AGONIST-INDUCED INTERNALISATION OF THE VASOACTIVE INTESTINAL POLYPEPTIDE RECEPTOR (VPAC$_2$)

Diane Dinnis

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University of Edinburgh

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I declare that the studies presented in this thesis are the result of my own independent investigation with the exception of: generation of the tagged and truncated $VPAC_2$ receptor constructs, which were made by Dr T.P.McDonald; radioiodinations and cAMP radioimmunoassays which were performed by Mr J.Bennie and Mrs S.Carroll.

This work is not currently being submitted for any other degree of professional qualification.

Diane Dinnis

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ABSTRACT

Vasoactive intestinal polypeptide (VIP) is a 28 amino acid peptide with a wide variety of functions in a number of biological systems. In the central nervous system VIP acts as a neurotransmitter or neuromodulator. Receptors for VIP belong to a subfamily of the classical seven transmembrane G-protein coupled receptors (GPCRs) which includes receptors for secretin, glucagon, calcitonin and parathyroid hormone. Currently two receptors for VIP have been cloned and named $VPAC_1$ and $VPAC_2$, they are differentially distributed in tissues but share similar pharmacology. These receptors are coupled to the stimulatory G-protein (G_S) and act via adenylyl cyclase to stimulate cAMP production. Prolonged exposure of GPCRs to agonist often results in reduced responsiveness to subsequent stimuli (desensitisation), an important mechanism in the regulation of intracellular signalling. A number of intracellular processes are thought to contribute to desensitisation; including phosphorylation, internalisation and down regulation.

To investigate agonist-induced internalisation of the $VPAC_2$ receptor we generated a C-terminal epitope-tagged receptor ($VPAC_2$-HA) and mutant receptors with serial C-terminal truncations. Addition of the epitope tag had no significant effect on the second messenger signalling or agonist binding properties of the $VPAC_2$ receptor stably expressed in the human embryonic kidney cell line (HEK293). Iodinated helodermin, a $VPAC_2$ receptor agonist, was rapidly internalised in cells expressing the $VPAC_2$ and $VPAC_2$-HA receptors following incubation at room temperature or at 37˚C. Internalisation kinetics of the $VPAC_2$ and $VPAC_2$-HA receptor were indistinguishable. In serum starved cells the epitope-tagged $VPAC_2$ receptor was predominantly located in the plasma membrane. Treatment with VIP caused a marked shift in receptor distribution from the plasma membrane to a single intracellular site. Receptor internalisation was dependent upon the concentration of agonist, incubation time and temperature. Removal of agonist resulted in the reappearance of receptor immunoreactivity at the plasma membrane; this movement was unaffected by the presence of the protein synthesis inhibitor cycloheximide, suggesting that the majority of receptors are recycled. Other work in our laboratory has demonstrated that truncation of the $VPAC_2$ receptor C-terminal intracellular domain prevents agonist-induced receptor phosphorylation. Nonetheless, truncated receptors were still able to internalise, indicating that phosphorylation is not an absolute requirement for $VPAC_2$ receptor internalisation. The truncated $VPAC_2$ receptor did not desensitise so we postulate that phosphorylation is necessary for this phenomenon.

To determine the mechanism underlying VPAC$_2$ receptor internalisation we used chemical treatments known to disrupt internalisation. Cells pretreated with hypertonic sucrose, an inhibitor of clathrin-coated vesicle formation, were unable to internalise the VPAC$_2$ receptor, whereas pretreatment with phorbol myristate acetate (PMA), an inhibitor of calveolae-mediated internalisation, had no effect on internalisation. In agreement with these studies it was shown that VPAC$_2$ receptors colocalise with transferrin receptors, which are internalised via clathrin pits and act as a marker of endosomes. No colocalisation was observed between the VPAC$_2$ receptor and a marker for the *trans*-golgi network, indicating that the receptor does not recycle through this compartment. This work provides the first direct evidence for agonist-induced internalisation of the VPAC$_2$ receptor. Interestingly, the receptor C-terminus does not appear to be actively involved in internalisation, although it is an important site for phosphorylation and desensitisation. In addition, these results indicate that the receptor is internalised via a clathrin-mediated mechanism and is recycled back to the cell surface, possibly in endosomes.

PUBLICATIONS ARISING FROM THIS THESIS

Dinnis, D. M., Morrison, C. F., Lutz, E. M., McDonald, T. P., and Harmar, A. J. (1997). Agonist-induced internalization of the VIP$_2$ receptor. European Congress for Molecular Cell Biology (ECBO) (Brighton, UK). *Meeting abstract.*.

Dinnis, D. M., Morrison, C. F., McDonald, T. P., and Harmar, A. J. (1998). Agonist-induced internalization of the VPAC$_2$ receptor. In 38th American Society for Cell Biology (San Francisco, CA). In Molecular biology of the cell, K. R. Yamamoto, ed.pp. 353a. *Meeting abstract.*.

McDonald, T. P., Dinnis, D. M., Morrison, C. F., and Harmar, A. J. (1998). Desensitisation of the human vasoactive intestinal peptide receptor (hVIP$_2$/PACAP R): evidence for agonist-induced receptor phosphorylation and internalisation. Annals of the New York Academy of Sciences *865*, 64-72.

McDonald, T. P., Morrison, C. F., Dinnis, D. M., and Harmar, A. J. (1997). Evidence for protein kinase A (PKA) dependent and independent desensitisation and phosphorylation of the the human vasoactive intestinal peptide receptor (VIP$_2$). Biochemical Society Transactions *25*, 442S.

ABBREVIATIONS

ABTS	2, 2, azino-bis (3-ethylbensthiasoline-6-sulfonic acid)
AC	adenylyl cyclase
ADNF	activity-dependent neurotrophic factor
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
$\alpha 2M$	$\alpha 2$macroglobulin
AP	adaptor or assembly protein
APS	ammonium persulphate
AR 4-2J	rat pancreatic acinar carcinoma cell line
ASGP	asialoglycoprotein
ATP	adenosine triphosphate
Baf A_1	bafilomycin A_1
BCA	bicinchoninic acid
B_2AR	β_2-adrenergic receptor
βARK	β-adrenergic receptor kinase
B_{max}	maximum binding
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
cAMP-TME	2'-O-monsuccinyladenosine 3',5'-cyclic monophosphate tyrosyl methyl ester
CCD	charge-coupled device
CCK	cholecystokinin
CCV	clathrin coated vesicle
cDNA	complementary DNA
CG	chorionic gonadotropin
CGRP	calcitonin gene-related peptide
CHL	chinese hamster lung
CHO	chinese hamster ovary
CNS	central nervous system
COS-7	green monkey kidney fibroblast
CRF	corticotrophin releasing factor
C-terminus	carboxy-terminus
DMEM	Dulbecco's modified eagle media
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EBSS	Earle's balanced salt solution

EC_{50}	excitation constant at half maximum
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis (β-amino ether) N,N,N',N'tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMR1	EGF module-containing mucin-like hormone receptor-1
FD	Farad
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GEB	glutathione elution buffer
GFP	green fluorescent protein
GH	growth hormone
GRF	growth hormone-releasing factor
GIP	gastric inhibitory polypeptide
GLP-1	glucagon like peptide-1
GLP-2	glucagon like peptide-2
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GRP	gastrin-releasing peptide
GST	glutathione S-transferase
GTP	guanosine triphosphate
HA	haemagglutinin
HCl	hydrochloric acid
HEK293	human embryonic kidney cell line
HIFCS	heat inactivated foetal calf serum
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HT-29	human adenocarcinoma cell line
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	inhibition constant at half maximum
IgG	immunoglobulin G
IL	interleukin
IP	inositol phosphate
IPTG	isopropyl β-D-thiogalactoside
K_D	equilibrium dissociation constant
KNRK	Kirsten murine sarcoma virus transformed rat kidney cell line

LH	luteinizing hormone
LDL	low-density lipoprotein
LB	luria bertani
M199	medium 199
mAB	monoclonal antibody
MAP	mitogen-activated protein
MEM	minimum essential medium
NaCl	sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
Na_3VO_3	sodium vanadate
NB-OK	human neuroblastoma cells
NG108-15	mouse neruoblastoma/rat glioma hybrid cells
NH_4Cl	ammonium chloride
NP-40	nonidet P-40
N-terminus	amino-terminus
OK	opossum kidney
pAB	polyclonal antibody
PACAP	pituitary adenylate cyclase activating peptide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PHI	peptide histidine isoleucine
PHM	peptide histidine methionine
PHV	peptide histidine valine
PKA	cAMP-dependent protein kinase
PKC	Ca^{2+}-dependent protein kinase
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
PRP	PACAP-related polypeptide
PTH	parathyroid hormone
PTHrP	parathyroid hormone releasing peptide

PTx	pertussis toxin
PVC	polyvinylchloride
Rhod	rhodamine
RIA	radioimmunoassay
RNA	ribonucleic acid
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulphate
SD	standard deviation
SEM	standard error of the mean
SSTR	somatostatin receptor
SUP-T1	Stanford University Pediatric T cell line 1
temed	N,N,N',N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
TfnR	transferrin receptor
TGN	trans-golgi network
TM	transmembrane
TRH	thyrotopin-releasing hormone
Tris	tris(hydroxymethyl)aminomethane
TRITC	rhodamine
TXR	Texas red
V	Volt
vATPases	vacuolar ATPases
VIP	vasoactive intestinal polypeptide

Standard abbreviations for amino acids

A	Ala	Alanine
B	Asx	Asparagine
C	Cys	Cysteine
D	Asp	Aspartic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Trptophan
Y	Tyr	Tyrosine
Z	Glx	Glutamine or glutamic acid

CHAPTER 1

General Introduction

1.1 Vasoactive intestinal polypeptide (VIP)

1.1.1 Isolation, structure and distribution

Vasoactive intestinal polypeptide (VIP) is a 28 amino acid peptide first isolated from the porcine intestine in 1970 (Said and Mutt, 1970a). VIP was originally identified as a vasodilator in the canine femoral artery (Said and Mutt, 1969) and subsequently found to induce smooth muscle relaxation in the lung (Piper et al., 1970). Shortly after its isolation the amino acid sequence of VIP was determined and it was synthesised (Bodanszky et al., 1973; Mutt and Said, 1974). The structure of VIP is well conserved throughout evolution with the majority of mammalian forms of the VIP having the same amino acid sequence (Campbell and Scanes, 1992b). VIP exhibits significant sequence homology with other peptides **(Table 1.1)**. This family of structurally related peptides includes secretin, gastric inhibitory peptide (GIP), glucagon, glucagon-like peptides (GLP-1 and GLP-2), growth hormone-releasing factor (GRF), helodermin, peptide histidine isoleucine (PHI), peptide histidine methionine (PHM) and pituitary adenylate cyclase activating polypeptide (PACAP) (Campbell and Scanes, 1992b).

Initially detected in endocrine cells of the gastrointestinal tract, VIP was subsequently shown to have a wide distribution in the cardiovascular, respiratory, immune and nervous systems. The discovery of VIP immunoreactivity in brain tissue and peripheral nerves led to the proposal that it may act as a neurotransmitter (Lorén et al., 1979; Said and Rosenberg, 1976). Colocalisation of VIP with acetylcholine (ACh) in sympathetic nerve endings of sweat glands (where VIP caused vasodilation and ACh stimulated secretion) provided one of the first clear examples for the coexistence of peptides with 'classical' neurotransmitters (Hökfelt et al., 1980). Both VIP and noradrenaline (NA) increase cAMP levels in the cerebral cortex and coapplication of these neurotransmitters causes a synergistic elevation in cAMP levels (Magistretti and Schorderet, 1984). In the central nervous system (CNS) the highest concentration of VIP-rich neurones are found in the cortex, hypothalamus and around the median eminence (Hökfelt et al., 1982). In the cerebral cortex VIP is contained in a homogenous population of radially oriented, bipolar neurones (Magistretti, 1990). Autoradiographic mapping of iodinated VIP binding sites in rat brain reveals a high density of receptors in the olfactory bulb, cerebral cortex (particularly layers I, II, IV and VI), dentate gyrus, subiculum, various thalamic and hypothalamic nuclei, superior colliculus, locus coeruleus, area postrema, subependymal layer and pineal gland (Martin et al., 1987). In addition, VIP has been

found in sensory neurones (Buck et al., 1982): expression of VIP in dorsal root ganglia is low under normal conditions but rises dramatically following nerve injury (Dickinson and Fleetwood-Walker, 1998). In the peripheral nervous system (PNS) VIP-containing fibres supply blood vessels, smooth muscle and glandular acini and ducts of a variety of organs and tissues (Sundler et al., 1988). VIP immunoreactivity has been observed in tumour cells derived from a number of different tissues (for a review see Reubi, 1996). VIP is rapidly metabolised in the liver resulting in low peptide levels in peripheral venous blood, however, enzymatic degradation of VIP can also occur in the brain, kidney and lungs (Said and Mutt, 1970b).

In common with other peptides belonging to this family, VIP is first synthesised in the form of a large precursor peptide (preproVIP) (Obata et al., 1981). The distribution of preproVIP mRNA in the rat CNS, revealed by *in situ* hybridisation, is in accordance with localisation of VIP described above (Dussaillant et al., 1992). Human preproVIP is a 170 amino acid polypeptide which is processed by proteolytic cleavage at mono- and di-basic residues into five peptide fragments, including preproVIP(22-79), PHM (peptide with N-terminal histidine and C-terminal methionine), preproVIP(111-122), VIP, and preproVIP(156-170) (Itoh et al., 1983; Ottesen et al., 1995) **(Figure 1.1)**. In some tissues an extended version of PHM, peptide histidine valine (PHV) (peptide with N-terminal histidine and C-terminal valine) is produced when a C-terminal cleavage site remains unprocessed (Yiangou et al., 1987). PHM is not found in other mammals which express an equivalent peptide, PHI (peptide with N-terminal histidine and C-terminal isoleucine). These peptides undergo post-translational processing and amide groups are added at their C-termini by hydroxylation of an additional glycine residue in an extended peptide prohormone (Prigge et al., 1997). C-terminal amidation is important for the biological activity of many peptides (for a review see Bradbury and Smythe, 1991). The ratio of preproVIP-derived peptides in some tissues is not always equimolar, indicating that tissue-specific processing may occur. One example is the processing of PHM in the male urogenital tract and nervous system (Fahrenkrug and Emson, 1989; Ottesen et al., 1995). Like VIP, PHM (PHI) and PHV are biologically active (Palle et al., 1990; Tatemoto and Mutt, 1981; Yiangou et al., 1987), however no specific receptors for PHM and PHV have been identified, and their effects are presumed to be mediated through receptors shared with VIP (Harmar et al., 1998).

1.1.2 Actions of VIP

Although originally identified and named on the basis of its strong vasodilator effect, VIP is involved in the regulation of many physiological processes and functions as a neurotransmitter or neuromodulator and hormone (Said, 1991; Said and Mutt, 1972b). VIP has a variety of actions in mammalian systems; an exhaustive description is beyond the scope of this chapter, but a summary is presented in **Table 1.2**, and a few putative actions of VIP are described below.

Work on cultured cells has demonstrated multiple long-term effects of VIP in stimulating mitosis, promoting differentiation of sympathetic neurones and enhancing their survival (Brenneman and Eiden, 1986; Pincus et al., 1990). The survival promoting action of VIP in spinal cord neurones appears to be mediated indirectly by VIP-stimulated release of neurotrophic factors from astroglia, these factors include protease nexin I, activity dependent neurotrophic factor (ADNF) and cytokines (Brenneman et al., 1996; Brenneman et al., 1987; Brenneman et al., 1997). The effects of VIP on neural cells vary, depending upon cell type and stage of development: VIP can either promote or inhibit proliferation (Waschek, 1996). VIP is involved in the early stages of development in the nervous system and has been reported to cause a dramatic increase in the growth of cultured mouse embryos (E9.5) *in vitro*. (Gressens et al., 1993). This data has been affirmed *in vivo* by experiments which found that VIP antagonists retard the growth of the nervous system of the mouse embryo (E9-E11) resulting in microcephaly (Gressens et al., 1994). However, these studies are controversial and recent experiments were unable to demonstrate a marked effect of VIP on embryo growth. (Sheward et al., 1998). VIP mRNA has not been detected in prenatal rodent CNS and current findings indicate that the peptide may be supplied to the embryo in maternal serum (Hill et al., 1996; Spong et al., 1999). VIP is also mitogenic for a number of different tumour cell lines and VIP antagonists have been shown to inhibit the growth and mitosis of tumour cells derived from neuronal and non-neuronal tissues (Lilling et al., 1994; Moody et al., 1993). Therefore, although VIP does not appear to be a direct promoter or inhibitor of cell growth, differentiation and survival, much evidence has accumulated for a modulatory role for VIP in these processes.

A considerable body of evidence is now available supporting a role for VIP in the regulation of circadian rhythms. Neurones within the suprachiasmatic nucleus (SCN) of mammals appear to function as a circadian clock, controlling diurnal variation in physiological systems (Meijer and Rietveld, 1989). A number of neuropeptides have

been found in the SCN, including VIP, PHI, vasopressin, neurotensin and substance P (Card et al., 1988). The first evidence for VIP involvement in mediating photic information came from a study of rats which displayed reduced VIP and PHI concentrations under constant light conditions (Albers et al., 1987). Subsequent studies have found a diurnal variation of VIP mRNA levels the in rat SCN (Albers et al., 1990; Okamoto et al., 1991a). Circadian variation in the expression of VIP receptor mRNA in rats over a 24 hr period has also been observed (Cagampang et al., 1998). The changing levels of receptor mRNA may provide a mechanism by which the rodent SCN can vary in its sensitivity towards VIP, however further studies are necessary to determine the precise function and mode of action of VIP in this system.

1.1.3 Involvement of VIP in disease and therapeutic potential

VIP acts as a potent relaxant of human airways *in vitro* (Said, 1991). Deficiencies in VIP levels are present in asthma, however VIP replacement therapy is impaired by rapid degradation of the peptide and clinical trials have found that its effects are less than conventional medicines (Morice and Sever, 1986; Ollerenshaw et al., 1989). To overcome this problem a long-lasting VIP analogue, Ro 25-1553, was synthesised. It has smooth muscle relaxant and anti-inflammatory actions and is currently undergoing clinical trials as a potential treatment for asthma (O'Donnell et al., 1994a; O'Donnell et al., 1994b; Tang et al., 1995).

VIP has been shown to protect against tissue and organ injury. It reduces acute lung injury induced by oxidative stress (Berisha et al., 1990; Pakbaz et al., 1993) and prevents post-ischaemic reperfusion damage in the heart (Kalfin et al., 1994). VIP also has neuroprotective properties which make it a strong candidate for the treatment of neurodegenerative disorders (Brenneman et al., 1998). The protective action of VIP is believed to stem from a number of its actions, including its affects on the immune system, its antioxidant activity and ability to counter nitric oxide toxicity (Misra and Misra, 1990; Said et al., 1995). The vasodilator activity of VIP makes it a potential treatment for disorders which require increases in regional or local blood flow, such as ischemia, male impotence and hypertensive disease (Said, 1991). As mentioned earlier VIP antagonists inhibit the growth of various non-small cell carcinoma cells lines, suggesting that these agents have a potential therapeutic use in preventing growth of tumour cells (Moody et al., 1993).

Table 1.1. Comparison of amino acid sequences of members of the secretin/glucagon family of peptides. Abbreviations; vasoactive intestinal polypeptide (VIP), peptide histidine methionine (PHM), peptide histidine isoleucine (PHI), pituitary adenylate cyclase-activating polypeptide (PACAP), gastric inhibitory polypeptide (GIP), growth hormone releasing factor (GRF). Amino acid residues indicated in red are identical to VIP.

Peptide	Species	Sequence
VIP	human	H S D A V F T D N Y T R L R K Q M A V K K Y L N S I L N
PHM	human	H A D G V F T S D F S K L L G Q L S A K K Y L E S L M
PHI	rat	H A D G V F T S D Y S R L L G Q I S A K K Y L E S L I
PACAP	human	H S D G I F T D S Y S R Y R K Q M A V K K Y L A A V L G K R Y K Q R V K N K
helodermin	gila	H S D A I F T E E Y S K L L A K L A L Q K Y L A S I L G S R T S P P P
secretin	human	H S D G T F T S E L S R L R E G A R L Q R L L Q G L V
glucagon	human	H S Q G T F T S D Y S K Y L D S R R A Q D F V Q W L M N T
GIP	human	Y A E G T F I S D Y S I A M D K I H Q Q D F V N W L L A Q K G K K N D W K H N I T Q
GRF	human	Y A D A I F T N S Y R K V L G Q L S A R K L L Q K I M S R Q Q G E S N Q E R G A R A R L

Figure 1.1. Schematic diagram of the structure and processing of human preproVIP. The putative signal peptide is contained within amino acids 1-21, and the precursor is processed into five peptide fragments, preproVIP(22-79) (the N-terminal flanking peptide), PHM, preproVIP(111-122) (the bridging peptide), VIP and preproVIP(156-179) (the C-terminal flanking peptide). PHV, the C-terminally extended form of PHM is also shown. Reproduced from Ottensen et al. (1995).
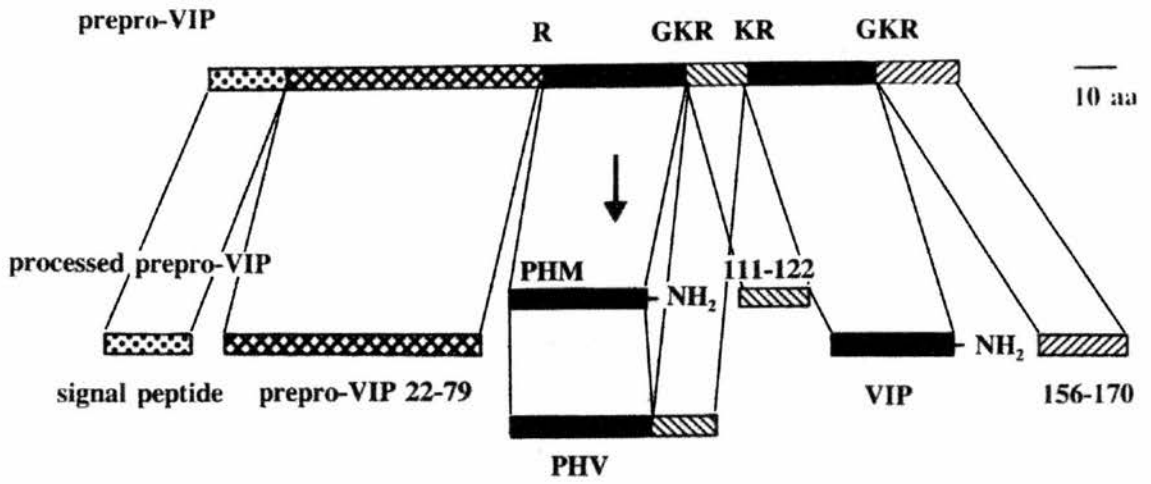
Table 1.2 Summary of the biological actions of VIP

	Actions
Cardiovascular system	Vasodilator of peripheral, splanchnic, coronary and cerebral vessels (Said and Mutt, 1970). Influences regional blood flow, total peripheral vascular resistance and arterial blood pressure (Said, 1983). Increases cardiac output (Said et al., 1972).
Respiratory system	Promotes pulmonary vasodilatation, bronchodilation and regulates bronchial secretions (Said, 1982)
Digestive system	Relaxation of enteric smooth muscle (Murthy et al., 1996). Controlling water, ion and protein secretion in epithelia and increased fluid secretion in the pancreas (Martin and Shuttleworth, 1996)
Metabolism	Stimulation of glycogenolysis and gluconeogenesis in the CNS (Magistretti et al., 1981) and liver (Feliu et al., 1983)
Endocrine/neuroendocrine	Modulates release of prolactin (Abe et al., 1985; Kato et al., 1978), GH, LH (Vijayan et al., 1979), CRH (Nussdorfer and Malendowicz, 1998), oxytocin and vasopressin (Ottesen et al., 1984). Stimulates melatonin release and serotonin-N-acetyltransferase activity in the pineal gland (Simonneaux et al., 1990; Yuwiler, 1983). Promotes release of catecholamines from the adrenal medulla (Malhotra et al., 1988). Promotes the release of glucose-induced insulin from the pancreas (Fahrenkrug et al., 1987; Schebalin et al., 1977)
Immune system	Modulates the immune system through effects on the production of cytokines (Ganea, 1996; Tang et al., 1995; Xin et al., 1997). Inhibits mitogen-induced proliferation of T-cell lymphocytes (Ottaway, 1987). Effects lymphocyte migration (Bondesson et al., 1991) and immunoglobulin production (Stanisz et al., 1986) (For review see Bellinger et al., 1996).
Reproductive system	Involved in the autonomic nervous control of motility and blood flow in the genitourinary tract (Fahrenkrug et al., 1988). Possibly an efferent transmitter in penile erection in man (Ottesen et al., 1984; Willis et al., 1983).

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1.2 Pituitary adenylate cyclase-activating polypeptide (PACAP)

1.2.1 Isolation, structure and distribution

Almost twenty years after VIP was isolated, a potent stimulator of adenylyl cyclase was isolated from ovine hypothalamus. This peptide was named pituitary adenylate cyclase-activating polypeptide (PACAP-38) in accordance with its potent activation of adenylyl cyclase in the pituitary (Miyata et al., 1989). PACAP displays striking sequence similarity with VIP, particularly in the N-terminal domain where 68% homology with ovine VIP is observed (Miyata et al., 1989) **(Table 1.1)** (Miyata et al., 1989). A second form of PACAP consisting of the N-terminal 27 amino acids of PACAP-38 was subsequently isolated from a side fraction of hypothalamic extract and named PACAP-27. PACAP-38 and PACAP-27 arise from alternative post-translational processing events (Miyata et al., 1990). The precursor to PACAP has been cloned from rat, sheep and human and consists of an open reading frame of 176 amino acids (Kimura et al., 1990; Ogi et al., 1990; Ohkubo et al., 1990). In common with preproVIP, the PACAP precursor contains sequences for multiple peptides, PACAP-38, PACAP-27 and PACAP-related polypeptide (PRP). Whether the latter is biologically active remains to be determined (Ohkubo et al., 1990).

Like VIP, PACAP is widely expressed in many central and peripheral neurones (Ghatei et al., 1993). Radioimmunoassays for PACAP-27 and PACAP-38 in rat brain reveal that these peptides are located predominantly in the hypothalamus and to a lesser extent in the cortex, hippocampus, cerebellum and posterior pituitary (Arimura, 1992a; Vertongen et al., 1992). In peripheral tissues, both peptides are present at significant levels in the testes, adrenal medulla and at lower levels in the female genital tract, lung, gastrointestinal tract, pancreas, the salivary glands and ocular tissues (Arimura, 1992a; Tobin et al., 1995; Wang et al., 1995). In the majority of tissues investigated the levels of PACAP-38 are higher than PACAP-27 (Arimura, 1992a).

1.2.2 Actions of PACAP

Many of the actions of PACAP are shared with VIP. In addition, the broad distribution of PACAP in nervous tissue suggests that it acts as a neurotransmitter or neuromodulatory peptide. In the CNS, PACAP potentiates the release of melatonin from the pineal gland (Simonneaux et al., 1998) and affects the release of prolactin, gonadotrophic hormones (Culler and Paschall, 1991; Nagy et al., 1993) and α-melanocyte-stimulating hormone from the pituitary (Koch and Lutz-Bucher,

1992). It also stimulates pituitary cells to release interleukin-6 (Tatsuno et al., 1991). Comprehensive reviews of PACAP structure and function have been published (Arimura, 1992a; Rawlings and Hezareh, 1996). PACAP potentiates glucose-induced insulin release from the pancreas (Kawai et al., 1992) and acts, more potently than VIP, on the chromaffin cells of the adrenal medulla stimulating catecholamine release (Wakade et al., 1992). *In vitro,* PACAP has been shown to regulate gastrointestinal motility via relaxation of smooth muscle (Katsoulis and Schmidt, 1996). PACAP acts as a neurotrophic factor stimulating growth, survival and differentiation in cultured neurones (Arimura et al., 1994; DiCicco-Bloom and Deutsch, 1992). In tumour cells PACAP has profound effects on the growth and differentiation of tumour cells derived from neuronal and non-neuronal tissues (Buscail et al., 1992; Hoshino et al., 1993).

1.3 Receptors for VIP and PACAP

1.3.1 G-protein coupled receptor (GPCR) families

G-protein coupled receptors (GPCRs) are a superfamily of glycoproteins with seven putative transmembrane (TM) spanning domains, which are cellular receptors for a diverse array of stimuli, such as; light, odours, neurotransmitters and hormones (monoamines, amino acids and peptides). These receptors are coupled through a variety of heterotrimeric G-proteins which stimulate or inhibit second messenger effectors within the cell. Rhodopsin, a photon receptor within rod cells of the retina was the first member of this receptor superfamily to be cloned. To date >200 GPCRs have been identified, mammalian GPCRs are divided, on the basis of their sequence similarities, into three classes; class I (rhodopsin-like receptors), class II (secretin receptor family) and class III (a diverse family of receptors which includes metabotropic glutamate and GABA$_B$ receptors and receptors for calcium ions and pheromones) (Birnbaumer, 1995; Segre and Goldring, 1993).

The secretin/glucagon/VIP family of peptides show striking similarities in their pharmacological, functional and molecular properties and had been postulated to act through a related set of receptors (Laburthe et al., 1983). The isolation of the rat secretin receptor (Ishihara et al., 1991), the first class II GPCR to be identified, prompted the use of homologous cloning strategies to identify other peptide receptors. This led to the cloning of receptors for VIP (Ishihara et al., 1992; Lutz et al., 1993), GRF (Mayo, 1992), GLP-1 (Thorens, 1992; Thorens et al., 1993), PACAP (Pisegna and Wank, 1993), glucagon (Jelinek et al., 1993; MacNeil et al., 1994), insect diuretic hormone (Reagan, 1994), GIP (Yamada et al., 1995) and GLP-2 (Munroe et al., 1999). Surprisingly, further additions to this family came from cloning of receptors for unrelated peptides, such as parathyroid hormone/parathyroid hormone releasing peptide (PTH/PTHrP) (Jüppner et al., 1991), calcitonin (Lin et al., 1991), corticotrophin-releasing factor (CRF) (Chen et al., 1993) and calcitonin gene-related peptide (CGRP) (Aiyar et al., 1996), which share 30-50% amino acid sequence with other secretin receptor family members. Other proteins which share significant sequence homology with the secretin receptor family, include the epidermal growth factor (EGF), module-containing mucin-like hormone receptor 1 (EMR1) (Baud et al., 1995) and CD97, an activation-induced antigen from leukocytes (Hamann et al., 1995). The TM domains of these proteins are particularly similar to the other class II GPCRs but they differ markedly in the presence of an expanded N-terminal extracellular domain containing EGF-like domains. Both

EMR1 and CD97 are thought to act as receptors for cells surface molecules (Baud et al., 1995; Hamann et al., 1995).

Receptors belonging to the secretin receptor family display little homology with the classical rhodopsin-like superfamily of GPCRs (<12%) (Ulrich et al., 1998). They differ in the presence of a signal peptide, a relatively large cysteine-rich amino terminus and multiple N-glycosylation sites (Ishihara et al., 1991; Laburthe et al., 1996). The general structural motifs of the class I and class II GPCRs are shown in **Figure 1.2**. There are six well conserved cysteine residues in the N-terminal extracellular domain (four of which are conserved in the EMR1 and CD97) and a conserved Cys-Trp motif in the second extracellular loop of class II GPCRs. The C-terminal intracellular domain is the most divergent region of the secretin receptor family (Laburthe et al., 1996). A recent study by Donnelly (1997) predicted differences in the arrangement of TM2, TM3 and TM7 between rhodopsin-like and secretin receptors which might affect their contact with the lipid bilayer (Donnelly, 1997). The genes encoding these receptors are also different; genes encoding rhodopsin-like receptors tend to be intronless, whilst genes for members of the secretin receptor family are complex and interrupted by a large number of introns, with a highly conserved organisation which probably emerged early during evolution (Laburthe et al., 1996). Recently, Barnes et al. (1998) postulated that *frizzled* proteins are evolutionarily close to the secretin receptor family, indeed they share significant degrees of primary sequence homology with these receptors, particularly in the putative TM domains and in the presence of a cysteine-rich amino terminus (Barnes et al., 1998). *Frizzled* proteins act as receptors for Wnts, signalling molecules involved in several aspects of embryo development. The mechanism of signal transduction for these receptors is not well documented, however evidence is accumulating for the involvement of G-proteins in Wnt signalling (Slusarski et al., 1997).

1.3.2 VIP and PACAP receptor classification

Studies of VIP binding sites revealed them to be heterogeneous. VIP receptors have been characterised using chemical cross-linking (reviewed by Laburthe and Couvineau, 1988) and photoaffinity labelling techniques (Robichon and Marie, 1987). Both types of experiment revealed two distinct classes of VIP receptor, that could be distinguished by their size following SDS-PAGE analysis and their affinity for VIP. Moreover, the molecular weight of solubilised VIP receptors displayed considerable variation depending upon the species and tissue studied. Some of the

differences in receptor size demonstrated using these biochemical methods were subsequently found to result from the levels of glycosylation resulting from post-translational modifications (Fabre et al., 1993; Luis et al., 1988). Nonetheless, radioligand binding experiments also suggested the presence of more than one type of VIP binding site in a number of tissues (Christophe et al., 1981; Leroux et al., 1984; Waelbroeck et al., 1981). The two sites were classified as high affinity (0.8 nM) and low affinity (9 nM) VIP binding sites (Waelbroeck et al., 1981). A third VIP binding site was distinguished using helodermin, a VIP-like peptide first isolated from Gila monster venom, which bound specifically to a single class of high affinity VIP receptors in rat liver membranes (Robberecht et al., 1984). Subsequently both PACAP-38 and PACAP-27 were found to share high affinity binding sites with VIP in membranes from lung (Lam et al., 1990), liver (Robberecht et al., 1991a) and with helodermin-preferring receptors in the human SUP-T1 lymphoblast cell line (Gourlet et al., 1991). PACAP-38 and PACAP-27 were also reported to bind at sites, which were distinct from VIP binding sites, in the rat CNS (Lam et al., 1990), cultured rat astrocytes (Tatsuno et al., 1990), human neuroblastoma (NB-OK) cells (Cauvin et al., 1990), rat adrenochromaffin cells (Watanabe et al., 1990) and rat cancerous pancreatic acinar cells (AR 4-2J) (Buscail et al., 1990). Radioligand binding studies using [^{125}I]-PACAP-27, distinguished two types of PACAP receptor in rat tissues, named PACAP types I and II (Shivers et al., 1991). PACAP type I binding sites display high affinity for PACAP but much lower affinity for VIP, whereas PACAP type II binding sites display similar affinities for both PACAP and VIP (Arimura, 1992b; Shivers et al., 1991). Further diversity of the PACAP type I binding sites was established by Shivers et al. (1991) who identified two classes of receptor, PACAP type I-A and PACAP type I-B, the former bound both peptides with equal affinity whilst the latter had a lower affinity for PACAP-27 (Shivers et al., 1991). Much of this conflicting literature has been resolved following the cloning and characterisation of individual receptor cDNAs encoding specific VIP/PACAP receptors. To date, one PACAP type I receptor with at least six splice variants and two PACAP type II (or VIP) receptors with distinct pharmacological profiles been isolated (see later for references).

The nomenclature for VIP/PACAP receptors has varied between laboratories and undergone several revisions as this family has expanded. The PACAP type I receptor has also been referred to as PVR1 (Rawlings et al., 1995). The first PACAP type II receptor was identified because of its high affinity for VIP, and named as a VIP receptor (Ishihara et al., 1992). A second VIP responsive receptor was cloned by

several different groups, who adopted different naming systems, thus these receptors were termed either; VIP_1 and VIP_2 (Lutz et al., 1993), PVR2 and PVR3 (Rawlings et al., 1995) or PACAPR-1 and PACAPR-3 (Inagaki et al., 1994). Recent alterations in this nomenclature have been made to consolidate the cloning developments and expanding knowledge of receptor pharmacology. Hence, the PACAP type I receptor is now termed PAC_1 and the two PACAP type II receptors are called $VPAC_1$ (previously VIP, VIP_1 or PVR2) and $VPAC_2$ (previously VIP_2 or PVR3) (Harmar et al., 1998), see summary in **Table 1.3**. These receptors are expressed endogenously in a number of different cell lines, some of these are presented in **Table 1.4**.

1.3.3 The $VPAC_1$ receptor

The first VIP/PACAP receptor was isolated by low stringency hybridisation of a rat lung cDNA library with secretin receptor cDNA (Ishihara et al., 1992). This receptor, now named $VPAC_1$, consists of 459 amino acid protein with a putative signal sequence located in first 30 N-terminal amino acids. The receptor contains seven TM domains and shares significant homology with other class II GPCRs. Northern blotting and *in situ* hybridisation revealed high levels of rat $VPAC_1$ mRNA in the lung, liver and intestine, with lower levels of expression in the thymus and brain (particularly in the cortex, hippocampus and olfactory bulb) (Ishihara et al., 1992; Usdin et al., 1994).

Human $VPAC_1$ receptor cDNAs were obtained by screening of the HT-29 colonic carcinoma cell line (Sreedharan et al., 1993) and jejunal epithelial cells (Couvineau et al., 1994). The cDNA encodes a 457 amino acid receptor (with a predicted molecular weight of 52 kDa) which shares 84% amino acid identity with its rat homologue (Sreedharan et al., 1993). The receptor N-terminal extracellular domain contains seven cysteine residues and three potential sites for N-linked glycosylation, with an additional glycosylation site on the second extracellular loop (Sreedharan et al., 1993). Northern blot analysis revealed mRNA transcripts for the human $VPAC_1$ receptor in lung tissues with a weaker expression in brain, heart, kidney, liver and placenta (Sreedharan et al., 1993). Wei and Mojsov (1996) used the more sensitive method of RNase protection to determine the tissue distribution of human $VPAC_1$ receptor mRNA and found high $VPAC_1$ mRNA expression levels in the liver, brain, heart and adipose tissues (Wei and Mojsov, 1996). $VPAC_1$ receptor cloning has enabled a detailed examination of both pharmacological and functional properties of this receptor. Both rat and human receptor homologues have been shown to specifically bind [^{125}I]-VIP and competitive displacement studies reveal that VIP,

PACAP-38, PACAP-27 and PHI have a similar affinity for the $VPAC_1$ receptor (Ciccarelli et al., 1994; Sreedharan et al., 1993; Usdin et al., 1994; Van Rampelbergh et al., 1996). VIP and related peptides stimulated a dose-dependent increase in cAMP accumulation in Chinese hamster ovary (CHO), COSGs1, COS-7, HEK293 cells and LVIP (cAMP reporter) cell lines transfected with rat or human $VPAC_1$ receptor cDNA, indicating that this receptor can couple to the stimulatory G-protein (G_s) and activate adenylyl cyclase (AC) (Ciccarelli et al., 1994; Couvineau et al., 1994; Ishihara et al., 1992; Sreedharan et al., 1993; Usdin et al., 1994). VIP treatment also resulted in accumulation of intracellular calcium ions $[Ca^{2+}]_i$ in COS-7 and HEK293 cells transfected with the human $VPAC_1$ receptor (Sreedharan et al., 1994). $VPAC_1$ receptors, stably transfected in CHO cells, stimulate inositol phosphate (IP) production in a pertussis toxin (Ptx)-sensitive manner, suggesting that this receptor can couple through an inhibitory G-protein (G_i/G_o) when transfected in CHO cells. The efficiency of receptor coupling to G_i/G_o is low compared with receptor coupling to G_s and maximal stimulation is dependent on the levels of receptor expression (Van Rampelbergh et al., 1997).

1.3.4 The $VPAC_2$ receptor

The rat $VPAC_2$ receptor was originally isolated from olfactory bulb and cerebral cortex cDNA libraries (Lutz et al., 1993; Usdin et al., 1994). The receptor cDNA encodes a protein of 437 amino acids (with a predicted molecular weight of 49 kDa) and shares ~50% sequence identity with rat $VPAC_1$ receptors. VIP, PACAP-38, PACAP-27 and PHI display a comparable affinity for the rat $VPAC_2$ receptor. These peptides were also able to stimulate cAMP production with similar potency, in COS-7 and LVIP cells stably transfected with rat $VPAC_2$ receptor cDNA (Lutz et al., 1993; Usdin et al., 1994). These results parallel those observed with the rat $VPAC_1$ receptor, with one important difference; micromolar amounts of secretin stimulate cAMP production in cells transfected with the $VPAC_1$ but are ineffective at the $VPAC_2$ receptor (Usdin et al., 1994). In the periphery, $VPAC_2$ receptor mRNA was detected in the stomach, duodenum, spleen, kidney, thymus, adrenal cortex, pancreas, testes, ovary and uterus (Usdin et al., 1994). In the CNS, $VPAC_2$ mRNA was detected in the SCN, thalamus, hippocampus, dentate gyrus, olfactory bulb, brain stem and at low levels in the pituitary (Lutz et al., 1994; Sheward et al., 1995). A detailed comparison of $VPAC_1$ and $VPAC_2$ mRNA distribution in the rat brain was carried out by Usdin and colleagues who found prominent levels of rat $VPAC_2$ mRNA in the hypothalamus, midbrain, brainstem, pituitary and olfactory bulb (Usdin et al., 1994). A summary of VIP/PACAP distribution in the rat brain is

presented in **Figure 1.3**. Distribution of $VPAC_1$ and $VPAC_2$ receptor mRNAs overlap but are often distinct and complementary, with the latter being less abundant but having a wider distribution in the periphery. A mouse $VPAC_2$ receptor cDNA cloned from an insulin-secreting β-cell line (MIN6) cDNA library encodes an identical amino acid sequence to the rat $VPAC_2$ receptor (Inagaki et al., 1994). The mouse receptor transfected into CHO cells displayed similar pharmacology, functional coupling and tissue distribution to the rat $VPAC_2$ receptor.

The human $VPAC_2$ receptor has been cloned from SUP-T1 lymphoblast (Svoboda et al., 1994), placenta (Adamou et al., 1995) and adipocyte (Wei and Mojsov, 1996) cDNA libraries. This receptor consists of 438 amino acids (with a predicted molecular weight of 49 kDa). The proximal 1-20 amino acids of the N-terminal domain are thought to be removed following cleavage at a putative signal peptide cleavage site (yielding a predicted molecular weight of 46.5 kDa for the mature receptor). The human $VPAC_2$ receptor shows marked amino acid homology (85%) with the rat homologue and 52% identity with the human $VPAC_1$ receptor. In common with the human $VPAC_1$ receptor, it has seven cysteine residues and three putative N-linked glycosylation sites at equivalent positions in the N-terminal extracellular domain downstream of the signal sequence (Adamou et al., 1995). Iodinated VIP and PACAP-27 ligands were used to characterise the affinity of peptides for the human $VPAC_2$ receptor transfected in COS-7, CHO and Chinese hamster lung (CHL) cells. The receptor has comparable affinities for VIP, PACAP-38 and helodermin, a slightly lower affinity for PACAP-27 and very low affinity (micromolar) for secretin (Adamou et al., 1995; Said and Mutt, 1969; Svoboda et al., 1994; Wei and Mojsov, 1996). These data indicate that the $VPAC_2$ receptor corresponds to the 'helodermin-preferring' receptor previously identified in rat liver membranes (Robberecht et al., 1984) and human SUP-T1 lymphoblasts (Robberecht et al., 1988). Stimulation of AC was also determined in the aforementioned cell lines, with VIP, PACAP-38 and PACAP-27 causing a dose dependent increase in cAMP with similar potencies, whilst secretin was ineffective (Svoboda et al., 1994; Wei and Mojsov, 1996). Thus secretin allows the $VPAC_1$ and $VPAC_2$ receptors to be distinguished pharmacologically as this peptide has a high affinity for $VPAC_1$ receptors and a relatively low affinity for $VPAC_2$ receptors (Usdin et al., 1994). Northern blotting and RNase protection assays detected human $VPAC_2$ receptor mRNA transcripts in skeletal muscle, heart, brain, placenta, stomach, kidney, pancreas and lung with low levels in adipose tissues and a complete absence in the liver (Adamou et al., 1995; Wei and Mojsov, 1996). The distribution

or $VPAC_2$ mRNA transcripts overlaps with that observed for the $VPAC_1$ receptor mRNA, although both receptors have areas of discrete expression, for example only $VPAC_1$ receptor transcripts are found in the liver, whilst $VPAC_2$ receptor transcripts are predominant in the pancreas and skeletal muscle.

1.3.5 The PAC_1 receptor

Six laboratories independently reported the cloning of PAC_1 receptor cDNA over a period of a few weeks in 1993. Receptor cDNA was isolated from rat brain cDNA libraries (Haft et al., 1994; Hashimoto et al., 1993; Hosoya et al., 1993; Morrow et al., 1993; Spengler et al., 1993) and a rat pancreatic acinar carcinoma cell line (AR4-2J) (Pisegna and Wank, 1993; Svoboda et al., 1993). The PAC_1 receptor consists of 495 amino acids; the N-terminal 19 amino acids act as a signal sequence, thus the mature receptor contains 476 amino acids (with a predicted molecular weight of 54 kDa). Significant sequence homology (51%) exists between the rat PAC_1 and $VPAC_1$ receptors, they are most divergent in their N-terminal extracellular domain, third intracellular loop and C-terminus. A few specific amino acids are conserved, including five cysteine residues in the N-terminus. There are five putative sites of N-linked glycosylation, three of which are located in the N-terminus and one each in the second and third extracellular loops (Pisegna and Wank, 1993). Cloning of the rat PAC_1 receptor revealed the existence of six cDNA splice variants. They differ in the presence or absence of an 84-base pair cassette (encoding 28 amino acids) in the C-terminal part of the third intracellular loop, there are two different cassettes named 'hip' and 'hop' (Spengler et al., 1993). In addition there are two possible variants of the hop cassette resulting from the presence of two consecutive splices sites in the rat PAC_1 hop exon. Insertion of one or both of these cassettes creates six potential isoforms of the PAC_1 receptor; PAC_1-short (without cassette), -hip, -hop1, -hop2, -hip-hop1 and -hip-hop2 (Journot et al., 1995; Spengler et al., 1993).

The human PAC_1 receptor was cloned by Pisegna and Wank (1996) from a frontal cortex cDNA library and consists of a 468 amino acid protein with 93% homology with the rat PAC_1 receptor (Pisegna and Wank, 1996). The gene organisation of the rat PAC_1 is highly conserved and homologous splice variants for the human PAC_1 receptor have been identified. The human PAC_1 splice variants have been named PAC_1-short, PAC_1-SV1, -SV2 and -SV3 (corresponding to rat PAC_1, PAC_1-hip, -hop1 and -hip-hop1). In addition their are two corresponding variants of SV2, SV2a and SV2b (corresponding to hop1 and hop2) which arise from splicing at one of two

acceptor splice sites (Pisegna and Wank, 1996). More recently a novel splice variant of both mouse and human PAC_1 receptors, missing a 21 amino acid sequence in N-terminal extracellular domain, has been isolated and named PAC_1-very short (Pantaloni et al., 1996).

Radioligand binding studies of the PAC_1-short receptor using $[^{125}I]$-PACAP-27 demonstrate that this receptor constitutes a type I PACAP binding site. Accordingly when stably transfected in COS-7, COSGs1 or CHO cells the receptor binds PACAP-38 and PACAP-27 with high (nM) affinity, but has a much lower affinity (μM) for VIP (Hashimoto et al., 1993; Hosoya et al., 1993; Pisegna and Wank, 1993). All of the human PAC_1 splice variants have a similar affinity for both PACAP-38 and PACAP-27 (Pisegna and Wank, 1996). However the PAC_1-very short receptor, which is missing 21 amino acids in the N-terminal extracellular domain has a higher affinity for PACAP-27 than the PAC_1-short form of the receptor (Pantaloni et al., 1996).

PAC_1 receptors are able to functionally couple to AC and PLC (Spengler et al., 1993). Rat PAC_1 receptor splice variants vary in their ability to activate these second messenger effectors. Examination of second messenger coupling for the PAC_1 splice variants revealed that PAC_1-short, PAC_1-hop1 and PAC_1-hop2 are all able to potently activate AC and PLC. In contrast PAC_1-hip is less effective at stimulating cAMP production and unable to activate PLC, whilst the PAC_1-hip-hop1 or -hip-hop2 splice variants display intermediate signalling properties (Journot et al., 1995; Spengler et al., 1993). Interestingly, the potencies of PACAP-38 and PACAP-27 to stimulate second messenger production via PAC_1 receptors vary. Both PACAP isoforms were equipotent in stimulating cAMP production in rat PAC_1 transfected LLC PK1 cells. In contrast, PACAP-38 was more potent than PACAP-27 at activating PLC and stimulating inositol phosphate (IP) production. In contrast, Pantaloni et al. (1996) found PACAP-38 and PACAP-27 were equally potent at stimulating IP production in cells transfected with the PAC_1-very short receptor (Pantaloni et al., 1996). The difference in efficacy of natural PAC_1 agonists in stimulating AC and PLC and the considerable diversity in signal transduction reported for PAC_1 splice variants forms the basis for complex regulation of signal transduction in these receptors. Whether the ability of PAC_1 receptors to couple to different G-proteins is determined by the different potencies of PACAP-38 and PACAP-27 for the receptor or the existence of PAC_1 splice variants remains controversial. Unlike the rat PAC_1 receptor, each human PAC_1 receptor splice

variant displayed a similar affinity for PACAP-38 and PACAP-27 and ability to activate AC, in common with the rat $VPAC_1$ receptor, they vary in their ability to activate PLC (Pisegna and Wank, 1996).

PAC_1 receptor mRNA is abundant in the brain, particularly in the olfactory bulb, thalamus, dentate gyrus, occipital cortex, hypothalamus, hippocampus and cerebellum (Hashimoto et al., 1993; Spengler et al., 1993). Low levels of PAC_1 mRNA transcripts have been detected in the adrenal gland, but are absent from other peripheral tissues. Spengler et al (1993) found that the expression level of PAC_1 splice variants is differentially regulated in tissues (Spengler et al., 1993). *In situ* hybridisation revealed that the PAC_1-short is most abundant in the brain, particularly in the pituitary, however PAC_1-hop mRNA is found in significant amounts in the olfactory bulb and in the adrenal gland. PAC_1-hip receptor transcripts have low expression levels and are generally found in the same areas as PAC_1-short mRNA (Journot et al., 1995).

1.3.6 Effector mechanisms of VIP/PACAP receptors

Early studies established the ability of VIP to stimulate AC and increase cAMP production in brain and other tissues (Quik et al., 1978; Robberecht et al., 1979). PACAP is also able to stimulate cAMP, with a 1000-fold higher potency than VIP in pituitary cells (Miyata et al., 1989). Solubilisation of a VIP receptor from rat liver membranes revealed its association with a 150 kDa protein (Couvineau et al., 1986) later found to be the stimulatory G-protein (G_s) (Couvineau et al., 1990; Kermode et al., 1992). Due to the relatively recently cloning of receptors for VIP and PACAP the involvement of specific receptors in signal transduction pathways is still being clarified. All of the VIP/PACAP receptors isolated to date have been found to couple to AC and increase cAMP production (see above) and this is generally considered as the primary signal transduction mechanism in most tissues.

Other class II GPCRs have been shown to stimulate alternative signal transduction pathways via direct G-protein coupling, examples include the calcitonin (Chabre et al., 1992; Force et al., 1992) and PTH/PTHrP receptors (Abou-Samra et al., 1992). VIP has been reported to stimulate turnover of phosphatidyl inositol in the rat adrenal medulla (Malhotra et al., 1988) and superior cervical ganglion (Audigier et al., 1986) and PACAP has a similar effect in mouse neuroblastoma cell lines (Taylor, 1996). Increased amounts of $[Ca^{2+}]_i$ in response to treatment with VIP has been reported for rat astrocytes (Fatatis et al., 1994) and in response to PACAP in

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pancreatic and neuronal cells (Canny et al., 1992; Yada et al., 1993). The involvement of PLC in the activation of IP production and increased $[Ca^{2+}]_i$ has been demonstrated *in vitro* for both PAC_1 (Spengler et al., 1993), $VPAC_1$ and $VPAC_2$ receptors (MacKenzie et al., 1996). PAC_1 receptors stimulate PLC via a pertussis toxin (PTx)-insensitive mechanism that probably involves the $G_{q/11}$ class of G-proteins (Van Rampelbergh et al., 1997). In contrast, $VPAC_1$ and $VPAC_2$ receptor stimulate PLC in a PTx-sensitive manner, suggesting the involvement of the $G_{i/o}$ class of G-proteins (MacKenzie et al., 1996; Van Rampelbergh et al., 1997). Indeed, VIP and PACAP have both been found to activate nitric oxide synthase (NOS) via a PTx-sensitive inhibitory G-protein (G_{i1-2}) in smooth muscle (Murthy and Makhlouf, 1994). Additional evidence for coupling of VIP receptors to the $G_{i/o}$ family proteins has been provided by covalent cross-linking experiments in the airway smooth muscle cells and alveolar macrophages (Shreeve et al., 1996). $VPAC_1$ and $VPAC_2$ receptors are also able to stimulate phospholipase D (PLD) (McCulloch et al., 1995).

1.3.7 Structure and function relationships of the VIP/PACAP receptors

A general role for the N-terminal extracellular domain as a site for peptide binding to VIP/PACAP receptors has been established (Cao et al., 1995; Holtmann et al., 1995; Lutz et al., 1996). The N-terminal domain and second and third extracellular loops of the VIP/PACAP receptor family contain cysteine residues many of which are highly conserved (see above). The importance of these residues for peptide binding has been investigated using point mutations or chimeric receptor constructs. Site-directed mutagenesis of individual cysteines in the N-terminal extracellular domain of the $VPAC_1$ receptor revealed that six of these residues are crucial for VIP binding. These residues are believed to form disulphide bonds which help to maintain receptor topology for ligand binding (Gaudin et al., 1995; Gaudin et al., 1996a). The importance of cysteine residues in the extracellular loops is controversial; for rhodopsin-like receptors a disulphide bond between the second and third extracellular domains is crucial for ligand binding, but mutation of these residues in the human $VPAC_1$ has been shown to have no effect on and to markedly decrease VIP binding affinity (Gaudin et al., 1995; Knudsen et al., 1997). Aspartate, tryptophan and glycine residues in the N-terminal extracellular domain of the human $VPAC_1$ receptor are also important determinants of ligand binding (Couvineau et al., 1995). Other residues, highly conserved in $VPAC_1$ and $VPAC_2$ receptors, were investigated by Nicole et al., (1998). Site-directed mutagenesis of a conserved glutamine residue in the N-terminal domain of both receptors completely prevented $[^{125}I]$-VIP binding. Surprisingly, mutation of conserved isoleucine or threonine

residues in this region decreased the binding affinity of VIP at the $VPAC_2$ receptor, but not at the $VPAC_1$ receptor, providing the first indication that these receptors may possess different determinants for VIP binding (Nicole et al., 1998). Collectively these studies indicate that VIP interacts mainly with the N-terminal extracellular domain of its receptors (Holtmann et al., 1996; Lutz et al., 1996; Vilardaga et al., 1995). Other functional domains are likely to be involved in ligand recognition, as demonstrated for members of the secretin receptor family (e.g. PAC_1 and secretin receptors), although data to support this hypothesis is limited (Couvineau et al., 1996b; Du et al., 1997).

GPCRs are often N-glycosylated. The addition of oligosaccharide chains can have a variety of functions, affecting ligand binding, signal transduction, trafficking and receptor conformation. VIP receptors are differentially glycosylated in a tissue and species-specific manner (Fabre et al., 1993). All of the VIP/PACAP receptors have three potential N-glycosylation sites in their N-terminal extracellular domain, $VPAC_1$ and PAC_1 receptors also have glycosylation sites in their second and third extracellular loops. Inhibiting N-glycosylation using tunicamycin or castanospermine in the human melanoma cell line (IGR39) prevented VIP binding by inhibiting cell surface expression of the endogenous VIP receptor (El Battari et al., 1991). Experiments using site-directed mutagenesis of the $VPAC_1$ receptor have demonstrated that either one of two asparagine residues (Asn^{58} and Asn^{69}) are necessary for correct delivery of the receptor to the plasma membrane, nevertheless, once solubilised these mutant receptors are still able to bind $[^{125}I]$-VIP (Couvineau et al., 1996a). Whether the equivalent asparagine residues in the PAC_1 and $VPAC_2$ receptors are of equal importance for membrane insertion remains to be determined.

Classically the third intracellular loop of GPCRs is considered as an important determinant for second messenger coupling. However, structural features which allow receptors to couple to more than one signal transduction mechanism have not yet been identified. The PAC_1 receptor provides an example of the importance of this region: the insertion of SV1 or SV2 cassettes into the third intracellular loop results in different second messenger coupling properties between the short form of this receptor and its splice variants. Other receptor regions important for regulation of receptor signalling will be considered later in this chapter.

