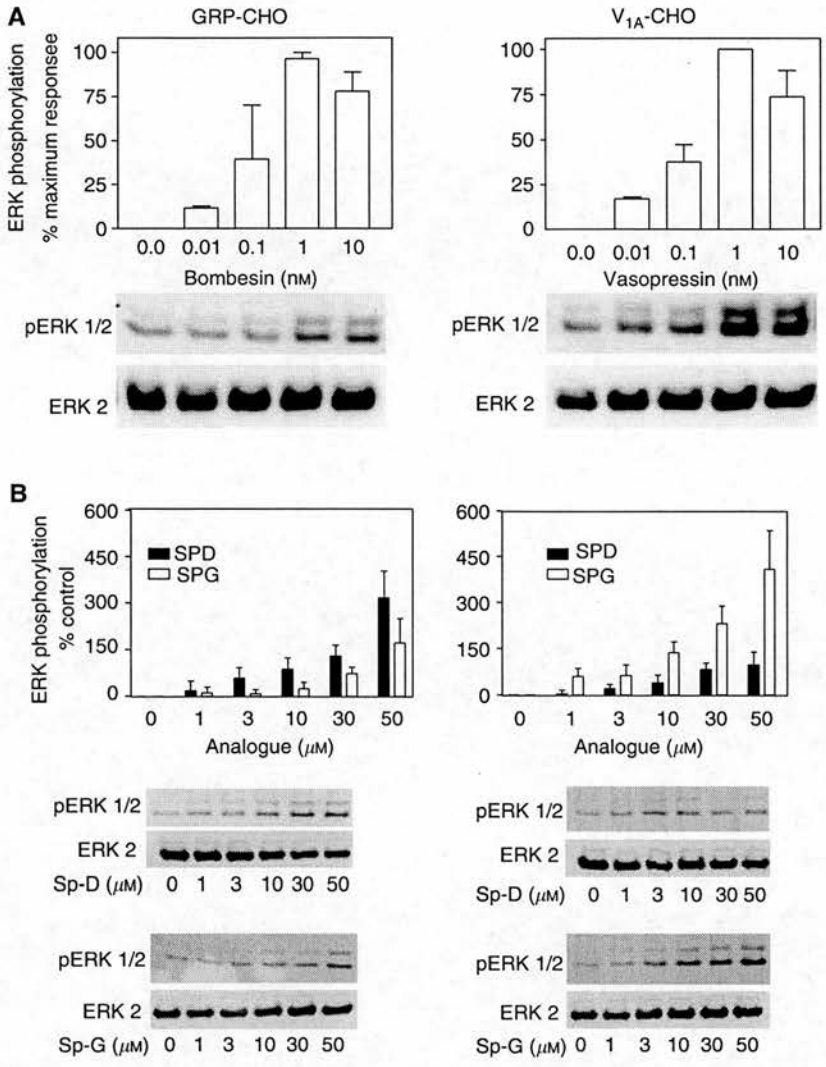


**Figure 6** (**A**) ERK phosphorylation by neuropeptides. Quiescent GRP (left) or $V_{1A}$ (right) receptor expressing CHO cells were stimulated for 5 min with neuropeptide at the indicated concentrations. Aliquots of cell lysate were resolved by SDS–PAGE and Western blots probed with monoclonal anti-pERK1/2 antibody (upper panel) or polyclonal anti-ERK2 antibody (lower panel). Representative Western blots are shown. Bar graphs represent the mean optical density (mean ± s.e.m.) of four separate experiments. (**B**) ERK phosphorylation by substance-P analogues. Quiescent GRP (left) or $V_{1A}$ (right) receptor expressing CHO cells were stimulated for 5 min with SP-D (filled bars) or SP-G (open bars) at the indicated concentrations. Aliquots of cell lysate were resolved by SDS–PAGE and Western blots probed with monoclonal anti-pERK1/2 antibody (upper panel) or polyclonal anti-ERK2 antibody (lower panel). Representative Western blots are shown. Bar graphs represent the mean optical density (mean ± s.e.m.) of four separate experiments.

receptors show some constitutive activity, as wild-type receptor expression alone was sufficient to increase transformation in the absence of exogenously added neuropeptide. The ability to observe constitutive GPCR activity is not only determined by the intrinsic molecular properties of the GPCR studied but also by the specific cellular background. Apparent constitutive activity of a wild-type GPCR may vary substantially in various systems. Differences in GPCR expression level, GPCR desensitisation, G-protein complement and GPCR/G-protein stochiometry can all contribute to such differences (Seifert and Wenzel-Seifert, 2002) This could explain why GRP receptors expressed in NCM460 cells, a nonmalignant human colon epithelial cell line, are constitutively active (Ferris *et al*, 1997) but are not in other cell types (Benya *et al*, 1994). Our findings are unlikely to be due to an overexpression artefact as the receptors in this study were expressed at levels not greater than those measured from a variety of other cancers (100–50 000 sites/cell, Jensen *et al*, 2001) So at these moderate levels, wild-type receptor was sufficient to activate downstream signals that promote cell survival under anchorage-independent conditions. The cells were, however, still able to respond to agonist, which resulted in a small but significant increase in resistance to the cytotoxic effects of etoposide. It is unclear how significant this change in sensitivity relates to the development of chemoresistance *in vivo*, but highlights an important area for future investigation.

Our previous work shows that sensitivity to AVP and GRP increases during the progression to chemoresistance in a set of SCLC cell lines (Waters *et al*, 2003). This would be analogous to the development of autocrine and paracrine growth loops in tumour development *in vivo*, with tumour cells becoming more dependent on neuropeptide growth factors for survival and subsequently developing chemoresistance. The mechanisms governing the survival advantage gained in neuropeptide receptor expressing


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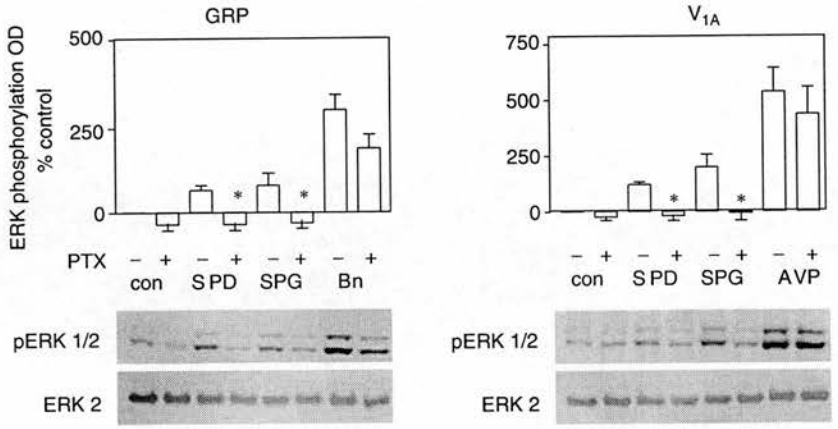


**Figure 7** Effect of pertussis toxin (PTX) on ERK phosphorylation. Confluent cultures of GRP (left) or $V_{1A}$ (right) receptor expressing CHO cells were quiesced overnight in the presence or absence of 100 ng ml$^{-1}$ pertussis toxin. Cells were stimulated for 5 min with 30 $\mu$M SP-D or SP-G or 1 nM neuropeptide as indicated. Aliquots of cell lysate were resolved by SDS–PAGE and Western blots probed with monoclonal anti-pERK1/2 antibody (upper panel) or polyclonal anti-ERK2 antibody (lower panel). Representative Western blots are shown. Bar graphs represent the mean optical density (mean ± s.e.m.) of four separate experiments (*significantly different from untreated control, $P<0.05$ ANOVA).

cells are poorly understood. During colon cancer progression, increased expression of GRP receptors leads to tumour cell differentiation, increased motility and adhesion to extracellular matrix via an increased activation of focal adhesion kinase (FAK) (Glover *et al*, 2004). Previous reports have shown that GRP receptors can activate insulin-like growth factor-1 receptors (IGF-R) leading to Akt activation in prostate cancer cells (Sumitomo *et al*, 2001), a mechanism which may underlie bombesin-mediated cell survival. In another study, human chorionic gonadotrophin (hCG) increased ovarian cell survival by up-regulating IGF-1 (Kuroda *et al*, 1998). Conversely, specific bombesin antagonists have been shown to inhibit growth and decrease EGF receptor expression in SCLC cells (Koppan *et al*, 1998) and breast cancer cells (Bajo *et al*, 2002). Bombesin antagonists in combination with GHRH antagonists have also been shown to down-regulate IGF-1, IGF-II, IGF receptors, GRP and EGF receptors in SCLC xenografts (Kanashiro *et al*, 2003). There is therefore potential for neuropeptides to regulate growth factor signalling pathways that control survival.

The present study shows that expression of GRP and $V_{1A}$ receptors in CHO cells also leads to an increased sensitivity to substance-P analogues. This was demonstrated by an inhibition of clonal growth by substance-P analogues only in receptor-transfected cells. We previously showed that substance-P analogues are more effective at inhibiting growth of chemoresistant tumour cells expressing high GRP receptor levels and can sensitise cells to etoposide-mediated cell death (Waters *et al*, 2003). Moreover, diverse tumour types expressing GRP receptors have a greater sensitivity to SP-D (Waters *et al*, 2003). Together, these data suggest that the increase in neuropeptide receptor expression that occurs as cells become more differentiated may contribute to the development of chemoresistance and may be exploited as a target for drug therapy. The potential role of neuropeptide receptors in the development of chemoresistance is an interesting finding that requires further study.

The mechanism of action of substance-P analogues was studied in receptor-transfected cells. Upon neuropeptide receptor transfection, the cells responded to the appropriate neuropeptide as expected for these predominately $G_q$-coupled receptors. The substance-P analogues behaved as antagonists for $Ca^{2+}$ release with potencies that reflected their affinities in receptor binding experiments, with SP-G showing some selectivity for $V_{1A}$ receptors. This may be useful in tailoring therapy to a specific tumour phenotype in as much as SP-G may be more beneficial in $V_{1A}$ receptor expressing tumours while SP-D may be better for tumours, which express both GRP and $V_{1A}$ receptors. Both neuropeptides and the substance-P analogues activated ERK in CHO cells expressing the GRP receptor and the $V_{1A}$ receptor. No ERK activation was observed in untransfected CHO cells suggesting that the activation is receptor mediated. These observations suggest that substance-P analogues act as dual efficacy receptor agonists directing receptor signalling via the GRP and the $V_{1A}$ receptor towards ERK activation while blocking liberation of intracellular calcium. Moreover, the substance-P analogues activate ERK via $G_i$ as the response is pertussis toxin sensitive and more prolonged than that observed with neuropeptide (data not shown). These data support a multitrack signalling complex leading from neuropeptide receptor to ERK activation; where one track is mediated by $G_q$ and most likely involves PKC, whereas another feeds into a $G_i$-mediated pathway (Wetzker and Bohmer, 2003). Although by far the majority of evidence suggests a preference for interacting with $G_q$, some previous work has shown $V_{1A}$ receptors coupling to $G_{i\alpha3}$ in quiescent fibroblasts and in hepatocytes (Strakova *et al*, 1997; Abel *et al*, 2000) There is also evidence that GRP receptors couple to $G_i$ in pancreatic acinar cells (Profrock *et al*, 1992), and that $G_i$ mediates bombesin-stimulated chemotaxis in monocytes (Djanani *et al*, 2003). Our data suggest that in the presence of substance-P analogues, the neuropeptide receptors exist in a conformation that is unable to activate $G_q$ but which is able to interact with $G_i$ and activate ERK. These analogues may act at a common region of the GPCRs perhaps at the receptor/G-protein interface, which favours coupling to $G_i$. Although activation of ERK is normally associated with a mitogenic response as is seen with the neuropeptides, deregulated ERK activation has also been shown to cause disruption of the cell cycle, growth arrest and apoptosis. In SCLC-activated raf-1, the upstream regulator of ERK causes growth arrest (Ravi *et al*, 1998). It is our hypothesis that deregulated ERK coupled with a decrease in intracellular $Ca^{2+}$ are important factors in the growth inhibitory/apoptosis inducing activity of substance-P analogues.

In summary, the results presented here show that neuropeptide receptor expression leads to the development of a transformed phenotype in CHO epithelial cells. These cells display increased sensitivity to substance-P analogues that parallels the situation observed in SCLC cells, which develop chemoresistance *in vivo*. These analogues activate GRP and $V_{1A}$ receptors in such a way as



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to cause activation of only a subset of possible downstream signals. They are competitive antagonists for PI hydrolysis mediated via $G_q$ but have agonist activity that results in activation of ERK via $G_i$. This presents a novel pharmacological mechanism whereby substance-P analogues utilise the normally mitogenic neuropeptide receptors to transduce an antiproliferative and apoptogenic cell signal. Moreover, the concentration of SPG used in this study was detected in patient plasma following administration with no noticeable toxicity (Clive *et al*, 2001), suggesting therapeutic doses can be achieved clinically. Development of these agents may provide a unique opportunity to treat SCLC and may be of particular benefit for tumours that have developed resistance to chemotherapy.

## ACKNOWLEDGEMENTS

UT-H was supported by a Cancer Research UK PhD studentship. This work was supported in part by the Scottish Hospitals Endowment Research Trust. TS is a recipient of a Wellcome Trust Research Leave Fellowship.

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**British Journal of Cancer (2003) 88,** 1808–1816
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# Increased gastrin-releasing peptide (GRP) receptor expression in tumour cells confers sensitivity to [Arg$^6$,D-Trp$^{7,9}$,$N^{me}$Phe$^8$]-substance P (6–11)-induced growth inhibition

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[Arg$^6$,D-Trp$^{7,9}$,$N^{me}$Phe$^8$]-substance P (6–11) (SP-G) is a novel anticancer agent that has recently completed phase I clinical trials. SP-G inhibits mitogenic neuropeptide signal transduction and small cell lung cancer (SCLC) cell growth *in vitro* and *in vivo*. Using the SCLC cell line series GLC14, 16 and 19, derived from a single patient during the clinical course of their disease and the development of chemoresistance, it is shown that there was an increase in responsiveness to neuropeptides. This was paralleled by an increased sensitivity to SP-G. In a selected panel of tumour cell lines (SCLC, non-SCLC, ovarian, colorectal and pancreatic), the expression of the mitogenic neuropeptide receptors for vasopressin, gastrin-releasing peptide (GRP), bradykinin and gastrin was examined, and their sensitivity to SP-G tested *in vitro* and *in vivo*. The tumour cell lines displayed a range of sensitivity to SP-G ($IC_{50}$ values from 10.5 to 119 $\mu$M). The expression of the GRP receptor measured by reverse transcriptase–polymerase chain reaction, correlated significantly with growth inhibition by SP-G. Moreover, introduction of the GRP receptor into rat-1A fibroblasts markedly increased their sensitivity to SP-G. The measurement of receptor expression from biopsy samples by polymerase chain reaction could provide a suitable diagnostic test to predict efficacy to SP-G clinically. This strategy would be of potential benefit in neuropeptide receptor-expressing tumours in addition to SCLC, and in tumours that are relatively resistant to conventional chemotherapy.
*British Journal of Cancer* (2003) **88,** 1808–1816. doi:10.1038/sj.bjc.6600957 www.bjcancer.com
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**Keywords:** gastrin-releasing peptide; SP-G; substance P analogues; tumour growth

Despite recent advances in cytotoxic drug development, it is a universal goal to develop novel cancer therapies that are more specific to cancer cells and produce minimal damage to nonmalignant cells. One approach to realising this goal is the development of drugs that are able to block the growth-promoting effects of cancer cell mitogens.