Figure 1.2 Comparison of structural motifs present in class I GPCRs (A) and class II GPCRs (B). Typical receptor features are shown in bold type. Both receptor families are thought to share the same overall plasma membrane topology, consisting of seven putative TM domains and a disulphide bond linking two cysteines in the first and second extracellular loops. These receptors have few other common sequence motifs. The class II GPCRs differ in the presence of an extended N-terminal domain containing numerous cysteine residues. Reproduced from Ulrich et al. (1998).
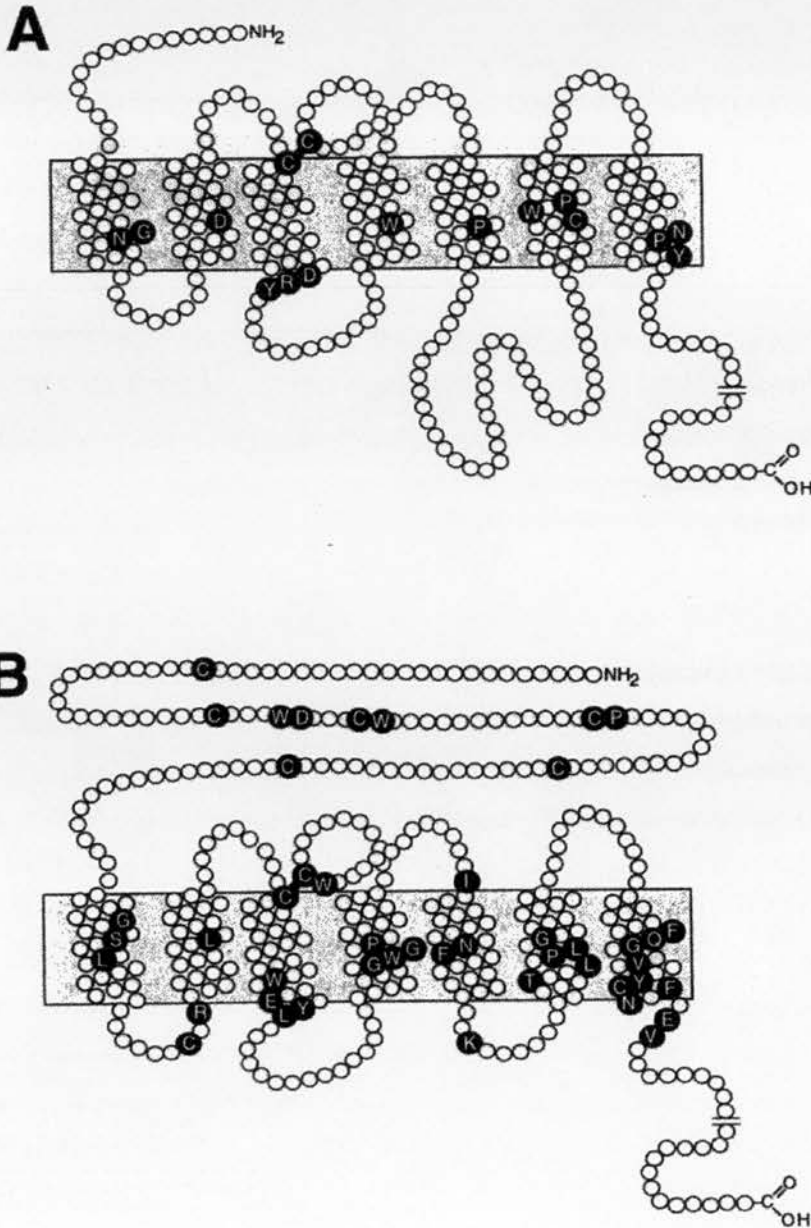
Table 1.3. Summary of nomenclature, pharmacology and tissue distribution of the VIP/PACAP receptor subtypes (see text for references).

Receptor subtype	Alternative nomenclature	Splice variants	Binding	Tissue Distribution
PAC_1	PACAP type I PVR1	PAC_1-v.short PAC_1-short PAC_1-hop1 PAC_1-hop2 PAC_1-hiphop1 PAC_1-hiphop2	PACAP-38 = PACAP-27 >> VIP > PHI	Brain (particularly olfactory bulb, thalamus, hypothalamus, hippocampus, pituitary and cerebellum) and adrenal medulla.
$VPAC_1$	PACAP type II VIP VIP_1 PVR2	none to date	VIP = PACAP-38 = PACAP-27 > PHI > helodermin > secretin	Lung, liver and intestine. Low expression in brain (particularly cortex, hippocampus and olfactory bulb), thymus, pancreas and muscle.
$VPAC_2$	PACAP type II VIP_2 PVR3 PACAPR3	none to date	VIP = PACAP-38 = PACAP-27 = helodermin > PHI >> secretin	Brain (particularly pituitary, thalamic nuclei; SCN and PVN, hippocampus and dentate gyrus), pancreas, lung, stomach, skeletal muscle, kidney, heart and placenta.

Table 1.4 Expression of endogenous VIP/PACAP receptors. Cell lines expressing a single type of VIP/PACAP receptors are shown for a number of different species. Receptor type is generally derived from pharmacological or molecular studies.

Receptor	Species	Cell line	Reference
VPAC$_1$	human	HT29 intestinal epithelial cells LoVo colonic adenocarcinoma Raji B-lymphoblastoma U-343 MG Cl 2:6 glioma	(Sreedharan et al., 1993) (Gourlet et al., 1997) (Sreedharan et al., 1993) (Nielsen et al., 1990)
VPAC$_2$	hamster mouse rat human	HIT-15 pancreatic insulinoma MIN6 pancreatic insulinoma RINm5F pancreatic insulinoma SUP-T1 lymphoblasts small cell lung carcinoma THP-1 monocytes	(Inagaki et al., 1994) (Inagaki et al., 1994) (Inagaki et al., 1994) (Svoboda et al., 1994) (Luis et al., 1990) (Gespach et al., 1989)
PAC$_1$	mouse rat human	N1E-115 neuroblastoma AR 4-2 J pancreatic acinar astrocytes PC12 adrenochromaffin αT3-1 gonadotroph-derived NB-OK neuroblastoma	(Chik et al., 1996) (Buscail et al., 1990) (Tatsuno et al., 1990) (Watanabe et al, 1990) (McArdle et al., 1997) (Cauvin et al., 1990)

Figure 1.3. Comparison of the major sites of expression of VPAC$_1$ (•), VPAC$_2$ (•) and PAC$_1$ (•) receptor mRNAs in rat brain.
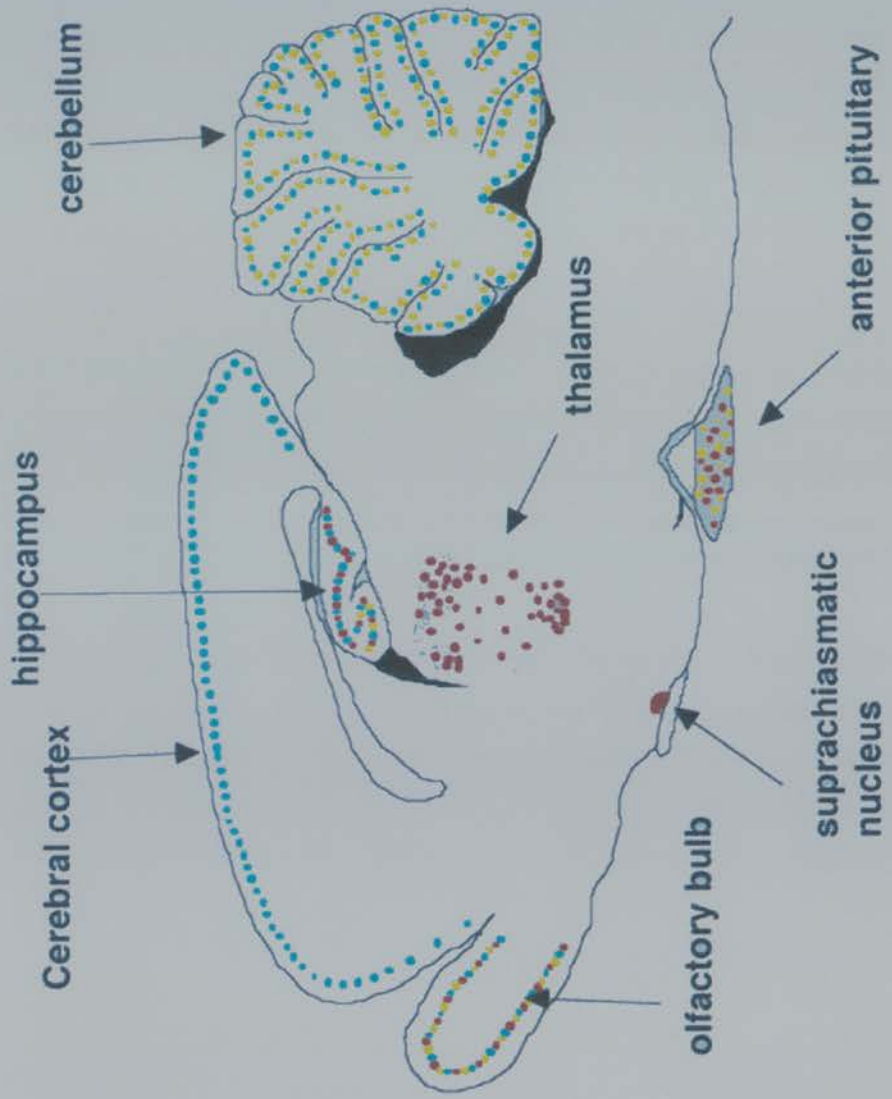
1.4 Regulation of GPCR signalling

1.4.1 Agonist-induced desensitisation

Prolonged exposure of receptors to neurotransmitters and hormones often results in a reduced responsiveness to subsequent stimuli, a phenomenon termed desensitisation. This is an important mechanism for regulating intracellular signalling and is mediated at the receptor level by several processes, including phosphorylation, internalisation and down regulation (Lohse, 1993). Phosphorylation and concomitant uncoupling of the receptor from G-protein occur rapidly following stimulation, resulting in receptor inactivation and cessation of signalling. Receptor internalisation physically removes the receptor from the cell surface thus preventing further stimulation. Long term effects on receptor availability occur via down-regulation, where total receptor number is lowered by a combination of protein degradation, transcriptional and post-transcriptional mechanisms (Hausdorff et al., 1990). Desensitisation is termed homologous when responsiveness to the original stimulus is reduced, or heterologous when the refractoriness is induced by stimuli acting through other receptors (Chuang et al., 1996a; Lefkowitz et al., 1990).

Extensive investigations of the involvement of phosphorylation and internalisation in desensitisation of class I GPCRs have been published (for review articles see Ferguson et al., 1996a; Ferguson et al., 1996c). The β_2-adrenergic receptor (β_2AR) has served as a prototype for studies of GPCR desensitisation. The comparatively recent cloning of class II GPCRs has precluded a thorough examination of the mechanisms underlying their desensitisation, nonetheless all of the secretin family receptors, studied to date, exhibit desensitisation in physiological systems (Holtmann et al., 1996). Whether data describing the molecular mechanisms of desensitisation for the rhodopsin-like receptor family can be applied to this new subfamily of GPCRs remains to be determined.

1.4.2 Agonist-induced phosphorylation of class I GPCRs

Early studies found that deletion or substitution of potential phosphorylation sites at serine and threonine residues in the C-terminal intracellular domain of the β_2AR markedly impaired its desensitisation (Bouvier et al., 1988; Hausdorff et al., 1991). It is now well established that phosphorylation has a fundamental role in β_2AR desensitisation. Two distinct types of serine/threonine kinase are involved, the second messenger-dependent kinases; protein kinase A (PKA) and protein kinase C (PKC) and β_2-adrenergic receptor kinase-1 (βARK1). βARK1 belongs to a family of

G-protein coupled receptor kinases (GRKs) which specifically phosphorylate agonist-occupied receptors. Classically, second messenger-dependent kinases have been implicated in heterologous desensitisation at low agonist concentration, whereas receptor specific kinases have been associated with homologous desensitisation at high agonist concentration (Benovic et al., 1986; Hausdorff et al., 1989). This model may be overly simplistic and evidence is now accumulating for desensitisation resulting from the concerted action of both types of kinase (Post et al., 1996).

The GRK family of protein kinases preferentially phosphorylate agonist-occupied GPCRs. Six mammalian cDNAs encoding GRKs have been identified to date, they are GRK1 (or rhodopsin kinase), GRK2 and GRK3 (or βARK kinases 1 and 2), GRK4, GRK5, GRK6 (Pitcher et al., 1998) **(Figure 1.4)**. GRK2, GRK3, GRK5 and GRK6 are widely distributed throughout body tissues, whereas GRK1 is almost exclusively located in rod and cone photoreceptor cells in the eye and GRK4 is confined to the testes and brain (Palczewski, 1997). All GRK proteins have a similar overall organisation and share 51-92% sequence similarity (Inglese et al., 1993). They are single subunit enzymes comprised of a central catalytic domain flanked by N- and C- terminal domains. The N-terminal region may be involved in receptor binding (Palczewski et al., 1993) whilst the C-terminal domain is thought to contain elements for membrane and receptor targeting (Premont et al., 1995). Several comprehensive review articles of GRK structure and function have been published (Palczewski, 1997; Pitcher et al., 1998; Premont et al., 1995). A general model of GRK action has been derived **(Figure 1.5)**. First, the GPCR binds to its ligand causing dissociation of G-protein subunits (G_α from $G_{\beta\gamma}$) and stimulation of second messenger production. The receptor kinase is then translocated to the membrane where it binds and phosphorylates serine and threonine residues in receptor C-terminal intracellular domains. For GRK2 and GRK3 the presence of free G-protein $\beta\gamma$-subunits plays a role in targeting to the membrane and may determine kinase selectivity (Inglese et al., 1993). Several lines of evidence suggest that GRKs interact with receptor regions which are distinct from their phosphorylation sites (summarised by Pitcher et al., 1998). GRK action can be regulated by a number factors including lipids, second messenger-dependent kinases and calcium-binding proteins (Pitcher et al., 1998).

It seems likely that many receptors are substrates for GRKs. The relatively large population of GPCRs, compared with the few identified GRKs, implies that these

enzymes may act promiscuously and are able to recognise a number of different substrates. Techniques using purified enzymes and reconstituted receptors have been used to investigate GRK phosphorylation *in vitro*. Using this method GRK2 (or βARK1) has been shown to phosphorylate receptors other than β_2AR; including the α_2-adrenergic (Benovic et al., 1987), β_1-adrenergic (Freedman et al., 1995), M_2-muscarinic (Kwatra et al., 1989), endothelin A and B (Freedman et al., 1997) and adenosine A_2 receptors (Mundell et al., 1997; Mundell et al., 1998b). To date few receptors, other than those belonging to the rhodopsin-like superfamily of GPCRs, have been directly tested as substrates for GRKs. The action of GRKs may have different consequences depending upon receptor type, for example GRK2 phosphorylation whilst having no effect on internalisation of the β_2AR, enhances internalisation of M_2-muscarinic receptors (Tsuga et al., 1994).

1.4.3 Role of agonist-induced phosphorylation in class I GPCR regulation

The question arises as to whether GPCR phosphorylation correlates with desensitisation. For the β_2AR, GRK2 phosphorylation specifically uncouples the receptor from the G-protein (Lohse et al., 1992). Nevertheless, for substantial β_2AR desensitisation to occur the presence of an accessory protein called β-arrestin is necessary (Palczewski et al., 1993). β-arrestin serves a dual regulatory role, firstly binding to GRK phosphorylated receptors and mediating rapid desensitisation and secondly targeting receptors for internalisation into endosomal vesicles (Ferguson et al., 1996b; Goodman et al., 1996; Lohse et al., 1990a). It is now well established that GPCR desensitisation, at least following phosphorylation by GRK1, GRK2 or GRK3 involves one of a family of arrestins (Ferguson et al., 1996b). Arrestins are cytosolic proteins whose expression is either restricted to the retina (visual and cone arrestins) or ubiquitous (βarrestin1 and βarrestin2). All of the arrestins share significant homology and normally have at least two mRNA splice variants (Freedman and Lefkowitz, 1996). The general structure of arrestins is shown in **Figure 1.6**. Other GPCRs shown to act as substrates for arrestin proteins include the rhodopsin and M_2-muscarinic receptors (Ferguson et al., 1996b). The last decade of research has seen many changes and developments in this field, the discovery of GRKs and arrestins is only the first step towards a definitive mechanism for β_2AR receptor desensitisation.

1.4.4 Agonist-induced phosphorylation of class II GPCRs

Several members of the secretin receptor family have been shown to undergo agonist-induced phosphorylation, including calcitonin (Nygaard et al., 1997), GLP-1

(Widmann et al., 1996), PTH/PTHrP (Blind et al., 1995), secretin (Ozcelebi et al., 1995) and VPAC$_2$ receptors (McDonald et al., 1998). In common with the β_2AR, the serine and threonine-rich intracellular C-terminal domains of these receptors have been identified as the main site of phosphorylation. The involvement of specific kinases in secretin receptor family phosphorylation has been investigated for a number of these receptors. For the PTH/PTHrP receptor Blind et al (1995) reported that, whilst PKA and PKC were actively involved in maintaining basal levels of receptor phosphorylation, their activity could not account for the observed level of agonist-stimulated phosphorylation. These results led them to postulate that GRKs may be involved in agonist-induced phosphorylation of PTH/PTHrP receptors (Blind et al., 1995). Work by the same group has since provided direct evidence for GRK2 phosphorylation of the PTH/PTHrP receptor *in vitro* (Blind et al., 1996). Non-second messenger dependent kinases are also believed to be involved in agonist-induced phosphorylation of the receptor for calcitonin (Nygaard et al., 1997). Current work by our group has shown that the VPAC$_2$ receptor, stably transfected in HEK293 cells, is constitutively phosphorylated and agonist treatment markedly enhances this phosphorylation (McDonald et al., 1998). Stimulation of PKA activity was unable to produce the same level of phosphorylation as that caused by VIP treatment, indicating that GRKs may also be involved in agonist-stimulated phosphorylation of the VPAC$_2$ receptor (McDonald et al., 1998).

1.4.5 Role of agonist-induced phosphorylation in class I GPCR regulation

The involvement of phosphorylation in desensitisation is not as well defined for the class II GPCRs as it is for the class I GPCRs. Conflicting results have been obtained for members of the secretin receptor family, and it is difficult to compare different studies because of the number of different methods employed to investigate this phenomenon. One example is the PTH/PTHrP receptor, this receptor activates both AC and PLC. Desensitisation of the cAMP response of this receptor was prevented in the presence of a PKC inhibitor, indicating a role for phosphorylation in desensitisation of the PTH/PTHrP receptor (Pernalete et al., 1990). In contrast, removal of PKC phosphorylation sites had no effect on the desensitisation of Ca^{2+} signalling (Malecz et al., 1998). This dichotomy could be due to the different signalling pathways being measured, indeed, truncation of the C-terminal tail of the PTH/PTHrP receptor enhances stimulation of AC but does not effect PLC (Iida-Klein et al., 1995). Overall these studies suggest that receptor phosphorylation may have variable effects on desensitisation for different signalling pathways of the same receptor. As mentioned above, evidence is accumulating for a role for GRKs in

PTH/PTHrP receptor phosphorylation, accordingly a recent study found that desensitisation of this receptor is influenced by coexpression with GRK2 (Fukayama et al., 1997).

The secretin receptor has also been the focus for studies of agonist-induced phosphorylation. A study by Holtmann et al (1996) revealed that removal of phosphorylation sites in the C-terminus of the secretin receptor caused a reduction in receptor desensitisation (Holtmann et al., 1996). Nonetheless, Shetzline et al (1998) showed that PKA and PKC inhibitors had no effect on desensitisation of cAMP stimulation following prolonged treatment with secretin. However, desensitisation of secretin receptor signalling was augmented by coexpression with GRKs in HEK293 cells (Shetzline et al., 1998). In contrast, coexpression of the secretin receptor with a dominant negative mutant of GRK2 in NG108-15 cells, had no effect on agonist-induced desensitisation (Mundell et al., 1997). Finally, agonist-induced phosphorylation of the GLP-1 receptor has been shown to correlate with desensitisation (Widmann et al., 1996). To date there are few published studies investigating agonist-induced phosphorylation of VIP receptors. Overall evidence is accumulating for a relationship between agonist-induced phosphorylation and desensitisation of secretin family receptors, discordant results outlined in this chapter may stem from variability in receptor systems, techniques or individual cell lines.

Figure 1.4 Structure of G-protein coupled receptor kinases (GRKs). Schematic diagram of GRK structure based on GRK1 (rhodopsin kinase) (**A**). The central catalytic domain contains a region of conserved residues found in all protein kinases. The N-terminus is the proposed site of substrate recognition, whereas the C-terminus is involved in targeting GRKs to the membrane. Both GRK2 and GRK3 have an extended C-termini which interacts with prenylated G-protein βγ-subunits and is likely to serve as a means of directing the GRKs towards activated GPCRs in the plasma membrane. Summary table of the GRK subfamilies and their tissue distribution (**B**). Reproduced from Bohm et al. (1997).

(A)
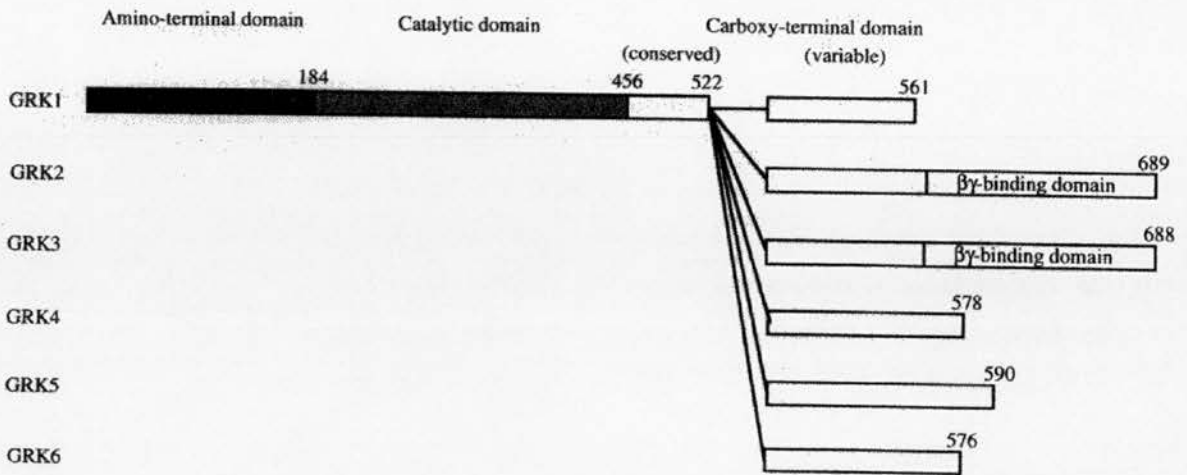
B)

Family name	Common name	Tissue distribution
GRK1	Rhodopsin kinase	retina, pineal
GRK2	βARK1	leukocytes, spleen, heart, lung, kidney
GRK3	βARK2	brain, spleen, heart, lung, kidney
GRK4	IT-11	brain, testis
GRK5	-	brain, skeletal muscle, pancreas, lung, kidney, placenta, liver, heart
GRK6	-	brain, skeletal muscle, pancreas, lung, kidney, placenta, liver

Figure 1.5 Stages in the desensitisation of G-protein coupled receptors in response to agonist stimulation. After agonist binding to the G-protein coupled receptor (GPCR) it is activated (GPCR*) and stimulates dissociation of Gα-subunits from Gβγ-subunits, which results in the activation of second messenger effectors. GPCR* is a substrate for phosphorylation by GRKs: after phosphorylation arrestin (Arr) binds to the receptor preventing the receptor from coupling to G-protein, resulting in desensitisation. The binding of arrestin also acts as a signal for receptor internalisation or sequestration. The internalised receptor is dephosphorylated and recycled back to the plasma membrane intact. Reproduced from Palczewski, 1997.
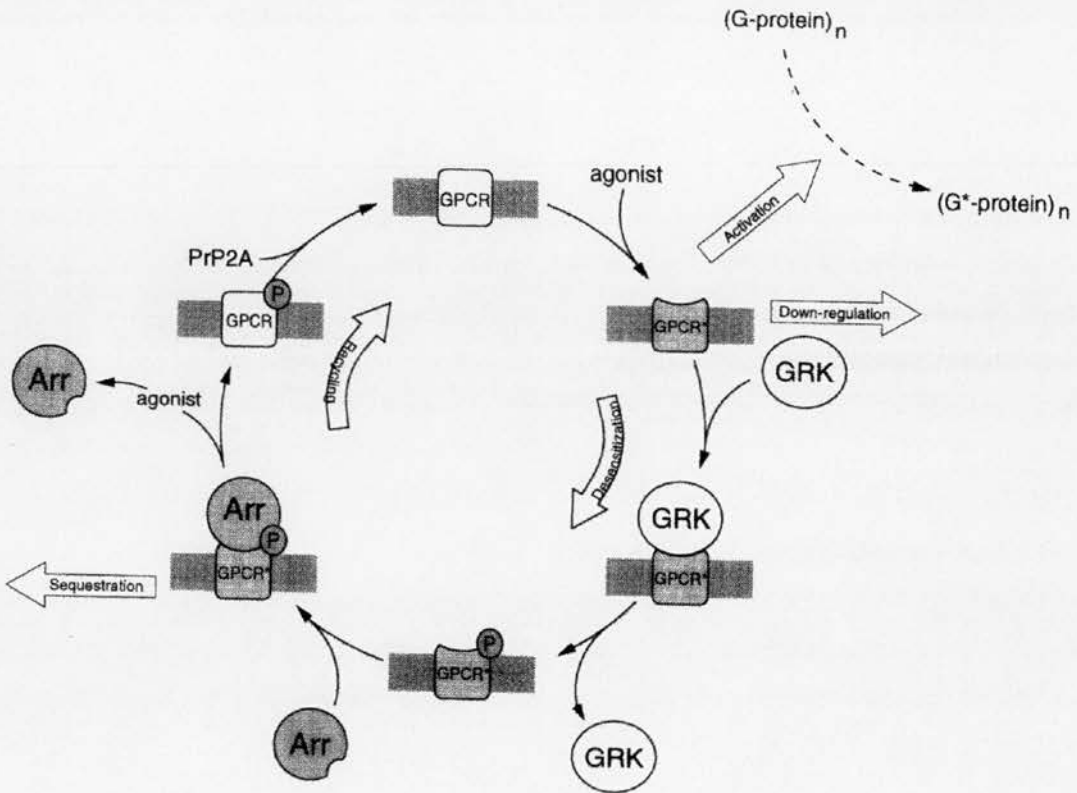
Figure 1.6 General structure of arrestin proteins. Schematic diagram of the general structure of arrestins, functional domains are indicated by shading (**A**). The N-terminal (R1 domain) consists of 25-29 amino acids that interact with the C-terminus (R2 domain). A is the putative region which recognises the activated receptor. P, is a positively charged region of ~20 amino acids which serves as a major site for phosphorylation. S, forms a secondary binding site, of 120-150 residues involved in receptor interaction in response to occupancy of other phosphorylation an activation-recognition binding sites. R2, the C-terminal 60-85 residues interact with R1. Reproduced from Bohm et al. (1997). Summary of arrestin protein families, their substrates and tissue distribution is also shown (**B**). Abbreviations; Rho (rhodopsin receptor), B$_2$AR (β_2-adrenergic receptor), M$_2$-musc (M$_2$-muscarinic receptor).

(**A**)

(**B**)

Family name	Receptor substrate	Tissue distribution
Visual arrestin	Rho>B$_2$AR>M$_2$-musc	retina, pineal gland
Cone arrestin	?	retina, pineal gland
β-arrestin1	B$_2$AR>M$_2$-musc>Rho	wide tissue distribution (particularly spleen, heart, liver and lung)
β-arrestin2(or arrestin3)	B$_2$AR=M$_2$-musc>>Rho	wide tissue distribution (particularly spleen, heart, liver and lung)

1.5 Agonist-induced internalisation of GPCRs

1.5.1 Receptor-mediated endocytosis

Endocytosis is an important mechanism in mammalian cells for the uptake of a variety of substances for cell maintenance, growth and regulation, including low density lipoprotein (LDL), transferrin, lysosomal enzymes, viruses, toxins and receptors for neurotransmitters, hormones and growth factors (Goldstein et al., 1979). Two general mechanisms for uptake have been determined, one involving non-coated membrane invaginations (Huet et al., 1980; Montesano et al., 1982) and the other coated pits (Goldstein et al., 1979). GPCR endocytosis has long been associated with clathrin-coated pits, although a few receptors internalise via cholesterol-rich regions of the membrane called calveolae (Anderson et al., 1992; Schmid, 1997).

A number of different techniques have been used to determine whether clathrin-coated pits are involved in endocytosis of GPCRs. Chemical treatments which disrupt clathrin formation, including hypertonic sucrose, mild acid, phenylarsine oxide and potassium depletion have been used to infer a clathrin-mediated mechanism of internalisation (Grady et al., 1995a; Moore et al., 1995). Immunofluorescence studies have been used to determine whether GPCRs colocalise with constitutively recycling receptors or with other molecular components of clathrin-coated pit internalisation (von Zastrow and Kobilka, 1992). Lastly, the expression of mutant or dominant negative proteins involved in different aspects of receptor-mediated endocytosis has been used to clarify the molecular mechanisms underlying this process. These techniques have been applied to the GPCR family to determine (albeit often indirectly) their mechanism of internalisation. The β_2AR has been shown to utilise both non-coated and clathrin coated vesicles (CCVs), in A431 cells and HEK293 cells respectively (Raposo et al., 1989; von Zastrow and Kobilka, 1992). M_1-muscarinic receptors have been shown to internalise in CCVs in HEK293 cells (Tolbert and Lameh, 1996) and in non-coated vesicles in CCL137 fibroblast cells (Raposo et al., 1987). Other class I GPCRs which appear to internalise via clathrin-coated pits include the human chorionic gonadotrophin hormone/luteinising hormone receptor (Ghinea et al., 1992), neurokinin-1 and -2 receptors (Garland et al., 1996) and the GRP receptor (Grady et al., 1995a). Overall, it appears that internalisation of the majority of GPCRs involves clathrin-coated pits and vesicles.

1.5.2 Mechanism of endocytosis

Clathrin was first purified from pig brain vesicles in 1975. It is the major constituent of coated pits and vesicles (Pearse, 1975). Clathrin coated pits have been extensively studied and comprehensive papers describing their structure and function are available (Pearse and Crowther, 1987; Pearse and Robinson, 1990; Schmid, 1997). Clathrin is a triskelion molecule composed of three heavy chains and three associated light chains, which spontaneously assembles to form a polyhedral lattice surrounding the coated pit (Pearse and Crowther, 1987). This structure provides the scaffolding for the mechanics of endocytosis **(Figure 1.7)**. The membrane localisation and receptor sorting components of endocytosis are mediated by clathrin-associated adaptor or assembly proteins (APs) (Pearse and Bretscher, 1981). There are four major AP complexes; AP-1 and AP-2 (which can be separated by hydroxyl-apatite chromatography and have also been named HA-1 and HA-2 adaptors) AP-3 (Pearse and Robinson, 1984; Simpson et al., 1996) and AP-4 (Dell'Angelica et al., 1999). AP-1, AP-3 and AP-4 participate in protein transport from the *trans*-golgi network (TGN) to the endosomal/lysosomal system, whilst AP-2 is involved in trafficking from the plasma membrane (Dell'Angelica et al., 1998). AP complexes are heterotetramers of differing molecular weights and function; each comprises two ~100 kDa subunits, one ~50 kDa subunit (μ) and one ~20 kDa subunit (σ) (Brodsky, 1997) **(Figure 1.8)**. AP-2 adaptors occur in aggregates on the plasma membrane; treatments which perturb clathrin-mediated endocytosis do not appear to affect this distribution, and formation and pinching off of CCVs is reestablished once the inhibiting treatment is removed (Hansen et al., 1993). This has led to the suggestion that adaptors may act as focal points on the membrane for clathrin association, although no direct evidence for this hypothesis has been obtained. Nevertheless, APs have been shown to interact with the terminal domain of the clathrin triskelion and are able to drive clathrin coat formation. Importantly APs also interact directly with endocytic sorting signals on transmembrane receptors and presumably help in directing cargo molecules into coated pits (Kirchhausen et al., 1997; Le Borgne and Hoflack, 1998).

The GTPase dynamin is another well established component of clathrin-mediated endocytosis. Dynamin has an important role in constriction and 'pinching off' of newly formed endocytic vesicles from the plasma membrane (for review articles see Damke, 1996; Schmid et al., 1998). GDP-bound dynamin is randomly distributed throughout the clathrin coat. GTP binding or GTP/GDP exchange prompts dynamin to form a helical collar at the neck of the pit which is required for vesicle budding.

The necessity of dynamin for CCV formation has been demonstrated from *in vitro* studies using dominant negative dynamin mutants (Damke et al., 1994). In addition a number of proteins involved in interactions between dynamin and clathrin have been identified, including amphiphysin and endophilin, and it is likely that more will be discovered before a model of the processes involved in clathrin-mediated endocytosis is complete (Schmid et al., 1998). A summary of the current proposed mechanism of internalisation via clathrin-coated pits is presented in **Figure 1.9**.

1.5.3 Receptor internalisation motifs

For endocytosis of a GPCR to occur the protein must be recognised by the endocytic machinery. This requires internalisation signals which are commonly found in the cytoplasmic domains of internalised proteins. These signal motifs tend to be short stretches of 4-6 amino acids, which share limited homology but possess common structural features and chemistry (Trowbridge, 1991). Analysis of the crystallographic structure of these sequences has shown that the majority of internalisation signals can form a surface exposed tight turn in the protein (Trowbridge et al., 1993). Examination of single TM receptors has revealed two important types of motif, one containing a critical tyrosine residue (tyrosine-based) and another including two leucine residues (dileucine-based).

The most common endocytic signals are tyrosine-based, their general motifs are either $NPX_{2-3}Y$ (where N is asparagine, P is proline, X is any amino acid and Y is tyrosine) or YXXØ (where Y is tyrosine, X is any amino acid and Ø is an amino acid with a bulky hydrophobic side chain: leucine, isoleucine, phenylalanine, methionine or valine). The $NPX_{2-3}Y$ motif in the cytoplasmic domain of LDL receptors (Chen et al., 1990) and tyrosine kinase receptors (Trowbridge, 1991) has been shown to act as an endocytic signal. There is a highly conserved $NPX_{2-3}Y$ in TM7 of the class I GPCR family, which was initially thought to be necessary for efficient internalisation of the β_2AR (Barak et al., 1994) and subsequently found to have numerous effects on this receptors properties (Barak et al., 1995b). For other class I GPCRs mutation of the essential tyrosine in this motif does not effect internalisation, examples include receptors for GRP (Slice et al., 1994) and angiotensin II (Thomas et al., 1995a). This motif is absent in class II GPCRs. The YXXØ motif has been shown to act as an internalization signal in several constitutively recycling receptors, including the transferrin receptor (TfnR), ASGP, mannose 6-phosphate and EGF receptors (for a review see Ohno et al., 1998). To date this motif has not been identified as an internalisation signal for any 7TM GPCRs. Dileucine motifs, which

were originally believed to be lysosomal sorting signals, have also been found to be involved in receptor-mediated endocytosis (Letourner and Klausner, 1992). Cytoplasmic dileucine motifs are necessary for internalisation of the T cell receptor (Dietrich et al., 1994) and human insulin receptor (Haft et al., 1994). A recent report found that mutation of a dileucine sequence in the C-terminus of the β_2AR impaired receptor internalisation indicating a role for this motif in GPCR endocytosis (Gabilondo et al., 1997).

There is now considerable evidence that tyrosine- and dileucine-based endocytic signals bind directly to the medium (μ) subunit of AP complexes resulting in concentration of specific membrane receptors in clathrin-coated pits (Marks et al., 1997; Ohno et al., 1998). Overexpression of chimeric proteins containing functional endocytic signal motifs can prevent receptor endocytosis indicating that this process is saturable, however tyrosine and dileucine-based motifs do not interfere with each other suggesting that they may bind to different sites on the APs (Marks et al., 1996). The amino acid composition of endocytic motifs is highly degenerate and can be influenced by the presence of acidic amino acid clusters which may modulate the AP binding (Kirchhausen et al., 1997). Post-translational receptor modifications, such as phosphorylation, palmitoylation or ubiquitination may also determine the accessibility of endocytic signals; these mechanisms are particularly relevant for receptors which undergo agonist-induced receptor internalisation (Le Borgne and Hoflack, 1998).

To date no endocytic motifs for the class II GPCRs have been identified, although the C-terminal intracellular domain has been shown to have a role in agonist-induced internalisation. C-terminal truncation mutants of the PTH/PTHrP receptor, for example, had both positive and negative effects on PTH/PTHrP receptor internalisation (Huang et al., 1995a). Of particular interest are three residues (EVQ), adjacent to TM7, which are highly conserved in the secretin receptor family. Alanine mutagenesis of these amino acids caused a 40% increase in receptor internalisation, indicating that these residues may constitute a negative signal for endocytosis (Huang et al., 1995a). Calcitonin receptor mutants truncated by 44 or 83 amino acids display impaired internalisation, however corresponding effects on signal transduction were observed which could account for the observed reduction in internalisation (Findlay et al., 1994). Unlike the PTH/PTHrP receptor, disruption of the EVQ motif in the calcitonin receptor did not enhance internalisation (Findlay et al., 1994). In contrast to all these findings, truncation of the C-terminal intracellular

domain of the secretin receptor had no effect on receptor-mediated endocytosis of secretin (Holtmann et al., 1996).

1.5.4 Endocytic pathways and receptor recycling

General models of receptor-mediated internalisation and trafficking have been derived from studies of single TM domain receptors, such as the nutrient receptors and tyrosine kinase receptors. Although receptors which internalise at clathrin-coated pits appear to be involved in similar processes they do show some differences. Firstly, receptors can differ in their membrane location: some receptors cluster in coated pits (e.g. LDL receptors), whereas others are distributed throughout the membrane and only migrate to coated pits following ligand binding (e.g. EGF and $\alpha_2$macroglobulin receptors) (Goldstein et al., 1979; Haigler et al., 1978; Willingham et al., 1984). Secondly, receptors and ligand are internalised into common endosomes from which they can be routed in several different ways, these are divided into four major types:

Type I, the receptor-ligand complex dissociates and receptors recycle to the plasma membrane whilst ligand is degraded.

Type II, both receptor and ligand recycle to the plasma membrane.

Type III, both receptor and ligand are degraded.

Type IV, receptor-ligand complexes are delivered to the opposite side of polarised cells where the ligand is then released (Shepherd, 1989).

One of the most extensively studied receptors which follows a type I endocytic pathway is the LDL receptor. LDL receptors are responsible for the transport of plasma cholesterol into cells (Anderson et al., 1982; Goldstein et al., 1979). LDL binds to receptors associated with clathrin-coated pits, the pits invaginate forming clathrin-coated vesicles (CCVs) which mature into endosomes through which LDL is transported to lysosomes and degraded, liberating cholesterol, whilst its receptor is recycled (Anderson et al., 1982). The transferrin receptor (TfnR) follows a type II endocytic route. Transferrin acts as a carrier for iron uptake in mammalian cells and is endocytosed via clathrin-coated pits. Once inside the cell the iron atoms are released and the receptor-transferrin complex is recycled back to the surface (Harding et al., 1983; Willingham et al., 1984). Type III and IV endocytic pathways are followed by epidermal growth factor (EGF) receptors (Carpenter and Cohen, 1976) and polymeric immunoglobulin A respectively (Mostov and Blobel, 1982).

The pathway taken by internalised materials involves a series of organelles which are responsible for their sorting and processing, including coated vesicles, sorting endosomes, recycling endosomes and lysosomes (Pastan and Willingham, 1983). The pH in these compartments varies and since receptors bind to their ligands with high affinity at neutral pH and weakly at mildly acidic pH, the acidification of endosomes promotes dissociation of ligand from receptor. The molecular basis for this change in binding affinity with pH is not clear, however acidity may induce a change in receptor conformation (DiPaola and Maxfield, 1984). The separation of ligand from receptor provides a means by which differential processing of endocytosed material can occur (Mellman et al., 1986). In addition, endosomal acidification induces a change in receptor conformation, which permits interaction with GPCR phosphatases (GRPs) and dephosphorylation (Krueger et al., 1997). Once dephosphorylated the receptor recycles back to the plasma membrane and is capable of responding to agonist **(Figure 1.5)**.

Sorting endosomes are essential for the efficient recycling of membrane components. Sorting endosomes consist of spherical vesicles with narrow diameter tubules on their surface. When the tubules bud off from the endosome they consist mainly of membrane with a relatively small lumenal content. These tubules then amalgamate to form a recycling endosome. After several rounds of budding from the sorting endosome the recycling endosome is rich in membrane proteins but contains few free ligands. This process has been termed iterative fractionation (Dunn et al., 1989). Receptors in the recycling endosome traffic back to the plasma membrane along with bulk membrane components and do not appear to be selectively recycled. This was demonstrated by Mayor et al (1993) who found that the recycling kinetics and location of a marker for bulk membrane and fluorescently labelled transferrin are identical, thus the route taken by recycling receptors is essentially a 'default' sorting pathway (Mayor et al., 1993).

The mechanism of delivery of lysosomally targeted ligands is controversial. Two models have been proposed: (1) the vesicle shuttle model and (2) the endosome maturation model (Helenius et al., 1983) **(Figure 1.10)**. In the vesicle shuttle model ligands are internalised into endosomes which fuse with pre-existing, stable sorting endosomes, from which receptors are channelled to recycling endosomes, as described above, whilst lysosomally targeted ligands are packaged into vesicles and transported to late endosomes (Griffiths and Gruenberg, 1991). In the endosome maturation model the compartment ligands targeted for degradation are retained and

accumulate within sorting endosomes which eventually lose their ability to fuse with endocytic vesicles and thus mature into late endosomes (Murphy, 1991). Fluorescence microscopy has demonstrated that an internalised fluid phase marker is not transferred from sorting to late endosomes in small units, favouring the endosome maturation model (Dunn and Maxfield, 1992). However, evidence for either model is limited and the actual mechanism of delivery of ligands for degradation requires further clarification.

1.5.5 Agonist-induced internalisation of class I GPCRs

Much is known about internalisation of single TM domain receptors, however the mechanism and function of seven TM domain receptors is not as well defined, although similarities in endocytic mechanisms and pathways have been identified. The β_2AR is one of the most extensively studied class I GPCRs. The first evidence for agonist-induced internalisation of this receptor was inferred from ligand binding studies (Kurz and Perkins, 1992). Direct visualisation of β_2AR internalisation, into small punctate accumulations within the cytoplasm, was subsequently demonstrated using immunofluorescence of epitope-tagged β_2ARs (von Zastrow and Kobilka, 1992). The time course for receptor internalisation determined by confocal microscopy was comparable with receptor sequestration as assessed in ligand binding studies. Internalisation was found to be dependent upon both agonist concentration, incubation time and temperature, and was reversible (von Zastrow and Kobilka, 1994; von Zastrow and Kobilka, 1992). Experiments using flow cytometry found that internalisation of β_2ARs is regulated by agonist efficacy: in the presence of agonist, receptors are in a dynamic steady state, cycling between endosomes and the cell surface (Morrison et al., 1996). In addition internalised β_2AR receptors were found to colocalise with TfnRs in HEK293 cells (von Zastrow and Kobilka, 1992). Recently a β_2AR-green fluorescent protein (GFP) fusion protein has been generated which permits internalisation and trafficking of the receptor to be observed in live cells (Barak et al., 1997; Kallal et al., 1998). β_2AR-GFP transfected HeLa cells were stimulated with agonist for different periods of time; in acutely treated cells (<30 mins) the internalised β_2AR colocalised with rhodamine-labelled transferrin, a marker for sorting and recycling endosomes. In contrast, following chronic treatment (>3 hrs) with agonist a large proportion of receptors colocalised with rhodamine-labelled dextran, a marker for late endosomes and lysosomes (Kallal et al., 1998). This latter study suggested that β_2AR internalisation may also contribute to receptor down-regulation, which has been confirmed by work from the same group (Gagnon et al., 1998).

Many other class I GPCRs have been found to undergo agonist-induced internalisation, including bombesin (Zachary and Rozengurt, 1987), thyrotropin-releasing hormone (Nussenzveig et al., 1993), neurokinin-1 (Garland et al., 1994), histamine H_2 (Smit et al., 1995), thrombin (Hoxie et al., 1993), M_2-muscarinic (Koenig and Edwardson, 1994), α_{1B}-adrenergic (Fonseca et al., 1995), μ-opioid (Arden et al., 1995) and dopamine D_1 receptors (Trogadis et al., 1995). The majority of these receptors follow an endocytic pathway similar to the β_2AR, involving phosphorylation, uncoupling of the receptor from G-protein (see section 1.4), followed by internalisation and recycling. Notable exceptions to this are the thrombin and proteinase-activated receptor-2 which are activated by thrombin and trypsin cleavage, respectively (Böhm et al., 1996; Hein et al., 1994). Cleavage exposes a tethered ligand which activates the receptor, the receptors are internalised and degraded in lysosomes. Thus recovery of receptors at the cell surface requires either synthesis of new receptors or mobilisation of existing receptors from intracellular stores. A comparison of these two types of endocytic pathways is shown in **Figure 1.11**.

1.5.6 Role of agonist-induced internalisation of class I GPCRs

The functional role of GPCR internalisation varies depending upon the receptor system studied. Initially, internalisation of the β_2AR was believed to contribute to receptor desensitisation (Cheung et al., 1989), however several lines of evidence have since shown that desensitisation of the β_2AR is independent of receptor internalisation. Blocking internalisation with pharmacological agents does not effect desensitisation (Hertel et al., 1985) and desensitisation is rapid and does not temporally match internalisation (Waldo et al., 1983). Mutant β_2ARs that do not desensitise are still able to internalise (Bouvier et al., 1988; Hausdorff et al., 1989; Lohse et al., 1990a) and *vice versa* (Barak et al., 1994). Nevertheless, current reports suggest that molecular intermediates required for receptor desensitisation are capable of initiating internalisation (Ferguson et al., 1998). Ménard et al (1997) found that preventing phosphorylation of the β_2AR and interaction with β-arrestin significantly reduced agonist-induced internalisation. Interestingly, this group found that different cultured cell lines varied in their expression levels of both these intermediates and internalisation correlated strongly with the amount of GRK2 and β-arrestin present (Ménard et al., 1996). Indeed dephosphorylation of a single serine residue in β-arrestin was found to be necessary for binding to clathrin and mediating β_2AR endocytosis but had no effect on agonist binding or desensitisation (Lin et al., 1997).

The currently favoured hypothesis is that $\beta_2 AR$ internalisation is essential for receptor dephosphorylation and subsequent resensitisation (Barak et al., 1994; Pippig et al., 1995; Yu et al., 1993; Zhang et al., 1997). In accordance with this finding rapid receptor internalisation of a number class I GPCRs has been found to be independent of desensitisation but may be involved in receptor resensitisation (Garland et al., 1996; Ng et al., 1995; Szekeres et al., 1998). In contrast, for irreversibly activated GPCRs, like proteinase-activated receptor-2 and thrombin receptor, internalisation is not involved in their resensitisation (Böhm et al., 1996; Hoxie et al., 1993).

1.5.7 Agonist-induced internalisation of class II GPCRs

Studies of internalisation of the secretin receptor family are still in their infancy due to the lack of experimental tools for studying peptides and their receptors. Diffuse binding determinants of peptides make it difficult to generate appropriately tagged ligands and a paucity of receptor specific antibodies has precluded direct labelling. Nonetheless, indirect evidence for internalisation of the members of the secretin receptor family has accumulated from studies using iodinated peptides. The development of a technique to separate externally and internally bound peptide using low pH has proved a valuable tool for investigating internalisation (Haigler et al., 1980). Using this method, iodinated peptides have been used to demonstrate internalisation of calcitonin (Ikegame et al., 1994), GLP-1 (Widmann et al., 1995), PTH/PTHrP (Teitelbaum et al., 1986), secretin (Izzo et al., 1989) and VIP receptors (Muller et al., 1985; Svoboda et al., 1988). However, these results should be interpreted with caution as insulation of labelled ligand from acidic washes does not necessarily provide a direct measure of receptor internalisation. During a study of cholecystokinin (CCK) receptor internalisation Roettger et al. (1995) found that, even though the radiolabelled ligand was no longer removed by acidic washes, the receptor remained in the plasma membrane (Roettger et al., 1995a). An alternative approach by Holtmann et al. (1996) used a fluorescently-labelled version of secretin to demonstrate peptide internalisation (Holtmann et al., 1996).

The majority of evidence for internalisation of VIP has come from studies of iodinated VIP binding to high affinity receptor sites in HT-29 cells, which express an endogenous $VPAC_1$ receptor. These studies all demonstrate a rapid, temperature-dependent movement of iodinated VIP from the cell surface to an intracellular compartment. $[^{125}I]$-VIP internalises rapidly at 37°C ($t^{1/2}=2$ mins), in a concentration and temperature dependent manner, before being degraded in

lysosomes and released into the surrounding medium (Luis et al., 1986; Muller et al., 1985). Simultaneous with VIP internalisation, the number of detectable cell surface binding sites was reduced, indicating a receptor-mediated mechanism for internalisation (Marvaldi et al., 1986). After internalisation the VIP-bound receptor was shown by quantitative autoradiography to be located in clear endosomal vesicles (Rosselin et al., 1988). Internalisation of VIP binding sites was reversible, as removal of VIP from the incubation medium resulted in a ~90% recovery of VIP binding sites at the cell surface ($t^{1/2}$=15 mins) (Luis et al., 1986). Recovery of functional VIP binding sites at the plasma membrane, in the presence a protein synthesis inhibitor, suggested that the $VPAC_1$ receptor was recycling (Boissard et al., 1986; Rosselin et al., 1988). This work led Luis et al (1988) to propose a dynamic model for VIP internalisation, where the ligand is degraded and the receptor recycled back to the cell surface **(Figure 1.12)**. Consistent with this model Turner et al. (1988) found that agonist pretreatment resulted in a shift of solubilised $VPAC_1$ receptors into a light vesicle fraction (as opposed to plasma membrane fraction) using a method involving sucrose density step gradients to provide a more direct measure of receptor movement (Turner et al., 1988). Evidence for $VPAC_2$ and PAC_1 receptor internalisation has been derived from indirect studies demonstrating the disappearance of [^{125}I]-peptide binding sites in SUP-T1 lymphoblast (Robberecht et al., 1989a) and AR 2-J rat pancreatic acinar cell lines (Svoboda et al., 1988) respectively.

The lack of specific VIP/PACAP receptor antibodies has prevented a thorough examination of receptor internalisation, nonetheless the results outlined above strongly support a mechanism for receptor-mediated endocytosis of VIP. The first direct evidence for internalisation and down regulation of the $VPAC_1$ receptor has been reported. Gaudin et al. 1996 have shown, using fluorescence microscopy, that an epitope-tagged version of the $VPAC_1$ receptor stably transfected into CHO cells was diffusely distributed over the plasma membrane in control cells, whereas 24 hr exposure to 10 nM VIP resulted in a shift in receptor location to an intracellular site (Gaudin et al., 1996b). To date there are no published studies which directly demonstrate agonist-induced internalisation of the PAC_1 or $VPAC_2$ receptors.

1.5.8 Role of agonist-induced internalisation of class II GPCRs

Determining the mechanism and function of internalisation of class II GPCRs may help to determine how second messenger signalling by these receptors is regulated. Holtmann et al (1996) demonstrated internalisation of wild type and C-terminal

truncated secretin receptors and provided evidence for internalisation as the major mechanism underlying desensitisation of this receptor (Holtmann et al., 1996). In support of this a recent report revealed that inhibition of internalisation, with concanavalin A, blocked receptor desensitisation of the endogenous secretin receptor expressed in NG108-15 cells (Mundell and Kelly, 1998a). However these findings are contentious and other studies indicate that the time course of desensitisation is too rapid for internalisation to play a significant role (Shetzline et al., 1998). Shetzline et al (1998) propose that secretin receptors, in common with adrenergic receptors, require phosphorylation to uncouple the receptor from G-protein and act as a trigger for internalisation and resensitisation. For the $VPAC_1$ receptor, internalisation of VIP in HT-29 cells occurs on a parallel time course with a reduction of cAMP stimulation (Boissard et al., 1986). However, a more specific model of VIP/PACAP receptor internalisation is required to confirm this relationship.

PROJECT AIM

The aim of this study was to provide direct evidence for agonist-induced internalisation of the human $VPAC_2$ receptor using an epitope-tagged $VPAC_2$ receptor for immunofluorescence studies. The role of the C-terminal intracellular domain of the receptor in internalisation was investigated. In addition the molecular mechanisms and endocytic pathways involved in internalisation were examined using agents which inhibit specific pathways of internalisation and by colocalisation of the $VPAC_2$ receptor with intracellular markers.

Figure 1.7 Schematic drawing of the structure of the clathrin triskelion. Each triskelion has three heavy chains and three light chains (**A**). The triskelions form a polyhedral lattice structure with the terminal domains projecting inwards forming another layer within the the lattice (**B**). Reproduced from Pearse and Robinson (1990).