Neuropeptides are important growth factors in a number of cancers including breast, colon, pancreatic, prostate, renal, gastric carcinoma and small-cell lung cancer (SCLC). SCLC cells proliferate in response to a range of neuropeptide growth factors, and in many cases these neuropeptides are involved in autocrine and paracrine growth loops that fuel unrestrained proliferation (Moody *et al*, 1985; Sethi and Rozengurt, 1991; Sethi *et al*, 1992, 1993; North *et al*, 1997). Drugs that target specific mitogenic neuropeptides have shown little promise. Monoclonal antibodies have been developed against circulating bombesin and one such antibody, 2A11, has been shown to inhibit the growth of SCLC *in vitro* and also as xenografts in nude mice (Chaudry *et al*, 1999); however, it has limited efficacy in human trials. Thus, 'broad-spectrum' neuropeptide receptor antagonists have been the main focus of research in this drug development strategy. Synthetic analogues of substance P, for example, [Arg$^6$,D-Trp$^{7,9}$,$N^{me}$Phe$^8$]-substance P (6–11) (SP-G), were initially identified as antagonists of substance P-mediated cellular effects and were subsequently found to also antagonise the cellular effects of bombesin (Jensen *et al*, 1984). When tested in SCLC cell lines, it was found that several substance P analogues inhibited calcium mobilisation stimulated by the neuropeptides: bombesin, bradykinin, gastrin, galanin, vasopressin, cholecystokinin and neurotensin (Woll and Rozengurt, 1988; Langdon *et al*, 1992; Sethi *et al*, 1992). They were also found to inhibit mitogenesis by the same range of neuropeptides in both Swiss 3T3 cells and SCLC cells (Woll and Rozengurt, 1988; Bepler *et al*, 1989; Sethi *et al*, 1992).

In addition to the *in vitro* growth-inhibitory effects of substance P analogues, these compounds inhibit the growth of tumours in xenograft models in nude mice (Langdon *et al*, 1992; Jones *et al*, 1997). SP-G has recently completed a phase I clinical trial and will be entering a phase II clinical trial in SCLC in the near future (Clive *et al*, 2001). The exact mechanism by which these compounds exert their antitumour effects are unknown, but we have previously shown that they act as 'biased' agonists inhibiting neuropeptide-stimulated growth while directly stimulating apoptosis (Tallett *et al*, 1996; MacKinnon *et al*, 1999, 2001). Understanding the exact manner in which substance P analogues modulate neuropeptide receptor signalling will allow for the rational design of more potent analogues. Identification of biological features in tumours that confer sensitivity to SP-G will elucidate the most effective use of this group of compounds in clinical practice.

In this study, we measured the expression of the mitogenic neuropeptide receptors for vasopressin ($V_{1A}$), gastrin-releasing peptide (GRP), bradykinin ($BK_2$) and gastrin by reverse

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Received 25 September 2002; revised 6 March 2003; accepted 6 March 2003

transcriptase–polymerase chain reaction (RT–PCR), and show that GRP-receptor expression predicts sensitivity to SP-G in a variety of tumours (SCLC, non-small-cell lung cancer (NSCLC), colorectal, ovarian and pancreatic) *in vitro* and *in vivo*. Our results suggest that an antineuropeptide growth factor strategy may be effective in a wider range of tumours and may also be of benefit in these neuropeptide-expressing tumours, which have acquired relative resistance to conventional chemotherapeutic agents.

## MATERIALS AND METHODS

### Materials

Rat-1a cells and rat-1a cells stably expressing the mouse bombesin/GRP receptor (GRPR) (BOR-15) were established by the Imperial Cancer Research Fund (ICRF, London, UK). RPMI-1640, Dulbecco's essential Eagle's medium (DMEM), bombesin and vasopressin were from Sigma (Poole, UK). SP-G was a kind gift from Peptec (Copenhagen, Denmark). All other reagents were of the purest grade available.

### Cell culture

Stocks were maintained in RPMI-1640 (tumour cell lines) or DMEM (nontumour cell lines) supplemented with 10% ($v\,v^{-1}$) fetal bovine serum (heat-inactivated at 57°C for 1 h) 50 U $ml^{-1}$ penicillin, 50 $\mu g\,ml^{-1}$ streptomycin and 5 $\mu g\,ml^{-1}$ L-glutamine in a humidified atmosphere of 5% $CO_2$:95% air at 37°C. For experimental purposes, cells were either grown in SITA medium (RPMI-1640 medium supplemented with 30 nM selenium, 5 $\mu g\,ml^{-1}$ insulin, 10 $\mu g\,ml^{-1}$ transferrin media supplement and 0.25% bovine serum albumin (tumour cell lines)) or with 0.1% ($v\,v^{-1}$) fetal bovine serum in DMEM (nontumour cell lines). Rat-1A fibroblasts stably transfected with the mammalian bombesin receptor (BOR-15 cells) were cultured in the presence of 400 $\mu g\,ml^{-1}$ G418-sulphate.

### Growth assays

Liquid growth was determined in SITA medium in the presence or absence of mediators. Cell number was determined using a Coulter Counter (model Z1, Beckman Coulter, Bucks, UK). Colony growth was determined in 0.3% agarose in SITA medium for 21 days as described by Sethi and Rozengurt (1991).

### Determination of intracellular $Ca^{2+}$ concentration

Intracellular $Ca^{2+}$ concentration was determined using the fluorescent indicator Fura-2-tetraacetoxymethylester AME (FURA-2-AM 1 μM) as described (Sethi *et al*, 1993) Ratiometric fluorescence was monitored in a Perkin-Elmer Fluorometric Spectrophotometer with dual excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm.

### Xenograft activity

Female *nu/nu* mice (6 weeks old) (ICRF, London, UK) were maintained in negative pressure isolators (Moredun Animal Research Unit, Edinburgh, UK). All xenografts used throughout were initially established by subcutaneous injection of $10^7$ cells from their respective cell lines and then maintained by serial passage of fragments of viable tumour as described previously (Langdon *et al*, 1992).

Mice were allocated into control and treatment groups containing six to eight mice. Treatment started when the xenografts reached a diameter of 3–10 mm with the first day of peptide administration designated as day 0. SP-G was dissolved in sterile distilled water at a concentration of 10 mg $ml^{-1}$ and administered by i.p. injection in a volume of 0.1 ml per 20 g of body weight to yield a dose of 50 mg $kg^{-1}$. The dose schedule employed was twice daily injections with an 8 h gap for a total of five injections. This schedule was chosen based largely on the pharmacokinetics of SP-G in *nu/nu* mice (Cummings *et al*, 1995). Controls received the same dose schedule of vehicle. Xenografts were measured either two or three times a week by means of calipers and tumour volume ($V$) was then calculated by the formula $V = \pi/6LW^2$, where $L$ is the longest diameter and $W$ the diameter perpendicular to $L$. Results were expressed as a relative tumour volume (RTV) which is defined simply as: $V$ on day $x/V$ on day 0, with RTV = 1 on day 0. Xenograft experiments were repeated on two or three separate occasions.

### Semiquantitative RT–PCR

RNA was extracted using Tri-Reagent (Sigma, Poole, UK) from exponentially growing cells, which had been in SITA medium for 3–5 days and was subsequently treated with DNase1 to remove any DNA contamination. cDNA was produced from this RNA using a first-strand synthesis kit (Boehringer Mannheim, Roche, UK). PCR primers used were: GRPR sense 5′-ATCTTCTGTACAGTCAAGTCC-3′, antisense 5′-GCTTTCCTCATGGAAGGGATA-3′; $V_{1A}R$ sense 5′-TACCTGCTACGGCTTCATCTGC-3′, antisense 5′-ACACAGTCTTGAAGGAGATGGC-3′; $BK_2R$ sense 5′-CCTGAGTGTCATCACCTTCTGC-3′, antisense 5′-TTGATGACACGGCAGAGG-3′; receptor for gastrin (gastrinR) sense 5′-CCTATCTCCTTCATTCACTTGC-3′, antisense 5′-AGTGTGCTGATGGTGGTGTAGC-3′; γ-actin sense 5′-ATGGCATCGTCACCAACTGG-3′, antisense 5′-ATGACAATGCCAGTG-GTGGC-3′. Polymerase chain reaction products were run on a 1.5% agarose gel containing 1 $\mu g\,ml^{-1}$ ethidium bromide. Stored images of the gels were analysed by densitometry using Gel Base/Gel Blot software. γ-Actin levels were measured as an internal control. Each product was expressed relative to the levels of γ-actin for the same cDNA batch and for each PCR reaction.

## RESULTS

### Neuropeptide sensitivity in the longitudinal cell lines GLC14, 16 and 19

The GLC14, 16 and 19 cell lines are SCLC cell lines established from one patient during longitudinal follow-up (Berendsen *et al*, 1988). During this period, the tumour changed from sensitive to resistant to chemotherapy, and the *in vitro* sensitivity to chemotherapeutic agents reflected the clinical resistance to treatment (de Vries *et al*, 1989 and T.S. observations (results not shown)). Initially, neuropeptides (at a concentration of 100 nM) in each of the three cell lines were screened for their ability to increase $[Ca^{2+}]_i$. Table 1 and Figure 1A show that the GLC14 cell line responded to bradykinin and serum resulting in a large increase in $[Ca^{2+}]_i$ and smaller responses were seen in case of neuropeptides CCK, GRP and neuromedin B. No $Ca^{2+}$ mobilisation was observed in response to neurotensin, substance P or vasopressin. However, in the GLC19 line, addition of neuropeptides bradykinin, CCK, GRP, neuromedin B and substance P caused large increases in $[Ca^{2+}]_i$. Vasopressin and neurotensin caused smaller but consistent increases in $[Ca^{2+}]$. The GLC19 was the only cell line in which neurotensin caused a measurable increase in $[Ca^{2+}]_i$.

One percent serum caused a rapid increase in $[Ca^{2+}]_i$ in all three cell lines of 100–150 nM, suggesting that the mobilisable $Ca^{2+}$ pools were equivalent in each line; however bombesin, neuromedin B and CCK showed an increased responsiveness in the GLC19 cells. Typical concentration response curves in GLC16 and 19 cells are shown in Figure 1B. Thus, during the tumour progression from GLC14, 16 to 19 cell lines, there is an increase in



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**Table 1** Neuropeptide $Ca^{2+}$ mobilisation in the SCLC GLC longitudinal cell line series

| $Ca^{2+}$ mobilising neuropeptides | GLC14 | GLC16 | GLC19 |
|---|---|---|---|
| Bombesin | 6±3 ($n=7$) | 25±59 ($n=7$) | 100±15 ($n=7$) |
| Bradykinin | 66±8 ($n=8$) | 105±7 ($n=8$) | 98±12 ($n=10$) |
| Carbamylcholine | 83±11 ($n=3$) | 104±18 ($n=5$) | 113±25 ($n=7$) |
| Cholecystokinin | 31±9 ($n=4$) | 32±4 ($n=3$) | 56±5 ($n=4$) |
| Neuromedin B | 7±4 ($n=4$) | 25±3 ($n=3$) | 150±20 ($n=4$) |
| Neurotensin | — ($n=3$) | — ($n=3$) | 43±12 ($n=5$) |
| Substance P | — ($n=3$) | 39±6 ($n=3$) | 72±10 ($n=3$) |
| Vasopressin | — ($n=3$) | 28±8 ($n=7$) | 42±9 ($n=7$) |
| Serum | 105±8 ($n=8$) | 109±15 ($n=8$) | 119±19 ($n=10$) |

The GLC19, 16 and 14 SCLC cell lines were loaded with 1 μM Fura2AME washed and resuspended in 2 ml electrolyte solution and the cell suspension placed in a quartz cuvette and stirred continuously. Neuropeptides were added at 100 nM concentration and fluorescence was recorded continuously as described in Materials and Methods. Results are expressed as change in intracellular calcium (nM). The following peptides were also tested at 100 nM–1 μM concentrations and no increase in $[Ca^{2+}]_i$ was ever observed: adrenocorticotrophin hormone, angiotensin I, atrial natriuretic peptide, calcitonin, chorionic gonadotropin, dynorphin, β-endorphin, endothelin, epinephrine, galanin, growth hormone-releasing hormone, gastric-inhibitory peptide, glucagons 5-hydroxytryptamine, Leu-enkephalin, neuropeptide-Y, parathyroid hormone, substance K, thyrotrophin-releasing hormone.

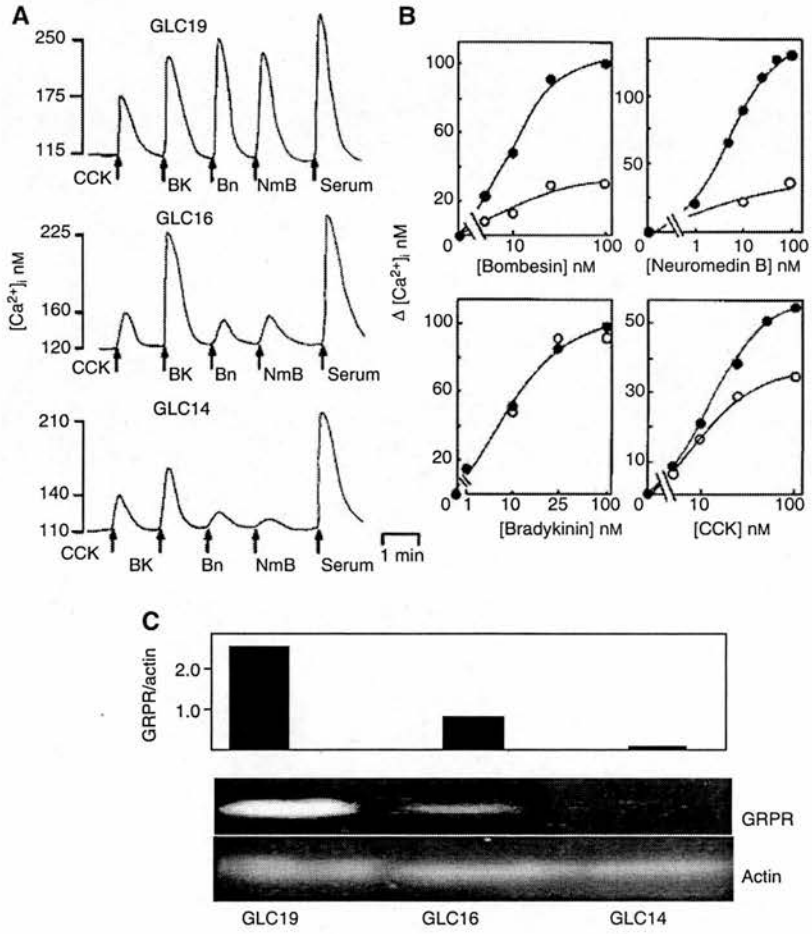


**Figure 1** Effects of bombesin, neuromedin B, bradykinin, cholecystokinin and serum on $[Ca^{2+}]_i$ in SCLC cell lines GLC14, GLC16 and GLC19. Cells loaded with FURA-2-AME were resuspended in electrolyte solution and placed in a quartz cuvette. Fluorescence was monitored and basal and peak $[Ca^{2+}]_i$ calculated as described in Materials and Methods. Panel A: All peptides were added at a final concentration of 100 nM. Abbreviations used: BN, bombesin; NmB, neuromedin B; BK, bradykinin; CCK, cholecystokinin. Serum was added at a final concentration of 1% $vv^{-1}$. Panel B: Dose-dependent effects of bombesin, neuromedin B, bradykinin and CCK in SCLC cell lines GLC16 and GLC19, open and closed circles, respectively. Peptides were added at the concentrations indicated. Representative concentration–response relations of three to five experiments are shown. Panel C: mRNA encoding the GRP receptor was detected by semiquantitative RT–PCR. γ-Actin mRNA levels were measured as an internal control for each PCR reaction. The results show a representative PCR reaction for the GLC14,16 and 19 cell lines. A bar chart showing relative receptor expression, calculated as density of PCR product/actin is shown for each cell line. The results represent the mean of two independent experiments.