(A)
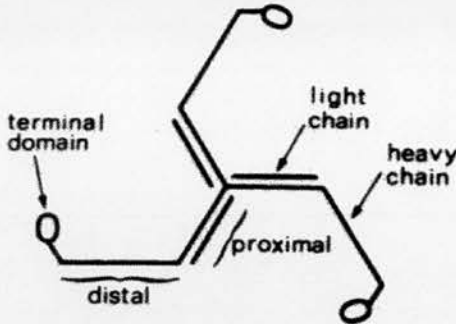
(B)
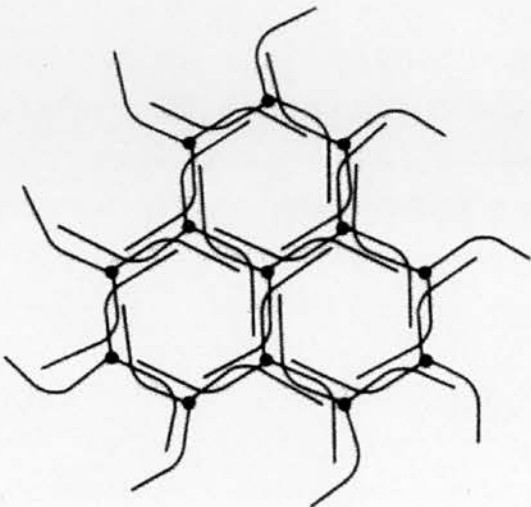
Figure 1.8 Schematic diagram of the structure of the adaptor proteins (APs). AP-1 (or HA-1) AP-2 (or HA-2), AP-3 and AP-4. Adaptor proteins (APs) have an unusual structure, consisting of two large 100 kDa units each flanked by a small appendage, a ~50 kDa unit and a small ~20 kDa unit. The β2-adaptin subunit of AP-2 contains a clathrin binding site. AP-2 (HA-2) and AP-1 (HA-1) are restricted to the coated pits in the plasma membrane and golgi region, respectively. Reproduced from Dell'Angelica et al. (1999).
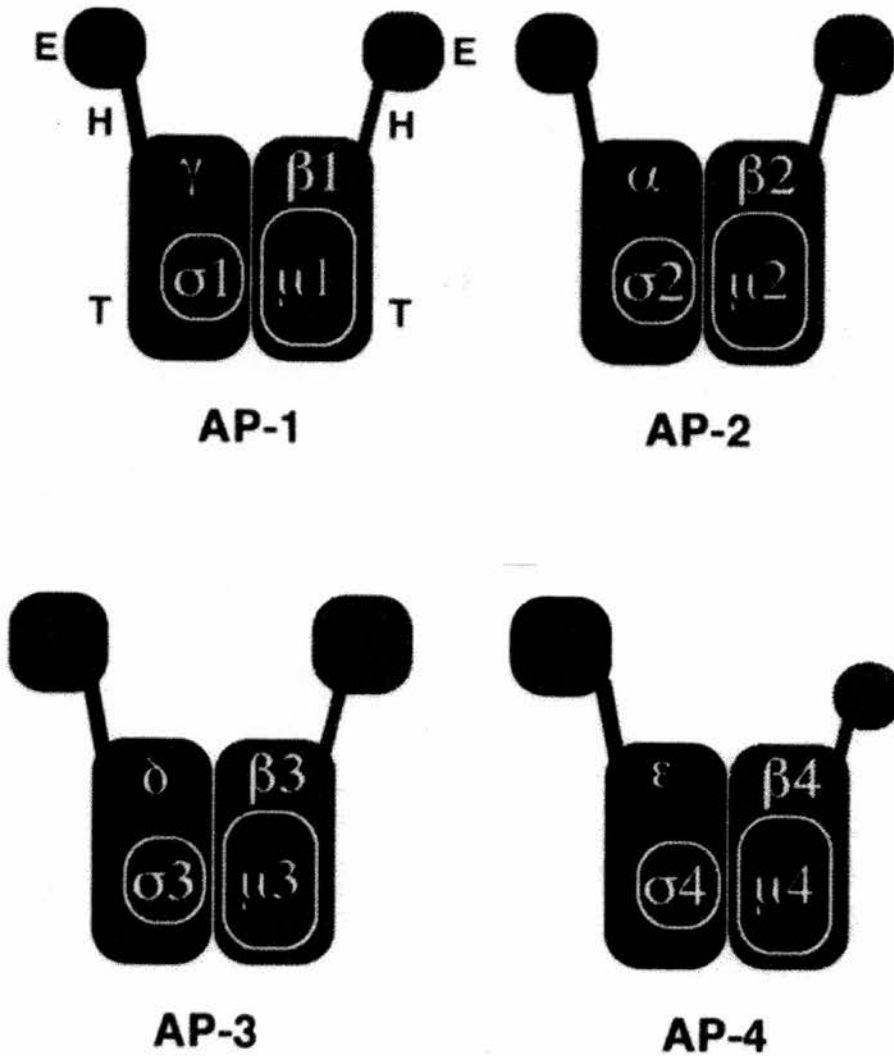
Figure 1.9 Simplified model of clathrin-mediated endocytosis. Clathrin, adaptor protein (AP-2) and amphiphysin assemble on the membrane, forming clathrin-coated pits. Agonist bound receptors accumulate at these sites. The coated pit invaginates, GTP binding or GTP/GDP exchange triggers assembly of a helical collar of dynamin at the neck of the pit. Dynamin undergoes a conformational change inducing 'pinching off' of the vesicle from the membrane. The clathrin coated vesicle (CCV) components are removed upon entry into the cell and are recycled for further rounds of endocytosis. Interestingly, dynamin is exclusive to the plasma membrane, however isoforms and splice variants of dynamin exist which may be involved in membrane- or tissue-specific vesicle budding. Other proteins such as amphiphysin (neuronal cells) and endophilins (non-neuronal cells) have been implicated in interactions between dynamin and the clathrin coat. These proteins contain proline-rich sequences, SH3 and pleckstrein homology domains which are known sites for protein-protein interactions. Reproduced from Schmid et al. (1998).
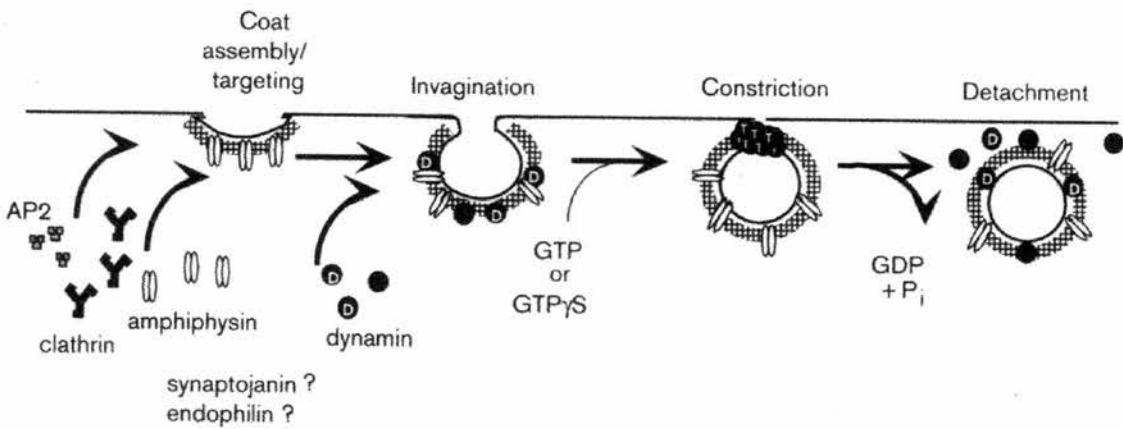
Figure 1.10 Schematic diagrams summarising the two proposed models for delivery of endocytosed material to late endosomes. Receptor and ligands are internalised into endocytic vesicles. These vesicles are directed towards a sorting endosome. The structure of the sorting endosome allows for efficient recycling of membrane components back to the plasma membrane, whilst ligands accumulate in the endosome lumen. In the vesicle shuttle model (**A**); ligands accumulate in a preexisting stable sorting endosome and are then transported by small carrier vesicles to late endosomes. In the endosome maturation model (**B**); the sorting endosome eventually loses its capacity to bud or fuse with endocytic vesicles and it matures into a late endosome. The late endosome can then be directed towards lysosomes where its components are degraded. Reproduced from Dunn and Maxfield (1992).

(A)

(B)

time
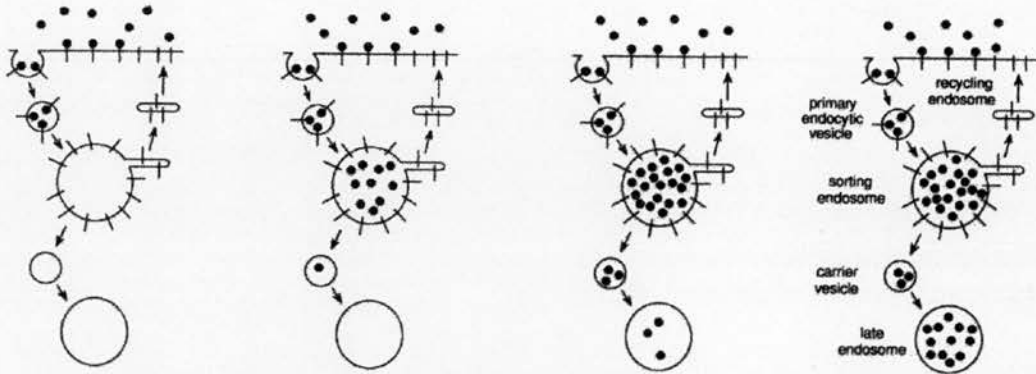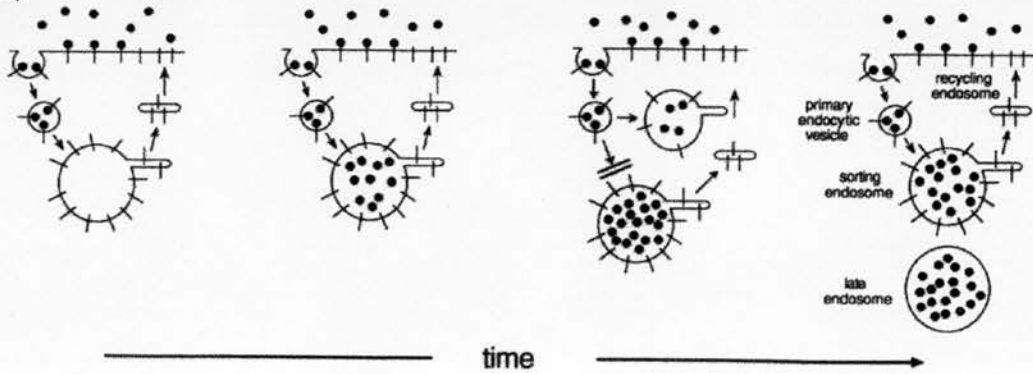
Figure 1.11 Pathways of endocytosis and intracellular sorting of GPCRs.
A typical pathway for endocytosis as followed by the B_2AR (**A**). The receptor binds
ligand, is phosphorylated by GRKs and uncoupled from G-proteins. Internalisation
occurs through clathrin-coated pits, ligand and receptor dissociate in an acidified sorting
endosome; the ligand is degraded whereas the receptor is recycled back to the cell surface.
A typical pathway for endocytosis of the proteinase-activated receptor-2 or thrombin
receptor (**B**). The receptor is activated when it is cleaved at its N-terminus exposing a
tethered ligand. The receptor is phosphorylated and uncoupled from G-proteins resulting
in desensitisaiton. Both receptor and ligand are degraded. Resensitisation requires
synthesis of new receptor or mobilisation of existing intracellular stores. Reproduced
from Bohm et al. (1997).
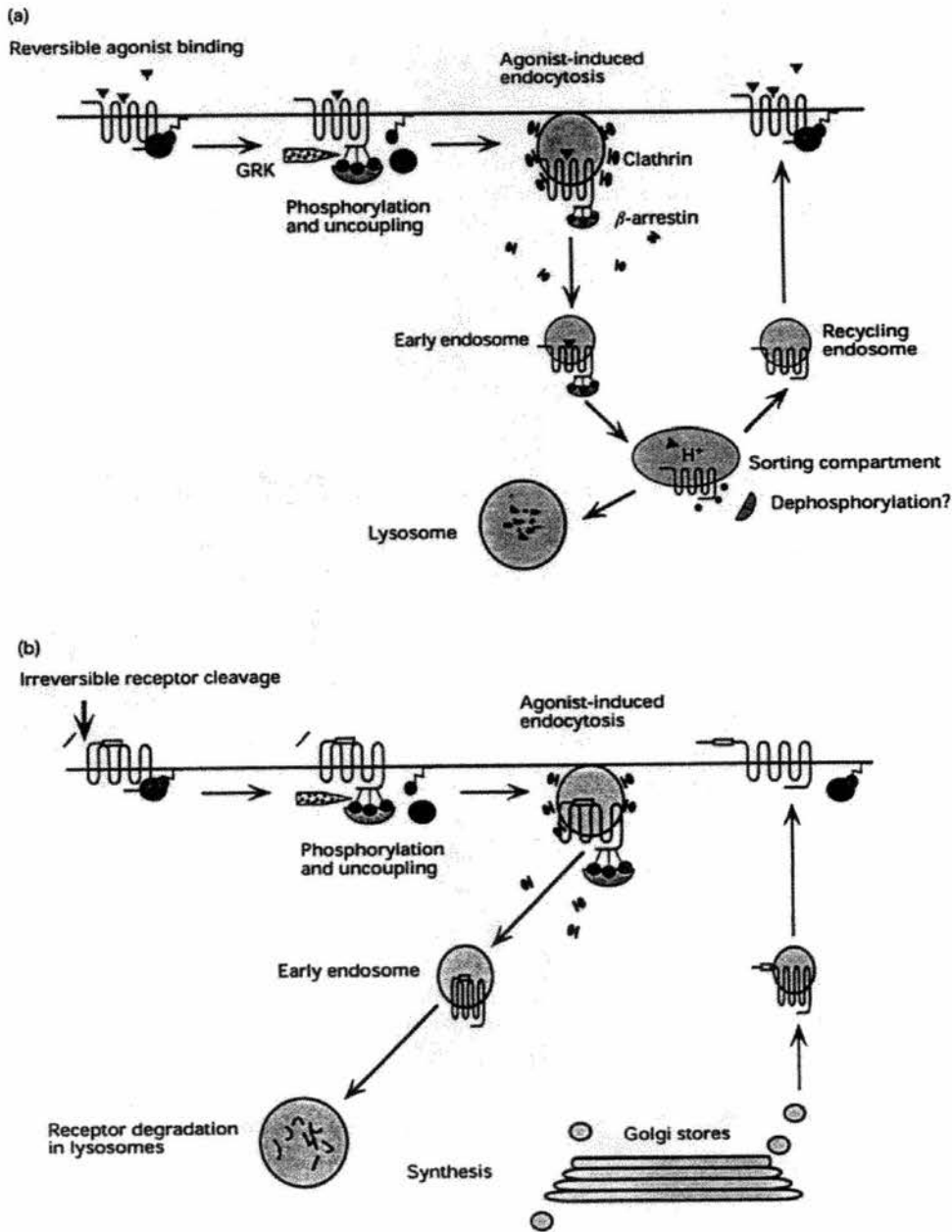
Figure 1.12 Model of agonist internalisation of VIP and its receptor. As proposed by Luis et al. (1988). The receptor is predicted to internalise rapidly with a $t^1/_2 \sim 2$ mins. Iodinated VIP can then be found in a clear vesicle membranes, probably endosomes. The ligand is degraded by lysosomes, whilst the receptor is recycled back to the membrane with a $t^1/_2 \sim 15$ mins, where it is again available for agonist stimulation. Reproduced from Luis et al. (1998).
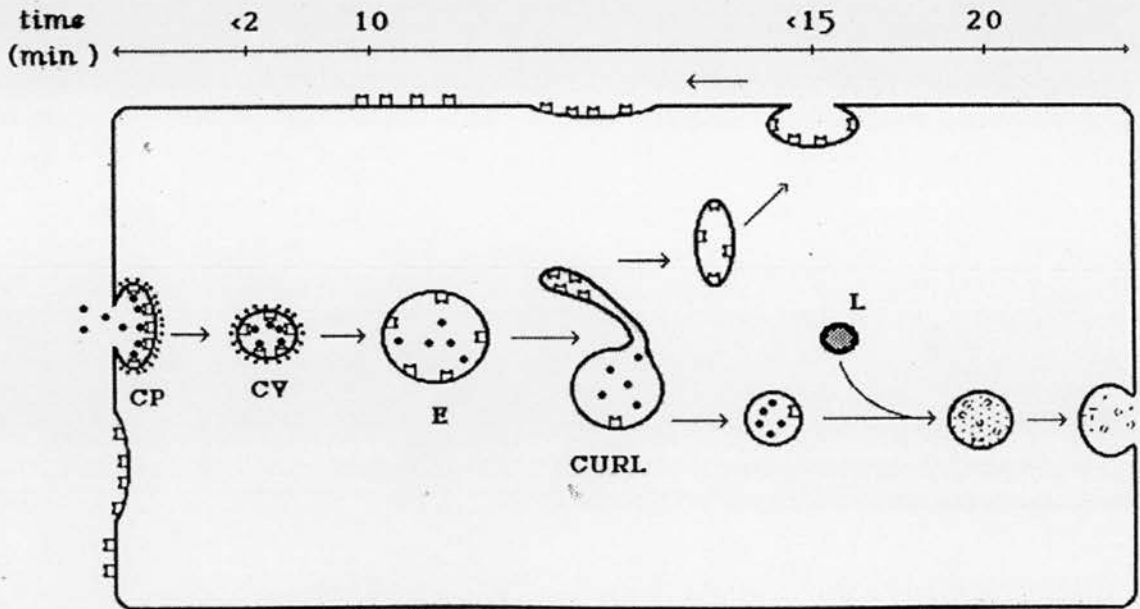
Dynamics of the VIP–receptor complex. Recent view of the fate of the VIP after ligand binding. **CP**: coated pit; **CY**: coated vesicle; **E**: endosome; **L**: lysosome. VIP; degraded VIP; VIP receptor; degraded receptor; clathrin-coated membrane.

CHAPTER 2

Materials and Methods

2.1 Generation of human VPAC$_2$ receptor cDNA constructs

2.1.1 Construction of epitope-tagged human VPAC$_2$ receptor cDNA

The human VPAC$_2$ receptor cDNA cloned into the pcDNA3 vector (Invitrogen, San Diego, CA) was used as the template for generating a haemagglutinin (HA) epitope-tagged VPAC$_2$ receptor (VPAC$_2$-HA). The terminal codon of the VPAC$_2$ receptor was altered from ATC to CTC by polymerase chain reaction (PCR), to introduce a *Xho*I site into the cDNA construct. Double restriction enzyme digest with *Xba*I (cutting in the multiple cloning site) and *Xho*I removed a 6.99-kb fragment containing the VPAC$_2$ receptor cDNA. The following oligonucleotide primers were synthesised, annealed together and ligated with the digested VPAC$_2$ receptor cDNA:

5'- TCGAGTACCCATACGATGTTCCAGATTACGCCTCCCTCTAGT-3'

5'- CTAGACTAGAGGGAGGCGTAATCTGGAACATCGTATGGGTAC-3'.

The resultant plasmid contained human VPAC$_2$ receptor cDNA with the last C-terminal amino acid changed from Ileu to Leu, followed directly by Glu, the HA epitope (YPYDVPDYASL) and a translational stop signal (TAC). DNA sequence was confirmed using an automated 373 DNA sequencer (PE Applied Biosystems, Warrington, UK).

2.1.2 Construction of C-terminal truncated and epitope-tagged VPAC$_2$ receptor cDNAs

C-terminally truncated, epitope-tagged VPAC$_2$ receptors were generated using the VPAC$_2$-HA receptor construct as a template. Truncations were generated after the following amino acids; Ser^{425}, Ser^{409}, Arg^{393}, Arg^{391} and Glu^{385}. Fragments of the VPAC$_2$-HA receptor cDNA were amplified by PCR using an oligonucleotide primer upstream of a unique *Hpa*I site (5' - CCATCTCAGTGCTGGTCAAGGACG - 3') in combination with one of the following downstream oligonucleotide primers (all of these primers contain a *Xho*I site underlined):

5' - CTG<u>CTCGAG</u>GGAGCCGCGGTGG - 3'

5' - GCG<u>CTCGAG</u>GGAGGAACCGCAGACCC -3'

5' - GT<u>CTCGAG</u>CCGGCTTCGCCATTTTCGC - 3'

5' - GGCA<u>CTCGAG</u>TCGCCATTTTCGCTTCAGC - 3'

5' - CG<u>CTCGAG</u>CTCGCACTGCACCTC - 3'.

The amplified products were separated by agarose gel electrophoresis and purified using the Qiaex II gel extraction procedure (Qiagen Ltd., Crawley, Sussex, UK) according to the manufacturers instructions. The VPAC$_2$-HA receptor construct was digested with *Hpa*I and *Xho*I and the purified PCR fragments were ligated in place,

thus, producing five C-terminally truncated and epitope tagged VPAC$_2$ receptor constructs. Sequences of the PCR products were verified using an automated 373 DNA sequencer (PE Applied Biosystems, Warrington, UK), no unintended base changes were observed. The truncated and tagged (TT) versions of the VPAC$_2$ receptors are referred to in the text as TT425, TT409, TT393, TT391 and TT385. All of the human VPAC$_2$ receptor constructs were generated by Dr T.P. McDonald.

2.2 Cell culture and transfection

2.2.1 Materials
All plasticware for cell culture was obtained from Corning Costar, High Wycombe, Bucks, UK. Cell culture media, freezing media and antibiotics were supplied by Gibco BRL, Life Technologies Ltd., Paisley, UK, with the exception of geneticin (or G418) which was purchased from Calbiochem-Novabiochem Ltd, Nottingham, UK. and foetal calf serum was obtained from Harlan Sera-lab Ltd., Crawley Down, Sussex, UK.

2.2.2 Maintenance of cultured cells
Cultured human embryonic (HEK 293) cells were maintained in Dulbecco's modified eagle media (DMEM) supplemented with 10% heat inactivated foetal calf serum (HIFCS) and 100 U/ml each of penicillin and streptomycin. Cells were maintained in 25 or 75 cm^2 flasks in a humidified atmosphere of 95% air/5% CO$_2$ with fresh medium added every 3-4 days. After 6-10 days in culture the cells were passaged using trypsin-ethylenediaminetetraacetic acid (EDTA) dispersal.

2.2.3 Long-term storage of cultured cells
Confluent cells in a 75 cm^2 flask were trypsinised with 2 mls trypsin-EDTA, resuspended in 6 mls DMEM (supplemented with 10% HIFCS and 100 U/ml each of penicillin and streptomycin) and decanted into a sterile 10 ml tube. The cell suspension was spun down at 210 g for 5 mins at 4°C in a Megafuge 2.0R (Heraeus Instruments, Ltd., Brentwood, Essex, UK). The pellet was resuspended in 2 mls freezing medium (Gibco BRL, Life Technologies Ltd., Paisley, UK), composed of dimethylsuphoxide in DMEM supplemented with foetal bovine and calf sera, 500 μl aliquots were placed at -70°C for at least 2 hrs before storage in a liquid nitrogen (N$_2$) container. To revive frozen cell stocks the cryovials were removed from liquid N$_2$, placed into warm water to thaw, 0.5 mls DMEM was added and the cell suspension was aseptically transferred into a flask containing culture media.

2.2.4 Transfection

An electroporation method was used to transfect cultured cells. HEK293 cells grown in 25 cm^2 flasks were dispersed with 2 mls trypsin-EDTA and resuspended in 6 mls DMEM (supplemented with 10% HIFCS and 100 U/ml each of penicillin and streptomycin). A sample of the cells was removed using a sterile glass pasteur pipette and cell numbers were counted on a haemocytometer. The cells were centrifuged at 210 g for 5 mins at 4°C in a Megafuge 2.0R (Heraeus Instruments, Ltd., Brentwood, Essex, UK), the supernatant was discarded and pellet resuspended in culture media to give a concentration of 5×10^6 cell/ml. 500 µl of this suspension was placed into a cuvette with 4 mm gap (Kramel Biotech, Cramlington, Northumberland, UK). 2-10 µg VPAC$_2$ receptor cDNA was added and the cuvette tapped gently to mix. Each cuvette was electroporated using a Gene pulser with capacitance extender (Bio-Rad, Laboratories, Hercules, CA) at 250 V with a capacitance of 960 µFD. These conditions allow cDNA to enter the cell. Efficient electroporation often results in marked cell death, hence electroporated cells with no added cDNA were used as controls for comparison. The contents of each cuvette was seeded into a 10 ml tissue culture dish containing culture medium. The cells were incubated at 37°C in a 95% air/5% CO_2 incubator for approximately 3 days. HEK293 cells transfected with cDNA were selected for with culture medium containing 400 µg/ml geneticin. The cells were grown to confluency and seeded into fresh dishes at a low density. Individual cells were isolated using sterile porcelain cloning rings, removed and grown further in selection media. Several clonal cell lines of each VPAC$_2$ receptor construct were obtained in this manner. Stably transfected HEK293 cells were maintained in DMEM supplemented with 10% HIFCS, 100 U/ml penicillin and streptomycin and 200 µg/ml geneticin.

2.3 cAMP stimulation assays

2.3.1 Materials

All plasticware was obtained from Corning Costar, High Wycombe, Bucks, UK. Assay media was supplied by Gibco BRL, Life Technologies. Phosphate buffered saline (PBS) was purchased in tablet form from Oxoid Ltd, Basingstoke, Gloucestershire, UK; the tablets were dissolved in distilled water (dH$_2$0) and autoclaved for 20 mins to sterilise. Rat tail collagen was obtained from Universal Biologicals, Stroud, Gloucestershire, UK. VIP was purchased from Calbiochem-Novabiochem Ltd, Nottingham, UK and Ro 25-1553 was a generous

gift from Dr P. Robberecht, Université libre de Bruxelles. Sodium iodide (Na^{125}I), with a specific activity of ~100 mCi/ml, was purchased from Amersham Biotech, Little Chalfont, Bucks, UK. All other reagents were obtained from Sigma, Poole, Dorset, UK unless indicated otherwise.

2.3.2 Assay for peptide-induced cAMP stimulation

Prior to the experiment, 24-well dishes were incubated with a 1% aqueous solution of rat tail collagen for approximately 2 hrs at room temperature, to provide a protein matrix for the cells to adhere to. The collagen was removed by aspiration and the wells washed twice with 500 µl sterile PBS. Trypsinised HEK293 cells were resuspended in culture medium (DMEM supplemented with 10% HIFCS, 100 U/ml each of penicillin and streptomycin and 200 µg/ml geneticin) and cell numbers were counted on a haemocytometer. The cells were seeded onto the coated dishes at 2×10^5 cells/well and incubated for a minimum of 48 hrs in at 37°C in an incubator gassed with 95% air/5% CO_2.

Cell monolayers were washed twice with 500 µl minimum essential medium (MEM) containing 0.25% bovine serum albumin (BSA) and incubated for 15 mins at 37°C with 200 µl MEM containing 0.25% BSA and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) an inhibitor of phosphodiesterase. 25 µl of peptide (either VIP or Ro 25-1553) was added to each well, at a range of concentrations (0.01-100 nM) in triplicate, using a Rainin repeating pipette (Rainin Instrument Co. Inc., Woburn, MA). The cells were incubated with peptide for 20 mins at 37°C in a 95% air/5% CO_2 incubator. The assay reaction was terminated by the addition of 200 µl 0.2 N HCl to each well and the dishes were stored at -70°C. Total cAMP levels were determined by radioimmunoassay of samples taken from each well (see section 2.3.4).

2.3.3 Assay for desensitisation of peptide-induced cAMP stimulation

HEK293 cells were prepared as described for the peptide dose response assay (see section 2.3.2). Prior to desensitisation, each well was washed twice with 500 µl MEM containing 0.25% BSA and incubated for 30 mins at 37°C with 200 µl MEM containing 0.25% BSA in the absence or presence of 1, 3 or 10 nM VIP. Cells were then washed gently three times with 500 µl MEM containing 0.25% BSA and incubated for a further 15 mins with MEM containing 0.25% BSA and 0.1 mM IBMX. The assay was performed as described (see section 2.3.2); with 25 µl VIP at a range of concentrations (0.01-100 nM) added to each well, in triplicate, and

incubated for a further 30 mins at 37°C in a 95% air/5% CO_2 incubator. The assay reaction was terminated by the addition of 200 µl 0.2 N HCl to each well and the dishes were stored at -70°C. Total cAMP levels were determined by radioimmunoassay of samples taken from each well (see section 2.3.4).

2.3.4 cAMP radioimmunoassay

Iodination of cAMP and radioimmunoassay (RIA) were carried out in our laboratories by Mr J. Bennie and Mrs S. Carroll. cAMP was iodinated by the chloramine-T method, adapted from the method of Greenwood et al. (1963). The following reaction mix was used;

> 0.15 µg 2'-O-monsuccinyladenosine 3',5'-cyclic monophosphate tyrosyl methyl ester (cAMP-TME)
>
> 40 µl 0.5 M sodium phosphate buffer, pH 7.5
>
> 5 µl sodium iodide (Na^{125}I) (3.7 GBq/ml; 100 mCi/ml)
>
> 5 µl chloramine-T (2 mg/2 ml H$_2$O).

The above ingredients were mixed gently for 1 min at room temperature and the reaction was stopped by the addition of 1 ml H$_2$O/0.1% trifluoroacetic acid (TFA). [^{125}I]-cAMP-TME was separated from its uniodinated form by reverse-phase liquid chromatography using a 1 ml 'Bond Elut' column (Anachem Ltd., Luton, Beds, UK). Radiolabelled cAMP was eluted with 40-50% methanol/0.1% TFA and stored in methanol at -40°C for a maximum of 8 weeks. Specific activities of 3 mCi/µg were routinely obtained.

All solutions for RIA were made up in 50 mM sodium acetate buffer (pH 6.0) containing 0.25% BSA. Rabbit RIB7 antiserum was used at a 1:166 000 titre. 100 µl rabbit antiserum and 50 µl [^{125}I]-cAMP (~10 000 cpm) (see above) were added to 3 ml polypropylene tubes with either standards (in triplicate) or assay samples (in duplicate) to give a total volume of 200 µl. Tubes were mixed gently and incubated overnight at 4°C. 200 µl donkey anti-rabbit IgG and non-immune rabbit serum (Scottish Antibody Production Unit, Carluke, Lanarkshire, UK) were added at 1:400 and 1:40 dilution, respectively. The tubes were mixed carefully and incubated on ice for 3 hrs. 600 µl 8.7% polyethylene glycol (PEG) in ice cold 0.01 M sodium phosphate buffered saline (pH 7.5) was added to each tube to assist the precipitation of [^{125}I]-cAMP/antibody complex and the tubes were spun at >1000 g for 1 hr at 4°C. The supernatant was aspirated from each tube and the precipitate counted for 90 secs on a Wallac 1470 gamma counter. The assay included a cAMP standard curve

from 1-512 nM. The cAMP concentration of unknown samples was determined by interpolation of the percentage bound counts and the log of the cAMP concentration, see below;

$$\% \text{ bound counts} = \frac{\text{unknown} - \text{nonspecific}}{\text{total} - \text{nonspecific}} \times 100$$

2.3.5 cAMP data analysis

Data was analysed using Pfit (Elsiever Biosoft, Cambridge, UK). A non-linear regression equation was applied to the data from cAMP assays. Half-maximal excitation values (EC_{50}), maximum values and curve slopes were estimated by this programme. All data were expressed as either mean ± standard deviation (SD) for individual experiments (in triplicate) or standardised to a percentage of the maximum and expressed as mean ± standard error of the mean (SEM) for a number of experiments. Non-parametric statistical analysis was applied to the data to determine significance. The GB-stat software (Dynamic Microsystems, Inc, Silverspring, MD) was used to perform a Wilcoxon rank test with Mann Whitney two independent variable assessment.

2.4 Radioligand binding assays

2.4.1 Materials

Assay media were supplied by Gibco BRL, Life Technologies. Helodermin for iodination was purchased from Calbiochem-Novabiochem Ltd, Nottingham, UK, whilst unlabelled helodermin for radioligand binding was obtained from Peninsula Laboratories, Inc, Belmont, CA. $Na^{125}I$ with a specific activity of 100 mCi/ml was supplied by Amersham Biotech, Little Chalfont, Bucks, UK. [^{125}I]-helodermin was synthesised in our laboratory (as described in section 2.4.2). 4-(2-aminoethyl)-bensenesulfonyl fluoride (AEBSF) was supplied by Alexis Corporation Ltd. Nottingham, UK. All other reagents were purchased from Sigma, Poole, Dorset, UK, unless otherwise stated.

2.4.2 Iodination of helodermin

Binding assays for peptide receptors often use iodinated peptide agonists as radioligands, mainly due to the lack of available receptor antagonists. Peptide iodination is performed by chemical substitution of hydrogen at tyrosyl residues. Iodination of helodermin was performed in our laboratory by Mr J. Bennie and Mrs

S. Carroll using a modified version of the chloramine-T method of Greenwood et al. (1963). The reaction mix was as follows;

> 5 μl helodermin (2.5 μg) in 0.1 M acetic acid
>
> 15 μl 0.5 M sodium phosphate buffer, pH 7.5.
>
> 5 μl Na^{125}I (3.7 GBq/ml; 100 mCi/ml).
>
> 10 μl chloramine-T (4 mg/2ml)

The reaction mix was incubated for 20 secs at room temperature, then 10 μl sodium metabisulphite (5 mg/ml) and 800μl potassium iodide (10 mg/ml) were added to terminate the reaction. Helodermin contains two residues where [^{125}I] can be substituted for hydrogen, thus the product of this iodination is an heterogeneous mixture of mono-iodinated and di-iodinated peptides, as well as unlabelled peptide. These molecules can be separated by chromatographic methods. The [^{125}I]-helodermin and free [^{125}I] were separated by high performance liquid chromatography (HPLC) according to the method of Harmar and Rosie (1984). [^{125}I]-helodermin eluted at 70% methanol/0.1%TFA. The specific activity was determined by counting the peak elute fraction on a desensitised gamma counter, where 10 000 counts/sec are approximately equivalent to 100 μCi. Specific activities of 200 μCi/μg were routinely observed. The elute was stored at -40°C and used within one month of synthesis.

2.4.3 Internalisation of [^{125}I]-helodermin in whole cells

12-well dishes were coated with an aqueous solution of 1% rat tail collagen for 2 hrs at room temperature. The collagen was removed by aspiration and each well was washed twice with 500 μl sterile PBS. Stably transfected HEK293 cells were seeded onto the dishes at 5×10^7 cells/well in culture medium and incubated for approximately 48 hrs at 37°C in a 95% air/5% CO_2 incubator. Each well was washed twice at room temperature in 500 μl medium 199 (M199) containing 0.2% BSA and 30 μg/ml bacitracin (assay medium) warmed to 37°C. Three wells/plate, reserved for protein assay, were washed in Earle's balanced salt solution (EBSS). 400 μl assay medium and 50 μl [^{125}I]-helodermin (1:100) was added to each well for varying incubation times (5, 10, 15, 20, 30, 45, 60 or 90 mins). Radioligand was added in the absence or presence of 1 μM unlabelled helodermin to provide a measure of total and non-specific binding values for each time point assessed. Each dish was incubated for 10 mins, preliminary assays were performed at 37°C, however high levels of [^{125}I]-helodermin were internalised even at the shortest time points measured, so subsequent experiments were performed at room temperature to reduce membrane fluidity and provide a clearer assessment of the time course of internalisation. Assay

medium was removed by aspiration and each well washed three times with 500 µl EBSS containing 0.1% BSA (with the exception of the wells reserved for protein assay where the medium was removed and no further washing was done). 500 µl acid strip buffer (0.2 M acetic acid and 0.5 M NaCl) was added to each well, incubated for 5 mins on ice before transferring into screw top eppendorf tubes (Sarstedt Ltd, Beaumont Leys, Leicester, UK) using a glass pasteur pipette. The acidic buffer strips radiolabelled ligand from the membrane, thus providing a measure of radioligand bound to receptors in the membrane (Haigler et al., 1980). The cells were then solubilised overnight at room temperature with 500 µl 4 M sodium hydroxide (NaOH) to release acid resistant or internal radioligand. These samples were also decanted into screw top eppendorfs using a glass pasteur pipette. All of the samples were counted on a Wallac 1470 gamma counter for 3 mins/tube. Protein samples were stored in 1 ml 1% sodium dodecyl sulphate (SDS) at -20°C before assay (see section 2.5.4).

2.4.4 [^{125}I]-helodermin membrane binding assay

Assay buffers

The following buffers were used for preparation of membranes and [^{125}I]-helodermin binding assays;

Tris-membrane buffer;

> 50 mM Tris-HCl (pH 7.4) containing 1 mM EGTA and a cocktail of protease inhibitors: 1 mM AEBSF, 2 µg/ml aprotinin, 4 µg/ml leupeptin, 2 µg/ml pepstatin, 1 mM sodium vanadate (Na$_3$VO$_3$), 1 mM sodium fluoride (NaF) and 50 µg/ml soybean trypsin inhibitor.

Tris-assay buffer;

> 50 mM Tris-HCl (pH 7.4) containing 5 mM MgCl$_2$, 0.5 mg/ml bacitracin, 0.1 mM AEBSF and 1% BSA.

Membrane preparation

Membrane preparations of HEK293 cells were made as follows. For each experiment two 75 cm^2 flasks of confluent cells were used per clonal cell line. Culture media was aspirated from the cells and the monolayer scraped into 2 mls Tris-membrane buffer (see above). Cells were pooled and then homogenised (20 secs, setting five) using an Ystral homogeniser (Scientific Industries International Ltd, Loughborough, Leics, UK). A 100 µl sample of the homogenate was taken for protein assay (see section 2.5.4). The cell homogenate was centrifuged at low speed (90 g) for 5 mins at 4°C in a Jouan MR812 to remove cell debris (Jouan, UK). The pellets were discarded supernatants centrifuged at 11600 g for 30 mins at 4°C, to isolate the cell membrane

fraction. These pellets were resuspended in 0.5 mls membrane buffer using a syringe and fine gauge needle and recentrifuged (11600 g for 30 mins at 4°C). The pellets were resuspended in 4.0 mls Tris-assay buffer (see above).

[125I]-helodermin binding assay

[125I]-helodermin binding to cell membrane preparations was assessed using a saturation assay performed in a final volume of 500 μl. The following assay components were added in triplicate: 300 μl Tris-assay buffer, 100 μl cell membrane suspension (final assay concentration of ~100 μg/ml) and 50 μl [125I]-helodermin in the absence or presence of 50 μl unlabelled helodermin at a range of concentrations (0.1, 0.3, 1, 3, 10, 30, 100 and 300 nM). Non-specific binding was determined in the presence of 1 μM unlabelled helodermin. The assay components were added to screw cap eppendorf tubes (Sarstedt Ltd, Beaumont Leys, Leicester, UK) and incubated for 10 mins at 37°C until equilibrium was reached. The binding assay was terminated by centrifugation at 11600 g for 20 mins at 4°C. The supernatant was removed by aspiration and each pellet washed carefully with 750 μl ice-cold Tris-assay buffer. Following complete removal of the wash buffer the samples were counted on a Wallac 1470 gamma counter for three mins/sample. 50 μl standards of radioligand (in triplicate) were included in each assay, these were used to calculate the specific activity of [125I]-helodermin (Ci/mmol). The specific activity was recalculated for each concentration of ligand in the assay allowing for the half-life of the radiolabel.

2.4.5 Radioligand binding data analysis

For some of the cultured cell membrane preparations high receptor expression levels, resulted in greater than 10% and up to as much as 50% of radioligand bound, which was particularly marked at low ligand concentrations. In order to make accurate estimates of the equilibrium dissociation constant (K_D) and maximum binding (B_{max}) for [125I]-helodermin binding the total free concentration of unlabelled and labelled ligand at equilibrium was calculated. The method of data analysis used is outlined below (see Appendix I):

1) Determine mean values for specific binding of the radioligand (in dpm). The amount of specific bound [125I]-helodermin (fmoles) can be determined from the specific bound (dpm) divided by the specific activity (fmoles/dpm). This value is normally calculated according to the amount of protein in each tube and expressed as fmoles/mg.

2) Calculate total ligand concentration from labelled and unlabelled concentrations added to each tube.

3) Calculate free ligand concentration (fmoles) by allowing for the amount of ligand specifically bound at equilibrium. Determining the free concentration of unlabelled ligand is done indirectly, assuming that the labelled and unlabelled ligands are chemically identical, the fraction of labelled helodermin that is free will equal the fraction of unlabelled helodermin that is free.

4) The specific bound radiolabel (fmoles/mg) can then be plotted against log free ligand concentration (nM).

5) Data were analysed using an iterative, non-linear least square curve fitting programme (Microcal Origin, Northampton, MA) to the logistic expression: $y=((A1-A2)/(1+x/x_0)^P$, where $A1$ = the initial binding maximum, $A2$ = the binding minimum (B_{max}), $x_0 = K_D$ and P = the slope. The curves were fitted to a minimum binding value fixed at zero. This programme determines the values for K_D, B_{max} and the slope of the curve, which should be equal to 1 for a single binding site with no cooperativity. The data are also presented as a Scatchard plot (Bound versus Bound/Free), for illustrative purposes only. Statistical analysis was performed using a students two-tailed t-test assuming unequal variances.

2.4.6 Protein assay

Protein assays were performed using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL). All samples were dissolved in 1 ml 1% SDS. A protein standard curve was established for each experiment using serial dilutions of 2 mg/ml BSA to give final concentrations of 25, 125, 250, 500, 750, 1000 and 2000 µg/ml. The assay was performed according to the manufacturers instructions. A working reagent of 50 parts Solution A (alkaline solution of BCA) to 1 part solution B (4% cupric sulphate) was used. The assay measures the reduction of copper ions (Cu^{2+}) in the presence of protein and alkali (the biuret reaction). Chelation of Cu^{1+} ions with BCA results in a colour change in the solution which increases linearly with protein concentration. 10 µl of each standard concentration or unknown protein sample were added to 96-well dishes (Greiner Labortechnik Ltd, Stonehouse, Gloucestershire, UK) in triplicate. 200 µl of working reagent was added to each well. The plates were covered and incubated at 37°C for 30 mins. Absorbance of the final solution was measured at 562 nm on a microplate Biokinetic reader EL312 (Bio-tek Instruments, Vermont, VT).

2.5 Immunfluorescence studies of the VPAC$_2$-HA receptor

2.5.1 Materials

All cell media, including opti-MEM® were purchased from Gibco Life Technologies, Ltd., Paisley, UK. Normal goat serum was supplied by Scottish Antibody Production Unit (SAPU), Carluke, Lanarkshire, UK. Phosphate buffered saline (PBS) tablets were obtained from Oxoid Ltd, Basingstoke, Hampshire, UK. PBS tablets were diluted in dH$_2$0 and autoclaved for 20 mins to sterilise. Paraformaldehyde was supplied by TAAB Laboratories, Aldermaston, Berks, UK. The source and catalogue number of the antibodies used are given in the text.

2.5.2 Indirect immunofluorescence of stably transfected HEK293 cells

Untransfected or stably transfected HEK293 cells were trypsinised, resuspended in culture media and counted on a haemocytometer. Cells were seeded onto sterile 13 mm glass coverslips (BDH, Merck Ltd, Lutterworth, Leics, UK) at 2x10^5 cells/ml in serum free medium (opti-MEM®) and incubated for >24 hrs in a 95% air/5% CO$_2$ incubator. Prior to fixation the cells were treated according to the required experimental conditions (see results sections for treatment regimens). Peptide dilutions in excess of 1 μM were made in 10x medium 199 (M199) in order to reduce acidity, whilst lower peptide concentrations were diluted in opti-MEM®.

The method for immunofluorescence was adapted from that of Harlow and Lane (1998). All of the following steps were performed at room temperature; between each stage the coverslips were removed carefully with a pair of fine watchmaker's forceps and rinsed three times in sterile PBS, before being placed into a fresh dish. Cells were fixed in 4% paraformaldehyde for 10 mins and permeabilised for 3-4 mins in PBS containing a mild detergent (0.2% Triton X-100). Non-specific antibody binding was blocked by incubating the cells in PBS containing 10% normal goat serum for a further 45 mins. For indirect immunofluorescence, the biotin-avidin system was used to enhance the fluorescence signal. Antibodies were added sequentially, each being incubated with the cells for 1 hr at room temperature. Prior to use the antibodies were diluted in PBS containing 10% normal goat serum and centrifuged at room temperature for 5 mins at 8500 g in a microcentrifuge (Eppendorf, 5415 C). After each antibody addition the coverslips were rinsed gently three times by immersion in PBS. The order of application of the antibodies used, their titre, supplier and catalogue number (#) are given below;

1)   Anti-HA mouse monoclonal antibody (mAb) @ 1:4000 dilution

2)   Goat anti-mouse IgG-biotin conjugate @1:300 dilution

3)   Avidin-FITC @1:100 dilution

Following antibody binding the cells were washed three times in PBS, once in dH_2O and mounted on microscope slides in Citifluor (PBS/glycerol) (City University, London, UK, patent pending). Immunofluorescence was observed using conventional fluorescence microscopy (see section 2.5.3). For preliminary experiments several different controls were used to assess the specificity of the observed fluorescence, (a) $VPAC_2$-HA transfected cells were substituted with untransfected HEK293 cells or HEK293 cells stably transfected with the $VPAC_2$ receptor, (b) the primary antibody was preincubated with blocking peptide (the original antigen), to remove all specific binding, (c) cells were not incubated with the primary antibody or (d) cells were not permeabilised prior to antibody addition. No specific staining was noted in any of these controls.

2.5.3 Dual antibody immunofluorescence of stably transfected HEK293 cells

In order to colocalise the $VPAC_2$-HA receptor with cellular markers, two primary antibodies were used in conjunction. Primary antibodies must originate from different species so they can be recognised independently by different secondary antibodies. As the majority of commercially available primary antibodies are raised in mice, it was necessary to substitute the mouse monoclonal antibody (mAb) against the HA epitope with a rabbit polyclonal antibody (pAb) to permit dual antibody labelling. The cells were fixed, permeabilised and non-specific antibody binding was blocked as described above and the following antibodies were added;

1)   Rabbit anti-haemagglutinin pAb @1:4000 dilution

2)   Goat anti-rabbit IgG-biotin conjugate @1:500 dilution

3)   Avidin-FITC @1:100 dilution

Preincubation of the blocking peptide (Santa Cruz Inc, Santa Cruz, CA. *#sc-805p*) with the anti-HA rabbit pAb was used as a control; no specific staining was observed. No difference was observed between the specific staining observed between the mouse mAb and rabbit pAB anti-HA antibodies. Therefore, the polyclonal anti-HA antibody was used in parallel with antibodies against an integral

membrane glycoprotein found in the *trans*-Golgi network (TGN46) or the transferrin receptor (TfnR).

Antibody labelling was carried out separately, or in tandem, to allow comparison of the location of the VPAC$_2$-HA receptor and marker (TGN46 or TfnR). Either of the following antibodies were used in separately or in tandem with the rabbit pAB anti-HA antibody. Colocalisation with the TGN46 was adapted from the method of Nilsson et al. (1991). The order of application of the antibodies used, their titre, supplier and catalogue number (#) are given below;

1)   Polyclonal sheep anti-human TGN46 @1:100 dilution

         (generous gift from Dr Ponnambalam, University of Dundee, UK).

2)   Donkey anti-sheep IgG-Texas red (TXR) conjugate @1:100 dilution

         (Jackson ImmunoResearch Labs., Inc., West Grove, PA; #713-075-147).

Colocalisation with TfnR antibody was performed according to the manufacturers instructions. The order of application of the antibodies used, their titre, supplier and catalogue number (#) are given below;

1)   Mouse mAB against anti human TfnR @1:50 dilution

         (Boehringer Mannheim Ltd, Lewes, Sussex, UK; *#1118 048*)

2)   Donkey anti mouse IgG-TXR conjugate @1:40

         (Jackson ImmunoResearch Labs., Inc., West Grove, PA; #715-075-150)

Immunofluorescence was observed using conventional fluorescence microscopy (see section 2.5.4). and digital imaging microscopy (see section 2.5.5). As previously described, a number of different controls were included for VPAC$_2$-HA receptor staining. In addition, cells were treated with both sets of antibodies in parallel or with individual sets of antibodies, to ensure that the emission spectra from the two different fluorophores did not overlap and produce a false signal. No fluorescence was observed with the FITC-conjugated antibody system when viewed under rhodamine excitation/emission filters and *vice versa*.

2.5.4 Immunofluorescence microscopy

Cells were viewed under phase contrast and fluorescence conditions on a Axiovert 135M inverted microscope (Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK) with x40 or x100 (oil immersion) objectives. Immunofluorescence was observed using the appropriate filters (**Table 2.1**). Photos of fluorescence were taken with a Contax 167MT camera attached to the microscope, using a 2 second exposure time and Ektachrome 160T (Kodak, UK) colour slide film. The pictures presented in

this thesis are reproductions of the original slides obtained by scanning them with a Nikon Cool scanIII film scanner in combination with Adobe Photoshop® software (Adobe systems, Inc.).

2.5.5 Digital imaging microscopy

Detailed analysis of immunofluorescence was performed using a Axiovert 135M (Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK) microscope attached to a CCD camera with a motorised stage and appropriate filters **(Table 1.2)** at x63 and x100 magnification **(Figure 2.1)**. Fluorescence images generated from conventional microscopy are two-dimensional, thus permitting small features in the same plane of focus (X/Y) to be resolved, but these images suffer from interference from fluorescence in X/Y planes which are not in focus and do not view the specimen as a three-dimensional object. For digital imaging microscopy a number of two-dimensional images were captured through the Z plane of focus for a single specimen. The focus drive was computer controlled to generate two-dimensional images at 1 μm steps throughout the specimen, using the digital Z capture programme (Digital Pixel Ltd., Brighton, UK). Each section was exposed twice under different filter conditions in order to measure the emission of both fluorophores used for double-labelling experiments. The sections form a stack of related images which were then restored. The image restoration or deconvolution process involves generation of a three-dimensional image; where the adjacent image sections are compared and out-of-focus fluorescence is removed. This process results in high resolution two-dimensional images with less haze from out-of-focus planes. In addition the image restoration algorithm can be applied to the stacked Z images and three-dimensional representations of the specimen can be displayed, three-dimensional images were generated but are not included in this thesis. Pictures were captured and deconvoluted using the Digital Z capture (Digital Pixel Ltd., Brighton, UK) in combination with IPlab software (Scanalytics Inc, Vienna, VA).

Table 2.1 Excitation and emission spectra of fluorophores. Parameters for single and triple fluorescent filter sets; as used for both single and double antibody labelling studies.

Fluorophore	Wavelength (nm)		Filter set		
	excitation	emission	exciter	dichroic	barrier
Fluorescein (FITC)	492	520	450-490	510	LP 515/520
Rhodamine (TRITC)	550	570	510-560	580	LP 590
Texas red (TXR)	596	620	510-560	580	LP 590
FITC/TRITC or TXR	492/550 or 596	520/570 or 620	400/495 or 570	410/505 or 585	460/530 or 610

Figure 2.1 Basic apparatus used for digital imaging microscopy of cells. Reproduced from Rizzuto et al., 1998. Images were captured and processed using the Digital Z pixel programme (Digital Pixel Ltd., Brighton, UK). Deconvolution and reconstruction were performed using IP lab software (Scanalytics Inc, Vienna, VA).
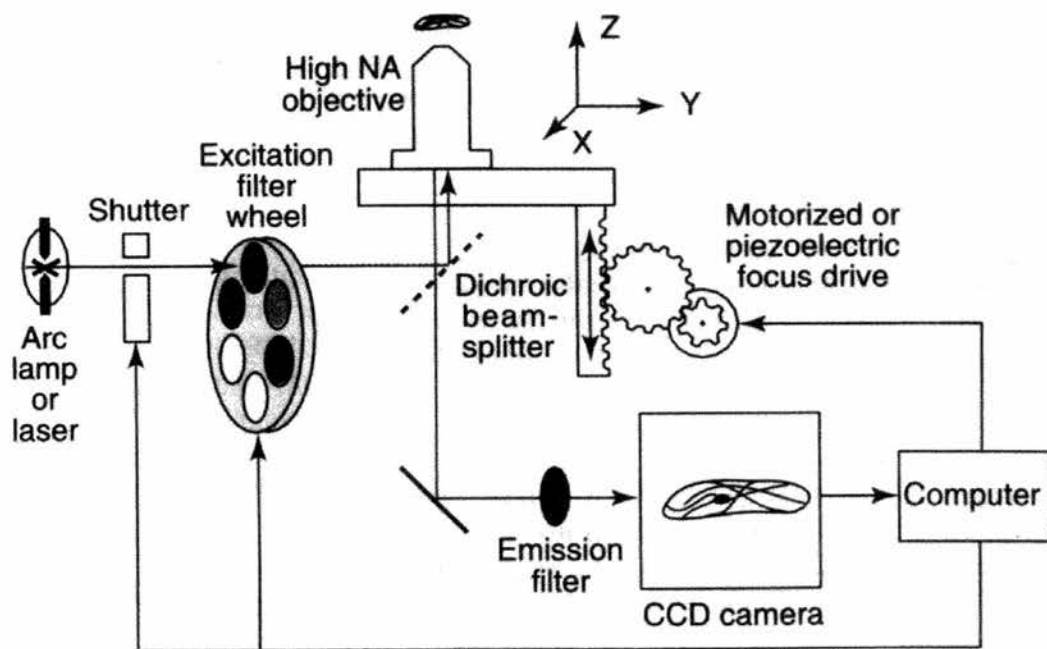
CHAPTER 3

Characterisation of wild type and epitope-tagged VPAC$_2$ receptors

3.1 Introduction

The cloning and expression of recombinant receptors in transfected cell lines has been instrumental in characterising their structure and function. Many methods used to characterise receptors require receptor-specific antibodies. However, antibodies against GPCRs are often difficult to generate due to low receptor expression levels and cross-reactivity; in addition antibody recognition can be perturbed by changes in receptor conformation which mask epitopes. This problem can be bypassed by generating recombinant receptor constructs with epitope or affinity tags which are recognised by commercially available antibodies. Epitope tags are typically small peptides of 6-13 amino acids; several different types of tag have been used in studies of GPCRs, examples include the c-myc (Ng et al., 1993; Ng et al., 1994), EYMPME (Arden et al., 1995; Wang et al., 1994), haemagglutinin (HA) (Smyth et al., 1996) and Flag epitopes (Garland et al., 1994; Smit et al., 1995; Zhang et al., 1997).

Incorporation of epitope tags into GPCRs has been used to aid their identification and purification (Gat et al., 1994; Gimpl et al., 1996). Epitope-tagged receptors have also been used for functional studies to demonstrate receptor phosphorylation (Ng et al., 1994; Smyth et al., 1996; Wang et al., 1994), localisation, trafficking (Ashworth et al., 1995; Grady et al., 1995b; Zhang et al., 1997) and to map the intracellular or extracellar distribution of different receptor domains (Xie and Abou-Samra, 1998). For these types of experiment it is important to demonstrate that the addition of the tag does not alter receptor properties.

Currently no antibodies against any of the VIP/PACAP receptors are available commercially. Epitope-tagged $VPAC_1$ receptors have been used for affinity cross-linking, Western blotting and confocal microscopy (Couvineau et al., 1996b; Gaudin et al., 1996b). In order to investigate regulation of the $VPAC_2$ receptor it was necessary to generate an epitope-tagged receptor construct. For class I GPCR epitope-tags are normally incorporated in the distal part of the N-terminal domain; this region is extracellular allowing the epitope to be detected in whole cells. Epitope-tags cannot be placed at the N-terminus of class II GPCRs as the distal part of this domain is cleaved to remove a signal peptide prior to receptor expression. Accordingly, an epitope-tag was incorporated after the last amino acid of the C-terminal intracellular domain of the $VPAC_2$ receptor. The human $VPAC_2$ receptor and haemagglutinin epitope-tagged $VPAC_2$ receptor ($VPAC_2$-HA) cDNA constructs were stably transfected in HEK293 cells. In this chapter the ability of these receptors

to stimulate second messenger production, their affinity for agonist and receptor expression levels are compared to determine whether addition of the epitope tag influences receptor function.

Many GPCRs exhibit a reduction in signalling, or desensitisation, following repeated or prolonged incubation with agonist (see chapter 1.4.1). Adenylyl cylase (AC) activation by VIP has been shown to desensitise in a number of different cell types expressing endogenous VIP/PACAP receptors (see discussion for references). Whilst many studies have characterised the ability of specific VIP/PACAP receptors to stimulate AC when transfected in cell lines, few studies have looked at desensitisation of this response. Therefore, experiments presented in this chapter determine whether $VPAC_2$ and $VPAC_2$-HA receptors are able to desensitise when stably transfected in HEK293 cells.

3.2 Results

3.2.1 VIP-stimulated cAMP formation in cells stably transfected with the $VPAC_2$ and $VPAC_2$-HA receptors

Recombinant human $VPAC_2$ and $VPAC_2$-HA receptors were transfected into HEK293 cells and several stable cell lines expressing the wild-type and epitope-tagged were isolated following antibiotic selection. The $VPAC_2$ receptor is coupled through the stimulatory G-protein (G_s) and activates AC resulting in cAMP production (Adamou et al., 1995; Lutz et al., 1993; Svoboda et al., 1994). Accordingly, all of the stably transfected clones displayed an increase in cAMP levels following stimulation with VIP. Representative clonal cell lines expressing the wild-type and epitope-tagged receptors were chosen and characterised in more detail. In whole cells transfected with the $VPAC_2$ and $VPAC_2$-HA receptors cAMP levels increase in a dose-dependent manner following receptor stimulation with VIP (0.01-100 nM) **(Figure 3.1)**. The concentration of VIP required for half-maximal stimulation (EC_{50}) of cAMP formation was not significantly different for the $VPAC_2$ and $VPAC_2$-HA receptors (1.2 ± 0.2 nM and 1.4 ± 0.2 nM, respectively). In addition no significant difference was observed between the maximal levels of cAMP stimulation for these receptors (876 ± 248 and 903 ± 262 pmoles/ml, respectively: n=4) **(Table 3.1A)**. These results indicate that the addition of the epitope-tag to the C-terminus of the $VPAC_2$ receptor has no effect on the ability of the receptor to couple to G-protein and stimulate second messenger production.

Untransfected HEK293 cells were included as controls in these experiments. These cells displayed a slight increase in cAMP levels following treatment with VIP, indicating the presence of an endogenous VIP responsive receptor in this cell line **(Figure 3.3)**. VIP stimulated cAMP formation in untransfected HEK293 cells with an EC_{50} of 0.1 ± 0.2 nM VIP. The maximal stimulation observed for untransfected HEK293 cells was approximately 10% of that seen in $VPAC_2$ receptor transfected cells (92 ± 19 pmoles/ml: n=4) **(Table 3.1A)**. This data is in agreement with previous studies which have identified an endogenous VIP responsive receptor in HEK293 cells (Simmons, 1990; Sreedharan et al., 1993). Sreedharan et al. (1994) proposed that the endogenous receptor is a $VPAC_1$ receptor.

3.2.2 Ro 25-1553-stimulated cAMP formation in cells stably transfected with the $VPAC_2$ and $VPAC_2$-HA receptors

The ability of Ro 25-1553 to stimulate cAMP formation in cells expressing the $VPAC_2$ and $VPAC_2$-HA receptors was compared. Ro 25-1553 is a synthetic VIP analogue which is a relatively specific agonist for $VPAC_2$ receptors (Bolin et al., 1995). Following stimulation of the $VPAC_2$ and $VPAC_2$-HA receptors with Ro 25-1553 (0.01-100 nM), cAMP levels increased in a dose-dependent manner **(Figure 3.2)**. No significant difference was observed between the EC_{50} values obtained for the $VPAC_2$ and $VPAC_2$-HA receptors (0.7 ± 0.1 and 0.5 ± 0.1 nM, respectively) or in the maximal levels of cAMP produced (611 ± 111 and 683 ± 199 pmoles/ml , respectively: n=4) **(Table 3.1B)**. These results provide further evidence that the addition of the epitope-tag to the $VPAC_2$ receptor has no effect on the ability of the receptor to couple to AC. In comparison with VIP, Ro 25-1553 was two-fold more potent at stimulating cAMP production. This finding is in accordance with previous reports that Ro 25-1553 was three-fold more potent than VIP at $VPAC_2$ receptors (Gourlet et al., 1997). Ro 25-1553 did not stimulate cAMP production in untransfected HEK293 cells **(Figure 3.3)**. The insensitivity of untransfected HEK293 cells to Ro 25-1553 is consistent with the proposal that these cells express an endogenous $VPAC_1$ receptor (Sreedharan et al., 1994), as Ro 25-1553 is 600-fold less potent that VIP at stimulating AC in cells which express the human $VPAC_1$ receptor (Gourlet et al., 1997).