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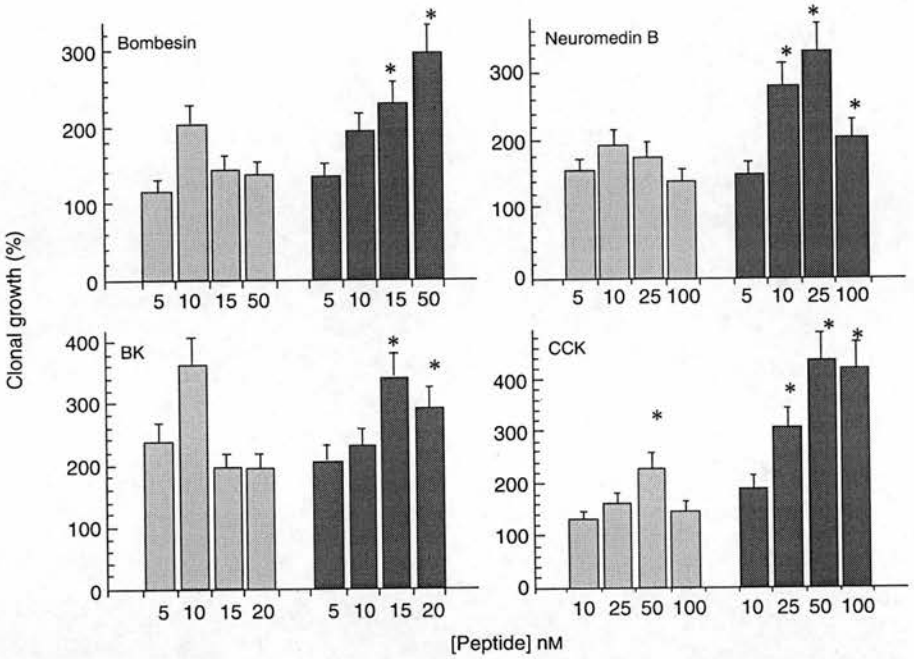


**Figure 2** Dose-dependent effects of bombesin, BK, CCK and neuromedin B on colony formation in GLC16 and GLC19 SCLC cells. GLC16 (hatched bars) and GLC19 (filled bars) cells, 3–5 days postpassage, were washed and $10^4$ viable cells per ml were plated in SITA medium containing 0.3% agarose on top of a base of 0.5% agarose in culture medium as described in Materials and Methods. Both layers contained additions at the nanomolar concentrations indicated. Cultures were incubated at 37°C in a humidified atmosphere at 5% $CO_2$:95% air for 21 days and then stained with nitro-tetrazolium blue. Spontaneous colony formation, that is, in the absence of any exogenously added peptide was $140 \pm 15$ ($n = 75$) and $160 \pm 20$ ($n = 85$), in the cell lines GLC16 and 19, respectively, and normalised to 100%. Each point represents the mean ± s.e.m. of two to four experiments each of five replicates. *Statistically different from neuropeptide concentration matched GLC16 cells (ANOVA, $P < 0.05$).

the number of neuropeptides able to induce a measurable increase in $[Ca^{2+}]_i$ and also an increase in the potency of individual neuropeptides. The expression of the GRP receptor was examined in the GLC cell lines by RT–PCR (Figure 1C). GRP receptor expression increased by six-fold in the GLC16 compared to the GLC14 cell line and by 11-fold in the GLC19 cell line, paralleling the increased responsiveness to GRP-induced $Ca^{2+}$ release.

CCK, bradykinin, neuromedin B and bradykinin stimulated a concentration-dependent increase in clonal growth in the GLC16 and 19 cell lines. There was a significant increase in the ability of bombesin, neuromedin B and CCK to stimulate clonal growth in the GLC19 SCLC cell line compared to the GLC16 cell line (Figure 2). Bradykinin was equally effective in stimulating clonal growth in both GLC16 and 19 cell lines. The ability of these neuropeptides to stimulate clonal growth in the GLC14 cell line was consistently less than that seen in the GLC16 cell line. Serum stimulated a 405–370% increase in clonal growth in the GLC19, 16 and 14 cell lines, and the cloning efficiency of all three SCLC cell lines was approximately 1.5%. Hence, the GLC19 cell line develops increased $Ca^{2+}$ responsiveness to the neuropeptides CCK, bombesin and neuromedin B, and this correlates with an increase in the ability of these neuropeptides to stimulate clonal growth.

SP-G inhibited $Ca^{2+}$ mobilisation induced by CCK, bombesin, neuromedin B and bradykinin in the GLC19 SCLC cell line (Figure 3A). SP-G also inhibited the growth and cloning efficiency of the GLC14 (not shown) 16 and 19 cells, but was most potent in the GLC19 cell line ($IC_{50}$ for the GLC14, 16 and 19 SCLC cells was 25, 25 and 15 $\mu$M, respectively, for liquid culture, and 25, 25 and 5 $\mu$M, respectively, for cloning efficiency, Figure 3B). Interestingly, SP-G was still effective in inhibiting the growth of the GLC19 SCLC line despite the development of resistance to conventional chemotherapeutic agents (de Vries *et al*, 1989). These findings suggest that increased neuropeptide receptor expression results in increased sensitivity to SP-G.

## Sensitivity to SP-G in a panel of tumour cell lines

A panel of tumour cell lines was selected that represents a spectrum of tumour types, NSCLC, ovarian, pancreatic and colon cancer, which express neuropeptide receptors in addition to SCLC. The characteristics of these cell lines and their sensitivity to SP-G are shown in Table 2. Growth inhibition by SP-G was not confined to tumour type. The two most sensitive cells were the H69 SCLC cell line ($IC_{50} = 10.5$ $\mu$M) and the HT29 colorectal cell line ($IC_{50} = 18$ $\mu$M). Four out of five SCLC cell lines had $IC_{50}$ values below the mean $IC_{50}$ value of 44 $\mu$M. Both the ovarian carcinoma and the non-small-cell carcinoma were sensitive to growth inhibition by SP-G, with $IC_{50}$ values of 31 and 33.5 $\mu$M, respectively. During the phase 1 clinical trial of SP-G, plasma levels of up to 40 $\mu$M were achievable with no dose-limiting toxicity (Clive *et al*, 2001). A total of 72% of the cancer cell lines tested had $IC_{50}$ values below or near this concentration. This suggests that 'broad-spectrum' neuropeptide antagonists such as SP-G may be effective antitumour agents in a variety of cancers other than SCLC.

## *In vivo* sensitivity to SP-G in a panel of tumour cell lines

Four members from the original panel of tumour cell lines were established as xenografts from their respective cell lines: H69 SCLC, HT29 colon carcinoma, PEO4 ovarian cancer and PANC-1 pancreatic cancer. Typical growth inhibition curves (Figure 4) are shown for each xenograft after i.p. treatment with 50 mg/kg SP-G. The HT29 xenograft showed a growth inhibition of 70.4%, 7 days after the commencement of treatment. In addition, this growth



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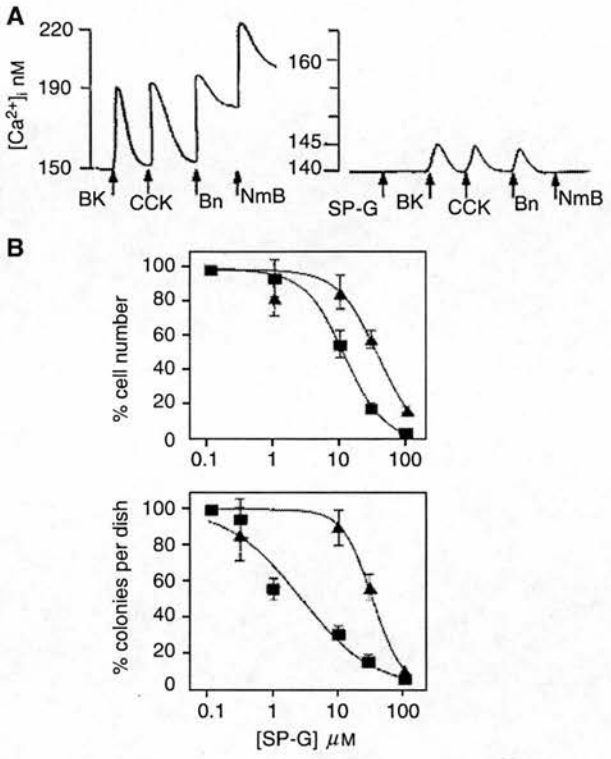


**Figure 3** (**A**) Effect of SP-G on neuropeptide-induced $Ca^{2+}$ mobilisation in GLC19 SCLC cells. Cells loaded with FURA-2-AME were resuspended in electrolyte solution and placed in a quartz cuvette. Fluorescence was monitored and basal and peak $[Ca^{2+}]_i$ calculated as described in Materials and Methods. Peptides were added at a final concentration of 30 nM and SP-G at a final concentration of 30 $\mu$M. (**B**) Effect of SP-G on GLC16 and GLC19 cell growth. Liquid growth (upper panel): GLC16 (triangles) and GLC19 (squares) SCLC cells were resuspended at a density of $5 \times 10^4$ cells in 1 ml SITA in the presence or absence of SP-G in triplicate. The controls (cells in absence of antagonist) after an initial lag period grew exponentially and then reached a plateau after 10 days (GLC19: $4.8 \pm 0.5 \times 10^5$ ($n = 6$) and GLC16: $5.7 \pm 0.5 \times 10^5$ ($n = 6$)). Each point represents the mean cell number after 10 days $\pm$ s.e.m. of two experiments each of three replicates. Colony growth (lower panel): GLC16 (triangles) and GLC19 (squares) SCLC cultures were washed, and $10^4$ viable cells/ml were plated in SITA medium containing 0.3% agarose on top of a base of 0.5% agarose in culture medium as described in Materials and Methods. Both layers contained additions as indicated. Cultures were incubated at 37°C in a humidified atmosphere at 5% $CO_2$ : 95% air for 21 days and then stained with nitro-tetrazolium blue. Each point represents the mean $\pm$ s.e.m. of two experiments each of five replicates.

inhibition was maintained over 17 days after the final injection (Figure 4). The PEO4 ovarian xenograft showed a maximum growth inhibition of 69.3% at day 7, and in common with HT29, growth inhibition was also maintained over 17 days beyond the final injection. The H69 xenograft showed a maximum growth inhibition of 39.9% to SP-G that was sustained for 10 days after treatment. The PANC-1 pancreatic tumour cell line was one of the least-sensitive cells lines to SP-G *in vitro* and proved to be the least-sensitive xenograft, with statistically significant growth inhibition recorded at only two time points, day 7 and day 10 (Figure 4). Overall, these data report, for the first time, that SP-G has marked *in vivo* xenograft activity in a variety of tumours including lung, colon, ovarian and pancreatic cancer, and in cancers such as ovarian cancer (with *in vitro* derived chemoresistance), NSCLC and colon cancer, which are relatively insensitive to standard conventional chemotherapy.

## Correlation of neuropeptide receptor expression with sensitivity to SP-G in a panel of tumour cell lines

The expression of GRP, gastrin, $BK_2$ and $V_{1A}$ receptors were examined by RT-PCR, in nine cell lines from the original panel representing a spectrum of sensitivity to SP-G. The results are shown in Table 2 and Figure 5 ($n = 4-5$ independent experiments). Receptors for vasopressin ($V_{1A}R$) were detected in four out of four lung cancer cell lines (three SCLC and one NSCLC). The WX330 SCLC cell line was the highest expresser of $V_{1A}R$. The pancreatic and ovarian cancer lines expressed low levels of the $V_{1A}R$ and in the colorectal cancer cell lines only HT29 cells expressed $V_{1A}R$. GRPRs were detected in three out of four lung cancer cell lines (two SCLC and one NSCLC). In the colorectal cell lines, the HT29 cell line showed high levels of GRPR expression and HCT116 cell line showed no detectable GRPR expression with intermediate expression in the HT18 cell line. The pancreatic cell line PANC1 had very low levels of GRPR expression. The PEO4 ovarian cancer cell line and H69 SCLC cell lines showed high expression of the GRPR (Table 2). Receptors for bradykinin were detected in all cells of the cell panel. Other studies have found an almost ubiquitous expression of bradykinin receptors in human lung cancers (Bunn *et al*, 1992). Gastrin receptors were detected in three out of four lung lines (two SCLC and one NSCLC) and low expression was detected in the ovarian line PEO4. The highest expression was in the SCLC cell line H510. Interestingly, none of the colorectal cell lines expressed gastrin receptors (Table 2). This finding confirms other studies, which show that the gastrin receptor is rarely expressed in colorectal cancer cell lines (Reubi *et al*, 1997). These results show that sensitivity to SP-G in a variety of tumours appears to correlate with the level of expression of neuropeptide receptors. In particular, of all the neuropeptide receptors examined, the correlation between relative GRP receptor expression (receptor/$\gamma$-actin) and sensitivity to SP-G was the most apparent (Spearmans correlation $R = -0.75$; $P = 0.026$, Figure 5).

## Effect of GRP receptor expression on SP-G sensitivity in fibroblasts

The importance of neuropeptide receptor status for cell sensitivity to SP-G was further tested in a rat-1 fibroblast system in which only one neuropeptide receptor (the mouse GRPR) was expressed at high levels ($K_d = 1$ nM, $B_{max} = 10^5$ receptors per cell) (Charlesworth *et al*, 1996). In native rat-1a fibroblasts, bombesin and other $Ca^{2+}$-mobilising neuropeptides, vasopressin, neurotensin, bradykinin and gastrin, failed to mobilise intracellular $Ca^{2+}$, suggesting absence of receptors in the parent cell line (Figure 6A). Rat-1a fibroblast cells stably expressing the mammalian bombesin receptor (BOR15) respond normally to bombesin stimulation in terms of signal transduction and stimulation of DNA synthesis (Charlesworth *et al*, 1996; MacKinnon *et al*, 2001). Bombesin stimulated a marked and rapid increase in $[Ca^{2+}]_i$ in BOR15 cells, which was blocked by Sp-G (Figure 6B). The effect of SP-G on the growth of rat-1a fibroblasts and BOR15 cells was therefore determined. The parent rat-1a cells were comparatively resistant to SP-G-mediated growth inhibition with only partial inhibition observed at the highest concentration of SP-G (41 + 12% inhibition at 100 $\mu$M, $IC_{50} > 100$ $\mu$M), whereas BOR15 cell growth was completely inhibited by 80 $\mu$M SP-G ($IC_{50}$ 44 $\mu$M, Figure 6). These results show that the presence of the bombesin receptor leads to increased sensitivity to growth inhibition by SP-G in fibroblasts.

## DISCUSSION

The GLC14, 16 and 19 are classic type SCLC cell lines derived from a 55-year-old female with SCLC. The GLC14 cell line was established from a supraclavicular lymph node before treatment.


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**Table 2** Growth inhibition by SP-G ($IC_{50}$) and neuropeptide receptor expression in a panel of tumour cell lines

| Cell line | IC50 (μM) | Relative NP receptor expression | | | |
|---|---|---|---|---|---|
| | | GRPR* | $V1_AR$ | $BK_2R$ | Gastrin R |
| H69 (SCLC, Moody *et al*, 1985) | 10.5 | 0.91±0.15 | 0.8±0.18 | 0.65±0.31 | 0.56±0.49 |
| HT29 (colorectal, Brattain *et al*, 1981) | 18 | 0.55±0.12 | 0.02±0.01 | 0.35±0.04 | — |
| H510 (SCLC, Moody *et al*, 1985) | 29 | 0.3±0.11 | 1.10±0.21 | 0.51±0.17 | 1.51±40.61 |
| PEO4 (ovarian, Lieber *et al*, 1975) | 31 | 1.31±0.25 | 1.10±0.02 | 0.24±0.10 | 0.2±0.05 |
| NX002 (NSCLC, Stark *et al*, 2001) | 33.5 | 1.01±0.21 | — | 0.65±0.23 | 0.56±0.28 |
| HRT18 (colorectal, Fogh *et al*, 1977) | 37 | 0.18±0.04 | — | 0.80±0.21 | 0.03±0.01 |
| W X330 (SCLC, Hay *et al*, 1991) | 42 | 0.09±0.01 | 3.71±0.91 | 0.22±0.12 | 0.27±0.18 |
| PANC1 (pancreatic, O'Hara *et al*, 1986) | 58 | 0.09±0.01 | 0.10±0.02 | 0.38±0.11 | — |
| HCT116 (colorectal, Langdon *et al*, 1988) | 129 | — | — | 0.65±0.25 | — |

RT–PCR products were analysed by densitometry and the relative expression level of four neuropeptide receptors are summarised. These values have been expressed as a ratio of the level of γ-actin expression for each cDNA batch and PCR run. Numbers represent the mean of four to six experiments. Spearman's rank correlation between $IC_{50}$ value and receptor expression level was performed. A significant correlation between sensitivity to SP-G and GRPR expression was confirmed (Spearman's $R$ value = −0.75, $P = 0.026$*).