3.2.3 [^{125}I]-helodermin binding to cell membrane preparations from HEK293 cells stably transfected with the $VPAC_2$ and $VPAC_2$-HA receptors

Membrane preparations of untransfected HEK293 cells or cells stably transfected with the $VPAC_2$ and $VPAC_2$-HA receptors were used for a saturation assay in which

the specific activity of $[^{125}I]$-helodermin was decreased by the addition of competing concentrations of unlabelled helodermin (0.1-300 nM). No specific binding was observed with membrane preparations of untransfected HEK293 cells (data not shown). A representative experiment is shown in **Figure 3.4**. The affinity of helodermin for $VPAC_2$ and $VPAC_2$-HA receptors was not significantly different, with equilibrium dissociation constants (K_Ds) of 2.2 ± 0.4 nM and 2.0 ± 0.4 nM, respectively **(Table 3.2)**. Hill coefficients did not differ from unity. A Scatchard transformation of the data produced a straight line, consistent with the presence of a single binding site **(Figure 3.4: inset)**. Both stably transfected cell lines expressed high numbers of binding sites, maximum binding (B_{max}) values of 6251 ± 2239 and 11197 ± 4936 fmoles/mg were obtained for the wild-type and epitope-tagged receptors, respectively **(Table 3.2)**. These data show that the addition of the epitope-tag to the C-terminus of the $VPAC_2$ receptor had no effect on its affinity for agonist.

3.2.4 Desensitisation of the $VPAC_2$ and $VPAC_2$-HA receptors

VIP treatment of HEK293 cells stably transfected with the $VPAC_2$ and $VPAC_2$-HA receptors, elicited a dose-dependent increase in cAMP levels. In order to establish whether this response was subject to desensitisation, cells were preincubated with VIP (0, 1, 3 or 10 nM for 30 mins) at 37°C, washed thoroughly and rechallenged with VIP at a range of concentrations (0.1-100 nM) in the presence of IBMX. Preincubation with VIP, at a concentration up to 10 nM, did not significantly effect basal cAMP levels. There was a reduction in maximal cAMP stimulation following preincubation with VIP for both the $VPAC_2$ **(Figure 3.5)** and $VPAC_2$-HA receptors **(Figure 3.6)**. A significant reduction in maximal cAMP stimulation was seen with both receptors after preincubation with 3 nM VIP ($P<0.05$) and 10 nM VIP ($P<0.01$). The greatest reduction (20%) in maximum cAMP levels was observed following pretreatment with 10 nM VIP. Reduction in maximal cAMP formation following preincubation with VIP was interpreted as agonist-induced desensitisation. When higher concentrations of VIP were used to elicit desensitisation elevated basal levels of cAMP formation were observed (>50%) which precluded curve fitting to the data. The concentration of VIP which caused half-maximal stimulation (EC_{50}) of cAMP levels was not significantly affected by pretreatment with VIP for either the $VPAC_2$ or $VPAC_2$-HA receptors **(Table 3.3)** These results demonstrate that the maximal cAMP response to VIP in HEK293 cells stably transfected with $VPAC_2$ and $VPAC_2$-HA receptors undergoes desensitisation. The reduction in cAMP formation

observed for both receptors is comparable indicating that the addition of the epitope-tag does not interfere with this desensitisation.
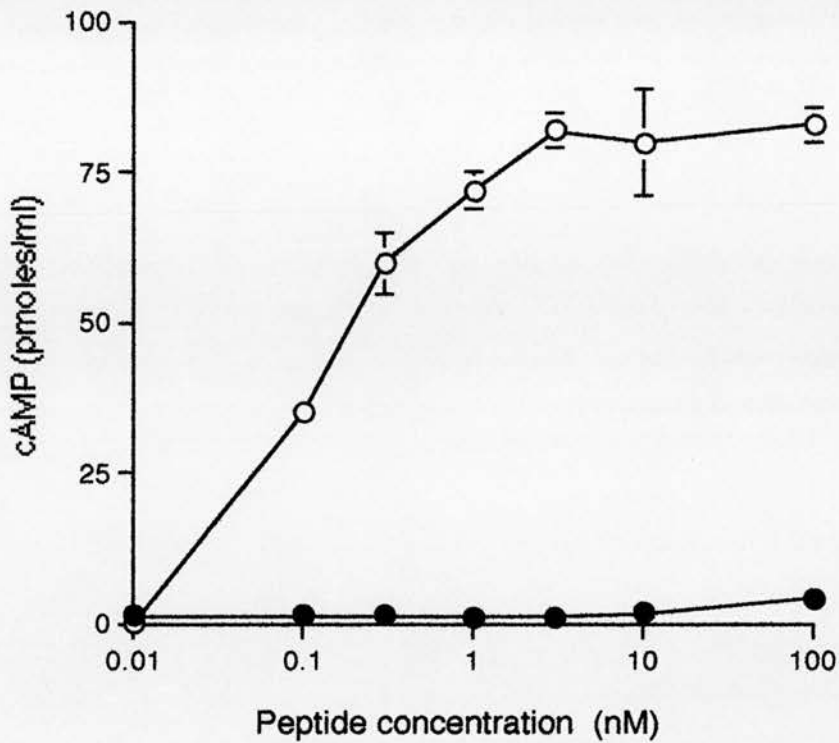
Figure 3.1   Stimulation of cAMP formation by VIP. cAMP levels in untransfected HEK293 cells (O) and cells stably transfected with $VPAC_2$ (□) and $VPAC_2$-HA receptors (■) following stimulation with VIP. Cells were incubated with VIP (0.01-100 nM) for 30 mins at 37°C, in the presence of IBMX. cAMP levels were determined by radioimmunoassay. Graph shows a typical experiment, performed in triplicate ± SD.
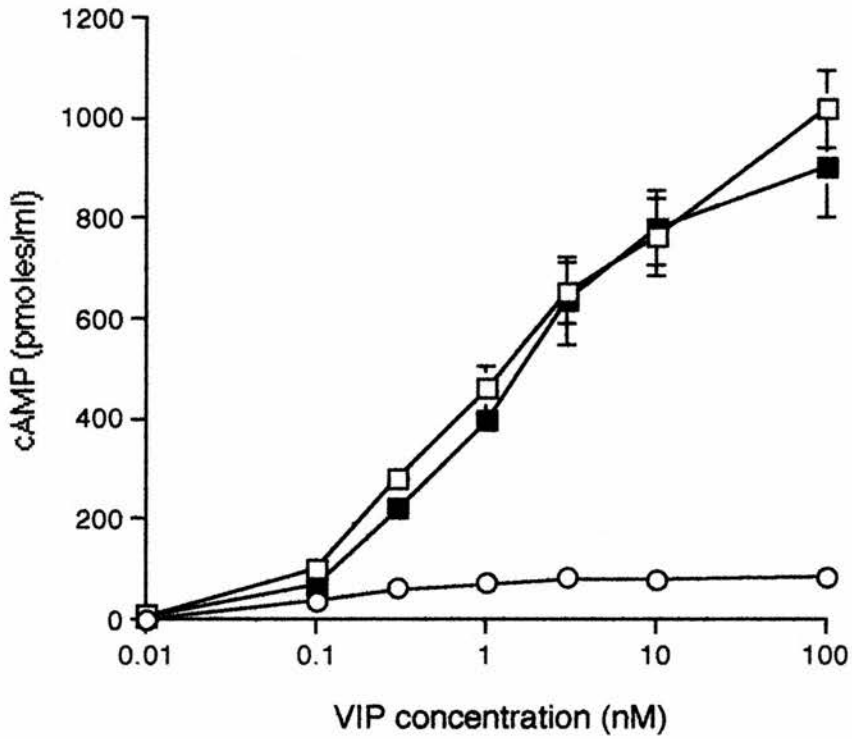
Figure 3.2 Stimulation of cAMP formation by Ro 25-1553. cAMP levels in untransfected HEK293 cells (O) and cells stably transfected with $VPAC_2$ (□) and $VPAC_2$-HA receptors (■) following stimulation with Ro-25 1553. Cells were incubated with Ro 25-1553 (0.01-100 nM) for 30 mins at 37°C, in the presence of IBMX. Levels of cAMP were determined by radioimmunoassay. Graph shows a typical experiment, performed in triplicate ± SD.
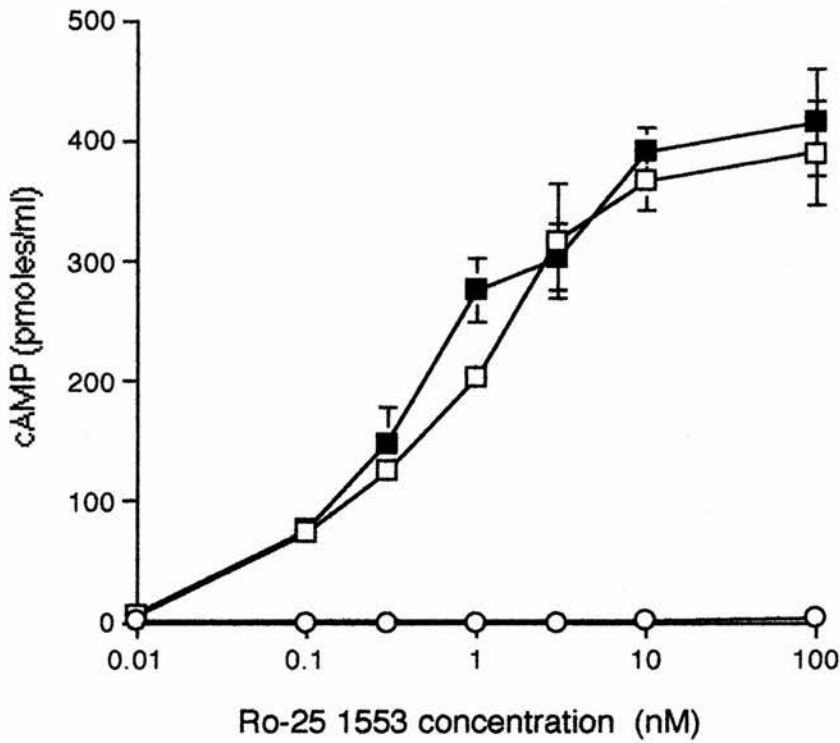
Figure 3.3 Stimulation of cAMP formation by VIP and Ro 25-1553 in untransfected HEK293 cells. cAMP levels in untransfected HEK293 cells following stimulation with VIP (○) or Ro 25 1553 (●). Cells were incubated with peptide (0.01-100 nM) for 30 mins at 37°C, in the presence of IBMX. Levels of cAMP were assessed by radioimmunoassay. Graph shows a typical experiment, performed in triplicate ± SD.

Table 3.1 Comparison of peptide-stimulated cAMP formation between the VPAC$_2$ and VPAC$_2$-HA receptors. HEK293 cells stably transfected with VPAC$_2$ and VPAC$_2$-HA receptors were treated with peptide (0.01-100 nM) and the levels of cAMP measured by radioimmunoassay. Concentration of VIP (**A**) and Ro-25 1553 (**B**) required for half-maximal stimulation of cAMP (EC$_{50}$) and maximum cAMP levels (pmoles/ml), results are means ± SEM for 3-4 separate experiments performed in triplicate.

(**A**)

Receptor	VIP EC$_{50}$ (nM)	Maximum cAMP stimulation (pmoles/ml)
Untransfected HEK293	0.1 ± 0.2	92 ± 19
VPAC$_2$	1.2 ± 0.2	876 ± 248
VPAC$_2$-HA	1.4 ± 0.2	903 ± 262

(**B**)

| Receptor	Ro 25-1553 EC$_{50}$ (nM)	Maximum cAMP stimulation (pmoles/ml)
Untransfected HEK293 cells	-	-
VPAC$_2$	0.7 ± 0.1	611 ± 111
VPAC$_2$-HA	0.5 ± 0.1	683 ± 199

Figure 3.4 **[125I]-helodermin binding to membrane preparations of cells stably transfected with the VPAC₂ and VPAC₂-HA receptors.** Representative saturation binding experiment for [125I]-helodermin in the presence of increasing concentrations of unlabelled helodermin (0.1-300 nM) for HEK293 cells stably transfected with the VPAC₂ (□) and VPAC₂-HA (■) receptors. Results are mean values for a single experiment performed in triplicate following a 10 min incubation at 37°C, until equilibrium was attained. **Inset;** A linear transformation of the data according to the method of Scatchard where bound/free versus bound is plotted.
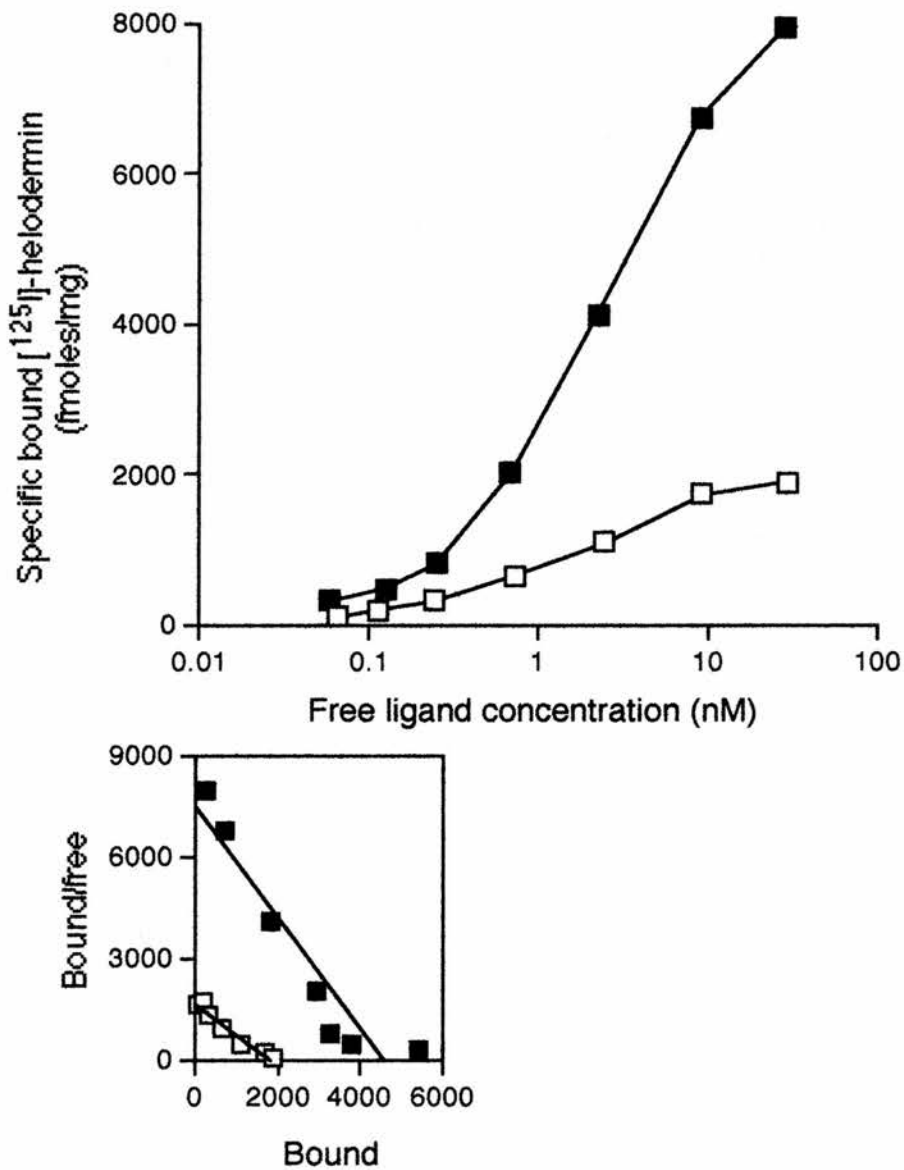
Table 3.2 **Comparison of K_D and B_{max} values obtained for homologous displacement of $[^{125}I]$-helodermin from membrane preparations of cells stably transfected with the VPAC$_2$ receptor and VPAC$_2$-HA receptor.** Results are a summary of 5-6 separate experiments performed in triplicate $\pm$ SEM.

Receptor	Equilibrium dissociation constant (K_D) (nM)	Maximum binding (B_{max}) (fmoles/mg protein)
VPAC$_2$	2.2 ± 0.4	6251 ± 2239
VPAC$_2$-HA	2.0 ± 0.4	11197 ± 4936

Figure 3.5   VIP-induced desensitisation of cAMP stimulation in HEK293 cells stably transfected with the VPAC$_2$ receptor. HEK293 cells stably transfected with the VPAC$_2$ receptor were pretreated with 0 (□), 1 nM (▲), 3 nM (▼) or 10 nM (○) VIP for 30 mins at 37°C. Desensitisation was evaluated by restimulating the cells with VIP (0.01-100 nM), in the presence of IBMX. Results are expressed as percentage of the maximal cAMP response for control cells not pretreated with VIP. Values are means ± SEM for 3-7 separate experiments performed in triplicate.
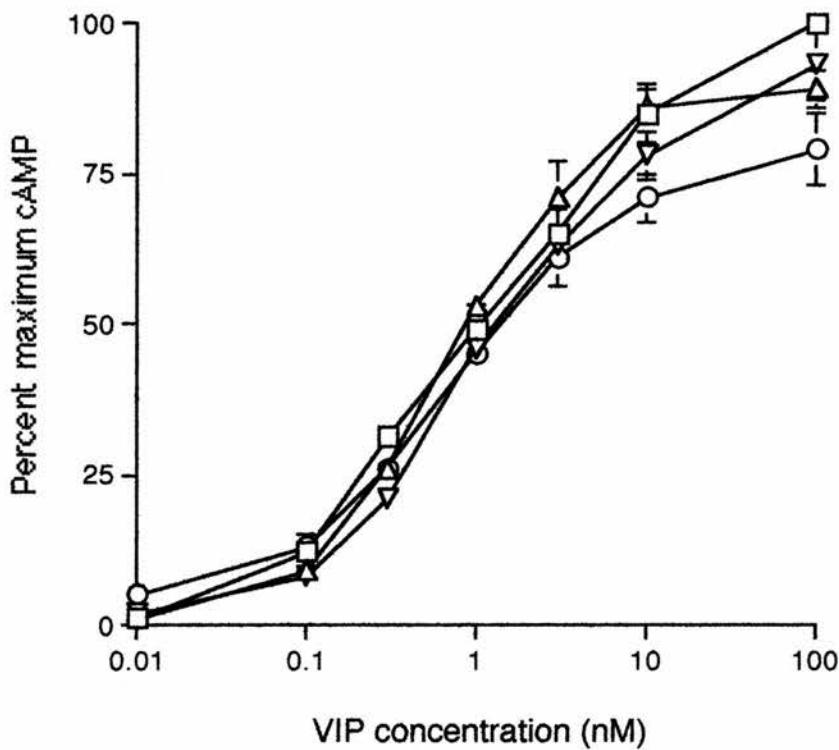
Figure 3.6   VIP-induced desensitisation of cAMP stimulation in HEK293 cells stably transfected with the VPAC$_2$-HA receptor. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were pretreated with 0 (■), 1 nM (▲), 3 nM (▼) or 10 nM (○) VIP for 30 mins at 37°C. Desensitisation was evaluated by restimulating the cells with VIP (0.01-100 nM), in the presence of IBMX for 30 mins at 37°C. Results are expressed as percentage of the maximal cAMP response for control cells not pretreated with VIP. Values are means ± SEM for 3-7 separate experiments performed in triplicate.
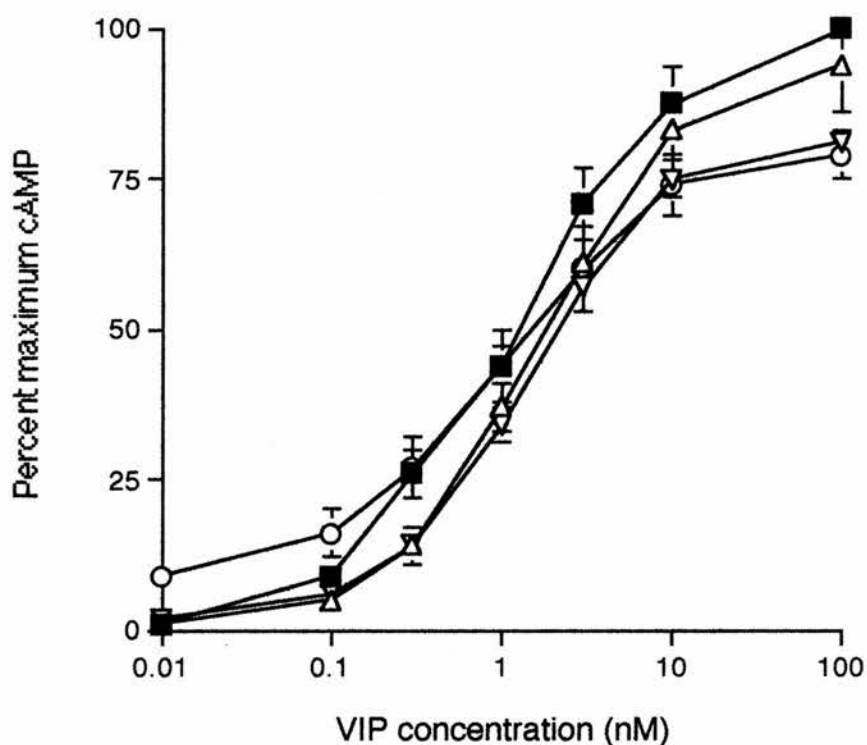
Table 3.3 Summary of VPAC$_2$ and VPAC$_2$-HA receptor desensitisation.
HEK293 cells stably transfected with the VPAC$_2$ (**A**) and the VPAC$_2$-HA receptors (**B**) were pretreated with 0, 1 nM, 3 nM or 10 nM VIP for 30 mins at 37°C, washed and incubated with 0.01-100 nM VIP for 30 mins at 37°C in the presence of IBMX. The concentration of VIP required for half-maximal stimulation (EC$_{50}$) and maximal cAMP levels are expressed as means ± SEM for 3-7 separate experiments performed in triplicate. Non-parametric significance testing using Mann-Whitney analysis for two independent groups was performed, significant differences are noted ($P<0.05$*, $P<0.01$**).

(A)

VPAC$_2$	EC$_{50}$ (nM)	Percent maximum cAMP stimulation (%)
Control	1.2 ± 0.2	108.6 ± 2.8
Pretreat with 1 nM	0.7 ± 0.1	90.1 ± 3.2*
Pretreat with 3 nM	1.6 ± 0.5	88.0 ± 5.2*
Pretreat with 10 nM	0.8 ± 0.1	81.3 ± 4.2**

(B)

VPAC$_2$-HA	EC$_{50}$ (nM)	Percent maximum cAMP stimulation (%)
Control	1.4 ± 0.3	104.5 ± 1.1
Pretreat with 1 nM	1.7 ± 0.3	95.9 ± 8.7
Pretreat with 3 nM	1.4 ± 0.1	82.2 ± 2.4*
Pretreat with 10 nM	1.6 ± 0.4	83.3 ±3.9**

3.3 Discussion

Comparison of second messenger stimulation between the VPAC$_2$ and VPAC$_2$-HA receptors

Experiments were undertaken to compare the ability of VPAC$_2$ and VPAC$_2$-HA receptors stably transfected in HEK293 cells to activate AC. Second messenger signalling properties of the VPAC$_2$ and VPAC$_2$-HA receptors were indistinguishable; both receptors displayed a dose-dependent increase in cAMP formation of equivalent potency in response to incubation with VIP or Ro 25-1553. The EC$_{50}$ value for VIP-stimulated cAMP formation was ~1.2 nM for both wild-type and epitope-tagged receptors. Previous determinations of the efficacy of VIP to stimulate AC in transfected COS-7 and Chinese hamster fibroblast (CHL) cells resulted in EC$_{50}$ values of 0.63 nM (Adamou et al., 1995) and 2.2 nM (Wei and Mojsov, 1996), respectively. Svoboda et al. (1994) reported EC$_{50}$ values of 5 and 10 nM for VIP stimulated cAMP formation in human VPAC$_2$ receptor transfected CHO cells, for high and low receptor expression levels, respectively (Svoboda et al., 1994). Overexpression in recombinant receptor systems alters receptor/G-protein stoichiometry which can influence receptor responsiveness. High levels of receptor may tend to produce saturation of signalling, thus limiting maximal responses, or it may lead to GPCR promiscuity where other second messenger systems are recruited which can complicate interpretation of the primary response (Kenakin, 1997b). It would be of interest to explore the effect of varying receptor number on the ability of VIP/PACAP receptors to activate AC in this system.

The EC$_{50}$ value for Ro 25-1553-stimulated cAMP formation determined in this study was ~0.6 nM for both the VPAC$_2$ and VPAC$_2$-HA receptors. Ro 25-1553 is a cyclic peptide analogue of VIP, it is efficacious, metabolically stable and has a prolonged duration of action in comparison with VIP (O'Donnell et al., 1994a). Ro 25-1553 was approximately two-fold more potent in stimulating cAMP at the VPAC$_2$ and VPAC$_2$-HA receptors than VIP. This is consistent with work by Gourlet et al. (1997) who found that Ro 25-1553 was three-fold more potent than VIP at stimulating AC in VPAC$_2$ receptor transfected CHO cells and SUP-T1 lymphoblasts (Gourlet et al., 1997). Previous studies have defined the general order of potency of VIP and related peptides for stimulation of adenylate cyclase in cells stably transfected with the human VPAC$_2$ receptor as PACAP-38 = helodermin >VIP $\geq$ PACAP-27 (Svoboda et al., 1994). However no studies to date have compared the potency of Ro 25-1553 with other VIP-related peptides at the VPAC$_2$ receptor.

Untransfected HEK293 cells contain an endogenous VPAC₁ receptor

Untransfected HEK293 cells were included as control cells for these experiments. Moderate VIP-induced increases in cAMP levels were observed in these cells, consistent with previous reports (Sreedharan et al., 1993; Sreedharan et al., 1994). Sreedharan et al. (1994) attributed this response to the presence of an endogenous VPAC₁ receptor. Support for this hypothesis was provided by Simmons (1990), who found an increase in cAMP stimulation in untransfected HEK293 cells incubated with high (µM) amounts of secretin, a relatively selective VPAC₁ receptor agonist (Simmons, 1990). The endogenous receptor in HEK293 cells constitutes a high affinity site for VIP with an EC_{50} of 0.1 ± 0.2 nM but does not respond to treatment with Ro 25-1553. This is in accordance with the low potency of Ro 25-1553 acting on cells stably transfected with the VPAC₁ receptor (Gourlet et al., 1997). Overall there is considerable evidence, from the data presented here and the work of other groups, that the endogenous VIP responsive receptor in HEK293 cells is a VPAC₁ receptor.

Affinity of [^{125}I]-helodermin for VPAC₂ and VPAC₂-HA receptors stably transfected in HEK293 cells

Iodinated ([^{125}I]-) and tritated ([^{3}H]-) peptides are both used in radioligand binding assays, although the former is more common as radioiodination generally produces ligands with high specific activity (Gammeltoft, 1990). Iodination can sometimes affect peptide affinity, however this has not been found for members of the VIP/PACAP family, where [^{125}I]-VIP (Van Rampelbergh et al., 1996), [^{125}I]-PACAP-27 and [^{125}I]-PACAP-38 all display similar affinities for receptors to their unlabelled counterparts (Robberecht et al., 1991b). Helodermin was chosen as the ligand for binding studies as it is readily iodinated, a potent agonist at VPAC₂ receptors and relatively stable at 37°C (Christophe et al., 1993). Robberecht et al. (1984) were the first group to use helodermin as a radioligand for VPAC₂ receptors. They found that [^{125}I]-helodermin bound rapidly and reversibly to rat liver membranes (Robberecht et al., 1984). Unlabelled peptides inhibited [^{125}I]-helodermin binding with the following order of potency; helodermin(1-35)-NH₂ = helodermin(1-27)-NH₂ > helospectin > VIP = PHI > [D-Ser²]VIP > [D-Asp³]VIP >> [D-His¹]VIP > [D-Ala⁴]VIP >> secretin = GRF (Robberecht et al., 1988). In our laboratory, peptides are iodinated by the chloramine-T method, where iodine is substituted for hydrogen at tyrosine residues. Helodermin contains two tyrosine residues, these residues can potentially be

iodinated twice, thus several species of helodermin can be produced. [^{125}monoiodo-Tyr^{10}]-helodermin and [^{125}monoiodo-Tyr^{22}]-helodermin predominate; these species can be separated by reverse phase chromatography. Christophe et al. (1993) found that radioligand binding assays performed with individual species of helodermin radiolabel produce similar results to non-purified [^{125}I]-helodermin (Christophe et al., 1993). Hence, for the experiments described in this chapter the mono- and di-iodinated helodermin species were not separated. The pharmacological properties of the VPAC$_2$ and VPAC$_2$-HA receptors were compared using a radioligand binding assay of stably transfected cell membrane preparations.

Peptide binding assays are notoriously problematic, due in part to the susceptibility of peptides and their receptors to degradation. This problem can be counteracted by the addition of peptidase and protease inhibitors to the assay medium. For the assays described here, bacitracin (a broad spectrum peptidase inhibitor) was added to the assay medium and a cocktail of protease inhibitors were included in cell membrane preparations to reduce degradation. Classically, receptor binding assays use radiolabelled antagonists to define receptor pharmacology, however this type of assay cannot often be applied to peptide receptors due to the lack of potent specific receptor antagonists. Therefore, agonists are commonly used for peptide receptor binding studies. Peptides have a strong tendency to bind non-specifically, indeed previous studies have shown that both [^{125}I]-VIP and [^{125}I]-helodermin are prone to high non-specific in SUP-T1 lymphoblasts (Robberecht et al., 1989b). This problem was reduced in the membrane binding assay by adding bovine serum albumin (BSA) to the medium and using a centrifugation method to separate bound from free ligand.

Radioligand binding assays revealed that the addition of the epitope-tag to the VPAC$_2$ receptor had no effect on affinity for helodermin (K$_D$~2.2 nM). The affinity of VPAC$_2$ receptors for helodermin, determined by homologous competition has previously been reported at a concentration of 3 nM in SUP-T1 lymphoblasts (Robberecht et al., 1988). Scatchard analysis of the data indicated the presence of a single binding site. VPAC$_2$ and VPAC$_2$-HA clonal cell lines expressed receptor at similar levels, although the VPAC$_2$-HA displayed a higher mean B$_{max}$ value than the wild type receptor, this difference was not significant. The data presented here indicate that the affinity of the VPAC$_2$ receptors for helodermin is unaffected by the addition of the epitope-tag to its C-terminus.

Desensitisation of the cAMP stimulation in VPAC$_2$ and VPAC$_2$-HA receptors stably transfected in HEK293 cells

The cAMP response in both VPAC$_2$ and VPAC$_2$-HA receptors desensitised following pretreatment with VIP (>1 nM) for 30 mins at 37°C. Desensitisation was moderate, with a 20% reduction in the maximum cAMP levels after pretreatment with 10 nM VIP. The concentration of VIP required for half-maximal stimulation was not effected by VIP pretreatment. A more marked (~50%) reduction in cAMP formation has previously been reported by Robberecht et al. (1989) in intact SUP-T1 lymphocytes preincubated with VIP or helodermin (10 nM for 15 mins at 37°C) (Robberecht et al., 1989c). However, there are a number of differences between the assay presented here and the assay used by Robberecht et al. (1989) which may account for the relatively small amount of desensitisation observed. Firstly, following the initial challenge with VIP the desensitised cells were incubated for 30 mins with the second dose of VIP, whereas Robberecht et al. (1989) rechallenged the cells for 45 secs only. The relatively long second incubation in the presence of phosphodiesterase inhibitor used for this study may have allowed higher levels of cAMP to accumulate which mask desensititsation. Secondly, it must be borne in mind that the VPAC$_2$ and VPAC$_2$-HA receptors are highly overexpressed in HEK293 cells. Receptor expression levels have been found to influence desensitisation of other GPCRs. For the GRP receptor expressed at low, medium and high levels in BALB 3T3 cells, increasing receptor number resulted in reduced desensitisation of the cAMP response (Tsuda et al., 1997). Similar results were obtained with the rat secretin receptor transfected in CHO cells, where pretreatment with secretin in cells expressing low levels of receptor resulted in a marked reduction in maximum cAMP production with a small increase in EC$_{50}$, whereas, cells expressing high levels of receptor displayed a relatively small decrease in maximum cAMP levels and a marked reduction in EC$_{50}$ (Vilardaga et al., 1994). Desensitisation of the GLP-1 receptor (Widmann et al., 1996) was unaffected by receptor number, although like the secretin receptor, the EC$_{50}$ values and maximum cAMP stimulation were differentially effected over a range of GLP-1 receptor expression levels (10-300 fmol/mg). Overall, it seems likely that the level of overexpression of the VPAC$_2$ and VPAC$_2$-HA receptors in HEK293 cells may have had an effect on the level of desensitisation observed, this possibility could be investigated using cells stably transfected with different amounts of receptor and would be an interesting avenue for future research. Surprisingly no effect on the half-maximal stimulation of cAMP was observed, this may be due to the assay method (as described above) or may be a feature of VPAC$_2$ receptor desensitisation,

it would be interesting to perform time course experiments for pretreatment and subsequent rechallenge with VIP to establish whether desensitisation has any impact on the potency of VIP to elicit cAMP production.

Persistent activation of adenylyl cyclase (AC)

Exposure of receptors to higher concentrations of VIP (>10nM) resulted in high basal levels of cAMP and persistent activation of AC, which hampered measurement of EC_{50} values and dose-response curve (data not shown). This problem has been found for the thyrotropin receptor overexpressed in HEK293 cells (Nagayama et al., 1994) and for several class II GPCRs, including calcitonin (Lamp et al., 1981), GLP-1 (Widmann et al., 1996) and $VPAC_1$ receptors (Turner et al., 1988). Several different methods have been used to counteract this problem, including using cell lines with lower expression levels, prolonged washing of preincubated cells with acidic wash buffers or using agonists with low affinity for the receptor. Turner et al. (1988) overcame this problem in HT-29 cells by pretreating them with PHI instead of VIP (Turner et al., 1988). PHI is a VIP agonist but has a lower affinity for VIP receptors and thus can be readily removed by washing. Future experiments using alternative low affinity ligands or employing more extensive wash conditions may enable cAMP desensitisation to be observed in the presence of higher agonist concentrations

Agonist-induced desensitisation of class II GPCRs

Many members of the class II GPCRs have been found to exhibit agonist-stimulated desensitisation in response to agonist pretreatment (Holtmann et al., 1996). Secretin receptor desensitisation was first demonstrated in rat gastric glands (Bawab et al., 1991) and has subsequently been reported in CHO and HEK293 cells transfected with the rat secretin receptor (Shetzline et al., 1998; Vilardaga et al., 1994) and in NG108-15 hybrid mouse neuroblastoma/rat glioma cells (Mundell et al., 1997). The receptor for GLP-1 also exhibits homologous desensitisation (Widmann et al., 1996; Widmann et al., 1997). The PTH/PTHrP receptor is able to couple to two distinct signalling pathways, one of which activates AC and increases cAMP accumulation and the second which activates phospholipase C (PLC) resulting in increased levels of intracellular calcium $[Ca_i^{2+}]$. Both of these signalling cascades are subject to regulation. Homologous desensitisation of PTH/PTHrP-stimulated cAMP production has been demonstrated in cultured bone cells (Teitelbaum et al., 1986) and human osteoblast-like SaOS-2 cells (Fukayama et al., 1992), whilst regulation of $[Ca_i^{2+}]$ levels has been found in osteoblast cells (Civitelli et al., 1989). The calcitonin

receptor also couples to multiple signalling pathways, mainly involving AC and PLC activation (Force et al., 1992). Homologous desensitization of calcitonin activation of AC and PLC has been demonstrated in osteoclasts and receptor-bearing cancer cells (Schneider et al., 1993; Wada et al., 1995). Desensitisation is much slower for the calcitonin receptor response than observed for the other class II GPCRs, which may be due in part to the poorly reversible binding of calcitonin or different molecular mechanisms involved in calcitonin receptor regulation (Schneider et al., 1993).

All VIP/PACAP receptors exhibit desensitisation but the physiological importance of this phenomenon still remains to be clarified. VIP exerts transient effects on a number of systems indicating that desensitisation of VIP signalling may be important at a physiological level. The first evidence for this phenomenon was shown following repeated iontophoretic application of VIP in the rat cerebral cortex *in vivo* (Phillis et al., 1978). VIP-induced desensitisation was also observed in isolated synaptosomes from the rat cerebral cortex, further supporting this finding (Staun-Olson et al., 1982). Rapid homologous desensitisation of VIP-stimulated cAMP production has been demonstrated in several cell lines, including HT-29 colonic carcinoma (Boissard et al., 1986), SUP-T1 lymphoblasts (Robberecht et al., 1989c), K-N-MC neuroblastoma (Olasmaa et al., 1987), IGR39 (a melanoma-derived cell line) (Martin et al., 1989) Ewing's sarcoma WE-68 (Van Valen et al., 1989), αT3-1 gonadotrophs (McArdle and Forrest-Owen, 1997), mononuclear lymphocytes (Wiik, 1988) and rat peritoneal macrophages (Pozo et al., 1995). HT-29 colonic adenocarcinoma cells express VPAC$_1$ receptors, SUP-T1 lymphoblasts express VPAC$_2$ receptors and αT3-1 gonadotrophs express PAC$_1$ receptors; the receptor type(s) in the other cell lines have yet to be defined. The majority of studies of VIP/PACAP receptor desensitisation have been performed using HT-29 cells where desensitisation of the cAMP response in HT-29 cells is rapid (within 5-10 mins), reversible and dependent upon agonist efficacy, concentration, temperature and incubation time (Boissard et al., 1986). VIP/PACAP receptors chronically stimulated with agonist (>6 hrs) are also subject to homologous desensitisation (Gespach et al., 1984; Robberecht et al., 1989a) which has been associated with concomitant down regulation of cell surface receptors (McArdle and Forrest-Owen, 1997). Down regulation of receptors persists after removal of the ligand and depends on the rate of receptor breakdown and new receptor synthesis (Boissard et al., 1986).

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Several mechanisms have been found to contribute to desensitisation of GPCRs, including phosphorylation, internalisation and down regulation. The relative contribution of these processes to desensitisation depends upon receptor type (see chapter 1.4). For VIP/PACAP receptors the role of phosphorylation, internalisation and down regulation in receptor desensitisation remains to be clarified. Recent data has provided the first evidence that the $VPAC_2$ receptor is phosphorylated in response to agonist treatment (McDonald et al., 1998) and work to determine the role of phosphorylation in desensitisation of the $VPAC_2$ receptor is currently ongoing in our laboratory. Classically internalisation was believed to have a primary role in VIP/PACAP receptor desensitisation (Rosselin et al., 1988) although data now suggests that internalisation may be insufficient to account for the level of desensitisation (Pozo et al., 1995; Wiik, 1988). The main focus of this thesis was to directly demonstrate agonist-induced internalisation of the $VPAC_2$ receptor, in order to examine mechanistic and functional aspects of this phenomenon. The epitope-tagged $VPAC_2$ receptor, characterised in this chapter appears to have identical properties to the $VPAC_2$ receptor and is therefore a useful tool for investigating $VPAC_2$ receptor internalisation.

Ultimately antibodies directed against the wild type receptor would be useful for *in vitro* and *in vivo* experiments. An attempt to generate rabbit polyclonal antibodies against the rat $VPAC_2$ receptor was made during the course of this work. The glutathione S-transferase (GST) fusion protein system was used to generate large quantities of proteins corresponding to the N- and C-terminal domains of the rat $VPAC_2$ receptor. Unfortunately immunisation of rabbits with these fusion proteins did not produce a significant amount of specific rat $VPAC_2$ receptor antibodies, for a description of the techniques used see Appendix II.

CHAPTER 4

Agonist-induced internalisation of the VPAC$_2$ receptor

4.1 Introduction

Evidence for agonist-induced internalisation of class II GPCRs has generally been derived from experiments using radiolabelled peptides as tracers for receptor movement (see chapter 1.5.7). Few studies of this family of receptors have monitored receptor movement directly. A considerable body of work supporting a mechanism for receptor-mediated endocytosis of VIP has been published. However, only one study using an epitope-tagged $VPAC_1$ receptor, has demonstrated internalisation and down regulation of this receptor *in vitro* (Gaudin et al., 1996a). To date, no direct evidence for $VPAC_2$ receptor endocytosis has been published. Therefore, in order to investigate whether the $VPAC_2$ receptor internalises in response to agonist treatment a C-terminally epitope-tagged version of this receptor was generated. In the previous chapter it has been demonstrated that wild type and epitope-tagged $VPAC_2$ receptors display identical affinities for ligand and that VIP stimulates second messenger formation through these receptors with similar potency. In this chapter agonist-induced internalisation of the $VPAC_2$ receptor was demonstrated using two different techniques. Firstly; by measuring $[^{125}I]$-helodermin binding to external and internal receptor populations in intact cell monolayers. Secondly; the epitope-tagged $VPAC_2$ receptor was used to localise the receptor in untreated and agonist stimulated cells using an immunofluorescence technique.

The majority of GPCRs have been shown to utilise clathrin coated pits to internalise, although some of these receptors are able to use other endocytic pathways *in vitro* (see chapter 1.5.2). A number of chemical treatments which disrupt endocytic pathways have been used in previous studies to determine the mechanism involved in GPCR internalisation. To prevent clathrin-mediated receptor endocytosis three methods are commonly used: (1) depletion of intracellular potassium (Larkin et al., 1983), (2) exposure of cells to hypertonic media (Daukas and Zigmond, 1985) and (3) acidification of the cytoplasm (Sandvig et al., 1987). Potassium depletion and hypertonicity both perturb endocytosis by inhibiting formation of the polyhedral clathrin lattice, resulting in small "microcages" of clathrin at inappropriate sites on the plasma membrane (Heuser and Anderson, 1989b). It has been suggested that these treatments may also prevent clathrin from interacting with APs (Hansen et al., 1993). Low cytosolic pH is believed to enhance clathrin lattice curvature, promote polymerisation of cytoplasmic clathrin and prevent coated pits from pinching off and forming coated vesicles (Heuser, 1989a; Sandvig et al., 1987). In addition, reagents

which prevent caveolae-mediated internalisation in several different cell types have been identified, one example is phorbol-12-myristate-13-acetate (PMA), an irreversible protein kinase C (PKC) activator (Smart et al., 1994a). In this chapter two methods known to inhibit clathrin-coated pit formation (hypertonicity and low pH) and PMA treatment were used in combination with [^{125}I]-helodermin binding and immunofluorescence experiments to evaluate the mechanism by which VPAC$_2$ receptors internalise.

Several different functions for agonist-induced internalisation of GPCRs have been postulated. For some receptors it is an important mechanism for desensitisation (e.g. thrombin receptors), whereas for others internalisation is required for receptor dephosphorylation and resensitisation (e.g. β_2AR) (see sections 1.5.6 and 1.5.8). In addition, recent studies have found that receptor-mediated endocytosis may have important implications for the activation of alternate second messenger effector systems (Daaka et al., 1998). For the VIP/PACAP receptors the role of agonist-induced internalisation in receptor function is poorly characterised. Establishing a chemical method to impair internalisation permits investigation of its role in desensitisation. Hence, preliminary experiments to evaluate the involvement of VPAC$_2$ receptor internalisation in desensitisation of cAMP signalling are presented here.

4.2 Results

4.2.1 Internalisation of [^{125}I]-helodermin in cells stably transfected with the VPAC$_2$ and VPAC$_2$-HA receptors

Internalisation of [^{125}I]-helodermin, a specific VPAC$_2$ receptor agonist, was assessed in HEK293 cells stably transfected with the VPAC$_2$ receptor and epitope-tagged VPAC$_2$ receptor. [^{125}I]-helodermin at low concentration (50-100 pM) binds both VPAC$_2$ and VPAC$_2$-HA receptors with similar affinity (see section 3.2.2). For the internalisation assay an acidic wash was used to strip bound [^{125}I]-helodermin from receptors at the cell surface, whilst acid resistant radiolabel was obtained by lysing the cells with NaOH. The amount of radiolabelled peptide bound to external and internal receptor populations was measured at different incubation times (2, 5, 10, 20 and 30 mins) at 37°C (**Figure 4.1**). Results are expressed as percentage of total bound [^{125}I]-helodermin which was at an acid resistant (or internal) site. Both VPAC$_2$ and VPAC$_2$-HA receptor transfected cell lines internalised radiolabel rapidly, with ~75% of the ligand in an acid resistant site

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following a 5 min incubation. The levels of internal [^{125}I]-helodermin reached a maximum of ~90% by 20 mins.

To allow a clearer examination of [^{125}I]-helodermin binding over an extended time course subsequent assays were performed at room temperature to reduce membrane fluidity and receptor movement. Levels of [^{125}I]-helodermin bound were measured over a longer time course (5, 10, 15, 20, 30, 45, 60 and 90 mins) **(Figure 4.2)**. Importantly, no significant difference between the internalisation kinetics of radiolabel were observed between the VPAC$_2$ and VPAC$_2$-HA receptors. [^{125}I]-helodermin was endocytosed rapidly during the first 30 mins of incubation with a $t\frac{1}{2}$ of ~20 mins. The rate of internalisation was reduced compared with that seen at 37°C, with only ~25% of the total [^{125}I]-helodermin internal after a 5 min incubation. The levels of internal [^{125}I]-helodermin reached a maximum of ~75% following 60 mins incubation at room temperature. These results indicate that HEK293 cells transfected with VPAC$_2$ and VPAC$_2$-HA receptors internalise peptides rapidly in a temperature dependent manner.

4.2.2 Effect of hypertonic media on internalisation of [^{125}I]-helodermin
Previous studies have provided considerable evidence for a receptor-mediated mechanism of VIP internalisation. Therefore, to establish whether a clathrin-dependent receptor mechanism was involved in the internalisation of [^{125}I]-helodermin, a chemical treatment to disrupt clathrin-coated pit formation was used. Increasing cell osmolarity with sodium chloride, sucrose or lactose has been shown to inhibit receptor-mediated endocytosis of a chemotactic peptide in polymorphonuclear leukocytes, without affecting fluid phase endocytosis (Daukas and Zigmond, 1985). Daukas and Zigmond (1985) reported that pretreatment with sucrose, at concentrations ranging from 0.45 M to 0.75 M, was able to rapidly inhibit internalisation, with maximal inhibition occurring at 0.75 M. For these experiments HEK293 cells stably transfected with the VPAC$_2$ and VPAC$_2$-HA receptors were preincubated with sucrose (0.45 M for 30 mins) at room temperature prior to radioligand binding. Pretreatment with hypertonic sucrose caused a marked reduction in the percentage of total [^{125}I]-helodermin which was internalised for both VPAC$_2$ and VPAC$_2$-HA receptors **(Figures 4.3** and **4.4)**. After a 90 min incubation in the presence of sucrose levels of internalised radiolabel were 43% and 34% of the total radiolabel compared with 75% and 68% in the absence of sucrose, for the VPAC$_2$ and VPAC$_2$-HA receptors respectively. Sucrose treated curves show considerable variation in the levels of internalised ligand over the first 20 mins of the

assay, where higher levels of internalised [^{125}I]-helodermin were observed. These fluctuations in internalised radiolabel could be due to alterations in cell structure or other non-specific effects induced by the high levels of sucrose present during the assay. These data indicate that clathrin-coated pits may be involved, at least in part, receptor-mediated endocytosis of [^{125}I]-helodermin.

4.2.3 VIP-induced internalisation of the VPAC$_2$ receptor

The effect of agonist stimulation on the distribution of the VPAC$_2$-HA receptor was investigated using an immunofluorescence technique. Stably transfected HEK293 cells were incubated in serum free medium for 24 hrs prior to fixing with paraformaldehyde and permeabilisation. A mouse monoclonal antibody directed against the HA tag was used in combination with a biotinylated goat anti-mouse IgG secondary antibody, which was in turn recognised by avidin conjugated to fluorescein isothiocyanate (FITC). Cells were viewed on an Zeiss inverted microscope, under phase contrast conditions **(Figure 4.5A,C,E)** or with the appropriate fluorescence filters **(Figure 4.5B,D,F)**. No specific fluorescent staining was obtained with untransfected HEK293 cells (data not shown) or cells stably transfected with the VPAC$_2$ receptor **(Figure 4.5B)**. In addition, no staining was observed when the primary anti-HA antibody was preincubated with the original antigenic peptide, if the primary antibody was excluded from the experiment or the cells were not permeabilised (data not shown). Specific staining was observed in HEK293 cells stably transfected with the VPAC$_2$-HA receptor, where the receptor was predominantly located in the plasma membrane **(Figure 4.5D)**. Pretreatment with VIP (10 μM for 30 mins) at 37°C resulted in a marked reduction in staining at the plasma membrane and an accumulation of receptor staining at a single intracellular site **(Figure 4.5F)**. Cells preincubated in serum free medium or VIP at a range of concentrations (0.01, 0.1, 1 or 10 μM for 30 mins) at 37°C, were viewed at x40 **(Figure 4.6A-E)** and x100 magnification **(Figure 4.7A-E)**, respectively. Following preincubation with 0.01 μM VIP the VPAC$_2$-HA receptor was predominantly located at the cell membrane, however, peptide concentrations in excess of 0.01 μM resulted in a visible shift in receptor distribution to a single intracellular site. A qualitative analysis of the staining for the VPAC$_2$ receptor indicated that the extent of receptor internalisation increased in a concentration dependent manner.

4.2.4 Ro 25-1553-induced internalisation of VPAC$_2$ receptor

The effect of other peptide ligands on $VPAC_2$ receptor internalisation was also investigated. Cells were pretreated with Ro 25-1553, a specific $VPAC_2$ receptor agonist, at a range of concentrations (0.01, 0.1, 1 or 10 μM for 30 mins) at 37°C prior to fixation. Ro 25-1553 stimulated internalisation of the receptor in a concentration dependent manner and the results were comparable with those obtained with VIP (**Figure 4.8A-E**). In contrast, the predominantly plasma membrane staining of the $VPAC_2$-HA receptor was unaffected in cells pretreated with secretin (10 μM for 30 mins) at 37°C (**Figure 4.9A,B**). Secretin has a modest affinity for the $VPAC_1$ receptor but does not bind to the $VPAC_2$ receptor (Svoboda et al., 1994). These results suggest that $VPAC_2$ receptor internalisation is dependent upon the specificity and concentration of agonist used; as VIP and Ro 25-1553 were both effective at causing internalisation, whereas secretin was not.

4.2.5 Effect of incubation time on VIP-induced internalisation of the $VPAC_2$ receptor

The time course of internalisation was examined by preincubating $VPAC_2$-HA transfected cells with VIP for varying lengths of time at 37°C. Cells were pretreated in serum free medium or with VIP (10 μM for 2, 10 or 30 mins) and viewed at x40 (**Figure 4.10A-D**) or x100 magnification (**Figure 4.11A-D**). Treatment with 10 μM VIP at 37°C resulted in a shift in receptor distribution which became more pronounced with increasing incubation time. Short incubations with peptide (<5 mins) had little observable effect on receptor localisation (**Figure 4.11B**). However, after a 10 min incubation with peptide at 37°C the receptor can be seen in punctate structures in the periphery of the cell (**Figure 4.11C**). By 30 mins there is a marked reduction in receptor staining at the plasma membrane and the majority of receptor is located within the cell at a single juxtanuclear site (**Figure 4.11D**). Preincubation with VIP over a longer incubation time was performed, HEK293 cells stably transfected with the $VPAC_2$-HA receptor were pretreated with VIP (10 μM for 5, 10, 20, 30, 60, 90 and 120 mins) at 37°C (**Figure 4.12A-H**). The results parallel those described above. Extending the incubation time with VIP did not appear to cause any further changes in the distribution of internalised $VPAC_2$ receptors, which remained confined to a single juxtanuclear site. These data indicate that agonist-induced internalisation is dependent on the length of incubation time with agonist and in the continued presence of VIP the receptor remains internalised.

4.2.6 Effect of incubation temperature on VIP-induced internalisation of the $VPAC_2$ receptor

The effect of temperature on $VPAC_2$ receptor internalisation was investigated. Lowering incubation temperature would be expected to reduce cell membrane fluidity and movement. Accordingly, no internalisation of the $VPAC_2$-HA receptor was observed in cells incubated with VIP (10 μM for 30 mins) at 4°C (**Figure 4.9C**). However, when incubated with VIP (10 μM for 30 mins) at room temperature the $VPAC_2$ receptor was still able to internalise (**Figure 4.9D**). Marked internalisation was observed following incubation with VIP (10 μM for 30 mins) at 37°C (**Figure 4.9E**). Although quantitative comparisons of the internalisation are not possible with this technique, receptor internalisation did appear to be more prominent at 37°C than at room temperature. These data indicate that agonist-induced $VPAC_2$ receptor internalisation occurs as a function of incubation temperature.

4.2.7 Effect of hypertonic media on agonist-induced internalisation of the $VPAC_2$ receptor

Immunofluorescence studies were also undertaken to determine the effect of hypertonic media on cell structure and localisation of the $VPAC_2$ receptor following agonist treatment. Hyperosmolar sucrose prevents clathrin coated pits from forming by disrupting the formation of clathrin polyhedral lattices (Daukas and Zigmond, 1985). Cells maintained in serum free media, were fixed or pretreated with sucrose (0.45 M for 10 mins at 37°C) prior to fixation. No difference was observed in the predominantly plasma membrane location of $VPAC_2$ receptor under conditions of high osmolarity (**Figure 4.13A,B**). Preincubation of cells with VIP (10 μM for 30 mins) at 37°C led to marked internalisation of the $VPAC_2$ receptor to a single juxtanuclear site which was abolished in the presence of hypertonic sucrose (**Figure 4.13C,D**). No obvious differences in cell structure could by noted from these studies. These data show that pretreatment with hypertonic sucrose is able to prevent agonist-internalisation of the $VPAC_2$ receptor, in agreement with the results for [^{125}I]-helodermin internalisation described earlier (see section 4.2.2). Thus providing further evidence to support a clathrin-mediated mechanism for $VPAC_2$ receptor internalisation.

4.2.8 Effect of acetic acid on agonist-induced internalisation of the $VPAC_2$ receptor

Acidification of the cytosol is another established method of preventing receptor-mediated endocytosis. This treatment causes hyperpolymerisation of clathrin triskelions (Sandvig et al., 1987). To investigate the effect of mild acid treatment on $VPAC_2$ receptor internalisation we preincubated cells with acetic acid

(5 mM in 0.5 M HEPES: pH=5.0) for 5 mins at 37°C prior to treatment with VIP (10 μM). Acid treatment had no effect in untreated cells where fluorescent staining was predominantly seen at the plasma membrane (**Figure 4.14A,B**). However, internalisation of the $VPAC_2$ receptor was prevented in the presence of acetic acid (**Figure 4.14C,D**). These results are consistent with those obtained using hypertonic media and further support a role for clathrin-coated pits in $VPAC_2$ receptor internalisation.

4.2.9 Effect of phorbol 12-myristate 13-acetate (PMA) on agonist-induced internalisation of the $VPAC_2$ receptor

Phorbol 12-myristate 13-acetate (PMA) is a protein kinase C (PKC) activator which has been shown to inhibit potocytosis by preventing invagination of caveolae in monkey kidney and mouse fibroblast cell lines (Smart et al., 1994a). PMA markedly inhibits caveolae invagination at concentrations in excess of 0.5 μM (Smart et al., 1994a) and has been used at millimolar (mM) amounts for studies of internalisation of the human muscarinic receptor (Tolbert and Lameh, 1996). $VPAC_2$-HA receptors stably transfected in HEK293 cells were pretreated with vehicle (1% ethanol) or PMA (1 mM in 1% ethanol) for 30 mins at 37°C prior to fixation. The $VPAC_2$ receptor was predominantly located at the plasma membrane in cells preincubated with ethanol or PMA (**Figure 4.15A,B**). Treatment with 10 μM VIP for 30 mins resulted in marked $VPAC_2$ receptor internalisation which was unaffected by the presence of ethanol or PMA (**Figure 4.15C,D**). These results suggest that caveolae are not involved in agonist-induced internalisation of the $VPAC_2$ receptor stably transfected in HEK293 cells.