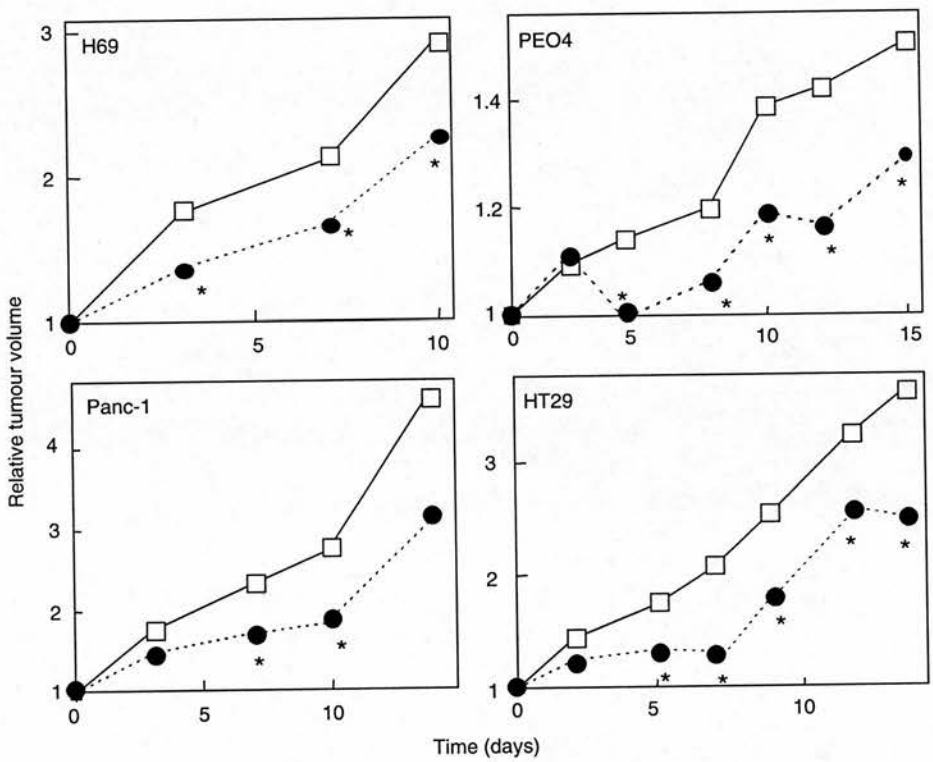


**Figure 4** Effect of SP-G on the growth of H69, HT29, PE04 and PANC-1 xenografts. Four cell lines from the original panel were established as xenografts from their respective cell lines: H69 SCLC, HT29 colon carcinoma, PEO4 ovarian cancer and PANC-1 pancreatic cancer. Typical growth curves are shown for each xenograft after i.p. treatment with 50 mg kg$^{-1}$ SP-G (●) or vehicle control (□) as five separate injections administered over 3 days as two injections on day 1 and 2 separated by a 6-h gap with the final injection given on day 3. The results represent the mean tumour volumes from a single study ($n = 6–8$). *Statistically significant from vehicle control (Students' $t$-test, $P < 0.05$).

After chemotherapy, the patient was in complete remission, 4 months later she relapsed. Further chemotherapy resulted in a partial response and the GLC16 line was established from a recurrence in the lung. After radiotherapy, the lung appeared tumour free; however, 3 months later tumour recurred in the lung from which the GLC19 cell line was derived. This was resistant to any further treatment and the patient died 2 months later (Berendsen *et al*, 1988; de Vries *et al*, 1989). *In vitro* sensitivity to chemotherapeutic agents reflected the clinically observed development of resistance to treatment (de Vries *et al*, 1989). We were also able to confirm these findings in the GLC cells used in this study (results not shown). One percent serum caused a rapid and equivalent increase in $[Ca^{2+}]_i$ in all three GLC SCLC cell lines suggesting that the mobilisable $Ca^{2+}$ pools were equivalent in each of the three cell lines. However, we show that in the progression to chemoresistance the GLC cells are able to respond to a greater



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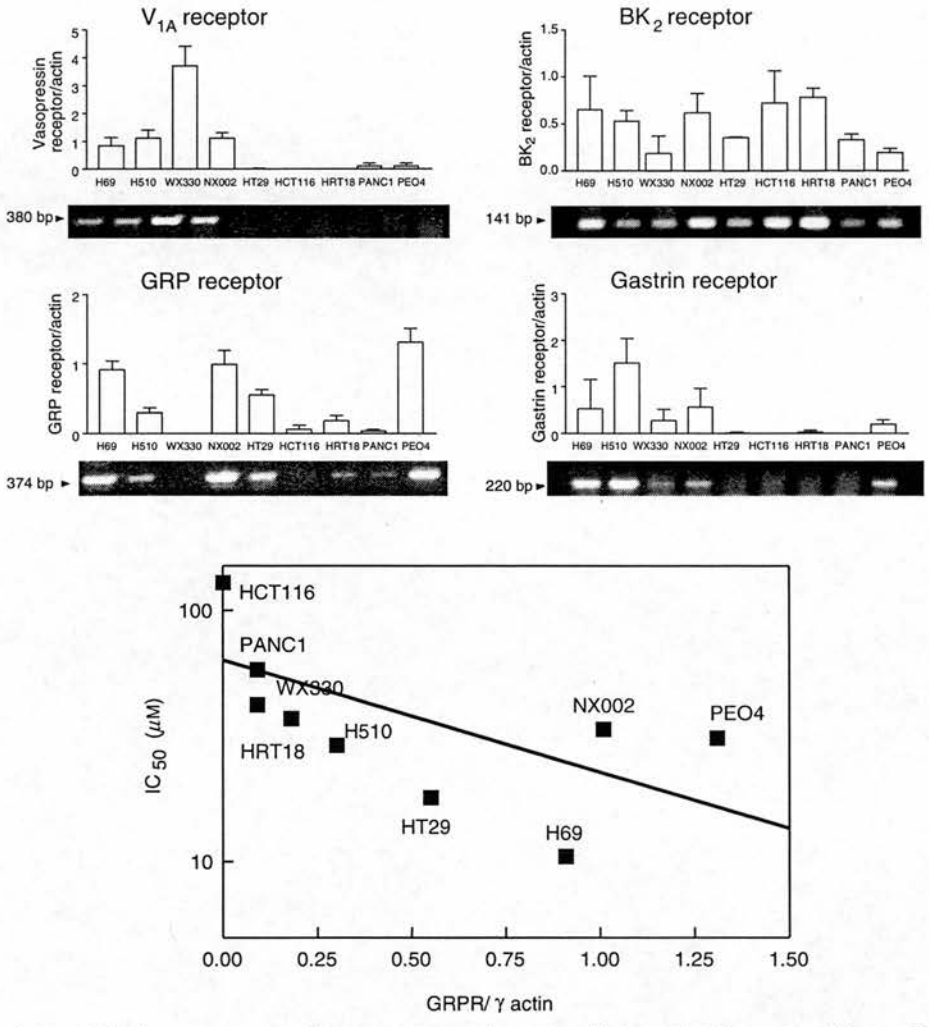


**Figure 5** Upper panel: Correlation between neuropeptide receptor expression and sensitivity to SP-G in tumour cell lines. mRNA encoding the GRP, $V_{1A}$, $BK_2$ and gastrin receptors was detected by semiquantitative RT–PCR. γ-Actin mRNA levels were measured as an internal control for each PCR reaction. The results show a representative PCR reaction for each of nine tumour cell lines, which represent a spectrum of sensitivity to SP-G. A bar chart showing relative receptor expression, calculated as density of PCR product/actin is shown for each receptor. The results represent the mean ± s.e.m. of four independent experiments. Lower panel: Correlation graph between GRPR expression and inhibition of cell growth ($IC_{50}$) for each of nine tumour cell lines as indicated (Spearman's *R* value = −0.75, $P = 0.026$*).

range of neuropeptides with increased potency, and are consequently more sensitive to growth inhibition by SP-G. The GLC19 cell line was more sensitive to SP-G both in liquid culture and in semisolid medium than the GLC16 and 14 cell lines. The $Ca^{2+}$-mobilisation and the clonal growth results suggest that this cell line may have greater neuropeptide dependence and this is reflected in its greater sensitivity to neuropeptide growth factor blocking agents such as SP-G. This led to the hypothesis that other neuropeptide-expressing tumours may also be sensitive to the growth-inhibitory effect of SP-G.

It is well known that neuropeptides can stimulate the growth of many types of cancers including pancreatic, colorectal, prostate, ovarian, breast and NSCLC (Bologna *et al*, 1989; Halmos *et al*, 1995; Aprikian *et al*, 1996; Ferris *et al*, 1997). A panel of 11 tumour cell lines, which represented a spectrum of tumour types, showed that sensitivity to SP-G differed across the panel with a range of >1 log order (10 μM to >100 μM). The sensitive cell lines (four SCLC, two colorectal carcinoma, one NSCLC and 1 ovarian) had $IC_{50}$ values that were in the range of physiologically obtainable plasma concentrations. Moreover, in the cell lines tested *in vitro* sensitivity correlated with sensitivity to SP-G *in vivo*. The results of this screen suggest that SP-G could be used therapeutically against several tumour types in addition to SCLC.

We have previously shown that SP-G augments etoposide-induced growth inhibition and apoptosis in SCLC cells and suggested that SP-G may be of increased benefit in patients following relapse or in conjunction with conventional chemotherapy (MacKinnon *et al*, 1999). It is of interest to note that the cell lines SCLC GLC19, ovarian cancer PEO4 and NSCLC NX022, which *in vitro* (and *in vivo*) are resistant to standard chemotherapeutic agents such as etoposide, are sensitive to the growth-inhibitory effects of SP-G. This raises the possibility that SP-G may be an effective anticancer agent in patients with neuropeptide-expressing tumours which are intrinsically resistant or have acquired resistance to conventional chemotherapy. A plausible extension of this hypothesis is that clinically aggressive drug-resistant SCLC


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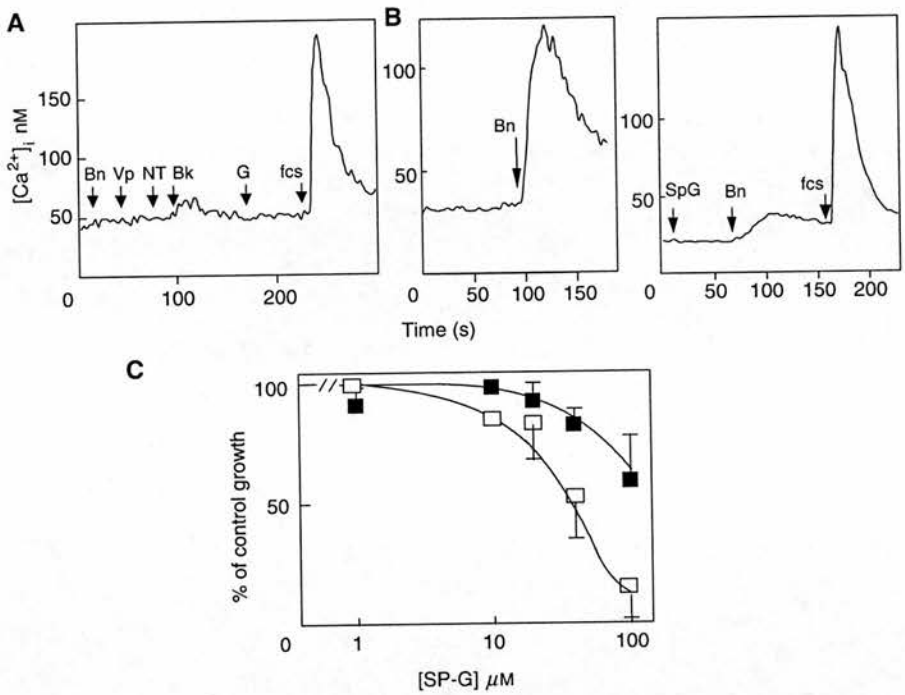


**Figure 6** Expression of the bombesin/GRP receptor in Rat-1 fibroblasts increases sensitivity to growth inhibition by SP-G. Rat-1a fibroblasts (**A**) or rat-1a fibroblasts expressing the bombesin receptor (**B**) were loaded with FURA-2-AME and resuspended in electrolyte solution. Fluorescence was monitored and basal and peak $[Ca^{2+}]_i$ calculated as described in Materials and Methods. (**A**) Rat-1a cells were stimulated with 10 nM each of bombesin (Bn), vasopressin (Vp), neurotensin (NT) and bradykinin (Bk), 10 $\mu$M SP-G or 1% FCS. The results are representative of three experiments. (**B**) (left) Response to 10 nM bombesin in BOR-15 cells and (right) effect of 10 $\mu$M SP-G on bombesin-stimulated $Ca^{2+}$ release in BOR-15 cells. (**C**) Rat-1 fibroblasts (closed squares) or Rat-1 fibroblasts expressing the bombesin receptor (BOR15, open squares) were grown in SITA media in the presence or absence of the indicated concentrations of SP-G. The cell number was determined and $IC_{50}$ values calculated following 7 days of growth.

cells that emerge after chemotherapy might have a more extensive network of neuropeptide regulation and therefore display increased sensitivity to neuropeptide antagonists. Further studies in longitudinal cell lines are required to investigate this hypothesis in further detail.

Although our data cannot indicate the expression of fully functional receptors, the RT-PCR approach was taken as it could be used as a possible diagnostic test in cancer patients to determine the potential tumour sensitivity to neuropeptide growth factor antagonist therapy. Of the four neuropeptide receptors tested, the most apparent correlation was between high expression of the GRP receptor and increased sensitivity to SP-G. This is in some ways surprising given that SP-G is more selective for the $V_{1A}R$ (Seckl *et al*, 1995) which is expressed on many lung cancer cells. However, GRP secretion and GRPR expression are the hallmarks of the neuroendocrine phenotype. The presence of the GRPR in particular may be a reflection of the general neuroendocrine phenotype of the cancer cells indicating greater neuropeptide dependence for growth. It would be interesting to look at the expression of other bombesin-like peptide receptors such as the neuromedin B receptor to assess its role in substance P-analogue-induced growth inhibition.

The GRPR has been shown to be oncogenic when transfected into the nonmalignant NCM460 colon epithelial cell line (Ferris *et al*, 1997). The increased proliferation was shown to be because of constitutive activation of the GRPR in that the receptors tonically coupled to $G_q$ in the absence of ligand. This gives a potential mechanism whereby the GRPR may act as an oncogene. In addition, many tumour types such as breast cancer (Halmos *et al*, 1995) and prostate cancer (Bologna *et al*, 1989; Aprikian *et al*, 1996) have also been shown to aberrently express GRPRs. We and others have previously shown that the expression of GRPRs in fibroblasts increases the ability of SP-G and other substance P analogues to activate the extracellular-signal-regulated kinase (ERK) and c-*jun*-N-terminal kinase (JNK) pathways leading to growth arrest and apoptosis (Jarpe *et al*, 1998; MacKinnon *et al*, 2001). Together, these data demonstrate that not only can GRPR expression transform cells, but these cells then become more sensitive to substance P-analogue-induced cell death.

These findings have important implications for the design of more advanced phase human clinical trials using substance P analogues. SP-G is currently entering a phase II clinical trial where its effectiveness will be tested in SCLC patients, but ultimately, compounds of this type may also be suitable for the treatment of a wide range of other tumour types and neuropeptide-expressing tumours that have become resistant to standard conventional chemotherapeutic agents. Screening tumour biopsy samples for neuropeptide receptor expression may provide insight into the likelihood of patients responding to treatment with substance P analogues, analogous to oestrogen receptor expression conferring efficacy to tamoxifen in breast cancer. It is suggested that tumours should be biopsied to select patients for substance P-analogue trials based on the expression of the GRPR and another neuropeptide receptor. Our results suggest that these tumours should show growth inhibition regardless of intrinsic or acquired resistance to standard chemotherapeutic agents. It is therefore proposed that in the first instance, patients with tumours that express the GRPR and another neuropeptide receptor who have failed conventional treatment should be randomised into two groups – best supportive care and treatment with substance P analogues.



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## ACKNOWLEDGEMENTS

CW and UT-H were supported by Cancer Research UK PhD studentships. This work was supported in part by the Scottish Hospitals Endowment Research Trust and the Melville Trust for the Care and Cure of Cancer. TS is a recipient of a Wellcome Trust Research Leave Fellowship.

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