4.2.10 Effect of hypertonic media on desensitisation of the $VPAC_2$ receptor

The above results demonstrate that both hypertonic sucrose and mild acid treatment are effective means of preventing agonist-induced internalisation of the $VPAC_2$ receptor. Therefore pretreatment with high osmolar sucrose was used in experiments to determine the role of $VPAC_2$ receptor endocytosis in desensitisation of second messenger signalling. HEK293 cells stably transfected with the $VPAC_2$ and $VPAC_2$-HA receptors were preincubated in the absence or presence of 0.45 M sucrose for 10 mins at 37°C prior to stimulation with VIP (0, 1, 3 or 10 nM for 30 mins) at 37°C. After removal of the desensitising stimulus the cells were challenged with VIP (3 nM) for a further 30 mins at 37°C in the presence of IBMX. Cells stably transfected with the $VPAC_2$, displayed a statistically significant reduction in cAMP stimulation following pretreatment with 1 nM ($P<0.01$), 3 nM ($P<0.01$) or 10 nM VIP ($P<0.01$)

in the absence or presence of hypertonic sucrose **(Figures 4.16)**. Cells transfected with the VPAC$_2$-HA receptor displayed signification desensitisation when pretreated with 10 nM VIP (P<0.01) in the absence or presence of hypertonic sucrose **(Figure 4.17)**. However, moderate amounts of desensitisation were observed following pretreatment with lower concentrations of VIP. Dose response curves for the desensitisation of cAMP response for both receptors are presented in the previous chapter. Higher doses of VIP could not be used in these experiments due to persistent activation of AC (see section 3.3). Overall desensitisation of the cAMP response following pretreatment with VIP appeared to be unaffected by the presence of hypertonic sucrose, for both the VPAC$_2$ and VPAC$_2$-HA receptors, although the effect was not as marked for the latter. These results indicate that agonist-induced internalisation of the VPAC$_2$ receptor is not required for desensitisation of second messenger signalling in HEK293 cells.

Figure 4.1 Internalisation of [125I]-helodermin at 37°C in VPAC$_2$ and VPAC$_2$-HA receptor transfected HEK293 cells. Comparison of [125I]-helodermin internalisation between HEK293 cells stably transfected with the VPAC$_2$ (□) and VPAC$_2$-HA (■) receptors. Results are expressed as a percentage of total specific [125I]-helodermin bound (acid susceptible plus acid resistant) which is acid resistant (or internal) at 37°C. Graph shows means ± SD for two separate experiments performed in duplicate.
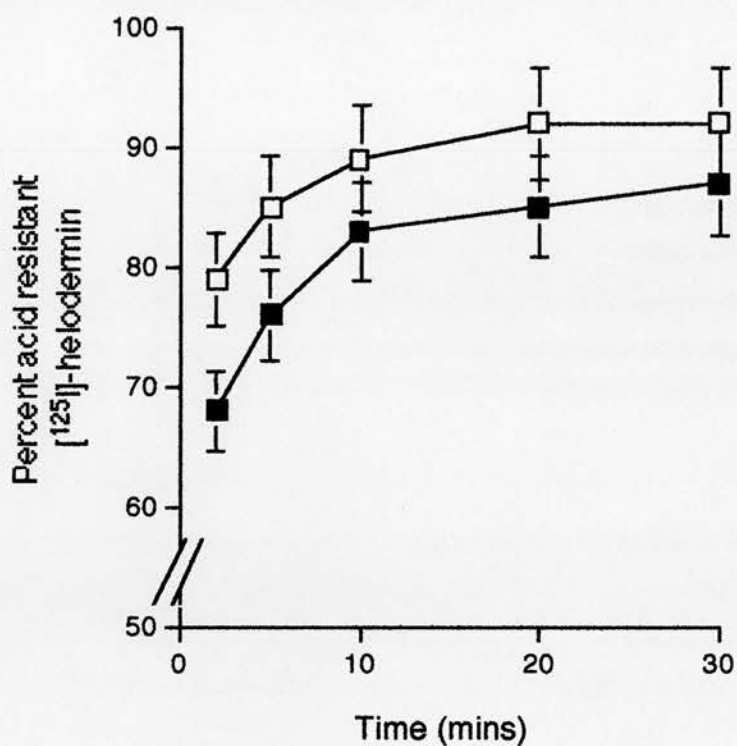
**Figure 4.2 Internalisation of [^{125}I]-helodermin at 18°C in VPAC$_2$ and VPAC$_2$-HA receptor transfected HEK293 cells.** Comparison of [^{125}I]-helodermin internalisation between HEK293 cells stably transfected with the VPAC$_2$ ($\square$) and VPAC$_2$-HA ($\blacksquare$) receptors. Results are expressed as a percentage of total specific [^{125}I]-helodermin bound (acid susceptible plus acid resistant) which is acid resistant (or internal) at room temperature. Values are means ± SEM for three separate experiments performed in duplicate.
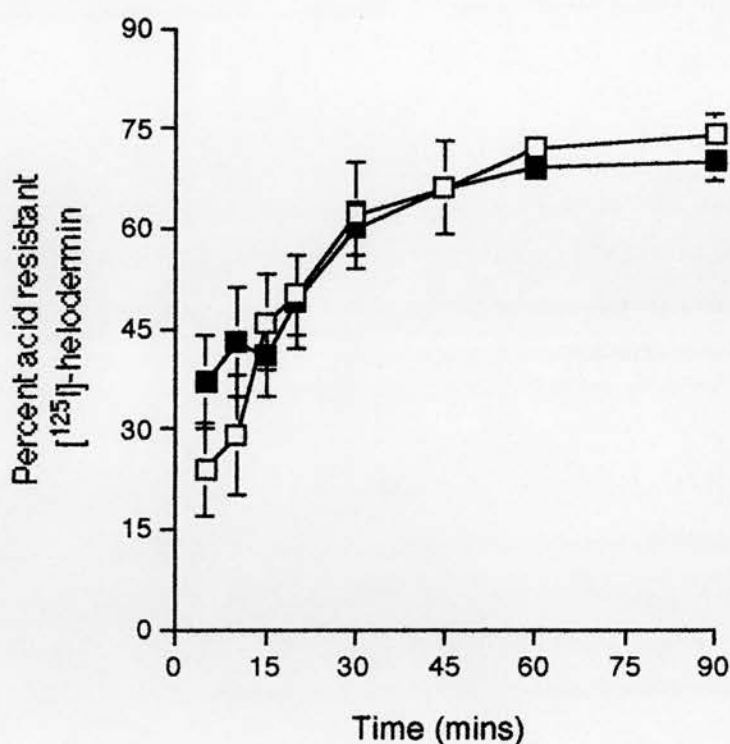
Figure 4.3 Effect of pretreatment with hypertonic sucrose on internalisation of [125I]-helodermin in VPAC$_2$ receptor transfected HEK293 cells. Comparison of internalisation of [125I]-helodermin in VPAC$_2$ receptor transfected HEK293 cells in the absence (□) or presence (△) of hypertonic sucrose. Cells were pretreated with sucrose (0.45 M for 30 mins) at room temperature prior to assay. Results are expressed as a percentage of total specific [125I]-helodermin bound (acid susceptible plus acid resistant) which is acid resistant (or internal). Graph shows means ± SEM for three separate experiments performed in duplicate.
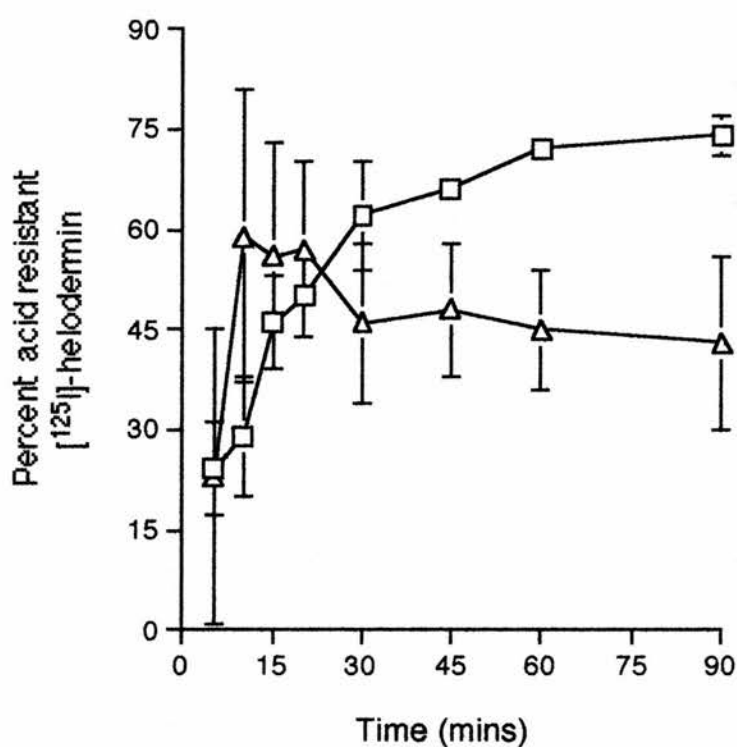
Figure 4.4 Effect of pretreatment with hypertonic sucrose on internalisation of [125I]-helodermin in VPAC$_2$-HA receptor transfected HEK293 cells. Comparison of internalisation of [125I]-helodermin in VPAC$_2$-HA receptor transfected HEK293 cells in the absence (■) or presence (▲) of hypertonic sucrose. Cells were pretreated with sucrose (0.45 M for 30 mins) at room temperature prior to assay. Results are expressed as a percentage of total specific [125I]-helodermin bound (acid susceptible plus acid resistant) which is acid resistant (or internal). Graph shows means ± SEM for three separate experiments performed in duplicate.
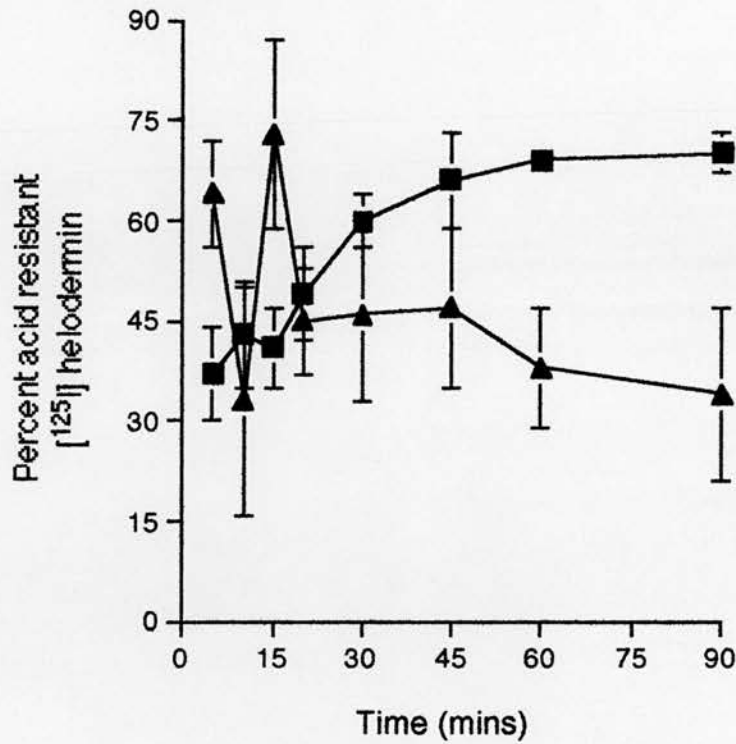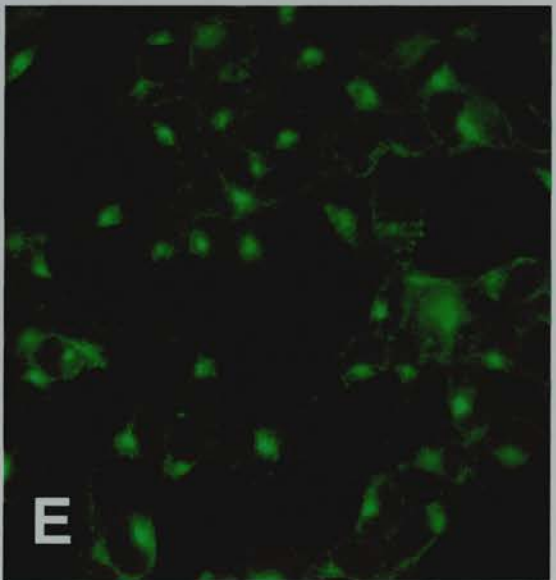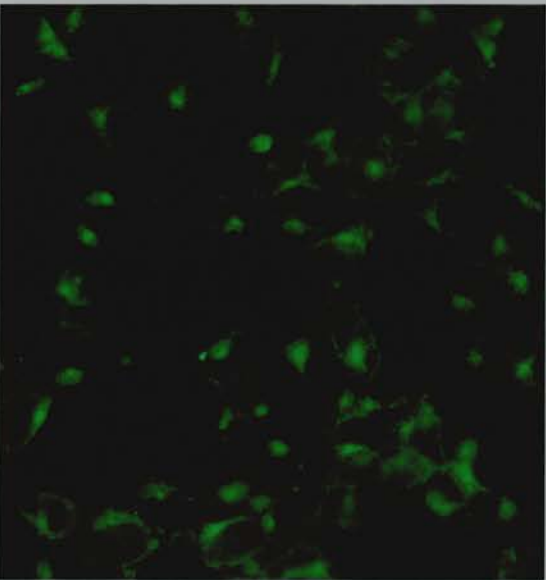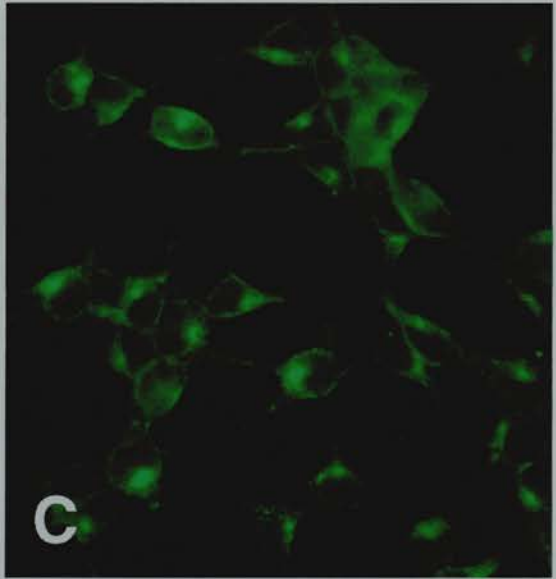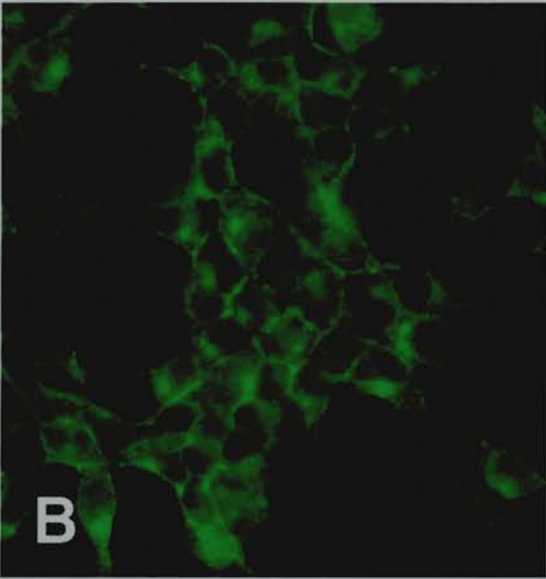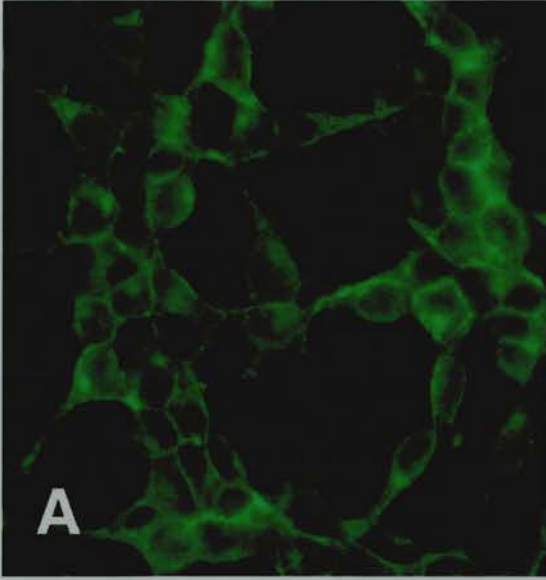
Figure 4.5 **Agonist-induced internalisation of the VPAC$_2$ receptor.**
HEK293 stably transfected with the VPAC$_2$ receptor (**A,B**) or VPAC$_2$-HA receptor
(**C,D**) were incubated in serum free medium at 37°C for 24 hrs prior to fixation and
viewed at x40 magnification under phase contrast (**A,C**) or fluorescence conditions
(**B,D**). VPAC$_2$-HA transfected cells were pretreated with VIP (10 μM for 30 mins) at
37°C prior to fixation and viewed at x40 magnification under phase contrast (**E**) or
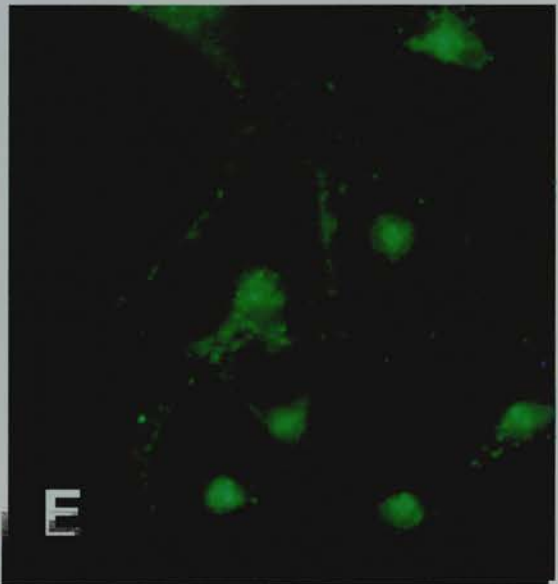fluorescence conditions (**F**).
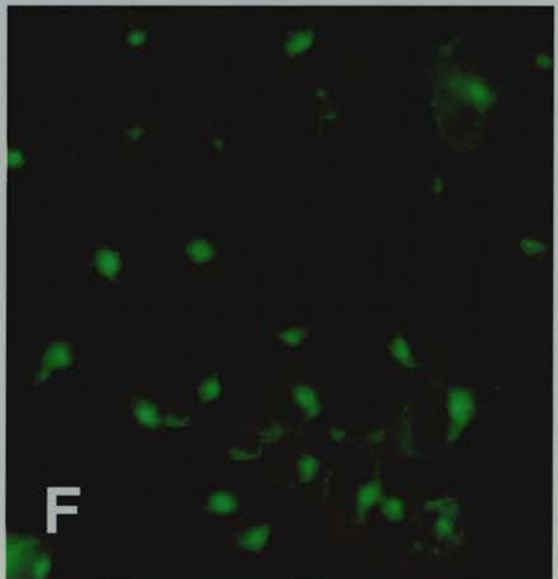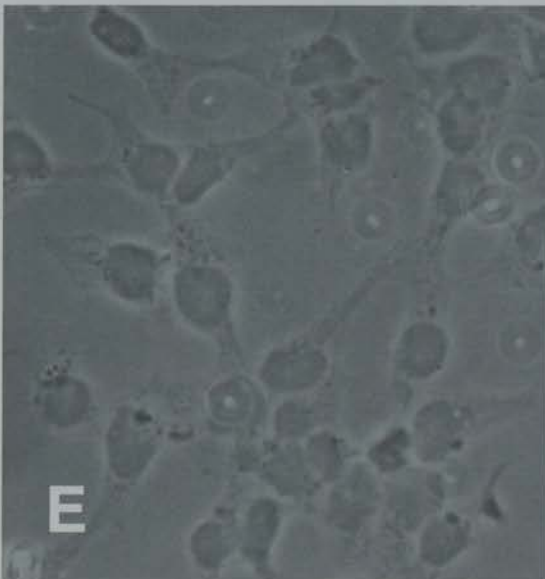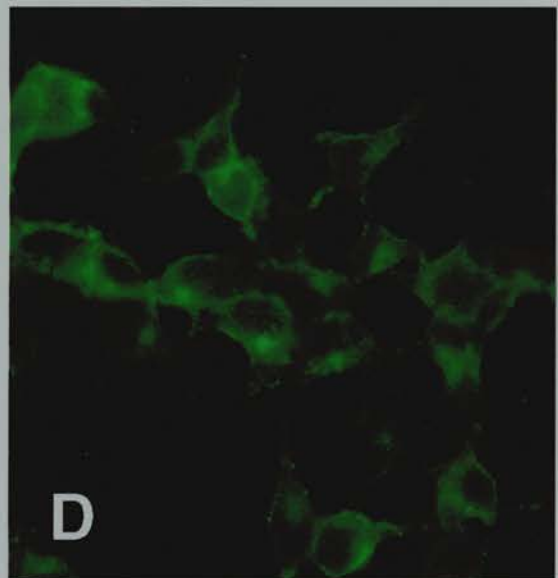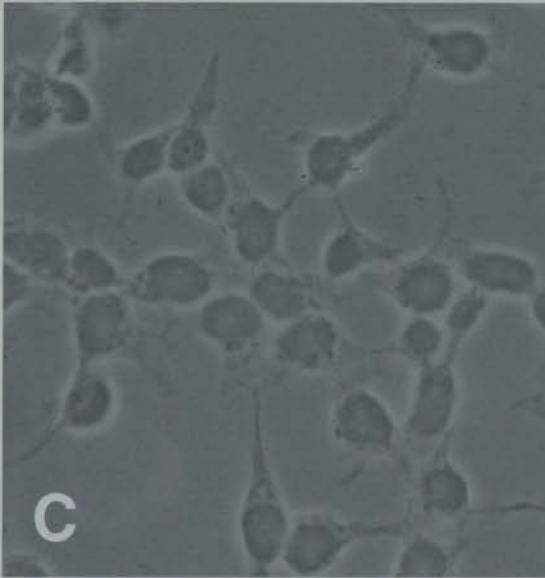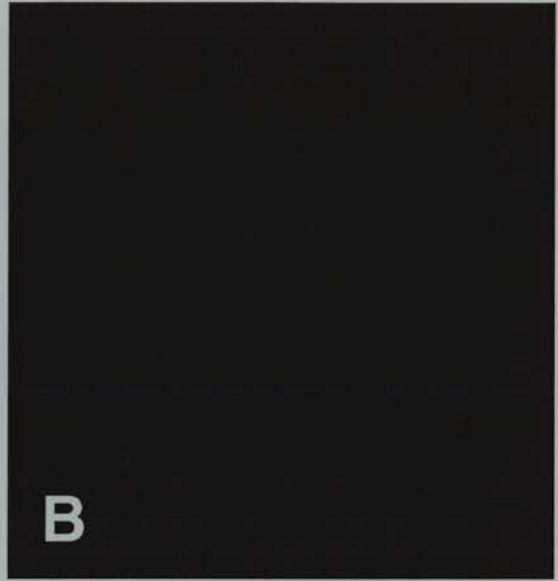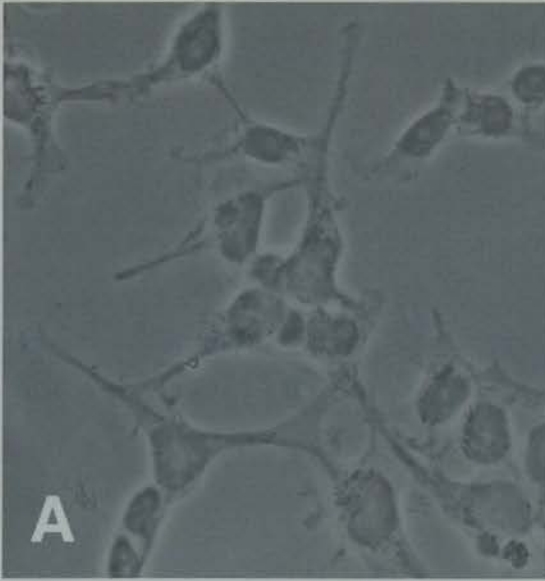
Figure 4.6   Effect of VIP concentration on agonist-induced internalisation of the VPAC$_2$ receptor. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated for 30 mins at 37°C with 0 (**A**), 0.01 μM (**B**), 0.1 μM (**C**), 1 μM (**D**) or 10 μM (**E**) VIP. Cells were fixed and viewed under fluorescence conditions at x40 magnification.

Figure 4.7 Effect of VIP concentration on agonist-induced internalisation of the VPAC$_2$ receptor. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated for 30 mins at 37°C with 0 (**A**), 0.01 μM (**B**), 0.1 μM (**C**), 1 μM (**D**) or 10 μM (**E**) VIP. Cells were fixed and viewed under fluorescence conditions at x100 magnification.
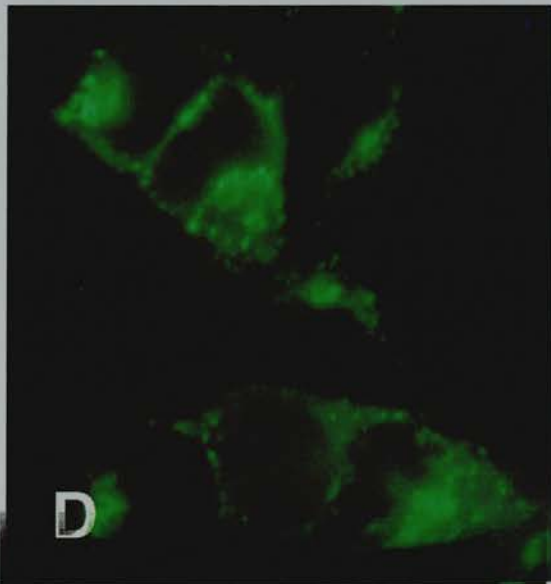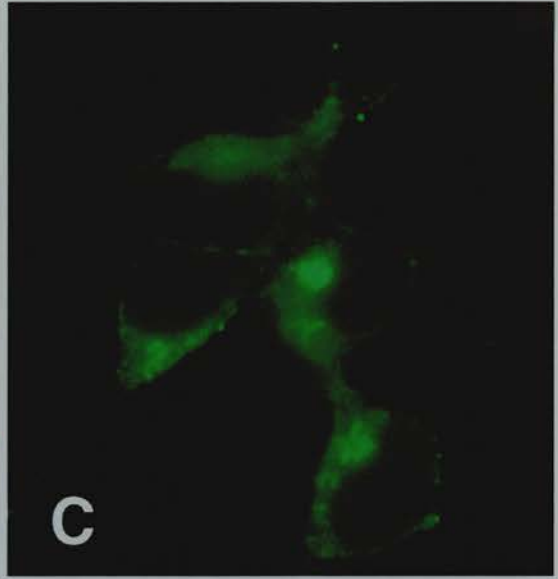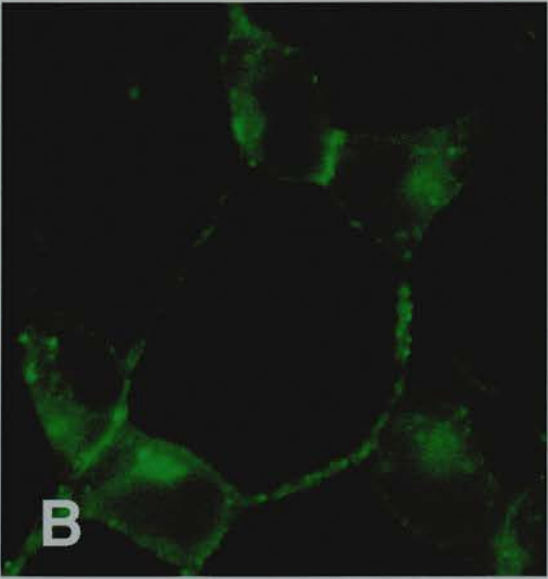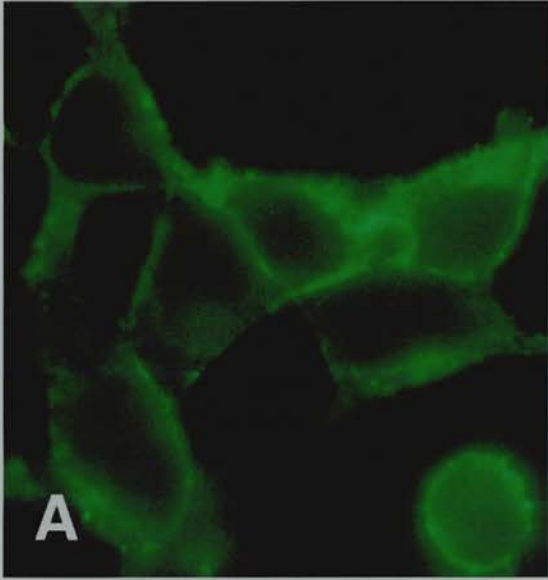
Figure 4.8 **Effect of Ro 25-1553 concentration on agonist-induced internalisation of the VPAC$_2$ receptor.** HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated for 30 mins at 37°C with 0 (**A**), 0.01 μM (**B**), 0.1 μM (**C**), 1 μM (**D**) or 10 μM (**E**) Ro 25-1553. Cells were fixed and viewed under fluorescence conditions at x40 magnification.
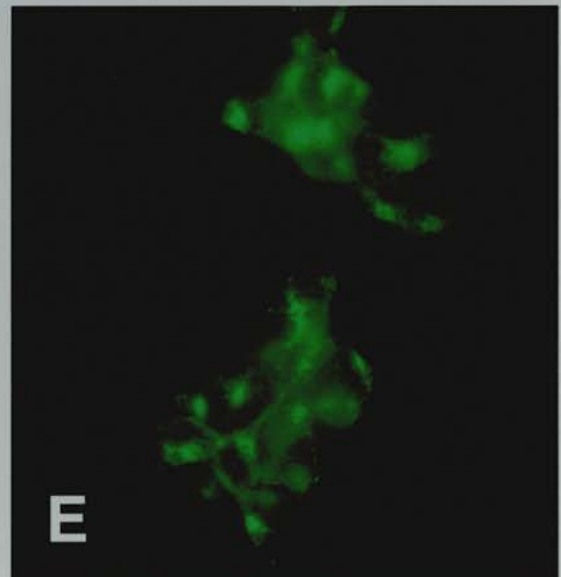
Figure 4.9 Effect of incubation temperature and pretreatment with secretin on localisation of the VPAC$_2$ receptor. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated for 30 mins at 37°C with 0 μM (**A**) or 10 μM (**B**) secretin or preincubated with 10 μM VIP for 30 mins at 4°C (**C**), room temperature (**D**) or 37°C (**E**). Cells were fixed and viewed under fluorescence conditions at x40 magnification.
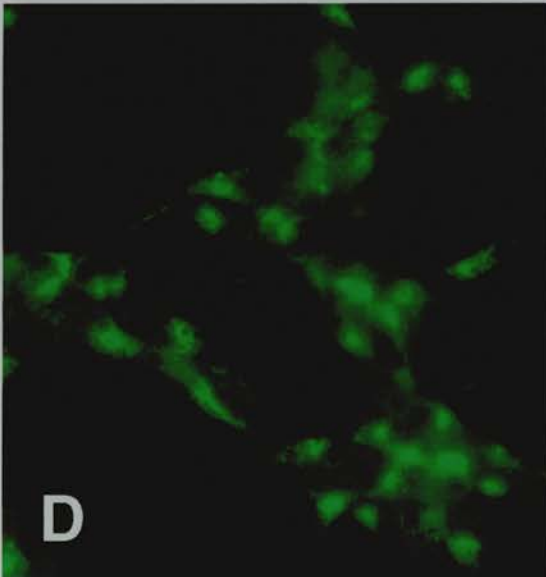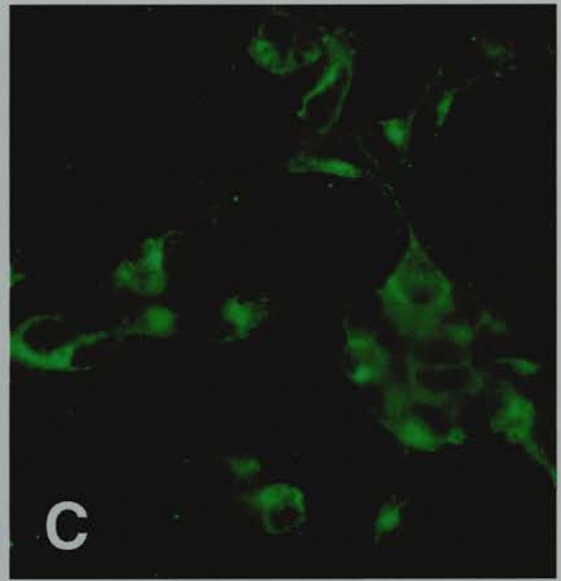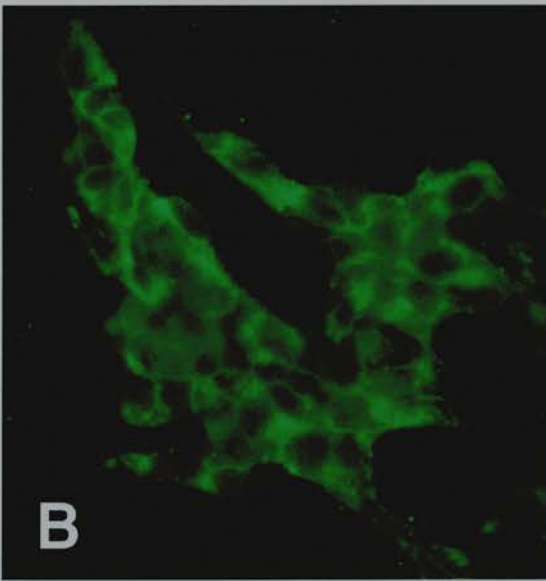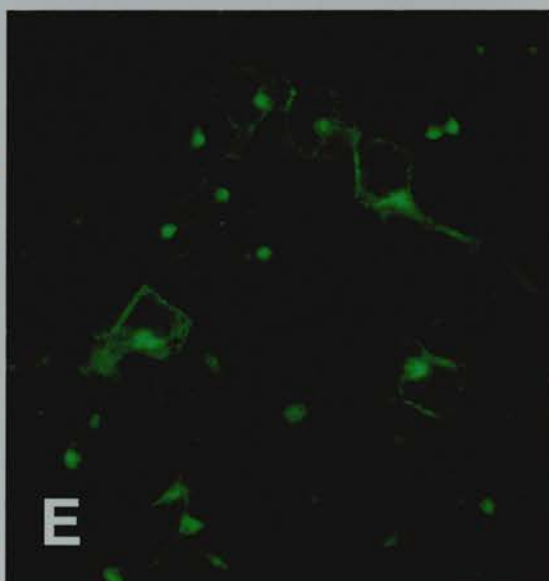
Figure 4.10   Effect of incubation time on agonist-induced internalisation of the VPAC$_2$ receptor. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with 10 μM VIP at 37°C for 0 (**A**), 2 mins (**B**), 10 mins (**C**) or 30 mins (**D**). Cells were fixed and viewed under fluorescence conditions at x40 magnification.
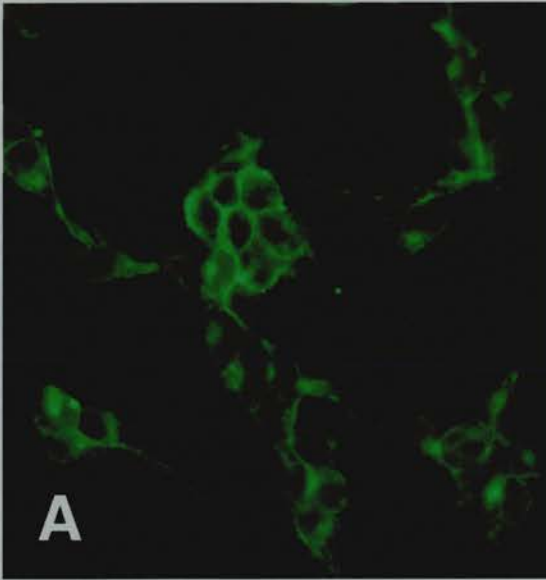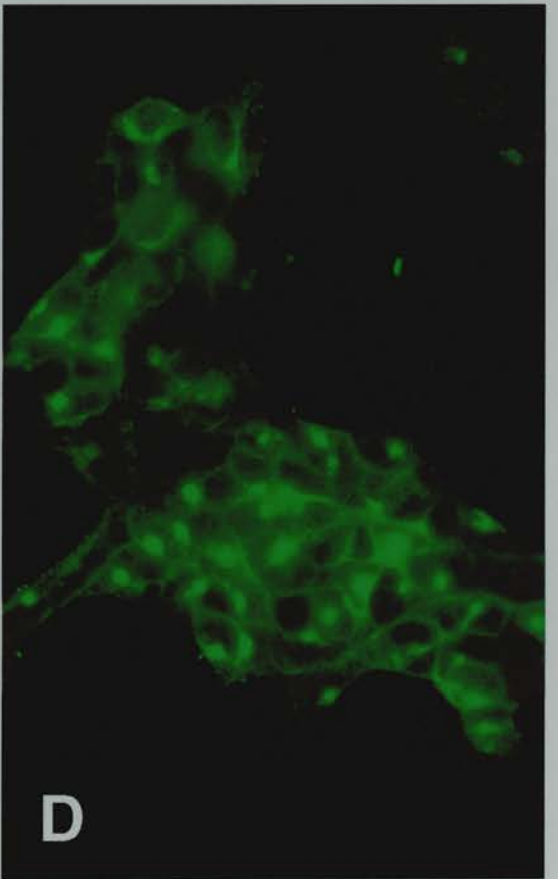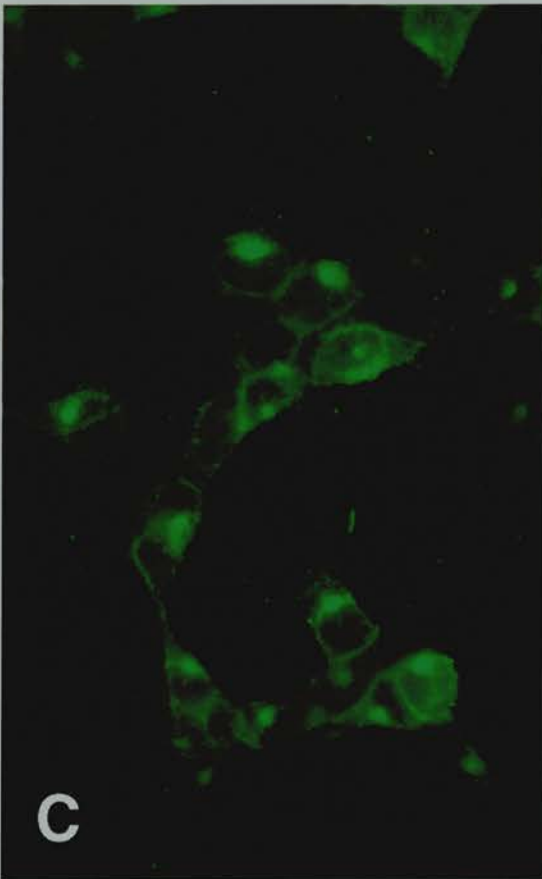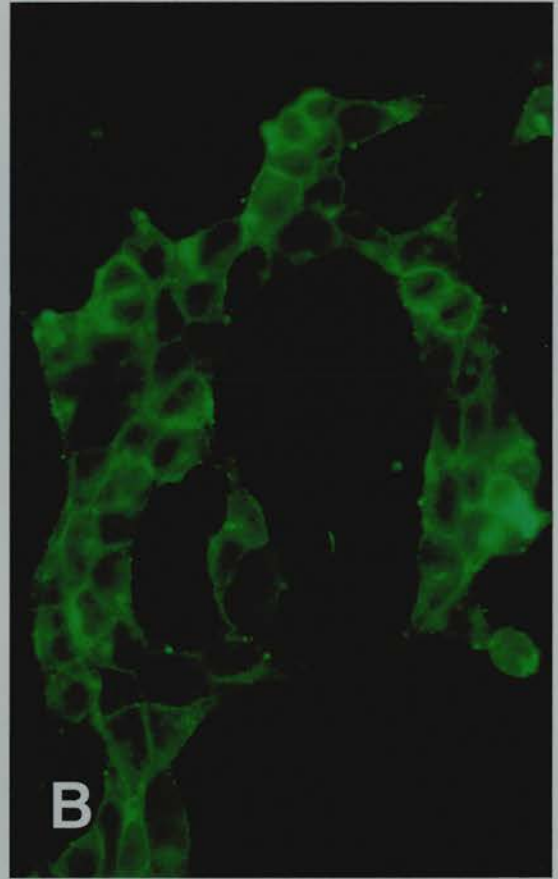
Figure 4.11   Effect of incubation time on agonist-induced internalisation of the VPAC$_2$ receptor. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with 10 μM VIP at 37°C for 0 (**A**), 2 mins (**B**), 10 mins (**C**), or 30 mins (**D**). Cells were fixed and viewed under fluorescence conditions at x100 magnification.
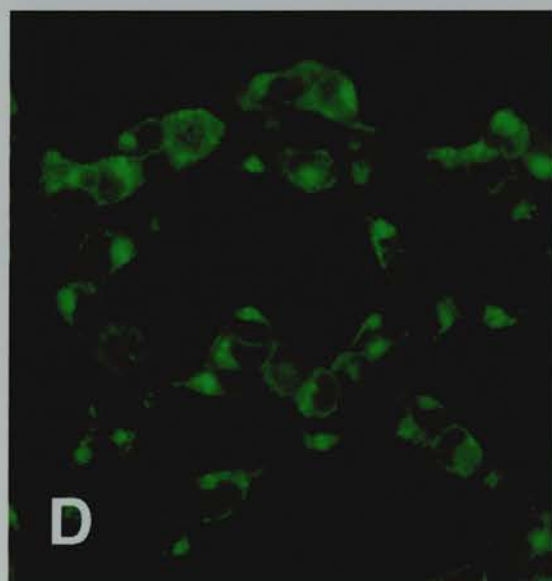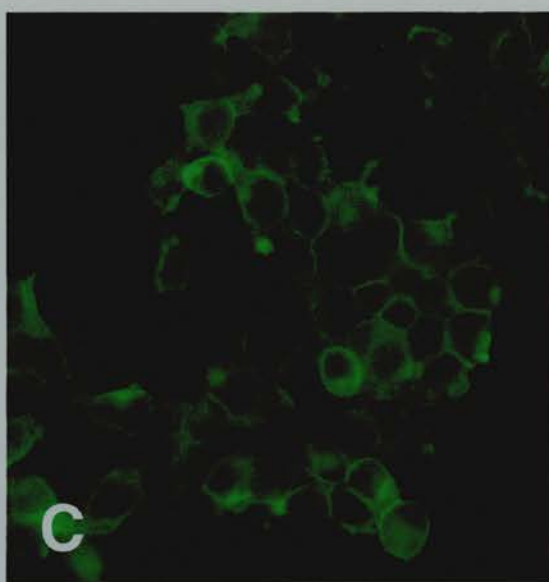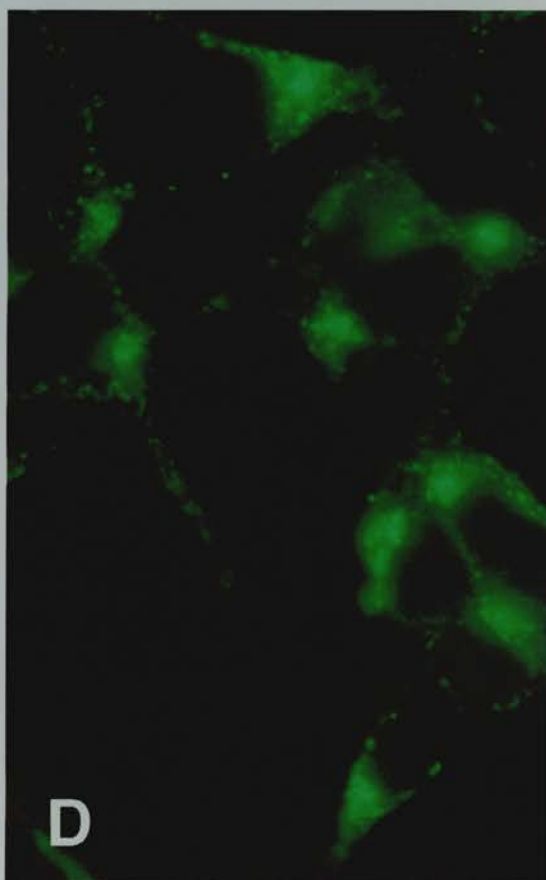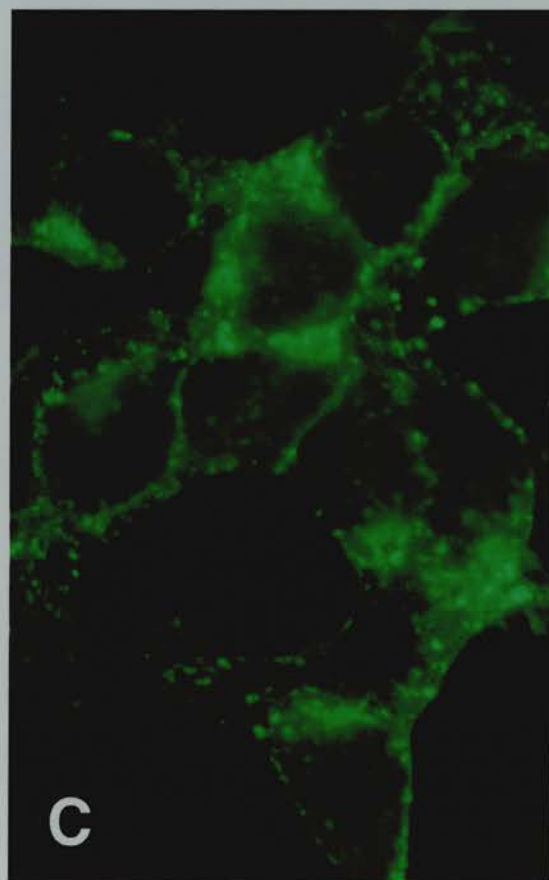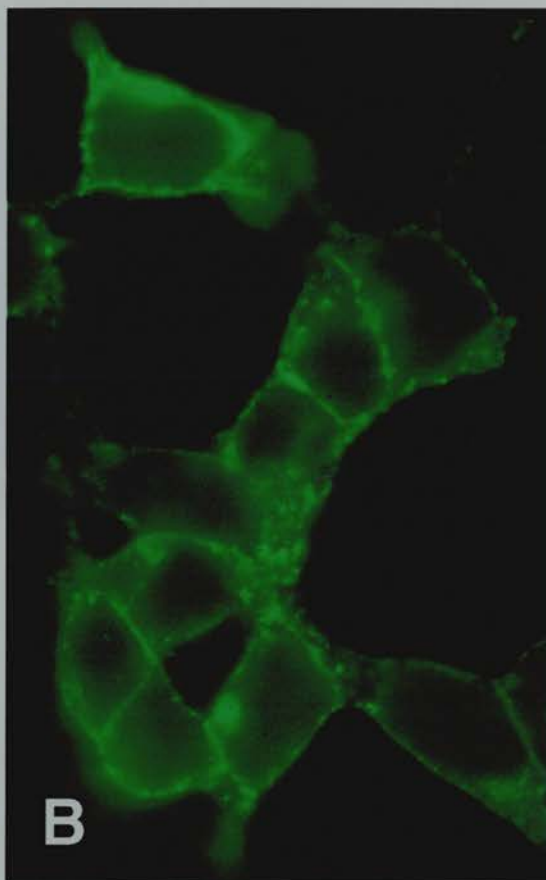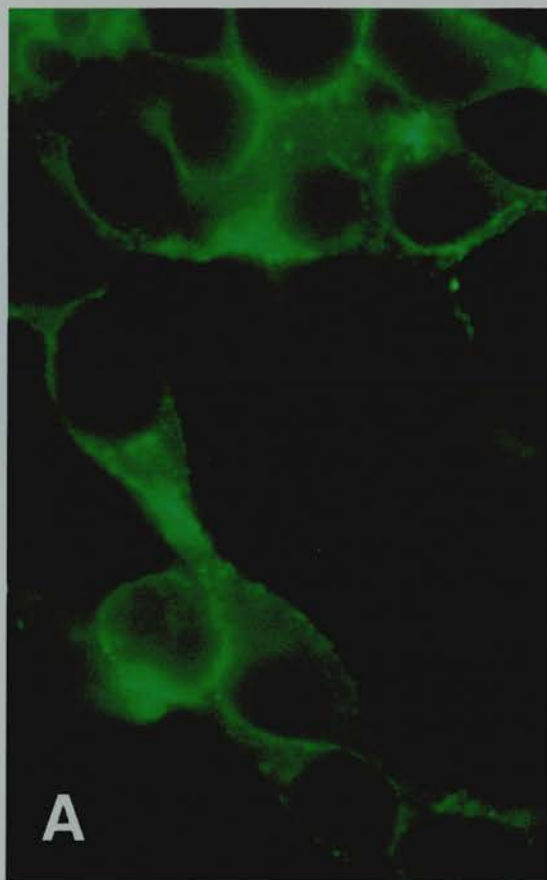
Figure 4.12   Effect of prolonged incubation time on agonist-induced internalisation of the VPAC$_2$ receptor. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with 10 μM VIP at 37°C for 0 (**A**), 5 mins (**B**), 10 mins (**C**), 20 mins (**D**), 30 mins (**E**), 60 mins (**F**), 90 mins (**G**) or 120 mins (**H**). Cells were fixed and viewed under fluorescence conditions at x100 magnification.
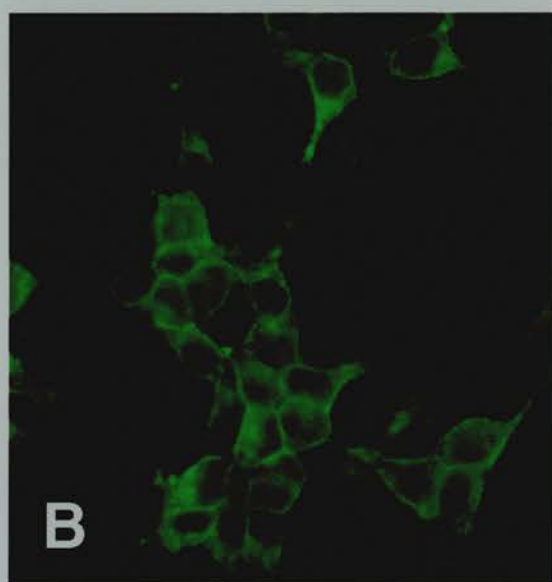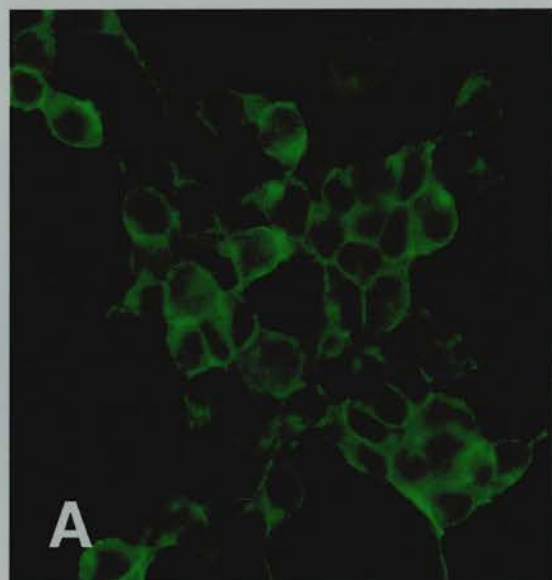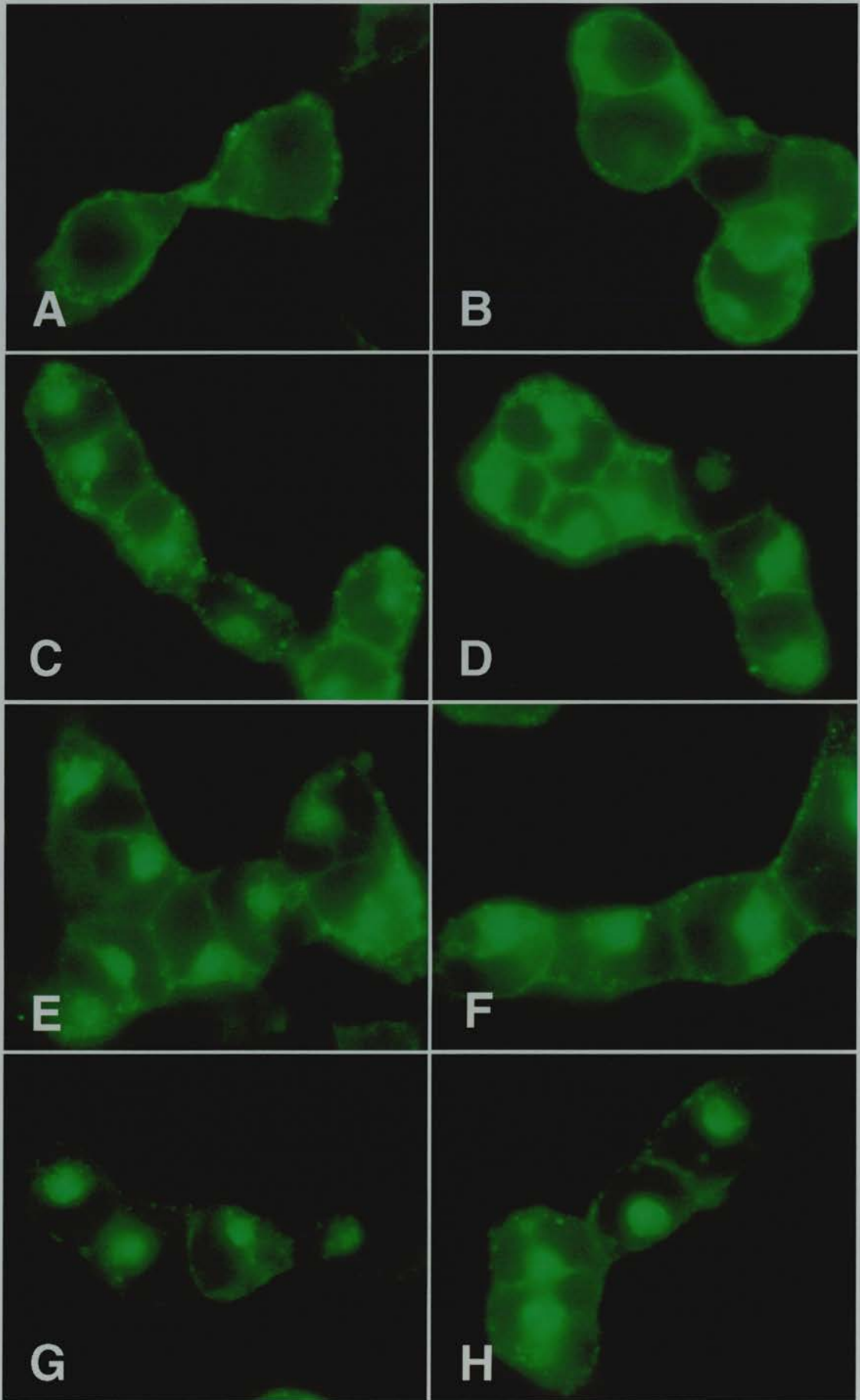
Figure 4.13 **Effect of hypertonic media on agonist-induced internalisation of the VPAC$_2$ receptor.** HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with serum free medium (**A**, **C**) or 0.45 M sucrose (**B**, **D**) for 10 mins at 37°C prior to stimulation with 0 (**A**, **B**) or 10 μM (**C**, **D**) VIP for 30 mins at 37°C. Cells were then fixed and viewed under fluorescence conditions at x100 magnification.
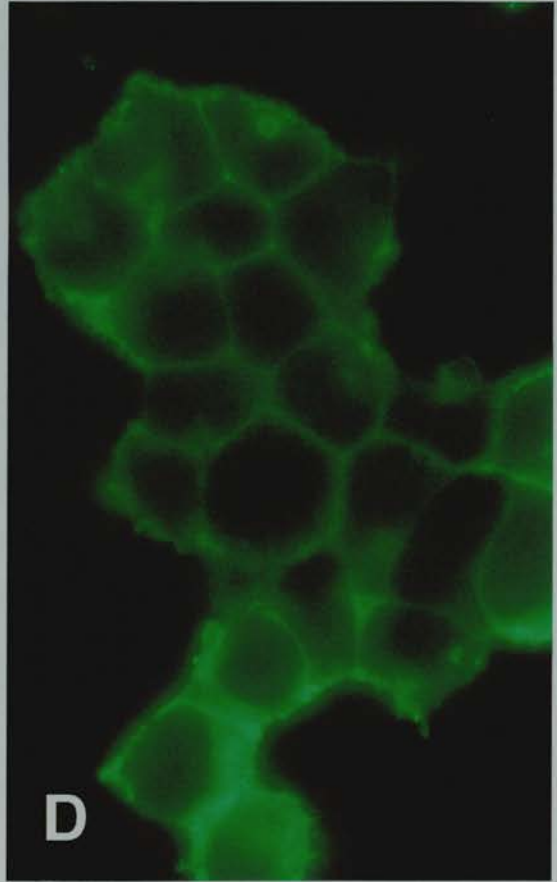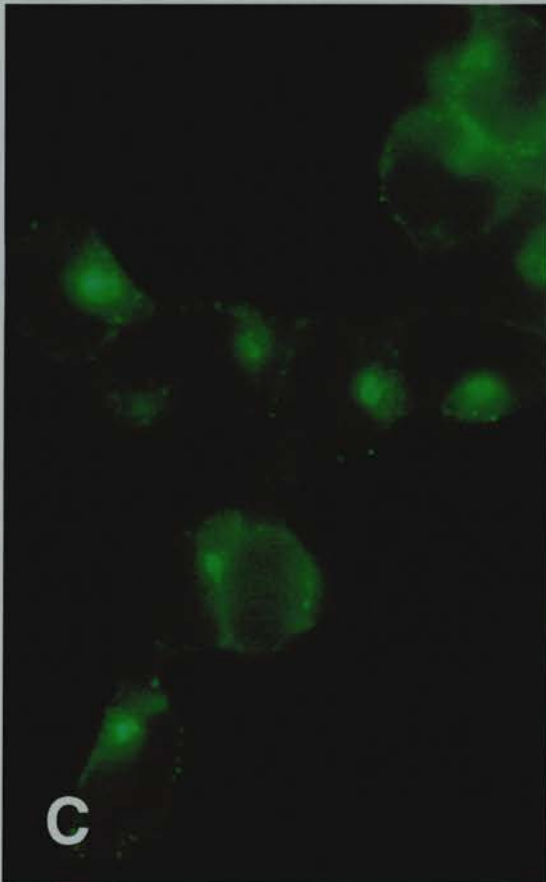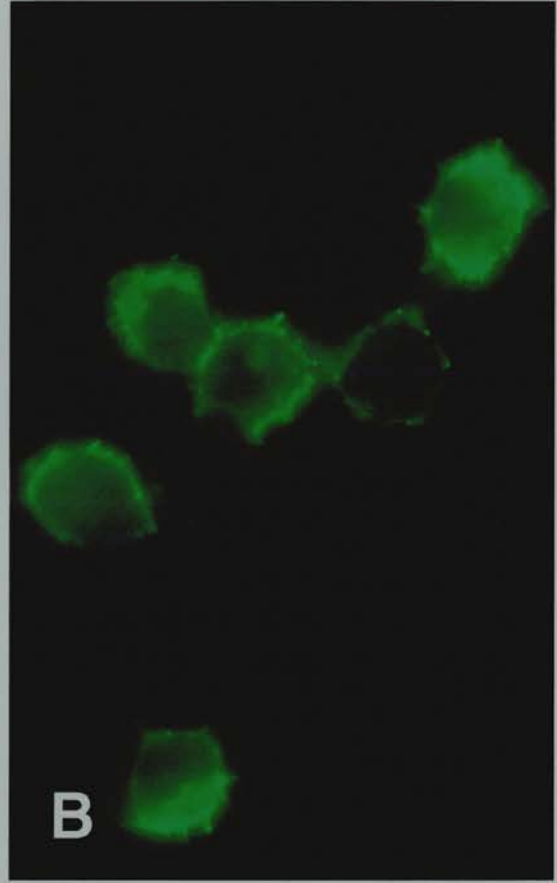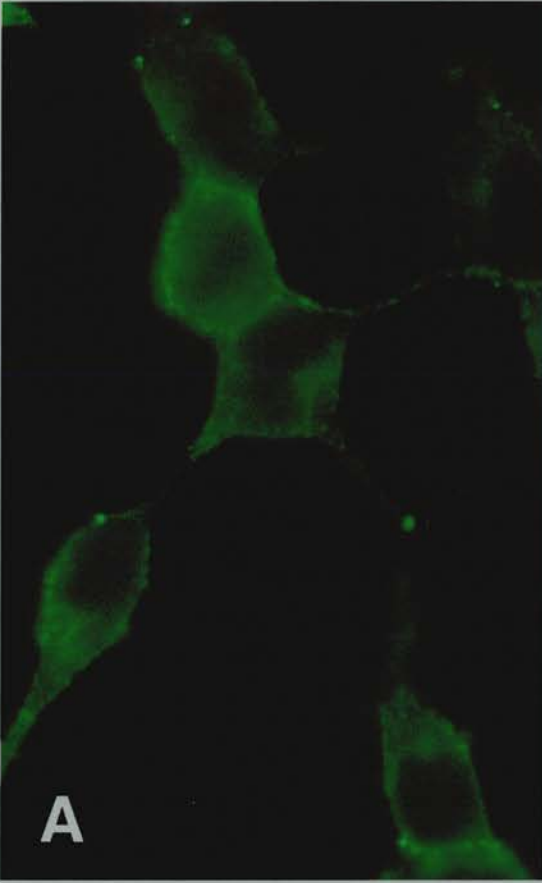
Figure 4.14 Effect of acetic acid on agonist-induced internalisation of the VPAC$_2$ receptor. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with serum free medium (**A, C**) or 5 mM acetic acid in 0.5 M HEPES (pH = 5.0) for 5 mins at 37°C (**B, D**), prior to stimulation with 0 (**A, B**) or 10 μM (**C, D**) VIP for 30 mins at 37°C. Cells were then fixed and viewed under fluorescence conditions at x100 magnification.
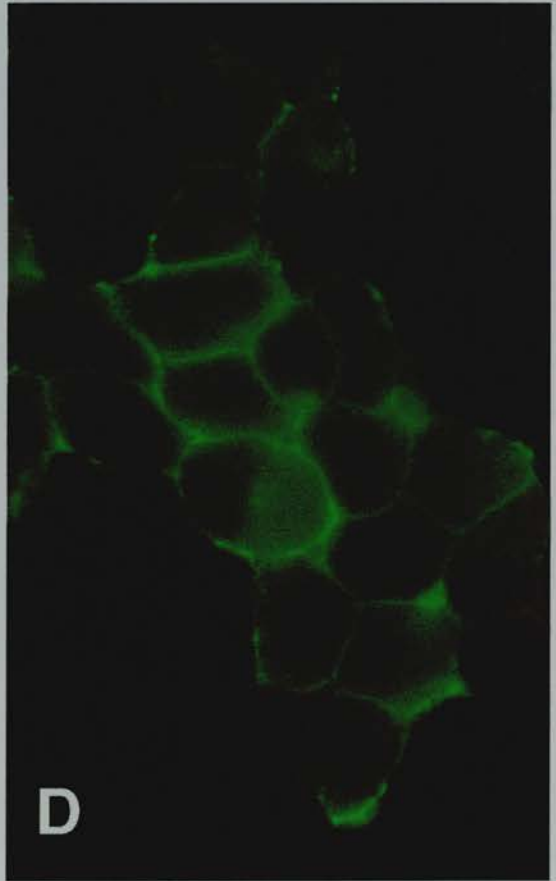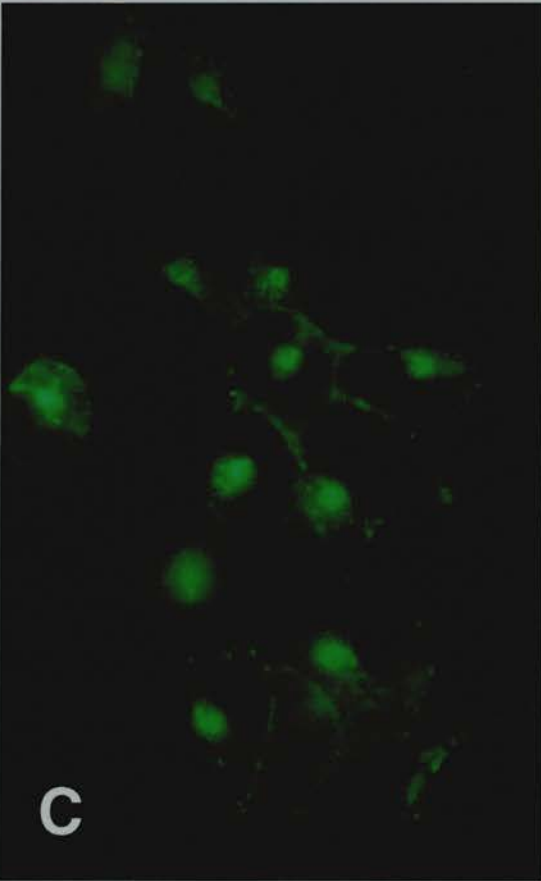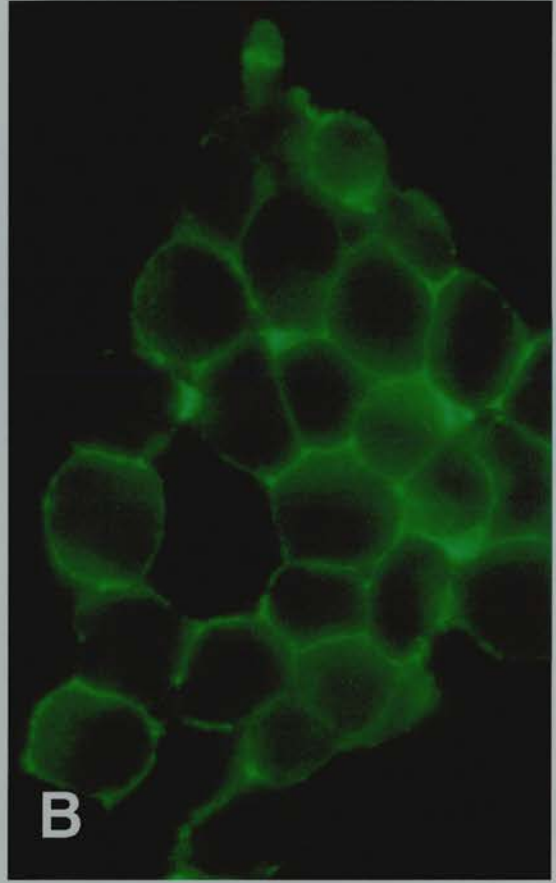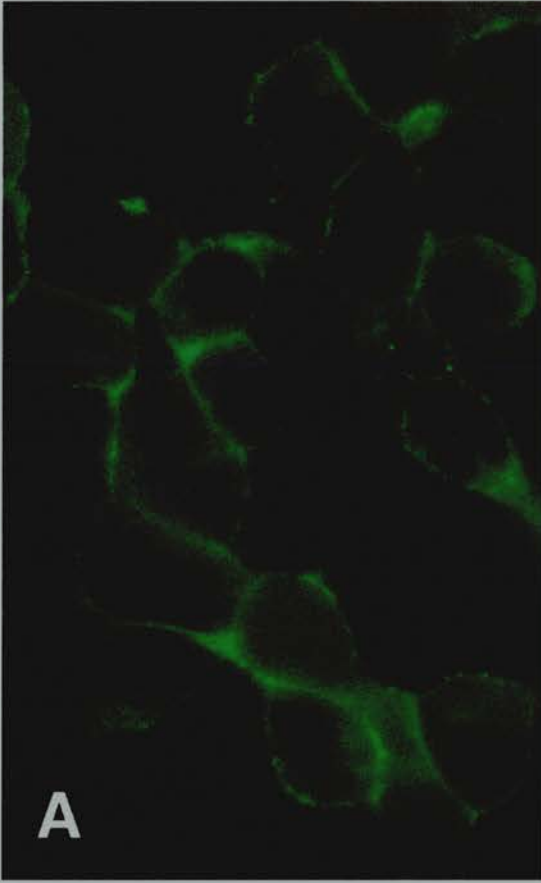
Figure 4.15 **Effect of phorbol 12-myristate acetate (PMA) on agonist-induced internalisation of the VPAC$_2$ receptor.** HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with vehicle (1% ethanol) (**A, C**) or PMA in 1% ethanol (**B, D**) for 30 mins at 37°C prior to stimulation with 0 (**A, B**) or 10 μM (**C,D**) VIP for 30 mins at 37°C. Cells were then fixed and viewed under fluorescence conditions at x100 magnification.
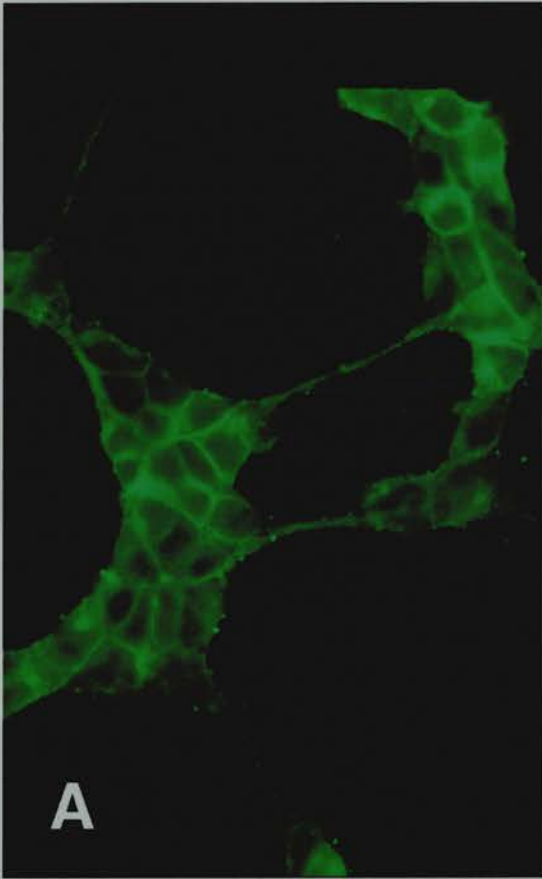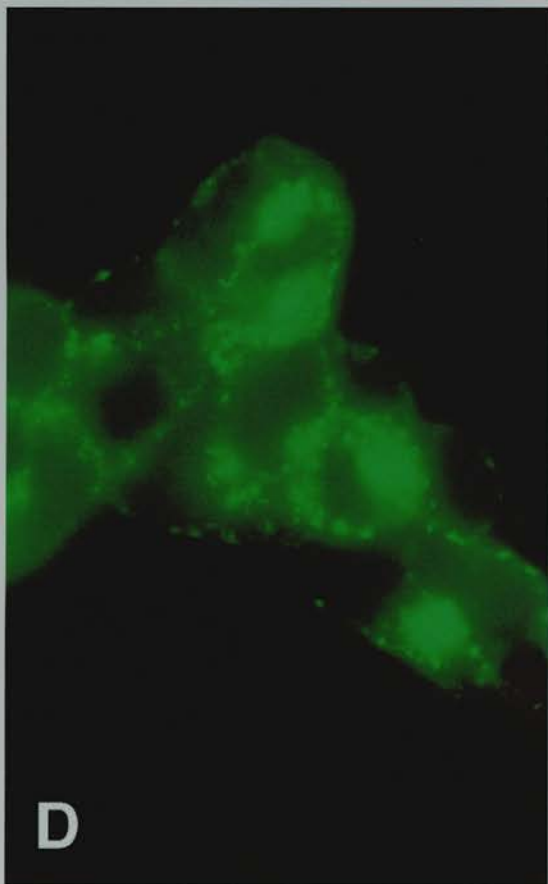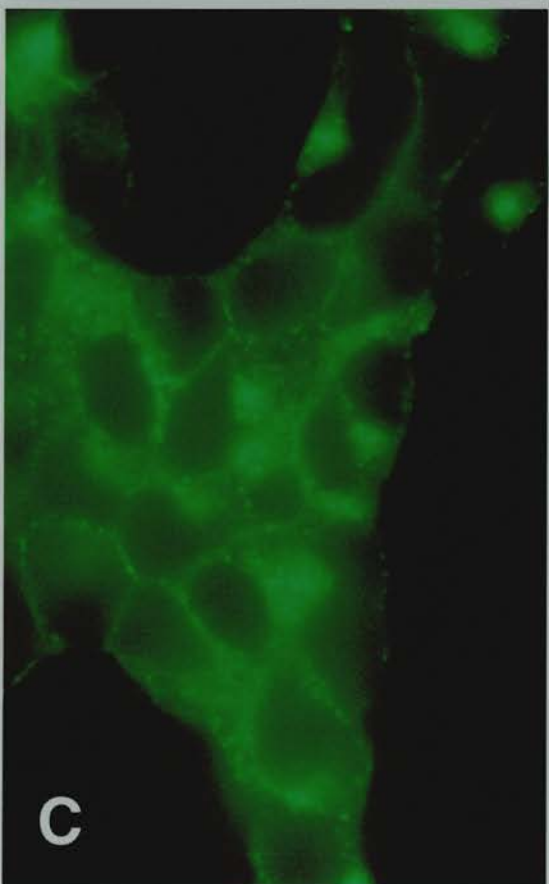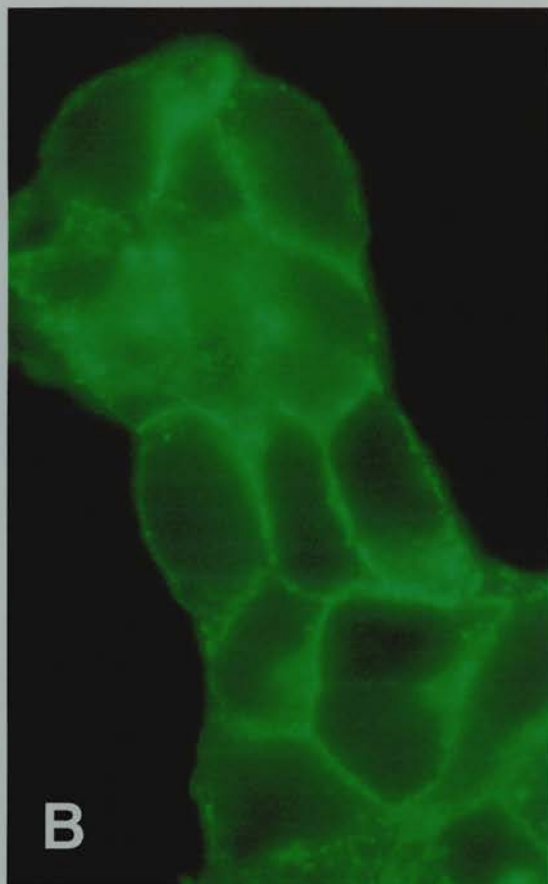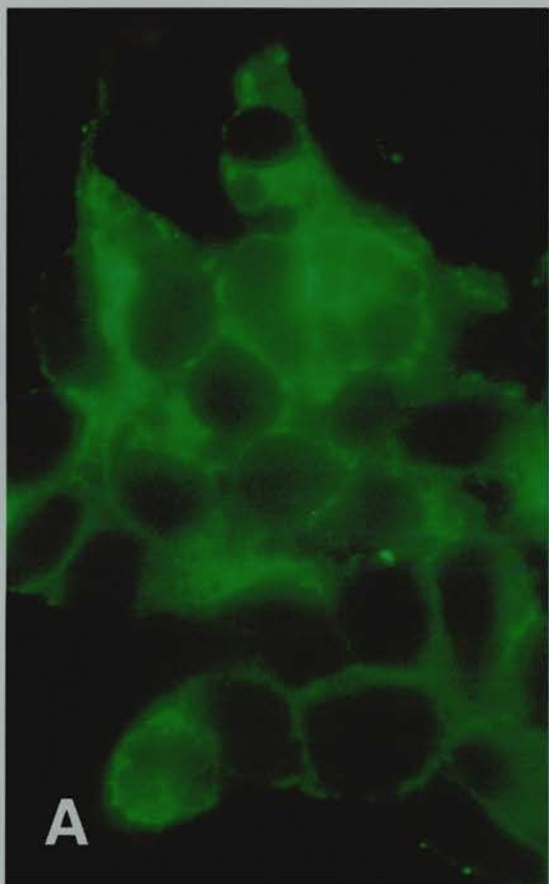
Figure 4.16   Effect of hypertonic sucrose on agonist-induced desensitisation of the cAMP response in HEK293 cells stably transfected with the VPAC$_2$ receptors. HEK293 cells stably transfected with the VPAC$_2$ receptor were pretreated with serum free medium (white columns) or 0.45 M sucrose (shaded columns) for 10 mins at 37°C, then incubated with 0, 1 nM, 3 nM or 10 nM VIP for 30 mins at 37°C in the absence or presence of hypertonic sucrose. The cells were washed extensively and desensitisation of the cAMP response was assessed by restimulating the cells with 3 nM VIP for a further 30 mins at 37°C in the presence of IBMX. cAMP levels were assessed via radioimmunoassay. Results are expressed as percentage maximal cAMP relative to control (untreated) cells and are means ± SEM for 3-4 experiments performed in triplicate (P<0.05*, P<0.01**).

Figure 4.17 Effect of hypertonic sucrose on agonist-induced desensitisation of the cAMP response in HEK293 cells stably transfected with the VPAC$_2$-HA receptor. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were pretreated with serum free medium (white columns) or 0.45 M sucrose (shaded columns) for 10 mins at 37°C, then incubated with 0, 1 nM, 3 nM or 10 nM VIP for 30 mins at 37°C in the absence or presence of hypertonic sucrose. The cells were washed extensively and desensitisation of the cAMP response was assessed by restimulating the cells with 3 nM VIP for a further 30 mins at 37°C in the presence of IBMX. cAMP levels were assessed via radioimmunoassay. Results are expressed as percentage maximal cAMP relative to control (untreated) cells and are means ± SEM for 3-4 experiments performed in triplicate ($P<0.05^*$, $P<0.01^{**}$).

4.3. Discussion

Internalisation of [^{125}I]-helodermin

Data presented in this chapter demonstrates that cells stably transfected with VPAC$_2$ and VPAC$_2$-HA receptors are able to internalise peptide rapidly at 37°C. It has previously been reported that reducing assay temperature slows the rate of VIP internalisation (Boissard et al., 1986). Hence the assay was repeated at room temperature to permit comparison of internalisation kinetics between the VPAC$_2$ and VPAC$_2$-HA receptors over a longer time course. Importantly, no difference was observed in the internalisation kinetics of [^{125}I]-helodermin between the VPAC$_2$ and VPAC$_2$-HA receptors. As the length of incubation with peptide increases the rate of internalisation slows, reaching a steady state after approximately 60 mins. This reduction in the rate of internalisation is predicted by the kinetic model of receptor endocytosis and recycling developed by Koenig and Edwardson (1997); where the rate of endocytosis is dependent upon the number of receptors at the cell surface. Therefore as cell surface receptor numbers decrease so does the endocytic rate and internalisation approaches steady state (Koenig and Edwardson, 1997). The internalisation of [^{125}I]-helodermin in VPAC$_2$ and VPAC$_2$-HA transfected HEK293 cells at 37°C, was remarkably rapid compared with previous studies of the internalisation of [^{125}I]-VIP (for reviews see Luis et al., 1988; Rosselin et al., 1988). This could be caused by inherent variability between different cell lines or the different pharmacology of these peptides. Accordingly Robberecht et al. (1989) found that helodermin dissociated more slowly from receptors than VIP and suggested that the latter may be preferable for investigating receptor internalisation (Robberecht et al., 1989a).

A model for receptor-mediated endocytosis of VIP has been derived, using a similar technique, by Luis et al. (1998) from studies of HT-29 cells. These cells express an endogenous VPAC$_1$ receptor. This group found that [^{125}I]-VIP internalises rapidly in HT-29 cells, with a $t\frac{1}{2}$=3 mins, approximately 60% of the peptide was intracellular following a 10 min incubation at 37°C (Muller et al., 1985). Similar studies have also reported rapid internalisation of [^{125}I]-VIP in AR 4-2 J cells, which express an endogenous PAC$_1$ receptor (Svoboda et al., 1988) and in human glioma cells, which express a helodermin-responsive receptor that is likely to correspond to the VPAC$_2$ receptor (Nielsen et al., 1990). These studies suggest that all of the VIP/PACAP receptors undergo agonist-induced internalisation. The time course for peptide internalisation in these cell lines is similar, which indicates that they may endocytose

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by a similar pathway. It would be interesting to compare peptide internalisation in the same cell line stably transfected cDNA constructs of each of the VIP/PACAP receptors.

Effect of an inhibitors of clathrin-dependent endocytosis on internalisation of [^{125}I]-helodermin

Preincubation with sucrose significantly reduced the extent of [^{125}I]-helodermin internalisation. Nonetheless some internalisation still occurred at early time points during the assay. Two explanations are possible: firstly, that the amount of sucrose used was not sufficient to completely block the endocytic pathway, or secondly, that internalisation can occur by a mechanism that does not require the formation of clathrin-coated pits. In view of the work from Daukas and Zigmond (1985), who found maximal inhibition with 0.75 M sucrose, it seems that the former explanation is most likely. However, it is impossible to exclude a clathrin-independent mechanism of endocytosis for the VPAC$_2$ receptor expressed in HEK293 cells or other cell lines. No studies of the effect of hypertonic media on VIP internalisation have been published to date, although a few studies have utilised alternative chemical agents to disrupt peptide internalisation. One report found that potassium depletion caused a moderate inhibition (~23%) of [^{125}I]-VIP internalisation in HT-29 cells; which is also indicative of a clathrin-mediated mechanism of receptor endocytosis (Phan et al., 1992). For future experiments it would be interesting to use higher concentrations of sucrose (0.75 M) or alternative inhibitors of clathrin-mediated endocytosis to confirm this finding. In addition, measurements of degraded [^{125}I]-helodermin released into the cell medium after internalisation could be made, these experiments would be pertinent to studies involving sucrose treatment as low levels of degraded peptide would be expected in the incubation medium if internalisation is completely blocked. Finally it would be interesting to establish whether peptide internalisation can be completely prevented by reducing the assay temperature further.

Agonist-induced internalisation of the VPAC$_2$ receptor

The immunofluorescence data presented in this chapter provides the first direct demonstration of agonist-induced internalisation of the VPAC$_2$ receptor. In cells maintained in serum free medium the VPAC$_2$ receptor is located predominantly at the plasma membrane. Treatment with agonist caused a shift in the distribution of the VPAC$_2$ receptor. After a 10 min incubation at 37°C the receptor appeared in punctate structures at the plasma membrane. These structures correspond to the description

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given for sorting endosomes by the work of other groups (Dunn et al., 1989). Continued incubation in the presence of agonist caused the receptor to shift dramatically from the plasma membrane to a discrete juxtanuclear site. $VPAC_2$ receptor internalisation was dependent upon incubation time, temperature and peptide concentration, in agreement with previous studies of iodinated peptide internalisation (see section 1.5.7).

Effect of inhibitors of clathrin-dependent and -independent endocytosis on agonist-induced internalisation of the $VPAC_2$ receptor

Pre-exposure of cells to hypertonic sucrose or acetic acid has previously been reported to prevent formation of the clathrin lattice (Daukas and Zigmond, 1985; Sandvig et al., 1987). Both of these treatments inhibited agonist-induced internalisation of the $VPAC_2$ receptor, suggesting that the $VPAC_2$ receptor is endocytosed via a clathrin-dependent mechanism. Agonist-induced internalisation of many class I GPCRs has been found to be inhibited by pretreatment with hypertonic sucrose or mild acid and these techniques are well established as indicators of a clathrin-mediated mechanism of internalisation. This technique has not been applied to many members of the class II GPCRs. One exception is the PTH/PTHrP receptor, where pretreatment with hypertonic sucrose was found to inhibit its internalisation (Huang et al., 1995a). PMA, an inhibitor of calveolae-mediated internalisation, had no effect on receptor internalisation indicating that calveolae are not involved in this process. Nonetheless, this does not exclude the possibility that calveolae or other protein coated or non-coated vesicles could be involved in the internalisation of $VPAC_2$ receptors in other cell lines or tissues. Indeed several GPCRs have been shown to utilise both clathrin-coated pits and calveolae-mediated mechanisms to internalise depending on the cell line they are expressed in (see section 1.5.1).

Role of agonist-induced internalisation of the $VPAC_2$ receptor

Inhibition of agonist-induced internalisation with hypertonic sucrose was used to investigate whether this process is involved in short term desensitisation of the cAMP response of the $VPAC_2$ receptor. Cells were desensitised with VIP and then restimulated in the absence or presence of hypertonic media and the levels of cAMP determined. The presence of hypertonic sucrose had no effect on the extent of desensitisation observed for both $VPAC_2$ and $VPAC_2$-HA receptors. This finding suggests that agonist-induced internalisation is not an absolute requirement for $VPAC_2$ receptor desensitisation. It is possible that sucrose treatment could have any number of effects on ligand binding or receptor G-protein coupling and so it would

be informative to confirm its effect on VPAC$_2$ receptor desensitisation using alternative methods. Several techniques could be applied to the VPAC$_2$ receptor to confirm that receptor internalisation is independent of desensitisation: (1) a comparison of the time course of desensitisation and internalisation processes; (2) alternative pharmacological agents could be used to block internalisation, examples include concanavalin A, phenylarsine oxide and mild acid treatment; (3) generating mutations of receptor or other components of these processes that can selectively effect either internalisation or desensitisation.

The role of internalisation in GPCR varies according to receptor type. For class I GPCRs the current hypothesis, based on studies of the β_2AR, is that receptor endocytosis is necessary for receptor dephosphorylation and resensitisation but not for desensitisation (Barak et al., 1994; Yu et al., 1993). In accordance with this reports suggest that receptor internalisation is not required for the desensitisation of other members of this family, including the A$_{2A}$-angiotensin (Mundell and Kelly, 1998a), TRH (Yu and Hinkle, 1998), neurokinin-1 (Sanders and LeVine, 1996) and dopamine D$_1$ receptors (Ng et al., 1995). Whether internalisation is necessary for the resensitisation of these receptors remains to be determined, to date the A$_{2A}$-angiotensin receptor is one of the few examples where blocking internalisation, with sucrose or concanavalin A has actually been shown to impair resensitisation of receptor signalling (Mundell and Kelly, 1998a). In contrast, several members of this receptor family require internalisation before desensitisation of second messenger signalling can occur, examples include the somatostatin (SSTR) (Beaumont et al., 1998), vasopressin V$_2$ (Pfeiffer et al., 1998) and M$_2$-muscarinic receptors (Tsuga et al., 1998). In addition endocytosis is essential for desensitisation of receptors which are irreversibly activated by agonist (for a review see Böhm et al., 1996). Fewer investigations of the role of internalisation of class II GPCRs have been performed. However, for studies of the secretin receptor internalisation appeared to be the predominant mechanism required for desensitisation of cAMP signalling in stably transfected CHO cells (Holtmann et al., 1996). In accordance with this Mundell et al. (1998) found that sucrose or concanavalin A pretreatment caused a moderate reduction in desensitisation of secretin receptors endogenously expressed in NG108-15 mouse neuroblastoma/rat glioma hybrid cells. Surprisingly this study found that, under normal conditions, the secretin receptor did not resensitise; this could be explained if secretin remained bound to its receptor or rapid down regulation occurs (Mundell and Kelly, 1998a).

Data presented in this chapter suggests that $VPAC_2$ receptor internalisation is not required for desensitisation. Therefore, it would be interesting to determine whether, like many class I GPCRs, $VPAC_2$ receptor internalisation is required for receptor resensitisation. A simple method of demonstrating this would be to examine cAMP levels following incubation with a desensitising stimulus, remove the agonist and monitor resensitisation, in the presence or absence of inhibitors of receptor internalisation.

Evidence for a physiological role of agonist-induced internalisation of the $VPAC_2$ receptor

Internalisation of the $VPAC_2$ receptor may have role in regulating VIP function in physiological systems. Receptor-mediated endocytosis of VIP has been shown to be necessary for bone resorption in cultured mouse calvaria (Hohmann et al., 1983), accumulation of adenosine 3',5'-monophospate in the rat pineal gland (Kaku et al., 1985) and hepatic clearance of VIP in the rat liver (Misbin et al., 1982). Establishing the immunofluorescence technique described here provides a model for investigating many different features of $VPAC_2$ receptor internalisation *in vitro*. Ultimately it would be interesting to establish whether this phenomenon is relevant *in vivo*. Current work in our laboratory has generated mice which are transgenic for the human $VPAC_2$ receptor. The $VPAC_2$ receptor construct used for this study also contains the haemagglutinin epitope-tag at its C-terminus, so that some of the procedures described here *in vitro* may be studied *in vivo* (Shen et al., in preparation). This would aid in delineation of the role of agonist-induced $VPAC_2$ receptor internalisation in whole animals.

CHAPTER 5

Role of the C-terminal intracellular domain in agonist-induced internalisation of the VPAC$_2$ receptor

5.1 Introduction

Agonist-induced internalisation of many GPCRs is influenced by signals contained within their C-terminal intracellular domains. The majority of studies investigating the role of C-termini in receptor-mediated endocytosis have used truncated or mutagenised receptors. The effect of these alterations vary according to the particular species variant and subtype. One of the most extensively characterised receptors is the β_2AR. Truncation of the C-terminal intracellular domain enhanced endocytosis of the human B$_2$AR but reduced endocytosis of the hamster β_2AR (Bouvier et al., 1988; Strader et al., 1987). Interestingly, the wild-type avian β_2AR does not internalise, however when its C-terminus is truncated it is able to internalise (Hertel et al., 1990). For other class I GPCRs shortening of the cytoplasmic tail has been shown to cause a reduction in endocytosis of the yeast α-factor (Reneke et al., 1988), GRP (Benya et al., 1993) and TRH receptors (Nussenzveig et al., 1993). In addition efficient endocytosis of the TRH receptor was found to require the presence of two cysteine residues in the C-terminal tail (Nussenzveig et al., 1993). In contrast, serial truncation of the luteinizing hormone/chorionic gonadotropin hormone (LH/CG) (Rodriguez et al., 1992) had both positive and negative effects on endocytosis, which showed no correlation with length of the C-terminal tail. A similar diversity has been observed for the role of the C-terminal intracellular domain in internalisation of class II GPCRs, examples include the PTH/PTHrP and calcitonin receptors which contain both positive and negative signals for endocytosis (Findlay et al., 1994; Huang et al., 1995a). In contrast, complete removal of the C-terminal tail of the secretin receptor had no effect on agonist-induced internalisation (Holtmann et al., 1996). Thus, the underlying role of the C-terminal intracellular domain in GPCR internalisation is difficult to define. This is perhaps not surprising considering the variability in amino acid sequence of the C-terminal intracellular domains of GPCR subtypes. Nonetheless, it appears that for many receptors, a combination of positive and negative signal motifs and a critical length of the C-terminal intracellular domain are necessary for efficient endocytosis.

Five epitope-tagged VPAC$_2$ receptor constructs were generated which were truncated at different amino acids in the C-terminal intracellular domain in order to remove potential phosphorylation sites in this region. Each receptor construct was stably transfected into HEK293 cells and characterised according to (1) its ability to couple to G$_s$, activate AC and stimulate cAMP formation, (2) its affinity for [^{125}I]-helodermin and (3) its expression level. The role of C-terminal intracellular

domain in agonist-induced internalisation of the $VPAC_2$ receptor was investigated by measuring the internalisation of $[^{125}I]$-helodermin and by localisation of truncated/tagged receptors in intact cells using immunofluorescence. In addition the ability of C-terminally truncated $VPAC_2$ receptors to desensitise was evaluated. Finally the effect of removal of the C-terminal tail on $VPAC_2$ receptor on desensitisation is compared with other results from our laboratory concerning the phosphorylation state of the truncated/tagged receptors.

5.2 Results

5.2.1 VIP-stimulated cAMP formation in cells stably transfected with truncated/tagged $VPAC_2$ receptors

Each C-terminally truncated $VPAC_2$ receptor construct generated for this study contained an epitope tag inserted after the last amino acid. The full length human $VPAC_2$ receptor consists of 438 amino acids, C-terminally truncated/tagged (TT) versions of the receptor are named according to their total number of amino acids (excluding the tag) as TT425, TT409, TT393, TT391 and TT385 **(Figure 5.1)**. All of the aforementioned receptor constructs were transfected into HEK293 cells and stable clones were isolated by antibiotic selection. A representative clone for each C-terminally truncated $VPAC_2$ receptor was chosen, according to its ability to couple to G_s and stimulate AC, and characterised further.

The potency of VIP to activate AC was assessed by measuring the levels of cAMP formation in whole cells. Cells transfected with TT425, TT409, TT393, TT391 and TT385 were incubated with VIP (0.01-100 nM for 30 mins) at 37˚C. Each clone displayed a dose-dependent increase in cAMP formation, with the exception of TT385, which displayed a similar response to that of untransfected HEK293 cells **(Figure 5.2)**. A comparison of cAMP formation between the $VPAC_2$, $VPAC_2$-HA and truncated/tagged receptors revealed differences in the potency of VIP to activate AC **(Figure 5.3)**. Mean EC_{50} values and maximal cAMP levels were calculated for each stably transfected clone; VIP-stimulated cAMP formation with a similar potency at the $VPAC_2$ and $VPAC_2$-HA receptors (EC_{50} values; 1.2 ± 0.2 nM and 1.4 ± 0.2 nM respectively). There were slight variations in the ability of VIP to stimulate cAMP formation in HEK293 cells expressing the truncated/tagged receptors. Compared with the wild type receptor, the EC_{50} values for AC activation by VIP were significantly lower for clones TT425 (0.7 ± 0.1 nM, $P<0.05$) and TT409 (0.4 ± 0.1 nM, $P<0.01$), comparable for clone TT393 (1.5 ± 0.2 nM) and significantly

higher for TT391 (4.0 ± 0.5 nM, $P<0.01$). No significant difference in maximal cAMP stimulation was found between $VPAC_2$, $VPAC_2$-HA and truncated/tagged receptors, with the exception of TT385 (**Table 5.1**). These data demonstrate that truncation of the C-terminal intracellular domain of the $VPAC_2$ receptor can influence the ability of this receptor to couple to G_s. The reduction in potency of VIP at TT391 is interesting as it is only two amino acid residues shorter than TT393, indicating that the loss of these residues may remove a critical length of the C-terminus or that the missing residues may be required for maintaining the receptor in an appropriate conformation for signalling.

5.2.2 [^{125}I]-helodermin binding to cell membrane preparations from HEK293 cells stably transfected with truncated/tagged $VPAC_2$ receptors

Saturation experiments with membranes prepared from HEK293 cells stably transfected with the truncated/tagged $VPAC_2$ receptors were used to determine their affinity for [^{125}I]-helodermin. No specific [^{125}I]-helodermin binding was observed in untransfected HEK293 cells or cells stably transfected with TT385 (data not shown). The affinity of [^{125}I]-helodermin for the truncated/tagged receptors was unaffected by truncation of the C-terminal domain; K_D values for the $VPAC_2$ and $VPAC_2$-HA receptors were 2.2 ± 0.4 nM and 2.0 ± 0.4 nM, respectively. No significant difference was observed between the affinity of helodermin for the wild-type or any of the truncated/tagged receptors; TT425 (5.5 ± 2.3 nM; n=4), TT409 (2.3 ± 0.6 nM; n=4) TT393 (1.7 ± 0.4; n=2) and TT391 (1.7; n=1) (**Figures 5.4** and **5.5; Table 5.2**). Only one determination of K_D was possible for TT391 (from three experiments), as low expression levels and high non-specific binding precluded curve fitting to the data. Hill coefficients did not differ from unity indicating the presence of a single binding site. Linear transformation of these data, according to the method of Scatchard, also confirmed the presence of a single binding site (**Figures 5.4** and **5.5; inset**). Maximum binding (B_{max}) values for each truncated/tagged receptor indicated variability in the levels of receptor expression; TT425 was expressed at levels comparable with the $VPAC_2$ and $VPAC_2$-HA receptors (7338 ± 188 fmoles/mg), whereas TT409, TT393 and TT391 exhibited approximately 5-20 fold less receptor expression (430 ± 52, 1405 ± 892 and $299 \pm$ nd fmoles/mg, respectively) (**Table 5.2**). Overall the affinity of helodermin for the $VPAC_2$ receptor was unaffected by truncation of the C-terminal intracellular domain. The truncated receptors showed a general trend of reduction in expression level with decreasing length of the C-terminal intracellular domain, however this is probably a feature of each particular

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clonal cell line selected for characterisation, rather than a result of C-terminal truncation.

5.2.3 Internalisation of [^{125}I]-helodermin in cells stably transfected with truncated/tagged VPAC$_2$ receptors

HEK293 cells stably transfected with TT393 and TT391 were tested for their ability to internalise [^{125}I]-helodermin. Both of these truncated/tagged receptors stimulated maximal levels of cAMP, which were comparable with the wild type receptor, although VIP was slightly less potent at TT391. [^{125}I]-helodermin had a similar affinity for TT393 and TT391. Intact cells expressing TT393 or TT391 were able to internalise [^{125}I]-helodermin (**Figure 5.6** and **5.7**, respectively). After 5 mins incubation at room temperature ~45% [^{125}I]-helodermin was in an acid resistant site (or internal) in cells expressing TT393 and TT391, whereas ~25% was internal in cells expressing VPAC$_2$ receptor. This result suggests that, at least initially, the truncated receptors internalised peptide more rapidly than the wild type receptor. However, the difference in internalisation did not persist and the rate of internalisation of [^{125}I]-helodermin appeared to reduce at later time points for the truncated/tagged clones. After >15 mins incubation with peptide the difference between the amount of internalised radiolabel for the wild type and mutant receptors was negligible. The levels of acid resistant [^{125}I]-helodermin attained steady state after 60 mins incubation. Thus, severe truncation of the C-terminal intracellular domain did not markedly effect the amount of peptide internalised, indicating that this domain is not required for agonist-induced internalisation. However the VPAC$_2$ receptor C-terminal tail may influence the rate at which peptides are internalised.

5.2.4 Agonist-induced internalisation of truncated/tagged VPAC$_2$ receptors

Internalisation of the truncated/tagged VPAC$_2$ receptors was assessed qualitatively using immunofluorescence. Each stably transfected clone was seeded onto glass coverslips and incubated in serum-free medium for ~24 hrs prior to fixation. Pretreatment of the VPAC$_2$-HA receptor with VIP (10 μM for 30 mins) resulted in a readily observable shift in receptor location from the plasma membrane to an intracellular site (see section 4.2.3). Therefore the same protocol was applied to each of the truncated/tagged receptors. Cells expressing TT425, TT409, TT393 and TT391 exhibited fluorescent staining predominantly in the plasma membrane (**Figure 5.8A,C,E,G**), whereas staining for TT385 in untreated cells did not show a discrete plasma membrane localisation (**Figure 5.8I**). Pretreatment with VIP (10 μM for 30 mins) caused these receptors to internalise to a discrete juxtanuclear site

(**Figure 5.8B,D,F,H**), with the exception of TT385 which appeared to be unaffected by pretreatment with VIP (**Figure 5.8J**). These experiments reveal that TT425, TT409, TT393 and TT391 are all able to undergo agonist-induced internalisation. The diffuse distribution of TT385 in intact cells combined with is inability to activate AC or bind [^{125}I]-helodermin suggest that this receptor is not inserted into the plasma membrane, therefore it is likely that the proximal region of the C-terminal tail is necessary for correct trafficking and insertion of receptor into the plasma membrane.

5.2.5 Desensitisation of truncated/tagged VPAC$_2$ receptors

The effect of C-terminal truncation of the VPAC$_2$ receptor on desensitisation of its cAMP response following pretreatment with VIP was investigated. TT391 is the most severely truncated receptor that retains the ability to bind helodermin. VIP has a slightly lower potency for activating AC through TT391 but is able to stimulate maximal levels of cAMP which are comparable with the wild type receptor. HEK293 stably transfected with TT391 were pretreated with VIP (0, 1, 3, 10 or 100 nM for 30 mins) at 37°C, then rechallenged with VIP (0.01-100 nM) in the presence of IBMX. Reduction in the maximum cAMP response following agonist pretreatment was interpreted as desensitisation. None of the VIP concentrations used induced desensitisation of second messenger signalling in cells expressing TT391 (**Figure 5.9**). These results contrast with those seen for the VPAC$_2$ and VPAC$_2$-HA receptors, where >3 nM VIP pretreatment was sufficient to cause a significant (>20%) reduction in maximum cAMP levels (see section 3.2.5). These data suggest that the presence of the C-terminal intracellular domain is required for desensitisation of the VPAC$_2$ receptor.

Figure 5.2. Schematic diagram of the membrane topology and amino acid sequence of the C-terminal intracellular domain of the VPAC$_2$ receptor and truncated/tagged VPAC$_2$ receptors. Serine and threonine residues are indicated in blue and the epitope-tag in red.

VPAC$_2$:   SEVQCELKRKWRSRCPTPSASRDYRVCGSSFSRNGSEGALQFHRGSRAQSFLQTETSVL

VPAC$_2$-HA: SEVQCELKRKWRSRCPTPSASRDYRVCGSSFSRNGSEGALQFHRGSRAQSFLQTETSVLEYPYDVPDYASL

TT425 :   SEVQCELKRKWRSRCPTPSASRDYRVCGSSFSRNGSEGALQFHRGSYPYDVPDYASL

TT409 :   SEVQCELKRKWRSRCPTPSASRDYRVCGSSYPYDVPDYASL

TT393 :   SEVQCELKRKWRSRYPYDVPDYASL

TT391 :   SEVQCELKRKWRYPYDVPDYASL

TT385 :   SEVQCEYPYDVPDVASL

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Figure 5.2 VIP-stimulated cAMP production of truncated/tagged VPAC$_2$ receptors. Representative experiment showing cAMP levels in untransfected HEK293 cells (○) and cells stably transfected with VPAC$_2$ (□) and VPAC$_2$-HA receptors (■), TT425 (▼), TT409 (▲), TT393 (●), TT391 (◆) and TT385 (▶) following stimulation with VIP. Cells were incubated with VIP (0.01-100 nM) for 30 mins at 37°C, in the presence of IBMX. cAMP levels were determined by radioimmunoassay. Graph shows a typical experiment, results are means ± SD for a typical experiment performed in triplicate.
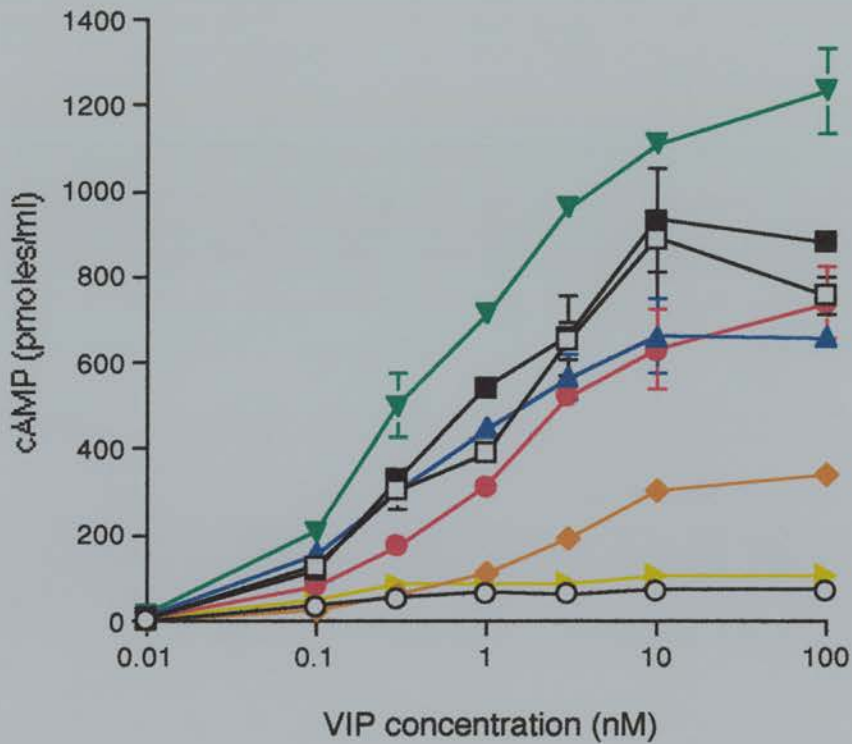
Figure 5.3 **Summary of VIP-stimulated cAMP production in truncated/tagged VPAC$_2$ receptors.** cAMP levels in cells stably transfected with VPAC$_2$ (□) and VPAC$_2$-HA receptors (■), TT425 (▼), TT409 (▲), TT393 (●) and TT391 (◆) following stimulation with VIP. Cells were incubated with VIP (0.01-100 nM) for 30 mins at 37°C, in the presence of IBMX. cAMP levels were determined by radioimmunoassay. Results are expressed as a percent of the maximal cAMP response for each individual receptor clone. Results are means ± SEM for 4-5 separate experiments performed in triplicate.
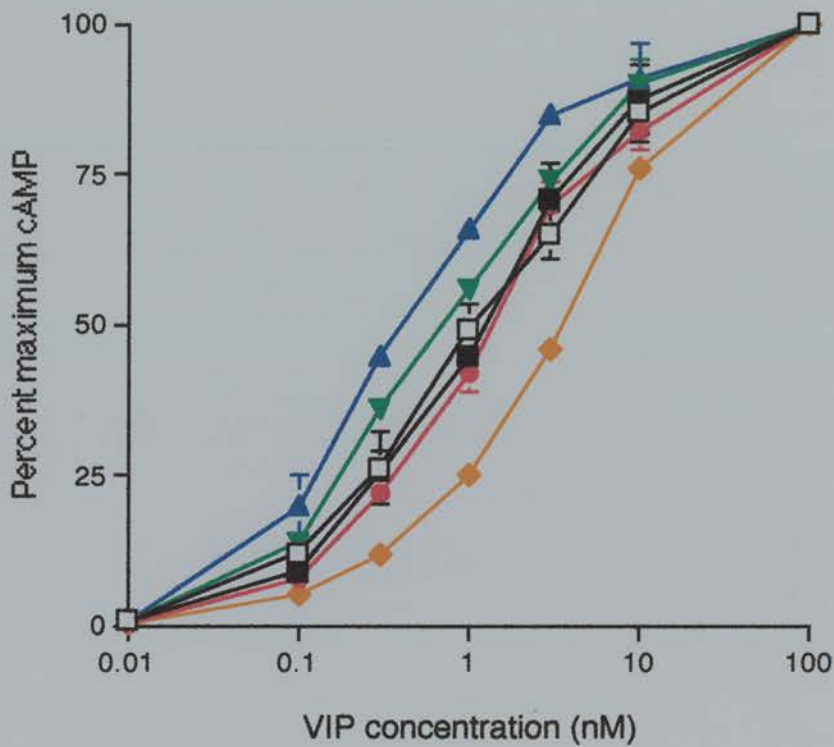
Table 5.1 Comparison of VIP-stimulated cAMP formation between the VPAC$_2$ receptor and C-terminal truncated and epitope-tagged VPAC$_2$ receptors. EC$_{50}$ values and maximum cAMP levels obtained by VIP stimulation of the VPAC$_2$ receptor and C-terminal truncated and HA-epitope tagged receptor constructs stably transfected in HEK293 cells. Values are expressed as means ± SEM for 4–5 separate experiments (P<0.05*, P<0.01**).

Receptor	EC$_{50}$ (nM)	Maximum cAMP stimulation (pmoles/ml)
VPAC$_2$	1.2 ± 0.2	928 ± 116
VPAC$_2$-HA	1.4 ± 0.2	1252 ± 345
TT425	0.7 ± 0.1 *	1315 ± 164
TT409	0.4 ± 0.03 **	923 ± 153
TT393	1.5 ± 0.2	1363 ± 301
TT391	4.0 ± 0.5 *	1024 ± 495
TT385	no cAMP stimulation	-

Figure 5.4 [^{125}I]-helodermin binding to membrane preparations of HEK293 cells stably transfected with the VPAC$_2$ receptor and truncated/tagged VPAC$_2$ receptors. Representative saturation binding experiment for [^{125}I]-helodermin in the presence of increasing concentrations of unlabelled helodermin (0.1-300 nM) for HEK293 cells stably transfected with the VPAC$_2$ (□), VPAC$_2$-HA receptors (■), TT425 (▼), TT409 (▲), TT393 (●) and TT391 (◆) receptors. Results are mean values for a single experiment performed in triplicate following a 10 min incubation at 37°C, until equilibrium was attained. **Inset:** Scatchard plot of bound/free versus bound for each of receptor construct.
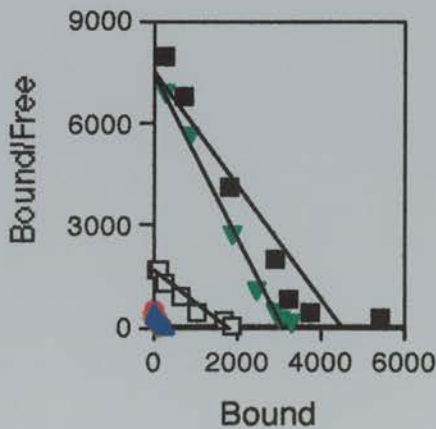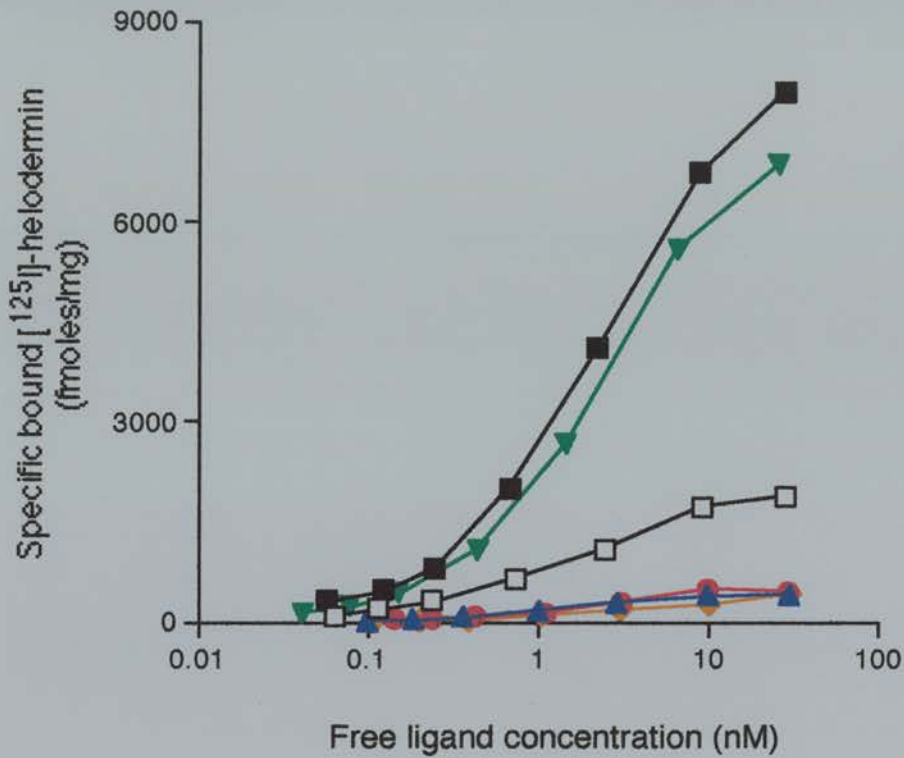
Figure 5.5 **[^{125}I]-helodermin binding to membrane preparations of HEK293 cells stably transfected with the VPAC$_2$ receptor and truncated/tagged VPAC$_2$ receptors.** Representative saturation binding experiment for [^{125}I]-helodermin in the presence of increasing concentrations of unlabelled helodermin (0.1-300 nM) for HEK293 cells stably transfected with truncated/tagged VPAC$_2$ receptors; TT409 (▲), TT393 (●) and TT391 (◆). Results are mean values for a single experiment performed in triplicate following a 10 min incubation at 37°C, until equilibrium was attained. **Inset:** Scatchard plot of bound/free versus bound for each of receptor construct.

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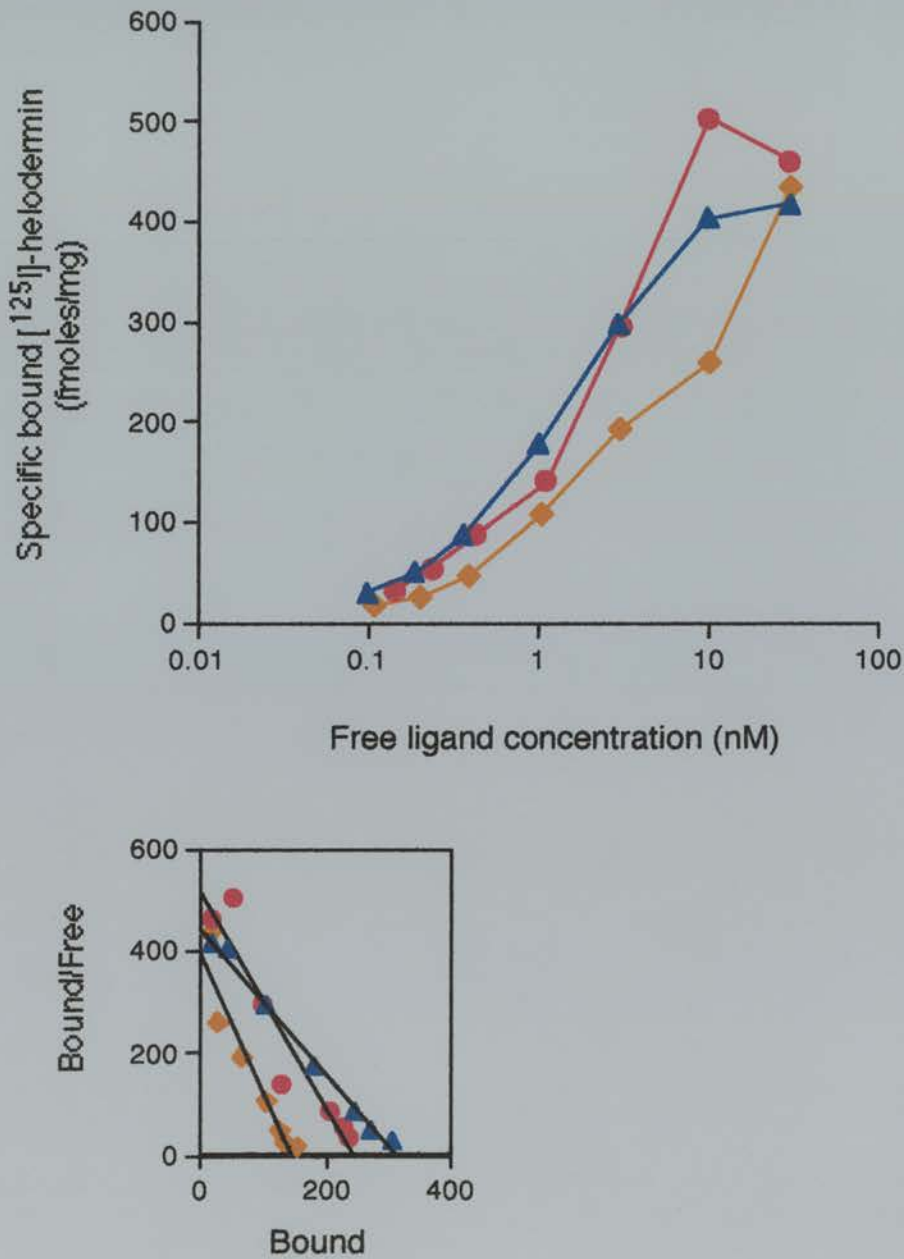
Table 5.2 Summary of [^{125}I]-helodermin membrane binding data for the VPAC$_2$ receptor and truncated/tagged VPAC$_2$ receptors. K_D and B_{max} values obtained from saturation assay of [^{125}I]-helodermin binding to membrane preparations of HEK293 cells stably transfected with the VPAC$_2$ receptor and C-terminal truncated and epitope-tagged VPAC$_2$ receptor constructs. Values are expressed as means ± SEM for 1-6 separate experiments (P<0.05*, P<0.01**).

Receptor	K_D (nM)	B_{max} (fmoles/mg)	n
VPAC$_2$ (438)	2.2 ± 0.4	6251 ± 2239	6
VPAC$_2$-HA	2.0 ± 0.4	11197 ±4936	7
TT425	5.5 ± 2.3	7338 ± 188	4
TT409	2.3 ± 0.6	430 ± 52	4
TT393	1.7 ± 0.4	1405 ± 892	2
TT391	1.7	299	1
TT385	no specific binding	-	2

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Figure 5.6 Internalisation of [^{125}I]-helodermin in HEK293 cells stably transfected with a truncated/tagged VPAC$_2$ receptor. Comparison of internalisation of [^{125}I]-helodermin in HEK293 cells stably transfected with the VPAC$_2$ (□) receptor and TT393 (●). Results are expressed as a percentage of acid resistant/total (acid susceptible plus acid resistant) specific [^{125}I]-helodermin bound at room temperature. Graph shows means ± SEM for 3 separate experiments performed in duplicate.
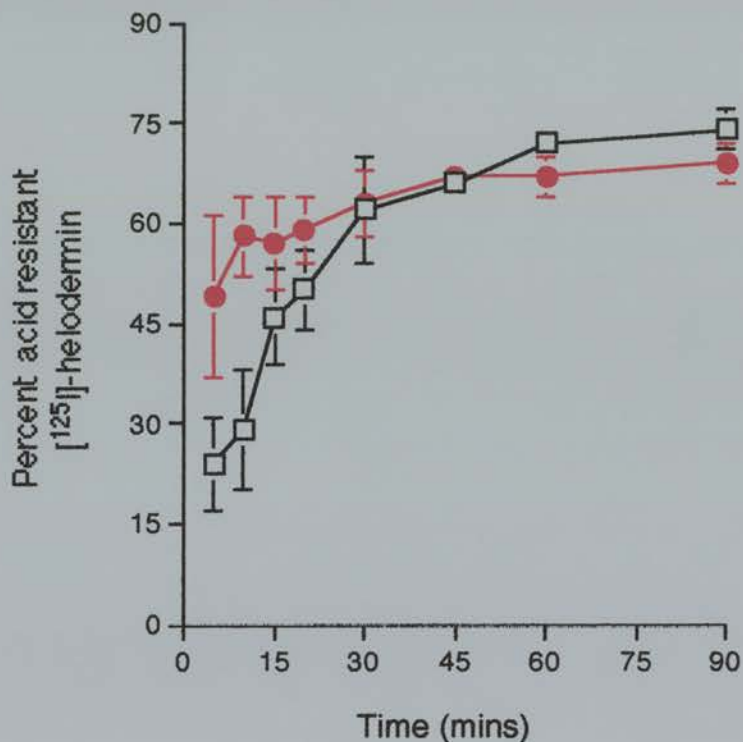
Figure 5.7 Internalisation of [^{125}I]-helodermin in HEK293 cells stably transfected with a truncated/tagged VPAC$_2$ receptor. Comparison of internalisation of [^{125}I]-helodermin in HEK293 cells stably transfected with the VPAC$_2$ (□) receptor and TT391 (◆). Results are expressed as a percentage of acid resistant/total (acid susceptible plus acid resistant) specific [^{125}I]-helodermin bound at room temperature. Graph shows means ± SEM for 3 separate experiments performed in duplicate.
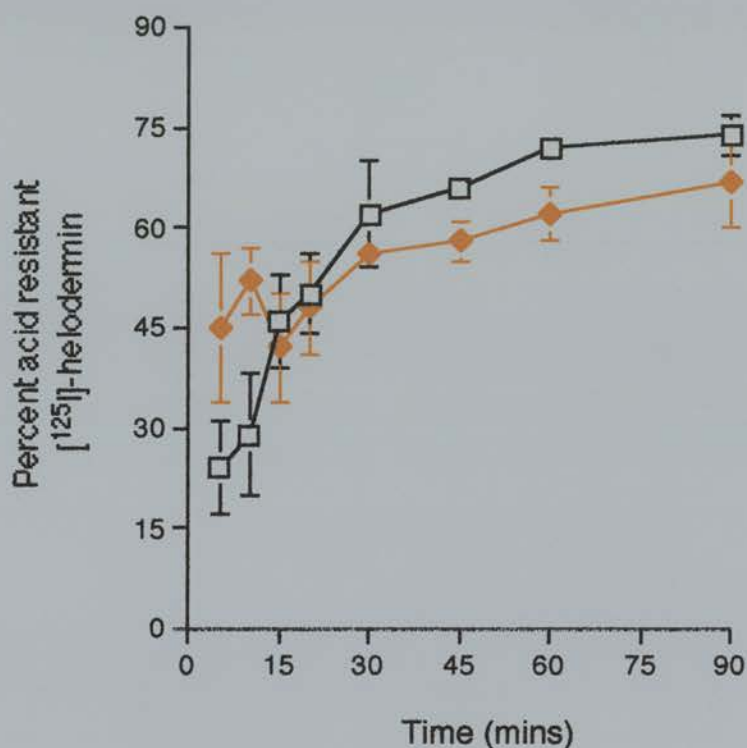
Figure 5.8 Agonist-induced internalisation of truncated/tagged VPAC$_2$ receptors. HEK293 cells stably transfected with truncated/tagged VPAC$_2$ receptors were incubated in serum free medium; TT425 (**A**), TT409 (**C**), TT393 (**E**), TT391 (**G**) or TT385 (**I**); or VIP (10 μM); TT425 (**B**), TT409 (**D**), TT393 (**F**), TT391 (**H**) or TT385 (**J**) for 30 mins at 37°C prior to fixing. Cells were viewed at x40 magnification under appropriate fluorescence conditions.
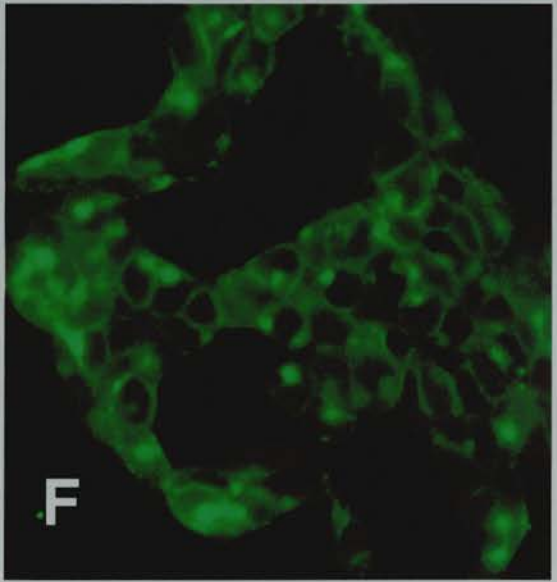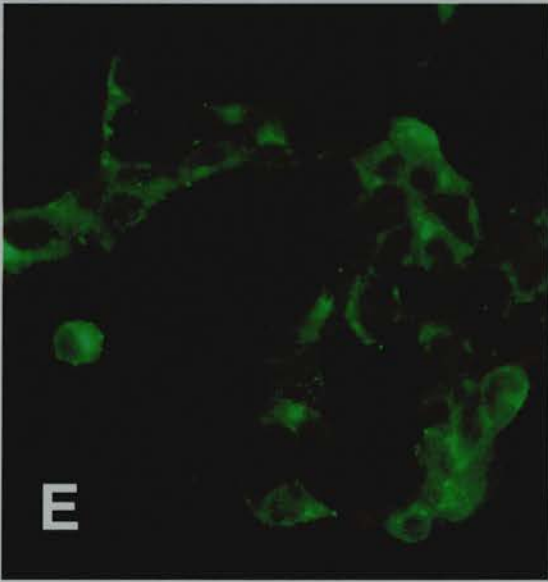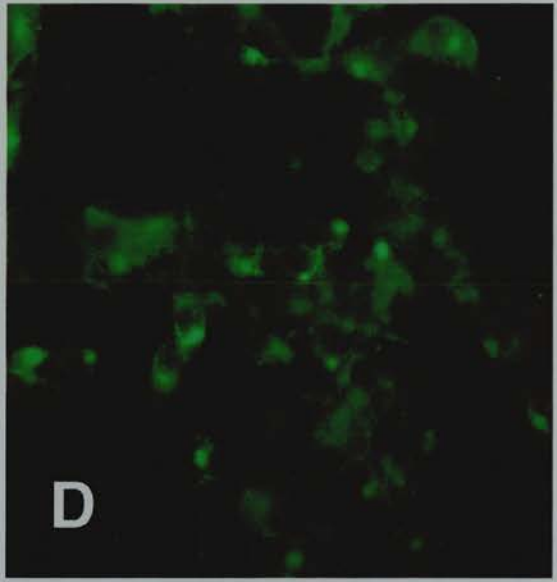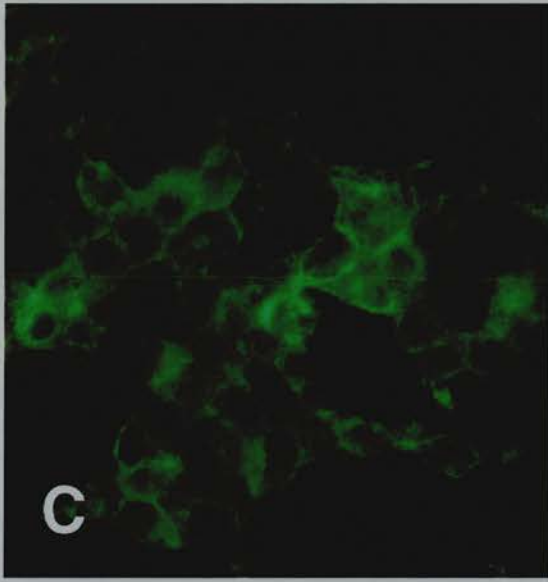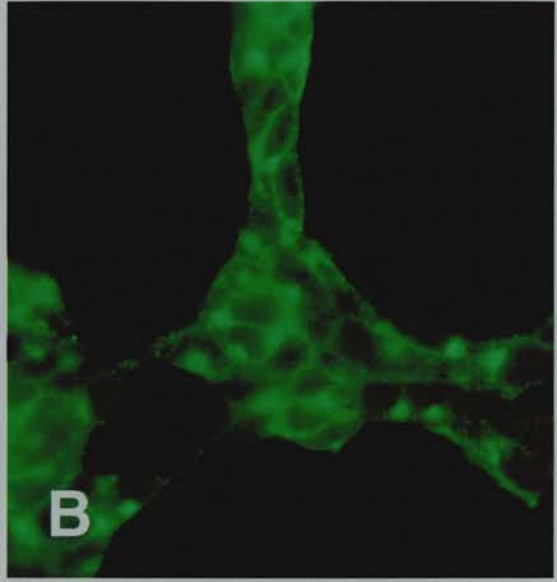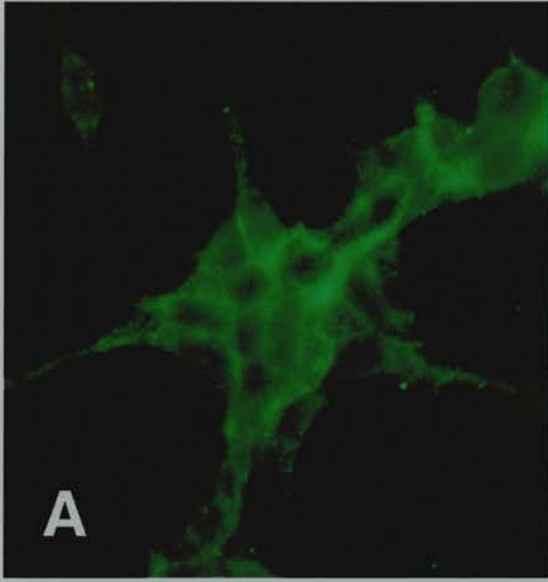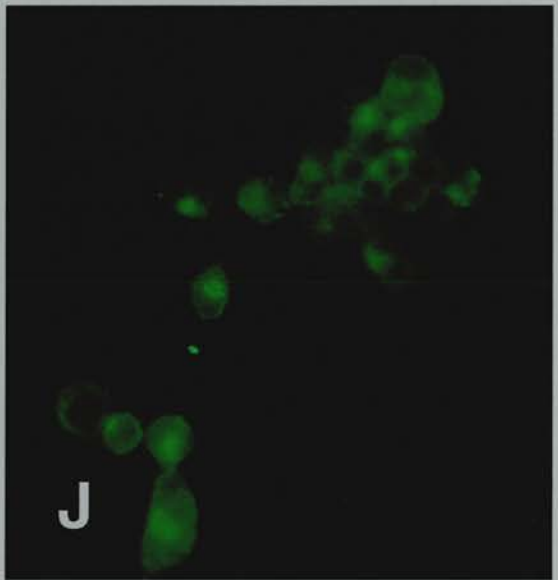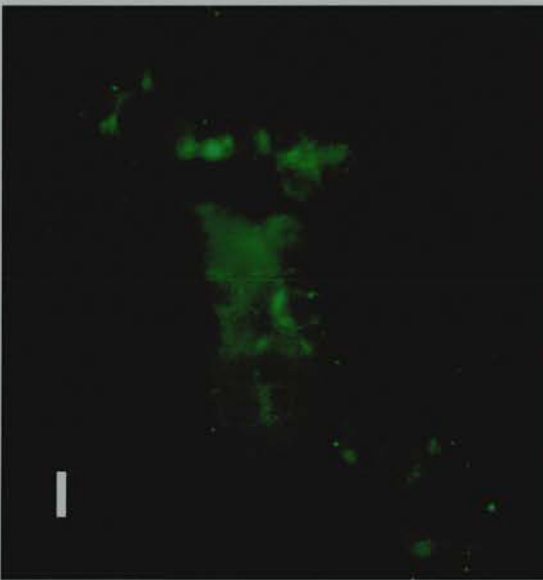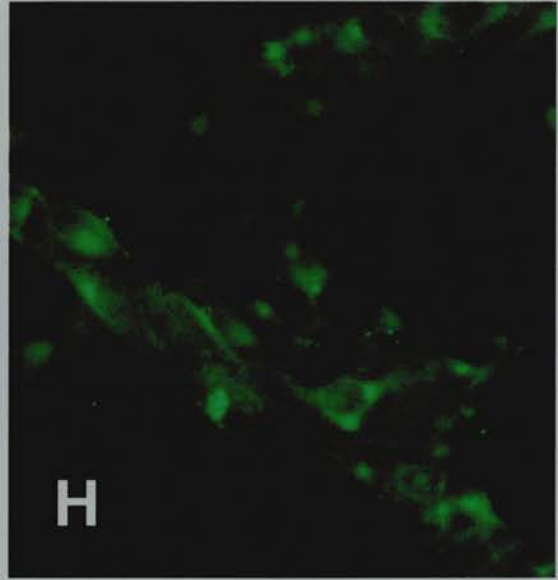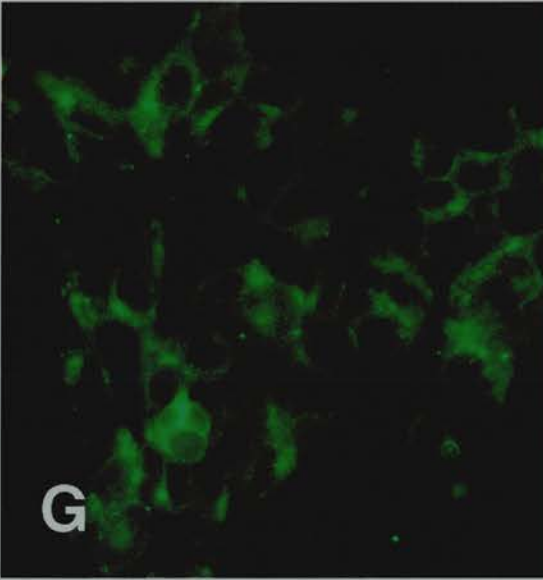
Figure 5.9 VIP-induced desensitisation of cAMP formation in HEK293 cells stably transfected with a truncated/tagged VPAC$_2$ receptor. HEK293 cells stably transfected with the TT391 were pretreated with 0 (◆), 1 nM (△), 3 nM (▽) 10 nM (○) or 100 nM (●) VIP for 30 mins at 37°C. Desensitisation was evaluated by restimulating the cells with VIP (0.01-10 nM), in the presence of IBMX. Results are expressed as percentage maximum cAMP response, relative to control (0 VIP) stimulation. Results are means ± SEM for 3-7 experiments performed in triplicate.
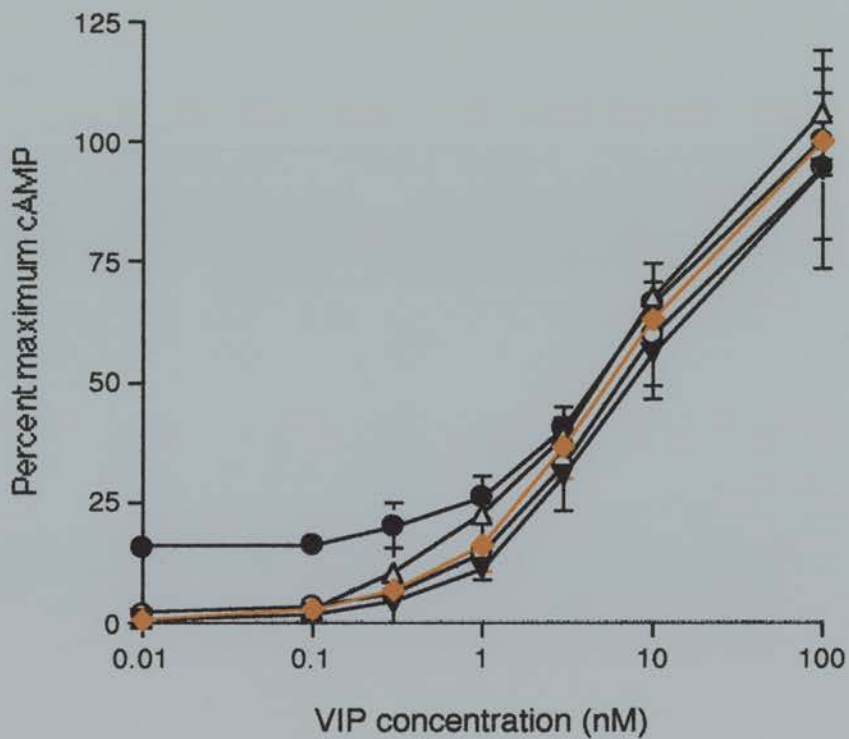
Table 5.3 Consensus motifs used to identify sites of phosphorylation by second messenger-dependent protein kinases. Phosphorylated residues are denoted with an asterisk (*). Where two amino acids function interchangeably they are separated with a slash (/). Neutral sequence positions are denoted by an X. As reviewed by Kemp and Pearson, (1990); Kennelly and Krebs (1991). Abbreviations; protein kinase C (PKC); protein kinase A (PKA); protein kinase G (PKG).

PKA phosphorylation motif	$R-X_{1-2}-S*/T*-X$
PKC phosphorylation motif	$(R/K_{1-3}, X_{2-0})-S*/T*-(X_{2-0}, R/K_{1-3})$ $S/T*-(X_{2-0}, R/K_{1-3})$ $R/K_{1-3}, X_{2-0})-S*/T*$
PKG phosphorylation motif	$(R/K)_{2-3}-X-S*/T*-X$

Figure 5.10 Predicted membrane topology and amino acid sequence of the VPAC$_2$ receptor and VPAC$_2$-HA receptor. A signal sequence in the first 20 amino acids of the N-terminal extracellular domain is removed. Putative N-glycosylation sites (#) and PKA (ψ) and PKC ($*$) phosphorylation sites are indicated. The positions at which stop codons were introduced into the C-terminal intracellular domain, in order to generate truncated VPAC$_2$ receptor mutants, are indicated by solid lines. An epitope-tag was introduced after the terminal amino acid of each truncated VPAC$_2$ receptor.

EXTRACELLULAR

INTRACELLULAR

HA tag
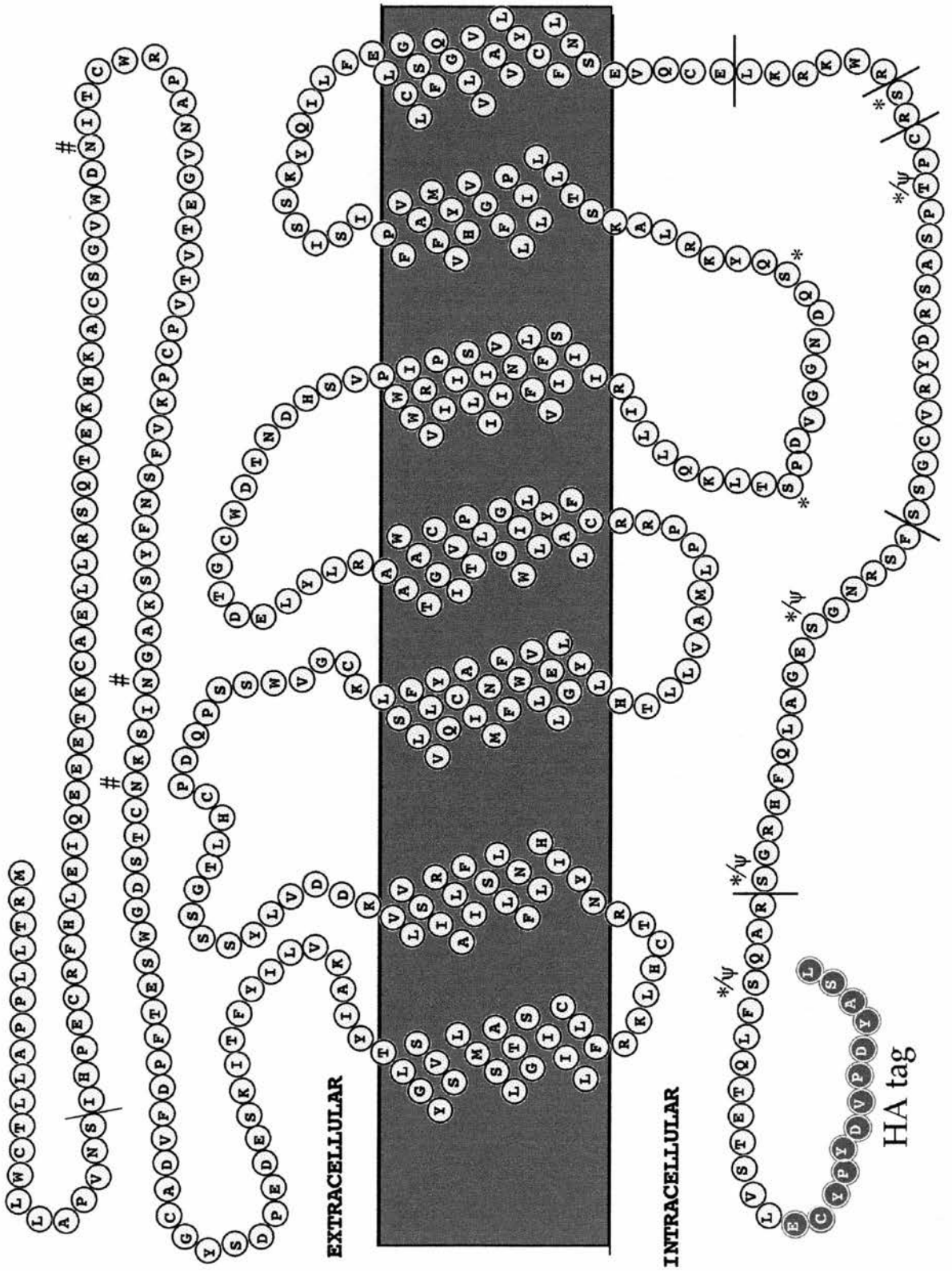
Table 5.4 Summary of data for the VPAC$_2$ receptor and truncated/tagged VPAC$_2$ recetors. [^{125}I]-helodermin internalisation, receptor internalisation and desensitisation were determined from work presented in this chapter and phosphorylation from the work of T.P.McDonald (in press). Abbreviations; nd=not determined.

Receptor	Peptide Internalisation	Receptor internalisation	Desensitisation (% reduction in max cAMP)	Agonist-induced phosphorylation
VPAC$_2$	Yes	Yes	~20%	Yes
VPAC$_2$-HA	Yes	Yes	~20%	Yes
TT425	nd	Yes	nd	Yes
TT409	nd	Yes	nd	Yes
TT393	Yes (altered kinetics)	Yes	0%	No
TT391	Yes (altered kinetics)	Yes	0%	No
TT385	No	No	no cAMP stimulation	No

5.3 Discussion

Effect of C-terminal truncation of the VPAC$_2$ receptor on second messenger signalling

To explore the role of the C-terminal intracellular domain in VPAC$_2$ receptor function five mutants progressively truncated in this domain were generated and characterised. All of these receptors coupled to G$_S$ and stimulated cAMP production, with the exception of TT385. VIP displayed a similar potency for stimulating cAMP levels in wild-type, epitope-tagged and TT393 receptor transfected cells, but was more potent at stimulating cAMP formation in cells transfected with TT425 and TT409 and less potent at TT391. These results suggest that the C-terminal domain of the VPAC$_2$ receptor influences second messenger signalling. This is an unusual feature of VPAC$_2$ receptor function; truncation of GPCRs does not generally effect coupling to G-proteins (see below for references). However, signalling of the calcitonin receptor, another class II GPCR, has been shown to be effected by truncation of the C-terminal intracellular domain (Findlay et al., 1994).

The maximal levels of VIP-stimulated cAMP formation for all of the truncated/tagged receptors (except TT385) were not significantly different from that observed with the wild type receptor. Maximal cAMP stimulation has previously been reported to depend upon the level of receptor expression in transfected cell lines, thus greater numbers of receptors would be predicted to display higher maximal cAMP stimulation in response to agonist (Kenakin, 1997b). This does not appear to be the case in this study. Two explanations are possible: either the difference in receptor expression is not great enough to cause a noticeable shift in maximum cAMP stimulation or receptor expression levels are not the limiting factor in this assay. In a stably transfected cell line where receptors are highly overexpressed the maximum level of cAMP stimulation may be circumscribed by components of the second messenger system (Kenakin, 1997b). Consistent with this report, experiments investigating the effect of C-terminal truncation of the opossum kidney (OK) PTH/PTHrP receptor found no effect on coupling to AC, when the receptor was highly overexpressed (Huang et al., 1995b). Whereas maximal AC activation was increased in C-terminally truncated rat PTH/PTHrP receptors, when the receptor was expressed at low levels, such that second messenger components were unlikely to limit signalling (Huang et al., 1995b; Iida-Klein et al., 1995). In view of these data, the results of this study, which showed no effect of C-terminal truncation of the VPAC$_2$ receptor on maximal cAMP stimulation should be

interpreted with some caution. Future experiments to determine the effects of C-terminal truncation on agonist stimulation of second messenger should use stably transfected cells lines with similar receptor expression levels and determine whether lowering receptor number has any effect on AC activation.

Effect of C-terminal truncation of the VPAC$_2$ receptor on agonist binding

The affinity of $[^{125}I]$-helodermin for the VPAC$_2$ receptor was unaffected by truncation of the C-terminal intracellular domain. This is in accordance with studies of other GPCRs where progressive truncation of their C-termini does not effect ligand affinity (see below for references). However a reduction in maximal receptor binding levels was observed with decreasing length of the C-terminal intracellular domain. This result is probably a function of the particular clones selected for characterisation. However it is possible that progressive truncation of the C-terminal tail may effect receptor expression. Indeed a study of the OK PTH/PTHrP receptor found that efficient trafficking and expression of this receptor required a critical length of the C-terminal tail (Huang et al., 1995b). Further experiments are necessary to determine whether progressive truncation of the VPAC$_2$ receptor has any effect on its expression at the cell surface. $[^{125}I]$-helodermin did not bind specifically to cells stably transfected with TT385, which lacked most of the C-terminal domain, indicating that this receptor is not expressed at the plasma membrane.

Effect of C-terminal truncation of class II GPCRs on second messenger signalling and agonist binding

Previous studies of class II GPCRs have yielded conflicting results concerning the involvement of the C-terminal intracellular domain in second messenger signalling and agonist binding. Complete removal of the C-terminal region had no effect on agonist binding or second messenger signalling of receptors for glucagon (Unson et al., 1995), GLP-1 (Widmann et al., 1996), PTH/PTHrP (Huang et al., 1995a; Iida-Klein et al., 1995; Schneider et al., 1994) and secretin (Ozcelebi et al., 1995). In contrast, a C-terminally truncated porcine calcitonin receptor displayed enhanced affinity for agonist and reduced ability to stimulate cAMP production (Findlay et al., 1994). Receptor regions involved in G-protein coupling for the secretin receptor family still remain to be defined; although the majority of evidence excludes the involvement of the C-terminus, the results presented in this chapter indicate that this region may influence second messenger signalling. It is possible that the changes in potency of VIP observed with the truncated/tagged VPAC$_2$ receptors may stem from

an indirect effect on interactions between the C-terminal intracellular domain and other receptor regions or regulatory elements involved in signalling.

Effect of C-terminal truncation of the VPAC$_2$ receptor on internalisation of [^{125}I]-helodermin

HEK293 cells stably transfected with TT393 and TT391 were able to internalise [^{125}I]-helodermin. These data suggest that the VPAC$_2$ receptor C-terminus is not involved in agonist-induced internalisation. Nonetheless, initial levels of internalised radiolabel were slightly higher that those seen for the full length receptor indicating that the VPAC$_2$ receptor C-terminus may contain residues which influence the rate of internalisation. This result is somewhat surprising as the truncated/tagged receptors are expressed at approximately 5- and 20-fold lower levels, respectively, than the full length receptor, and thus would be predicted to endocytose more slowly (Koenig and Edwardson, 1997). A direct comparison of peptide internalisation between cells expressing similar levels of receptor is necessary to clarify these findings.

Effect of C-terminal truncation of the VPAC$_2$ receptor on agonist-induced internalisation

Agonist-induced internalisation of truncated/tagged VPAC$_2$ receptors was demonstrated using immunofluorescence. All of the truncated/tagged receptors endocytosed in response to treatment with VIP, with the exception of TT385. These experiments support the data from [^{125}I]-helodermin internalisation studies, indicating that truncation of the VPAC$_2$ receptor C-terminal tail does not markedly effect endocytosis. However, this technique is not quantitative and measurements of radiolabelled peptide internalisation are still necessary to confirm this finding for TT425 and TT409. These findings are similar to those obtained for a C-terminally truncated secretin receptor which retained its ability to internalise in response to agonist (Holtmann et al., 1996). TT385 did not have a discrete membrane localisation in untreated cells and was unaffected by VIP pretreatment indicating that it is not inserted into the plasma membrane. This receptor lacks only six more residues that TT391, which still retains the ability to bind [^{125}I]-helodermin, indicating that these residues (LKRKWR) may constitute a signal for receptor trafficking or expression.

If the C-terminus of the VPAC$_2$ receptor is not involved in agonist-induced internalisation the question remains as to which receptor regions are required.

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General endocytic motifs for receptors have been identified (see section 1.5.3). A tyrosine-based motif, $NPX_{2-3}Y$, in the seventh TM domain of the β_2AR, has been found to be required for receptor endocytosis, however this motif is absent in the secretin receptor family (Barak et al., 1994). Another tyrosine-based endocytic motif, YXXØ (where Y is tyrosine, X is any amino acid and Ø is any amino acid with a bulky hydrophobic side chain; leucine, isoleucine, phenylalanine, methionine or valine) has been shown to be involved in internalisation and targeting of a number of receptors to endosomes, lysosomes or the *trans*-Golgi network (for reviews see Marks et al., 1997). This endocytic signal has been shown to interact with the medium subunits ($\mu1$, $\mu2$ and $\mu3$) of adaptor proteins (APs) (for a review see Ohno et al., 1998). Interestingly a YXXØ motif (YKRL) is located in the third intracellular loop of the $VPAC_2$ receptor. This motif could potentially mediate interactions with APs and facilitate internalisation. In addition, the $VPAC_2$ receptor has two dileucine motifs; one each in the second and third intracellular loops of the $VPAC_2$ receptor. Dileucine motifs have been implicated in the endocytosis of several single TM domain receptors (Dietrich et al., 1994; Haft et al., 1994) and in β_2AR internalisation (Gabilondo et al., 1997). Additional mutagenesis studies are necessary to determine whether any of these putative signal motifs are involved in $VPAC_2$ receptor internalisation. To date no other general endocytic motifs for GPCRs have been identified.

Effect of C-terminal truncation of the VPAC2 receptor on agonist-induced desensitisation

VIP-stimulated cAMP formation in a dose dependent manner in HEK293 cells stably transfected with TT391 (the shortest functional $VPAC_2$ receptor), however this response did not desensitise after agonist pretreatment. These data indicate that the C-terminal intracellular domain of the $VPAC_2$ receptor is required for desensitisation. The ability of the other truncated/tagged to internalise was not investigated. One might predict that progressive truncation of the C-terminal tail will have intermediate effects on receptor desensitisation, compared with wild type receptors and TT391. However the method used in this study to demonstrate desensitisation is not particularly robust as evidenced by the moderate (~20%) reduction in maximum cAMP levels compared with other studies (Boissard et al., 1986; Robberecht et al., 1989c). Therefore an alternative measure of receptor desensitisation may be useful. Preliminary experiments in our laboratory by Mrs C.F.Morrison have measured cAMP accumulation over time following treatment with VIP in the presence of IBMX. These experiments show a rapid accumulation in

cAMP during the first 20 mins of exposure to agonist, after which time cAMP levels either fall rapidly (VPAC$_2$ and VPAC$_2$-HA receptors), fall slightly (TT425 and TT409) or continue to rise, up to 4 hrs after the initial stimulation (TT393 and TT391) (C.F.Morrison; personal communication). These results suggest that whilst TT393 and TT391 do not desensitise, TT425 and TT409 are still able to desensitise, albeit to a lesser extent than the wild type receptor.

A role for phosphorylation in desensitisation of the VPAC$_2$ receptor

Data from our laboratory has provided the first evidence for agonist-induced phosphorylation of the VPAC$_2$ receptor (McDonald et al., 1998). Phosphoamino acid analysis revealed that phosphorylation of the VPAC$_2$-HA receptor in stably transfected HEK293 cells occurs predominantly on serine residues, with a comparatively small amount of threonine phosphorylation (T.P.McDonald; in press). An analysis of protein kinase phosphorylation sites in the cytoplasmic domains of the VPAC$_2$ receptor was conducted using published consensus motifs (Kemp and Pearson, 1990; Kennelly and Krebs, 1991) (**Table 5.3**), these sites are shown in **Figure 5.10**.

Evidence is accumulating for a phosphorylation-dependent mechanism of VPAC$_2$ receptor desensitisation. When HEK293 cells stably transfected with the VPAC$_2$ and VPAC$_2$-HA receptors were pretreated with an inhibitor of PKA (H-89) the maximum VIP-stimulated cAMP formation was enhanced, whereas an inhibitor of PKC (bisindolymaleimide) had no effect (McDonald et al., 1998). These results suggest that PKA may be involved in desensitisation of this receptor. PKA phosphorylation sites are mainly located in the C-terminal intracellular domain, as removal of this region prevents desensitisation it seems reasonable to hypothesise that phosphorylation may be involve in receptor/G-protein uncoupling.

Interestingly recent work in our laboratory by Dr T.P.McDonald found that TT425 and TT409 are phosphorylated to a similar extent as the VPAC$_2$-HA receptor in response to agonist treatment, whereas TT393, TT391 and TT385 do not undergo agonist-induced phosphorylation (T.P.McDonald; in press). These data suggest that the majority of phosphorylation occurs in the cytoplasmic tail between residues Cys^{394} and Ser^{409}. These data cannot exclude the possibility that agonist-induced phosphorylation occurs in the distal portion of the C-terminal tail between Phe^{410} and Leu^{438}, which is compensated for by phosphorylation at alternative sites in the truncated/tagged receptors. The complete absence of agonist-induced

phosphorylation in TT393, TT391 and TT385 suggests that serine residues in other cytoplasmic domains are not major sites for VPAC$_2$ receptor phosphorylation. The inability of cell transfected with TT391 to desensitise in response to pretreatment with VIP may be a reflection of the lack of phosphorylation of this receptor. Ultimately the identification and mutagenesis of specific phosphorylation sites may prove a more informative method of determining the relationship between these two phenomena.

Phosphorylation and desensitisation of class I and class II GPCRs involves second messenger-dependent and -independent kinases

A relationship between receptor desensitisation and phosphorylation is now well established for the class I GPCR family. The C-terminal intracellular domains of GPCRs have been identified as important sites for agonist-induced phosphorylation as they are often rich in serine and threonine residues (see section 1.4.2 and 1.4.3). The β_2AR is a well characterised example, its desensitisation is dependent upon agonist-induced phosphorylation by second messenger-dependent kinases and GRKs (Bouvier et al., 1988; Lefkowitz et al., 1990). Evidence from studies of class II GPCRs have found a similar phosphorylation-dependent mechanism for desensitisation (Holtmann et al., 1996; Pernalete et al., 1990; Widmann et al., 1996). Moreover, for many of these receptors GRK mediated phosphorylation influences their desensitisation (Fukayama et al., 1997; Nygaard et al., 1997; Shetzline et al., 1998). Thus phosphorylation appears to be a prerequisite for desensitisation of both class I and class II GPCRs.

Role of second messenger-dependent and -independent kinases in VPAC$_2$ receptor phosphorylation and desensitisation

Analysis of the extent of phosphorylation of the VPAC$_2$ receptor in response to treatment with forskolin (which stimulates AC) or VIP, revealed that the action of PKA was unable to account for all of the observed phosphorylation (McDonald et al., 1998). Thus a second messenger-independent kinase may be required for VPAC$_2$ receptor phosphorylation. GRKs are likely candidates as they specifically phosphorylate agonist-occupied receptors. The desensitisation of several class II GPCRs has recently been shown to be influenced by the presence of GRKs (Fukayama et al., 1997; Nygaard et al., 1997; Shetzline et al., 1998). Experiments to determine whether the VPAC$_2$ receptor is a substrate for GRKs would be interesting. Several methods are possible, including looking at the effect of inhibitors of second messenger dependent kinases on agonist-induced phosphorylation, previous studies

have used staurosporine - which is able to inhibit both PKA and PKC - to demonstrate the involvement of an alternative kinase (Nygaard et al., 1997). More direct methods involve cotransfection of VPAC$_2$ receptor expressing cells with GRKs or dominant negative mutant GRKs (Fukayama et al., 1997; Mundell et al., 1997; Mundell et al., 1998b; Shetzline et al., 1998). Unlike second messenger-dependent protein kinases, there are no clear consensus sequences for GRK recognition, although pairs of acidic amino acids C-terminal and N-terminal to phosphorylation sites favour substrate phosphorylation by GRK1 and GRK2, respectively (Onorato et al., 1991). *In vitro* GRK phosphorylation occurs at multiple sites in the distal portion of the C-termini of the β$_2$AR and rhodopsin receptor (Fredericks et al., 1996) and in the third intracellular loop of the M$_2$-muscarinic receptor (Nakata et al., 1994). Ultimately direct amino acid sequencing of the radiolabelled receptor will be necessary to confirm phosphorylation at specific residues in the VPAC$_2$ receptor C-terminus.

Summary

The results presented in this chapter and phosphorylation data previously obtained by Dr T.P.McDonald for the full length and truncated/tagged VPAC$_2$ receptors is summarised in **Table 5.4**. Overall these results reveal that the VPAC$_2$ receptor C-terminal intracellular domain is not involved in agonist-induced receptor internalisation, although it can influence the rate of endocytosis. In contrast severe truncation of the C-terminal intracellular domain did effect VPAC$_2$ receptor desensitisation. From this data and other experiments in our laboratory considerable evidence is accumulating for a phosphorylation-dependent mechanism of VPAC$_2$ receptor desensitisation. Overall it appears that internalisation and desensitisation are regulated by different regions of the VPAC$_2$ receptor. Indeed distinct structural determinants for these two properties have been shown for a number of class II GPCRs (Holtmann et al., 1995; Widmann et al., 1997). Further work is required to establish specific regions of the VPAC$_2$ receptor which are involved in these processes. Finally, TT391 has been shown to internalise but does not desensitise lending further weight to the argument that receptor internalisation is not a requirement for the cessation of intracellular signalling.

CHAPTER 6

Intracellular trafficking and recycling of the VPAC$_2$ receptor

6.1 Introduction

The majority of GPCRs undergo agonist-induced internalisation. Once internalised the ligand/receptor complexes can have a number of different fates (see section 1.5.3). Internalised GPCRs are predominantly recycled to the cell surface, whereas their ligands are degraded (this pattern of internalisation has been categorised as a type I endocytic pathway: Shepherd, 1989). GPCRs are capable of undergoing many rounds of endocytosis and recycling without impairment of their binding or signalling capabilities (Mellman et al., 1986). The proportion of receptors that are recycled, compared to those that are degraded, may be influenced by the concentration of agonist and incubation time. To date evidence from studies of $VPAC_1$ and $VPAC_2$ receptors (expressed in HT-29 and SUP-T1 lymphoblasts respectively), indicates that both of these receptors recycle whilst VIP is degraded intracellularly. Accordingly, studies investigating internalisation of $[^{125}I]$-VIP in HT-29 cells found that the internalised peptide accumulates in lysosomes where it is degraded and then released into the surrounding medium (for reviews see Luis et al., 1988; Rosselin et al., 1988). Recycling of internalised VIP/PACAP receptors has been determined indirectly by measuring loss and recovery of VIP binding sites at the cell surface in HT-29 cells and SUP-T1 lymphoblasts. This type of experiment reveals that the number of binding sites decreases in a dose-dependent manner following stimulation with VIP, this process is reversible and receptor number recovers upon removal of agonist (Boissard et al., 1986; Robberecht et al., 1989a).

In order to directly demonstrate $VPAC_2$ receptor recycling; immunofluorescence studies of receptor localisation were performed using cells treated with VIP then allowed to recover in the absence of peptide. This experiment was performed in the absence and presence of a protein synthesis inhibitor to establish whether new protein synthesis is required for receptor recycling. In addition, the intracellular location of internalised $VPAC_2$ receptors was compared with TGN46 a marker for the *trans*-Golgi network (TGN) (Prescott et al., 1997). The TGN is a distinct compartment of the Golgi complex located on its *trans* surface and is a site for protein sorting and release. Some constitutively recycling receptors have been shown to enter this compartment following endocytosis (see section 6.3). Intracellular trafficking of the $VPAC_2$ receptor was also compared with the constitutively recycling transferrin receptor (TfnR), which is as a marker for sorting and recycling endosomes.

As VIP and its receptor have different intracellular fates, they must dissociate at some point along the endocytic pathway. Endosomal acidification has been proposed as a means of separating ligand from receptor (for a review see Maxfield and Yamashiro, 1987). Accordingly, inhibition of endosome acidification has been found to prevent recycling of a number of GPCRs. The role of endosome acidification in $VPAC_2$ receptor recycling was investigated using bafilomycin A_1 (baf A_1) a specific inhibitor of vacuolar ATPases which are responsible for the low pH found in endosomes.

6.2 Results

6.2.1 Recycling of the $VPAC_2$ receptor

The immunofluorescence technique described previously, was used to determine whether internalised $VPAC_2$ receptors recycle back to the cell surface following removal of agonist. HEK293 cells stably transfected with the $VPAC_2$-HA receptor were incubated with VIP (10 µM for 30 mins) at 37°C, the stimulus was removed by washing and the cells incubated for a further 1-2 hrs in serum free medium (**Figures 6.1A-D** and **6.2A-D**). Treatment with VIP caused the receptor to shift to a single intracellular site (**Figure 6.2B**). Removal of VIP followed by a 1 hr incubation in the absence of stimulating peptide had little effect on the pattern of staining observed for the internalised $VPAC_2$ receptor (**Figure 6.2C**). After a 2 hr incubation in the absence of stimulating peptide $VPAC_2$ receptor staining reappeared back at the cell surface, however this staining was punctate in comparison with untreated cells suggesting that the recycling of the receptor was not complete (**Figure 6.2D**). These experiments demonstrate that $VPAC_2$ receptor internalisation is reversible. Longer incubations in the absence of peptide would be required to define the complete time course of receptor reappearance back at the cell surface.

6.2.2 Effect of cycloheximide on internalisation and recycling of the $VPAC_2$ receptor

There are two possible mechanisms to account for the reappearance of $VPAC_2$ receptor staining at the cell surface: either internalised receptor is recycled or new receptor is synthesised and transported to the plasma membrane. The protein synthesis inhibitor, cycloheximide was used to evaluate whether the latter mechanism is involved in recycling. The concentration of cycloheximide used has previously been reported to be an effective inhibitor of protein synthesis in cells expressing endogenous VIP/PACAP receptors in experiments from other

laboratories (Luis et al., 1987; Robberecht et al., 1989a). VPAC$_2$-HA transfected cells were treated with VIP (10 μM for 1 hr) at 37°C, in the presence of cycloheximide (40 μM), before being washed and incubated in serum free medium with or without cycloheximide. The pattern of staining for the VPAC$_2$ receptor in untreated and VIP treated cells was unaffected by preincubation with cycloheximide **(Figure 6.3A,B)**. In agonist treated cells, which were washed and allowed to recover for 2 hrs after incubation with peptide, staining for the VPAC$_2$ receptor was predominantly observed at the plasma membrane **(Figure 6.3C)**. This reappearance of receptor back to the cell surface was unaffected by the presence of cycloheximide **(Figure 6.3D)**. These data suggest that newly synthesised receptors do not form a major part of the recycling VPAC$_2$ receptor population.

6.2.3 Localisation of the VPAC$_2$ receptor and a marker for the *trans*-Golgi network

An antibody against the integral membrane glycoprotein TGN46, an established marker for the *trans*-Golgi network (TGN), was used in immunofluorescence studies to investigate the intracellular location of the internalised receptor. Cells were preincubated in serum free medium or with VIP (10 μM for 30 mins) at 37°C, fixed and incubated with either FITC-conjugated anti-VPAC$_2$-HA or TXR-conjugated anti-TGN46 antibodies, or both. No specific staining was observed when the primary antibodies were excluded from the experiment and specific fluorescent staining for a single marker was only observed with the appropriate fluorescence filters (data not shown). As shown previously the VPAC$_2$ receptor was predominantly observed at the plasma membrane in untreated cells **(Figure 6.4A)** and at a single intracellular site following incubation with VIP **(Figure 6.4B)**. In the same cells, TGN46 was confined to a discrete juxtanuclear site in both untreated **(Figure 6.4C)** and VIP pretreated cells **(Figure 6.4D)**. From this experiment the site of internalised VPAC$_2$ receptor appears to colocalise with TGN46. These cells were also viewed using a dual fluorescence filter, permitting staining for both fluorochromes to be observed simultaneously. In untreated cells the VPAC$_2$ receptor was located mainly in the plasma membrane and TGN46 staining was within the cells, little overlap between the signal was observed **(Figure 6.5A,B)**. After treatment with VIP both markers were located intracellularly and in close proximity to each other, however there were few sites at which colocalisation was apparent, as would be evidenced by a yellow staining pattern **(Figure 6.5C,D)**.

To verify these findings cells were analysed using digital imaging microscopy. This technique removes out-of-focus fluorescence and provides a clearer picture of the precise location of fluorescent markers. In addition this technique permits three-dimensional reconstruction of the cells (data not shown). In untreated cells the $VPAC_2$-HA receptor was localised to the membrane, whilst the TGN46 marker was intracellular and appeared to form a ring-like structure (**Figure 6.6A,B**). This structure is similar to the tubulo-vesicular structure described in electron micrographs of the rat homologue of TGN46 (Luzio et al., 1990). The internalised $VPAC_2$ receptor and TGN46 marker did not colocalise in cells preincubated with VIP (10 µM for 30 mins) at 37°C (**Figure 6.7A,B**). The pattern of fluorescence for the internalised $VPAC_2$ receptor was adjacent to the TGN46 receptor but the two fluorescence signals did not overlap. The lack of colocalisation of these antibodies indicates that, following short term incubation with VIP, the internalised $VPAC_2$ receptor is not targeted to the TGN.

6.2.4 Localisation of the $VPAC_2$ receptor and a marker for the transferrin receptor

The transferrin receptor (TfnR) is a constitutively recycling receptor which is expressed in many cell lines, including HEK293 cells (Fonseca et al., 1995; Moore et al., 1995). The TfnR and its ligand have been used as markers for sorting and recycling endosomes. A mouse anti-TfnR antibody was used in combination with the a TXR-conjugated secondary antibody, whilst a rabbit anti-HA tag antibody recognised by FITC-conjugated secondary antibodies was used to stain for the $VPAC_2$-HA receptor. HEK293 cells stably transfected with the $VPAC_2$-HA receptor were incubated in serum free medium or VIP (10 µM for 30 mins) at 37°C prior to fixing. These cells were then processed for immunofluorescence with antibodies against either the $VPAC_2$-HA or TfnRs, or both. No specific staining was observed when the primary antibodies were excluded from the experiment. Specific fluorescent staining for a single marker was only observed with the appropriate fluorescence filters (data not shown). In untreated cells the majority of $VPAC_2$ receptor fluorescence was located in the cell membrane (**Figure 6.8A**), whereas the TfnR was found intracellularly with a few areas of punctate staining in the cell membrane (**Figure 6.8B**). VIP pretreatment caused the $VPAC_2$ receptors to internalise to a single juxtanuclear site, which appeared to correspond with the intracellular location of the transferrin receptor (**Figure 6.9C,D**). Both sets of antibody staining were viewed simultaneously using a dual fluorescence filter. In untreated cells some colocalisation of the $VPAC_2$-HA and TfnR staining was

observed **(Figure 6.10A,B)**. In VIP-stimulated cells there was a marked overlap in staining for the VPAC$_2$-HA and TfnRs, visible as yellow staining pattern **(Figure 6.10C,D)**.

This finding was confirmed using digital imaging microscopy. Fluorescence in untreated cells was predominantly observed in the cell membrane for the VPAC$_2$ receptor and intracellularly for the TfnR, although some staining could be seen in punctate structures at the cell surface **(Figure 6.11A)**. In cells treated with VIP (10 µM for 30 mins) the internalised VPAC$_2$ receptor staining overlapped with staining for the TfnR resulting in yellow staining **(Figure 6.11B)**. These data indicate that the VPAC$_2$ receptor and TfnR colocalise and may therefore share a common route of intracellular trafficking.

6.2.4 Effect of bafilomycin A$_1$ on recycling of the VPAC$_2$ receptor

Bafilomycin A$_1$ (baf A$_1$) is a macrolide antibiotic which inhibits vacuolar ATPases (vATPases) and prevents endosome acidification. Baf A$_1$ is effective at a relatively low concentrations, in previous experiments 0.25 µM baf A$_1$ has been shown to inhibit endosome acidification and prevent receptor recycling in living cells of several types (Johnson et al., 1993). This compound was used in combination with immunofluorescence to establish whether endosome acidification is necessary for VPAC$_2$ receptor recycling. Staining for the VPAC$_2$ receptor in control cells was predominantly located at the cell surface, when these cells were washed for 2 hrs the cell surface staining was punctate compared with non washed cells **(Figure 6.12A)**. In cells preincubated in vehicle (0.3% DMSO) for 30 mins at 37°C the VPAC$_2$ receptor location was not different from normal under any of the experimental conditions (data not shown). Pretreatment with baf A$_1$ (1 µM in 0.3% DMSO) for 30 mins at 37°C, had no effect on the cell surface distribution of the VPAC$_2$ receptor in untreated cells (data not shown) or on internalisation of receptor in cells treated with VIP (10 µM for 30 mins) **(Figure 6.12C)**. After a 2 hr wash in the absence of stimulating peptide the VPAC$_2$ receptor has been shown to recycle back to the cell surface **(Figure 6.12B)**. However when baf A$_1$ treated cells were washed and incubated in the absence of peptide for 2 hrs the internalised receptor no longer recycled back to the cell surface **(Figure 6.12D)**. These data suggest that endosome acidification is necessary for recycling of the VPAC$_2$ receptor back to the plasma membrane.

Figure 6.1   Redistribution of internalised VPAC$_2$ receptors following removal of agonist and incubation in serum free medium. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with 0 (**A**) or 10 μM (**B,C,D**) VIP for 30 mins at 37°C, the cells were washed three times in PBS and fixed (**A,B**), or incubated for a further 1 hr (**C**) or 2 hrs (**D**) in serum free medium at 37°C prior to fixation. Cells were viewed at x40 magnification under appropriate fluorescence conditions.
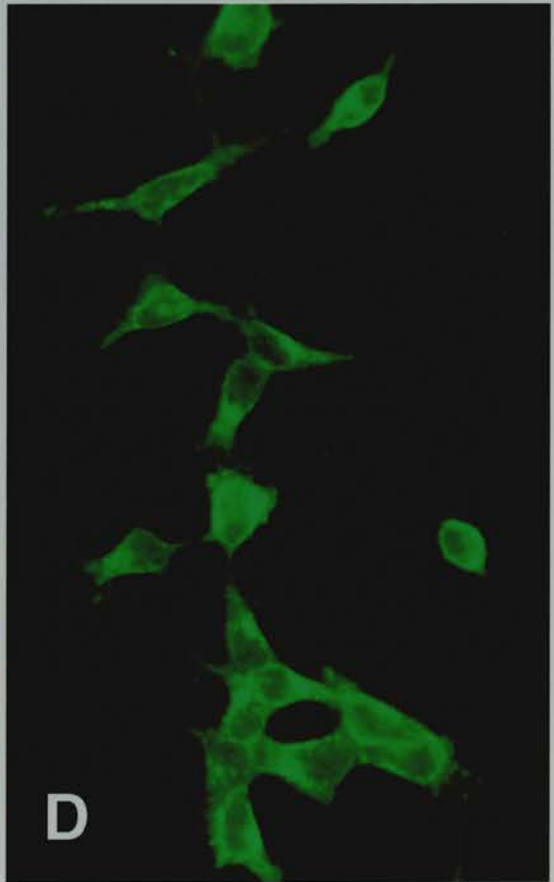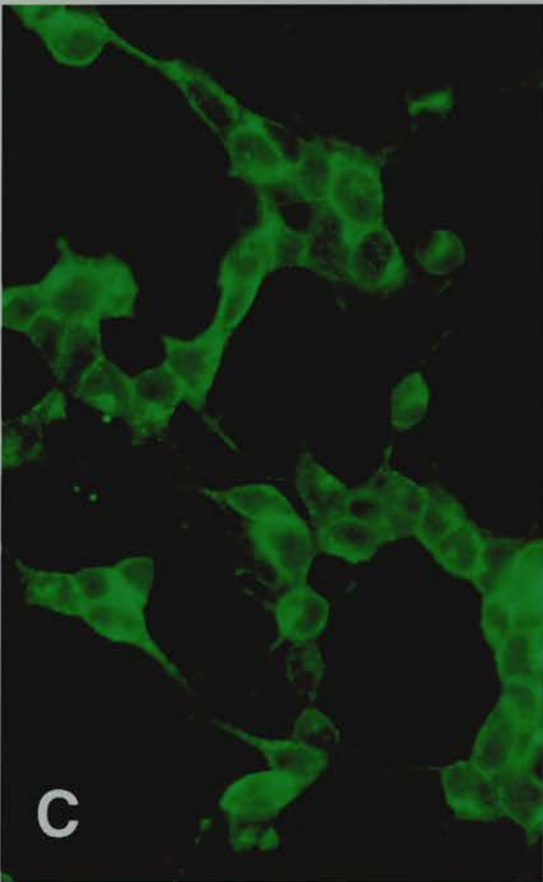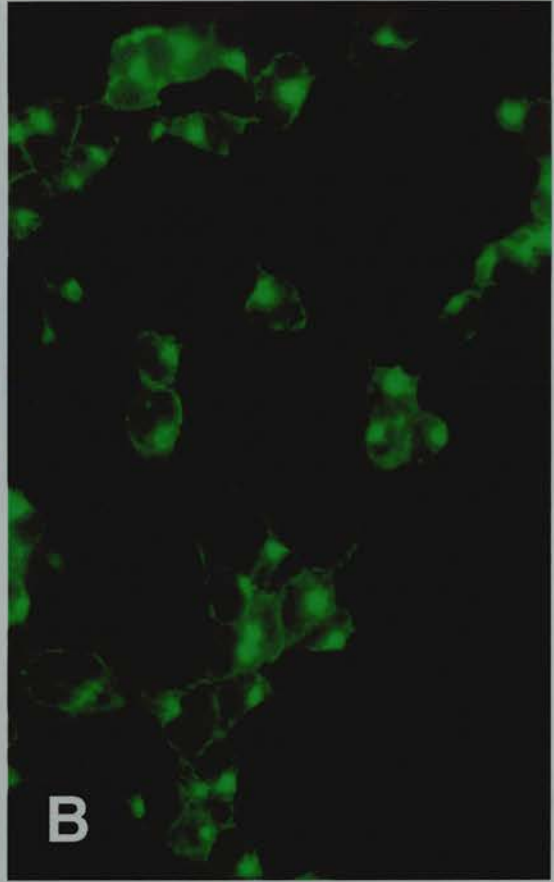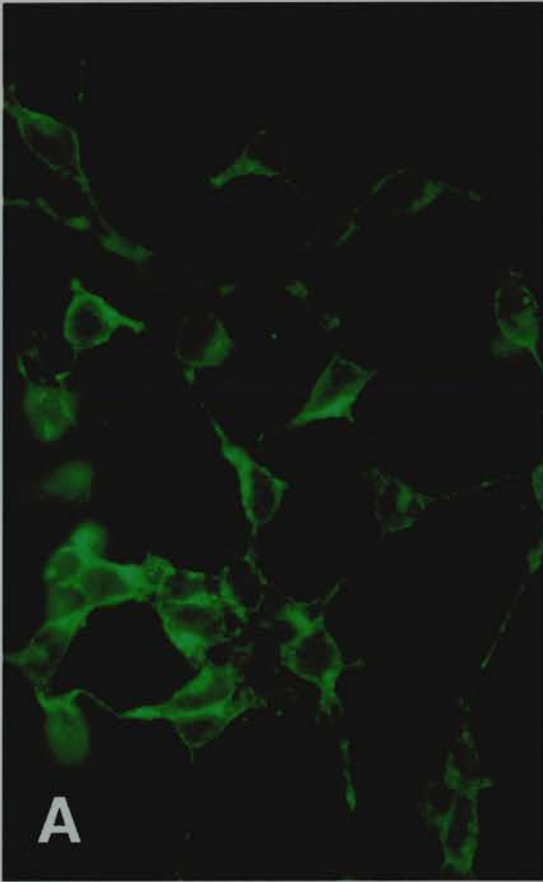
Figure 6.2 Redistribution of internalised VPAC$_2$ receptors following removal of agonist and incubation in serum free medium. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with 0 (**A**) or 10 μM (**B,C,D**) VIP for 30 mins at 37°C, the cells were washed three times in PBS and fixed (**A,B**), or incubated for a further 1 hr (**C**) or 2 hrs (**D**) in serum free medium at 37°C prior to fixation. Cells were fixed and viewed at x100 magnification under appropriate fluorescence conditions.
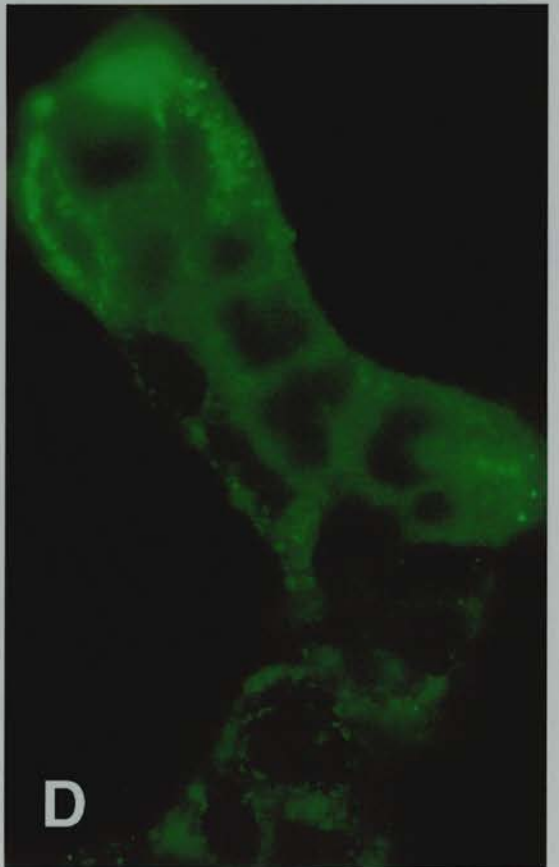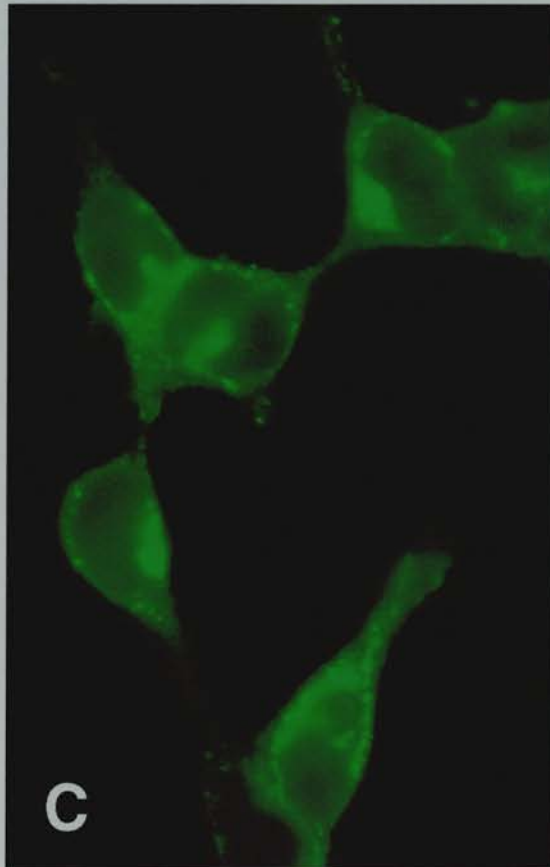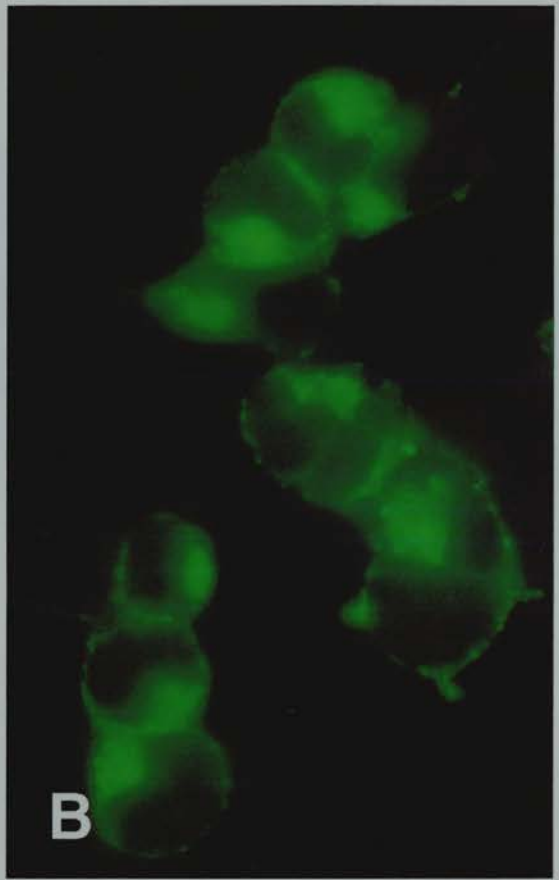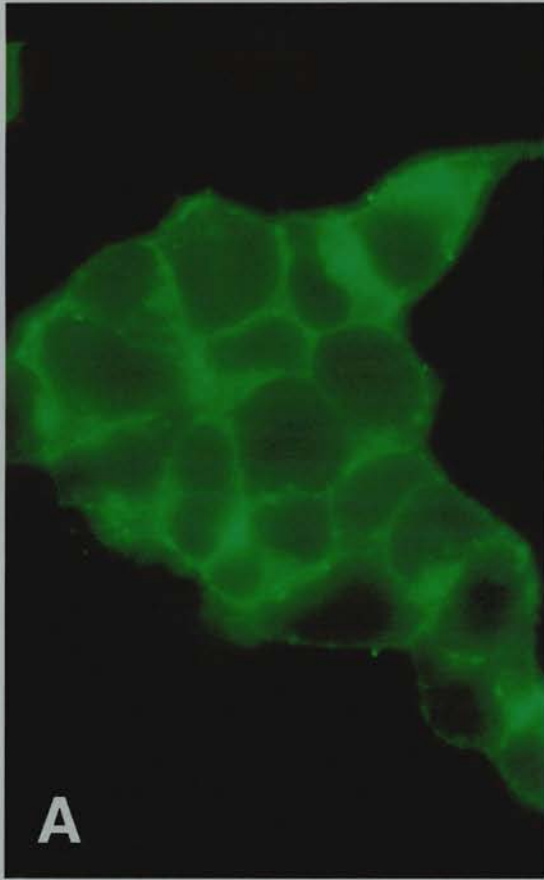
Figure 6.3 **Effect of cycloheximide on recycling of the VPAC$_2$ receptor.** HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with 40 μM cycloheximide (**A**) or 10 μM VIP and 40 μM cycloheximide (**B,C,D**) for 30 mins at 37°C, washed three times with PBS and fixed (**A,B**) or incubated for a further 2 hrs in serum free medium (**C**) or serum free medium containing 40 μM cycloheximide (**D**). Cells were fixed and viewed at x100 magnification under appropriate fluorescence conditions.
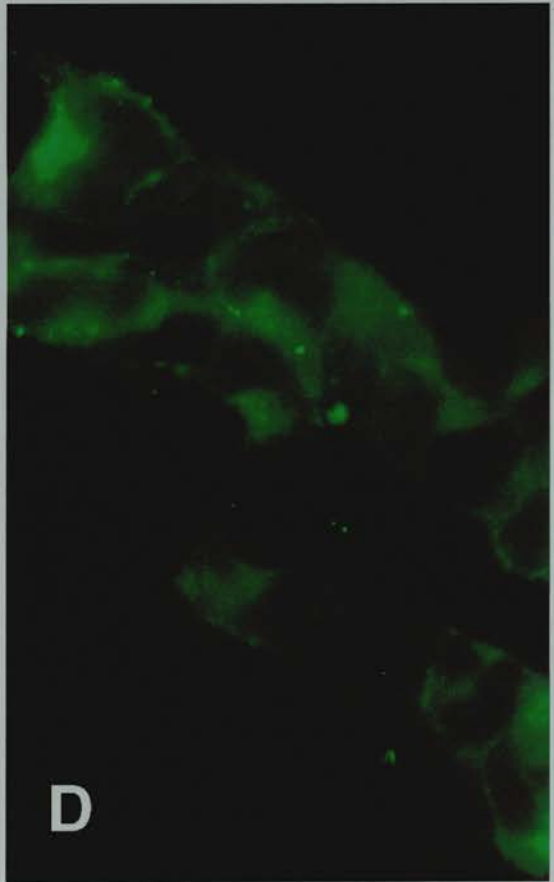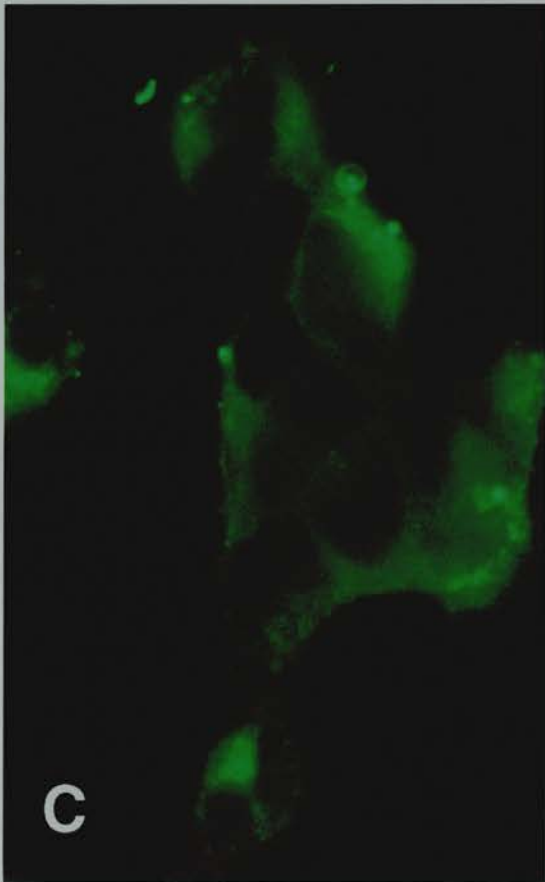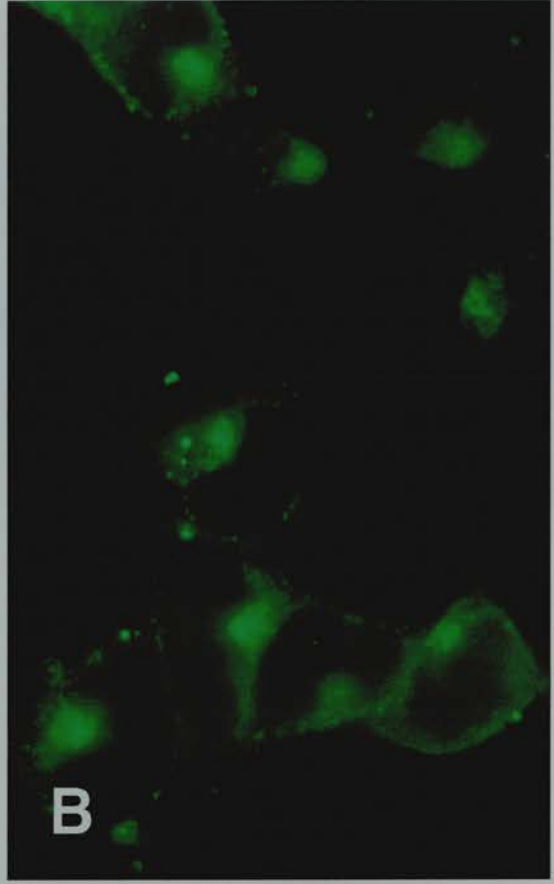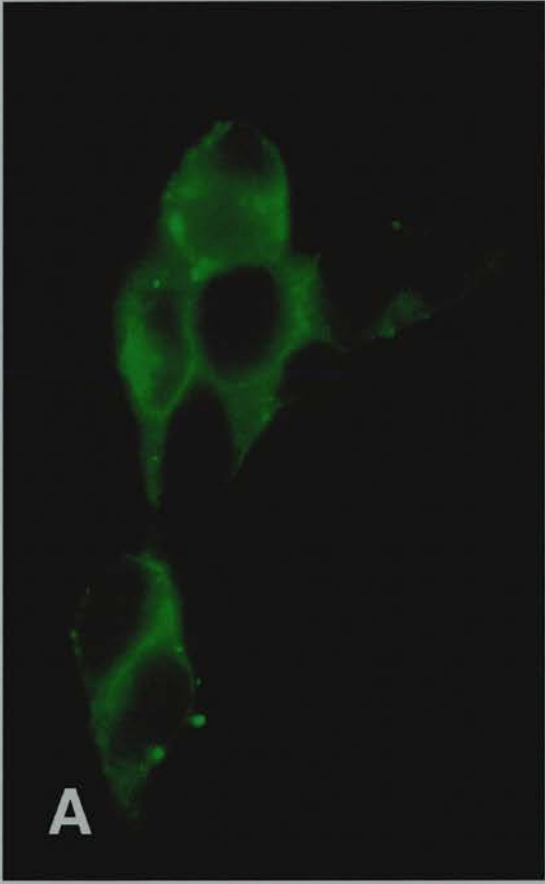
Figure 6.4 Localisation of the VPAC$_2$ receptor and a marker for the trans-Golgi network (TGN46) in HEK293 cells. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with serum free medium (**A,B**) or 10 μM VIP (**C,D**) for 30 mins at 37°C. Cells were fixed and incubated with antibodies against the VPAC$_2$ receptor conjugated to FITC (green) and TGN46 conjugated to Texas red (red). Fluorescence pictures of corresponding cells show location of the VPAC$_2$ receptor and TGN46 in untreated cells (**A,B**), respectively and cells pretreated with VIP (**C,D**), respectively. Cells were viewed at x100 magnification, under the appropriate fluorescence conditions. Arrows indicate comparable areas in each cell.
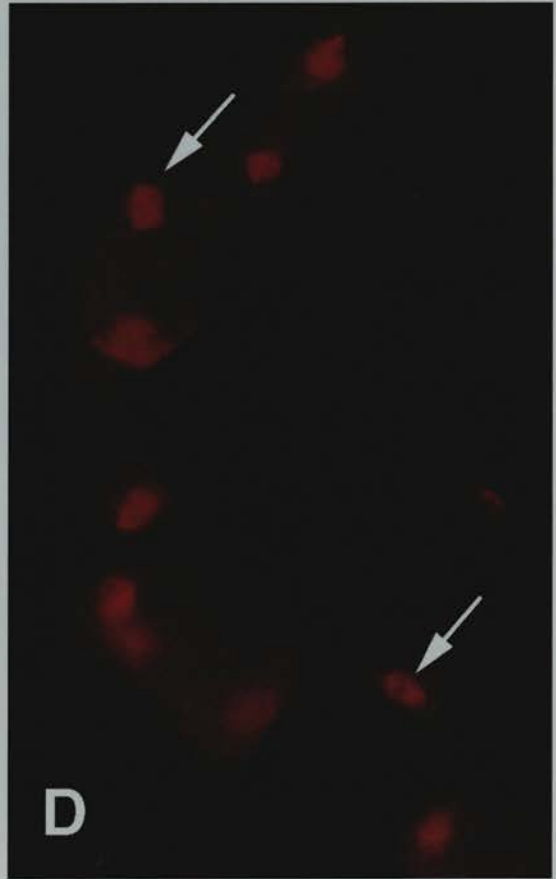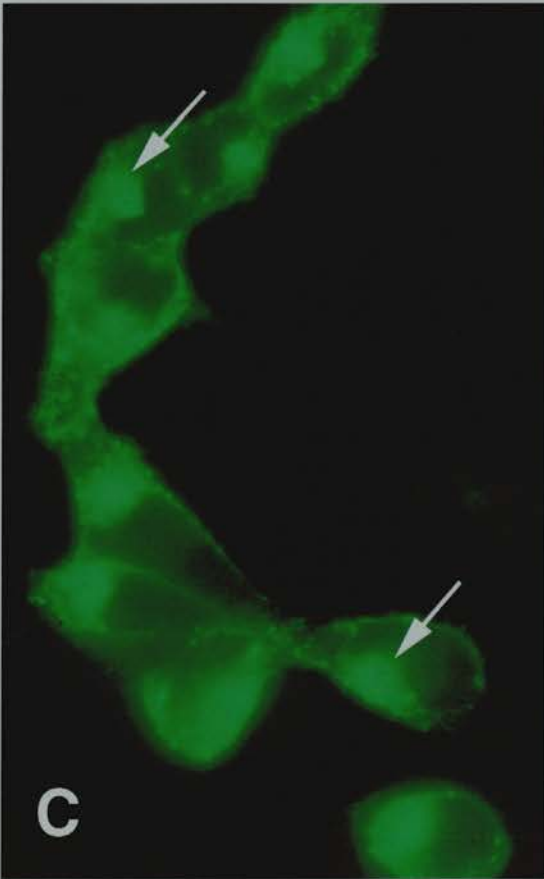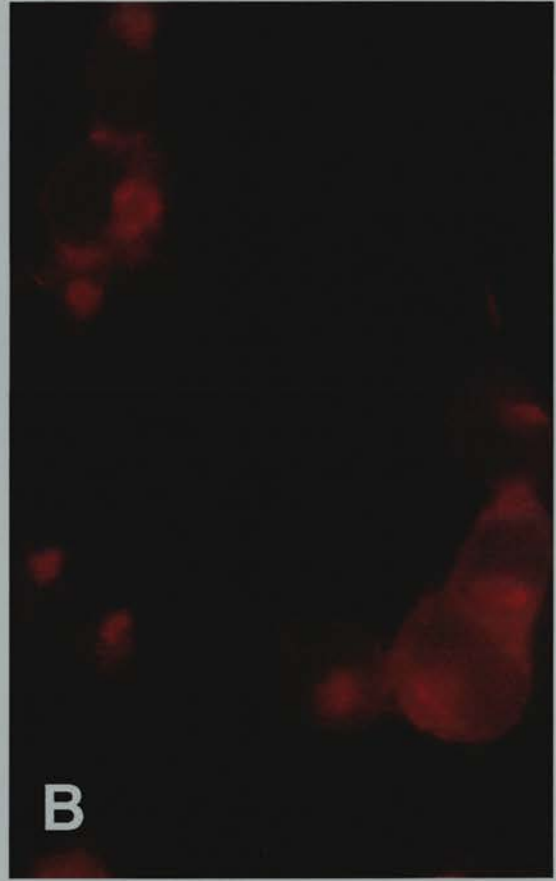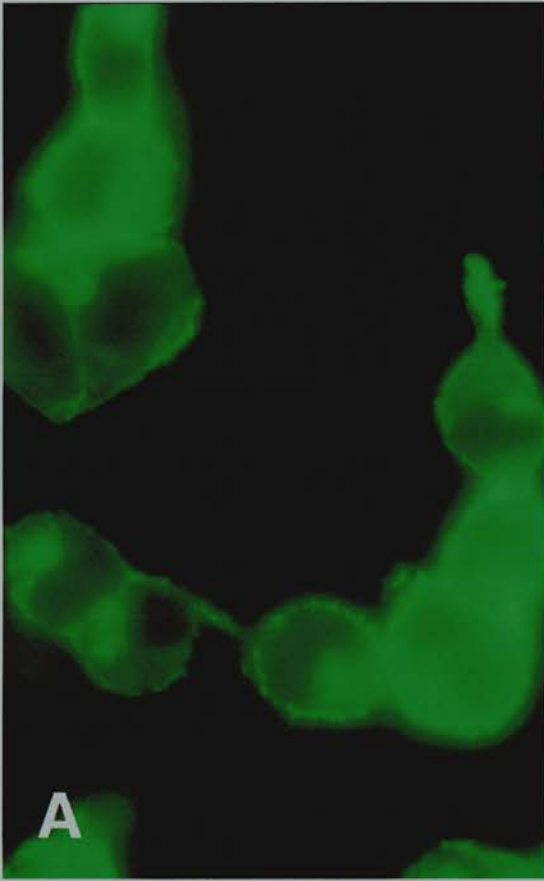
Figure 6.5   Localisation of the VPAC$_2$ receptor and a marker for the *trans*-Golgi network (TGN46) viewed using dual antibody fluorescence filters. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated in serum free medium (**A**,**B**) or 10 μM VIP (**C**,**D**) for 30 mins at 37°C. Cells were fixed and incubated with antibodies against the VPAC$_2$ receptor conjugated to FITC (green) and TGN46 conjugated to Texas red (red). A dual fluorochrome filter was used so that specific staining by both antibodies could be observed simultaneously. Cells were viewed at x100 magnification under appropriate fluorescence condition. Two examples of the staining pattern are given, for untreated cells (**A**,**B**) and for 10 μM VIP pretreated cells (**C**,**D**).
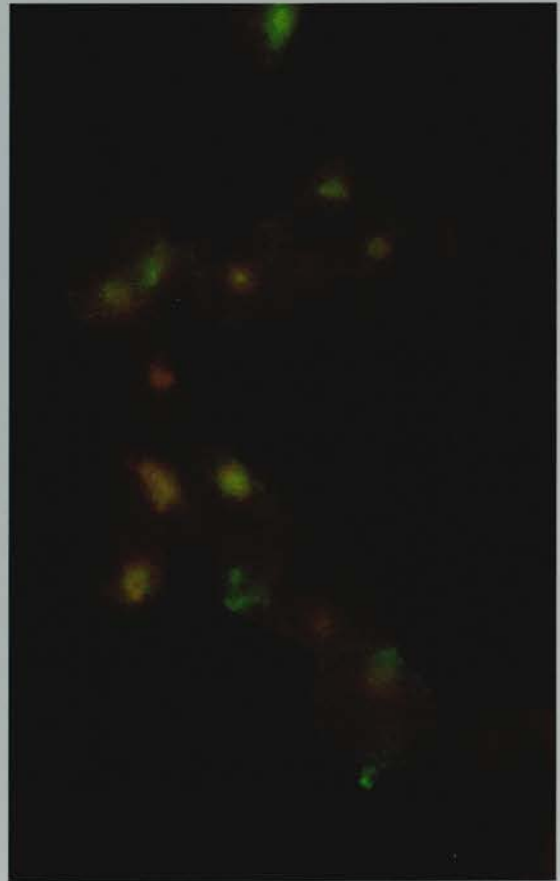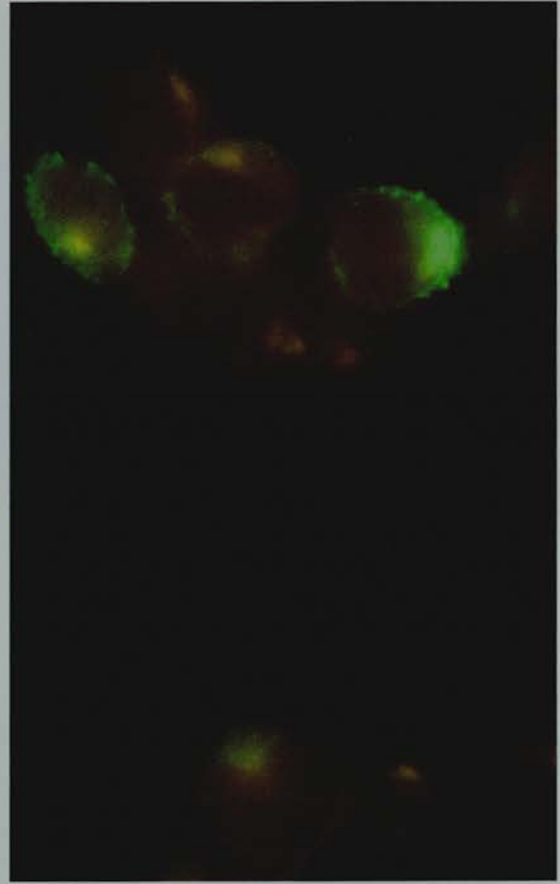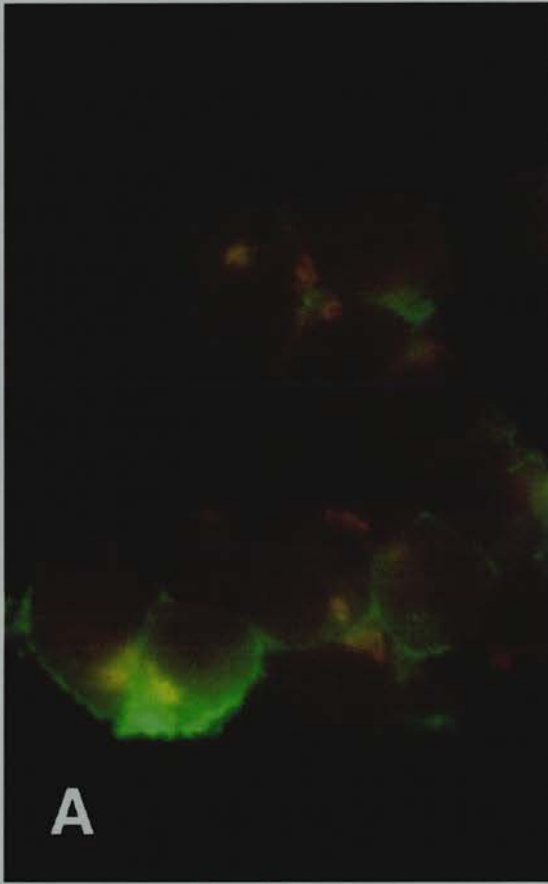
Figure 6.6 Localisation of the VPAC$_2$ receptor and a marker for the *trans*-Golgi network (TGN46) in HEK293 cells using digital imaging microscopy. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with serum free medium for 30 mins at 37°C. Cells were fixed and incubated with antibodies against the VPAC$_2$ receptor conjugated to FITC (green) and TGN46 conjugated to Texas red (red) and viewed using digital imaging. Images through the Z plane were taken every 1 μm using a Zeiss inverted microscope connected to a CCD camera. These images were reconstructed and deconvoluted to remove out-of-focus fluorescence from adjacent images. A single image through the centre of the cells is presented here at x63 (**A**) and x100 (**B**) magnification.
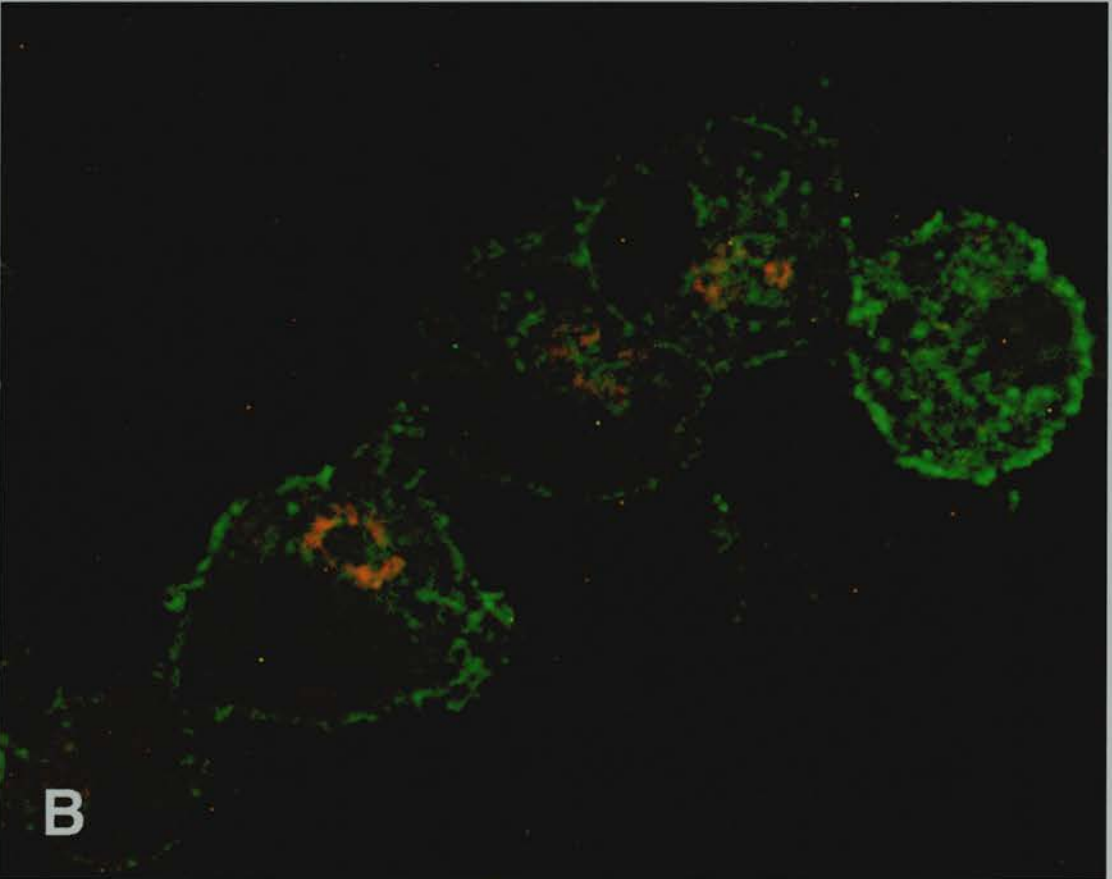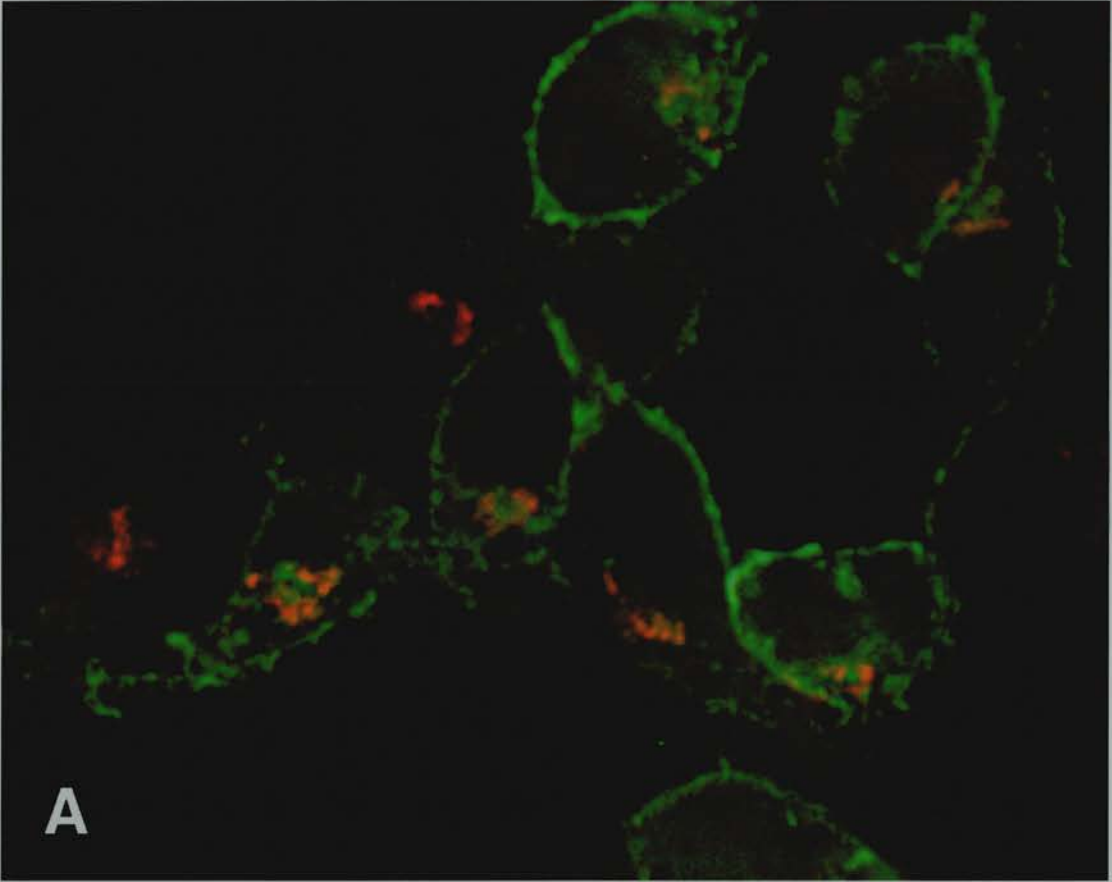
Figure 6.7 Localisation of the VPAC$_2$ receptor and a marker for the *trans*-Golgi network (TGN46) in HEK293 cells pretreated with VIP using digital imaging microscopy . HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with 10 μM VIP for 30 mins at 37°C. ells were fixed and incubated with antibodies against the VPAC$_2$ receptor conjugated to FITC (green) and TGN46 conjugated to Texas red (red) and viewed using digital imaging. Images through the Z plane were taken every 1 μm using a Zeiss inverted microscope connected to a CCD camera. These images were reconstructed and deconvoluted to remove out-of-focus fluorescence from adjacent images. A single image through the centre of the cells is presented here at x63 (**A**) and x100 (**B**) magnification.
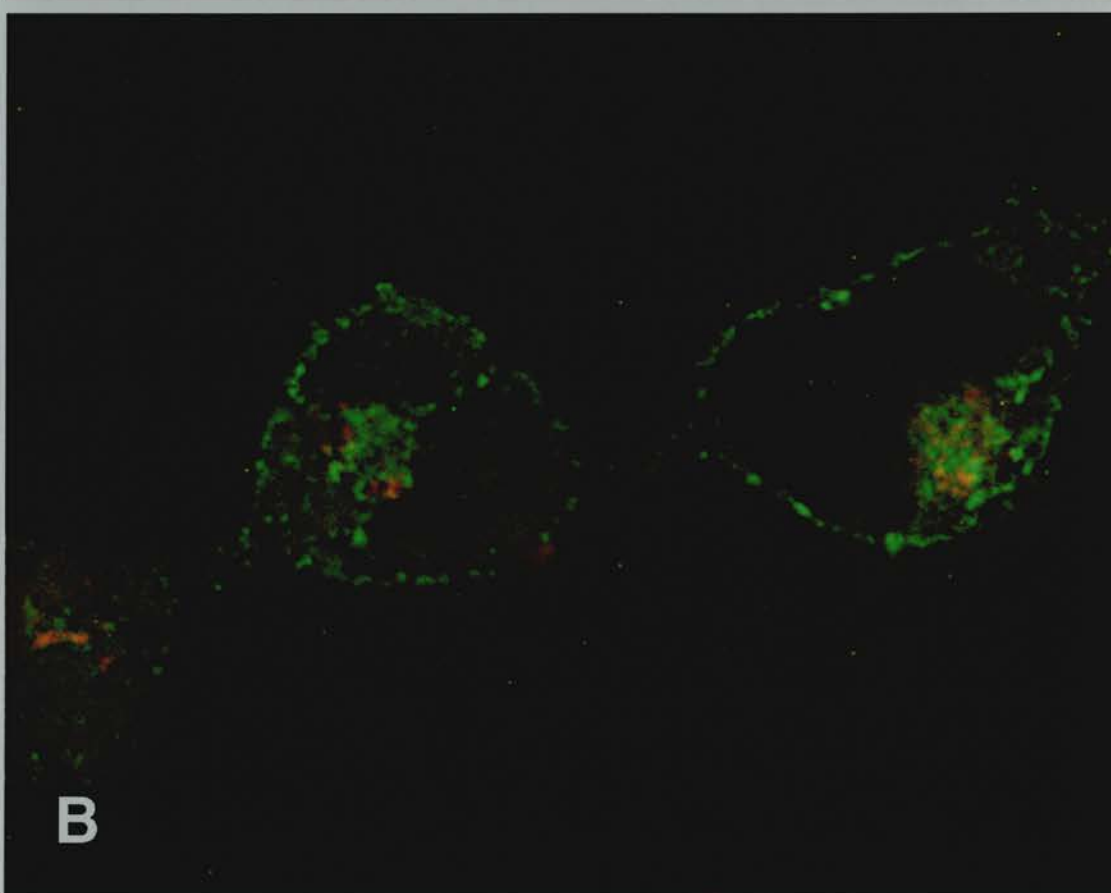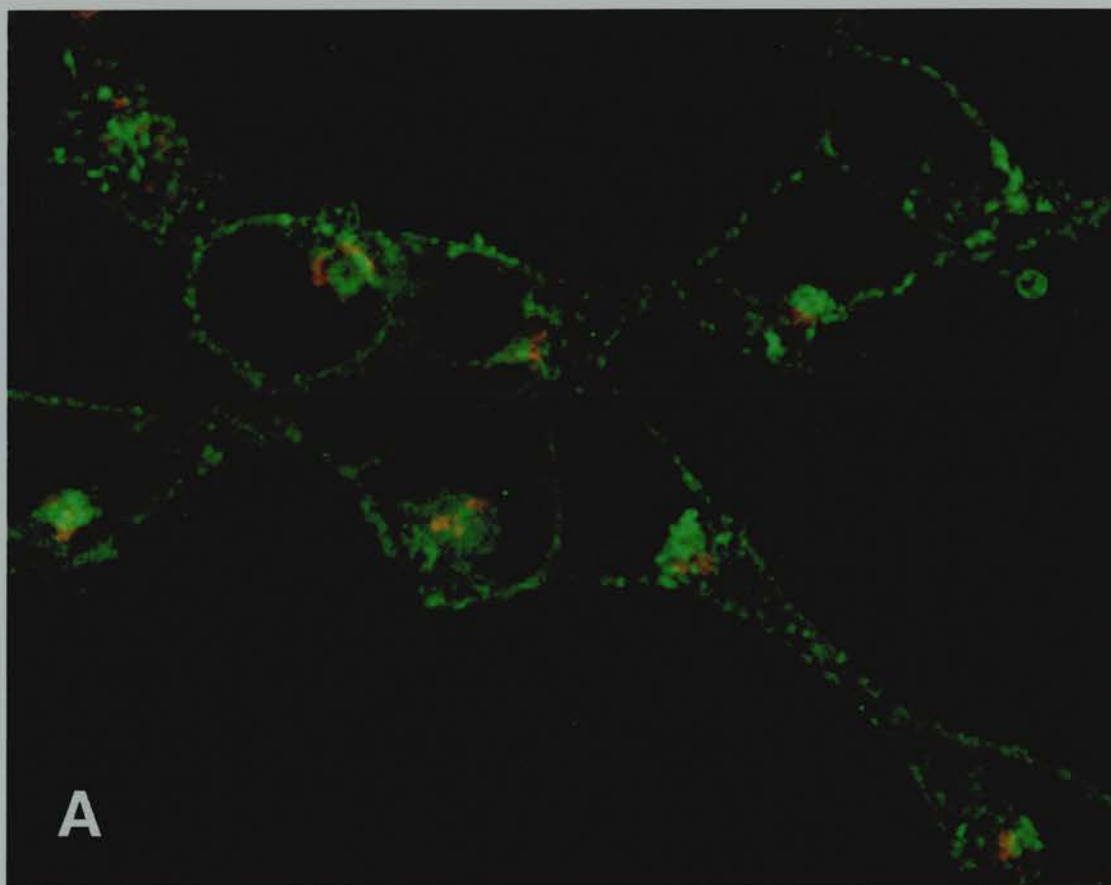
Figure 6.8 Localisation of the VPAC$_2$ receptor and a marker for the transferrin receptor (TfnR). HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with 0 (**A,B**) or 10 μM VIP (**C,D**) for 30 mins at 37°C. Cells were fixed and incubated with antibodies against the VPAC$_2$ receptor conjugated to FITC (green) and TfnR conjugated to Texas red (red). Fluorescence pictures of corresponding cells show location of the VPAC$_2$ receptor and TfnR (**A,B**) in untreated cells and cells pretreated with 10 μM VIP (**C,D**), respectively. Cells were viewed at x100 magnification under appropriate fluorescence condition. Arrows indicate points at which specific staining for both markers overlap.
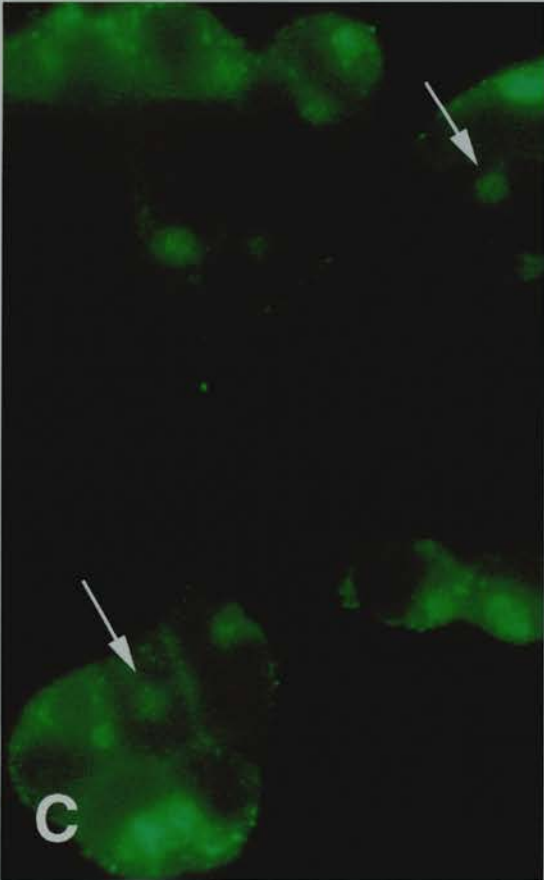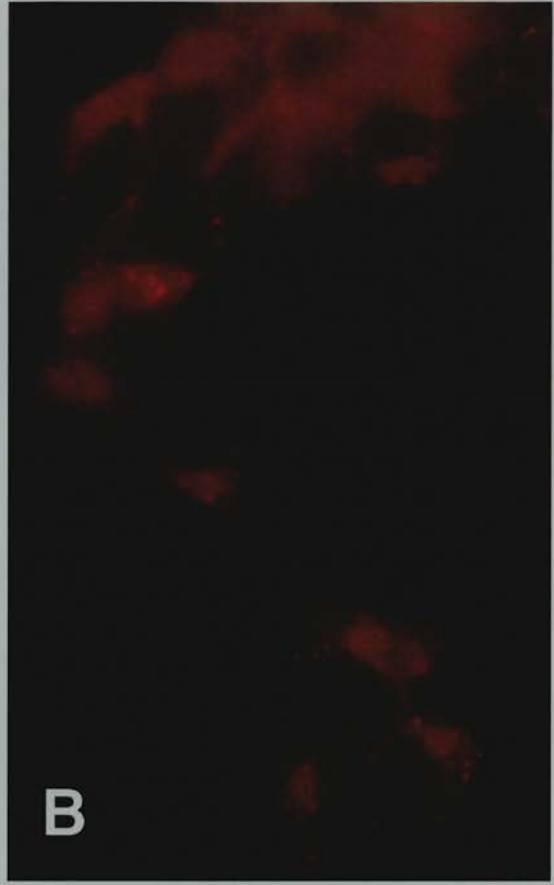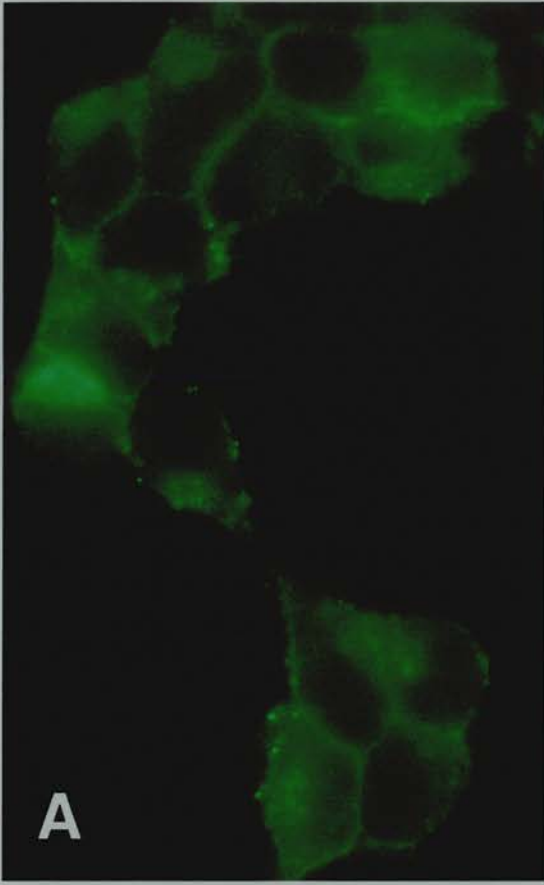
Figure 6.9 Localisation of the VPAC$_2$ receptor and a marker for the transferrin receptor (TfnR) viewed using dual antibody fluorescence filters. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were incubated in serum free medium (**A,B**) or 10 μM VIP (**C,D**) for 30 mins at 37°C. Cells were fixed and incubated with antibodies against the VPAC$_2$ receptor conjugated to FITC (green) and the TfnR conjugated to Texas red (red). A dual fluorochrome filter was used so that specific staining by both antibodies could be observed simultaneously. Cells were viewed at x100 magnification under appropriate fluorescence condition. Two examples of the staining pattern are given, for untreated cells (**A,B**) and for 10 μM VIP (**C,D**) pretreated cells.
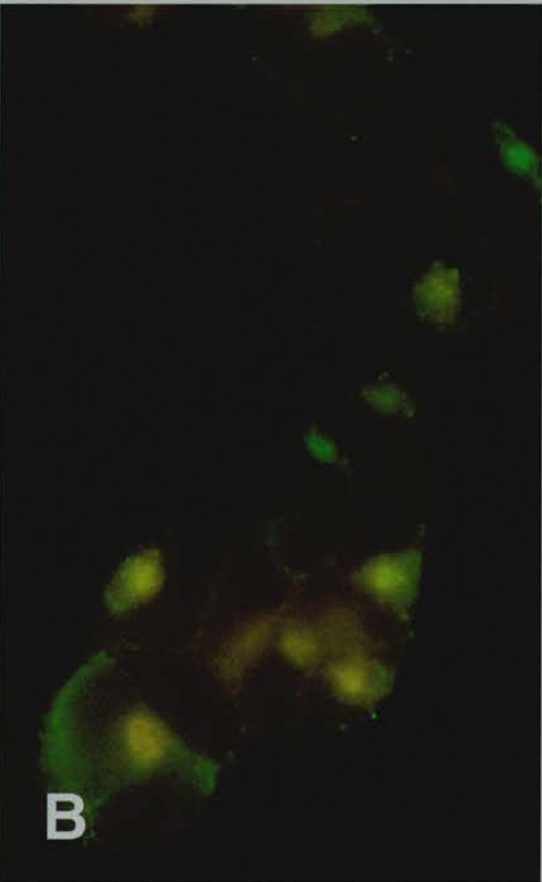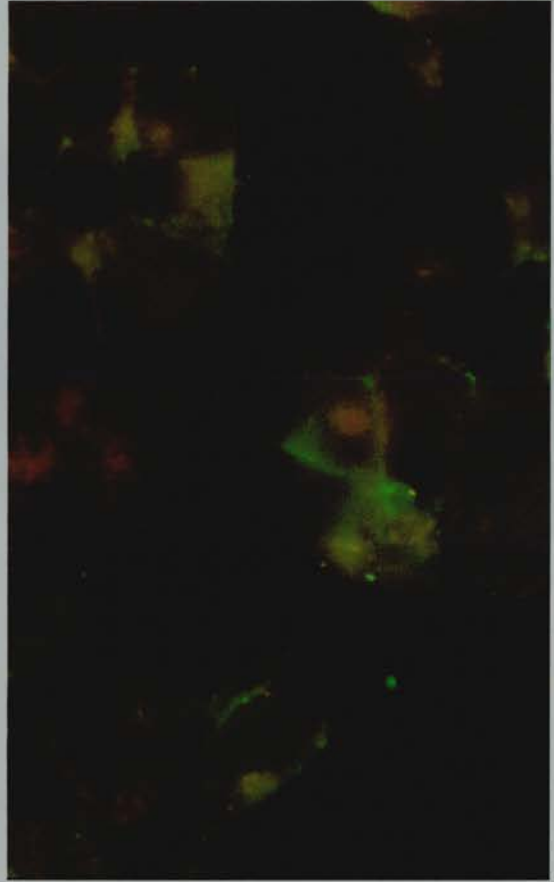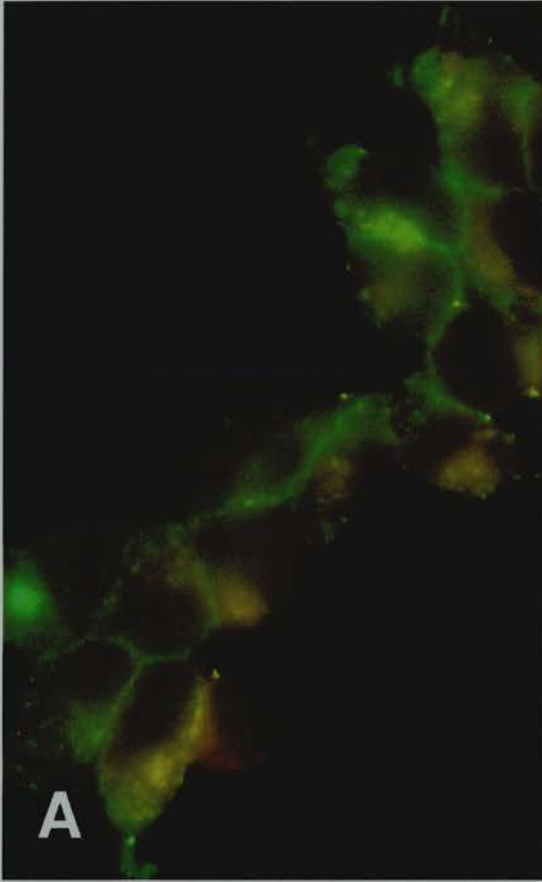
Figure 6.10 Localisation of the VPAC$_2$ receptor and a marker for the transferrin receptor (TfnR) using digital imaging microscopy. HEK293 cells stably transfected with the VPAC$_2$-HA receptor were preincubated with serum free medium (**A**) or 10 μM VIP (**B**) for 30 mins at 37°C. Cells were fixed and incubated with antibodies against the VPAC$_2$ receptor conjugated to FITC (green) and the TfnR conjugated to Texas red (red). The cells were fixed and viewed using a digital imaging apparatus. Images through the Z plane of focus were taken every 1 μm using a Zeiss inverted microscope connected to a CCD camera. These images were reconstructed and deconvoluted to remove out-of-focus fluorescence from adjacent images. A single image through the centre of the cells is presented here at x63 magnification for untreated (**A**) and 10 μM VIP treated cells (**B**). Arrows indicate points at which specific staining for both markers overlap.
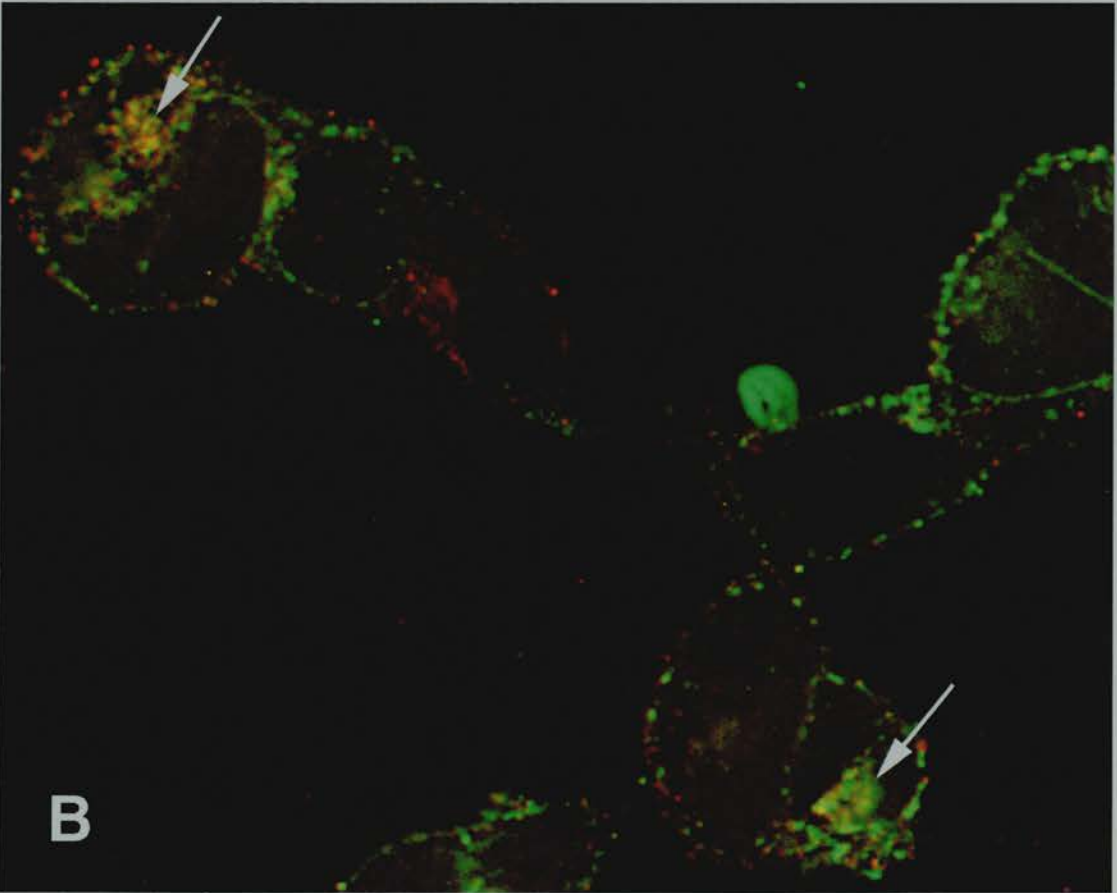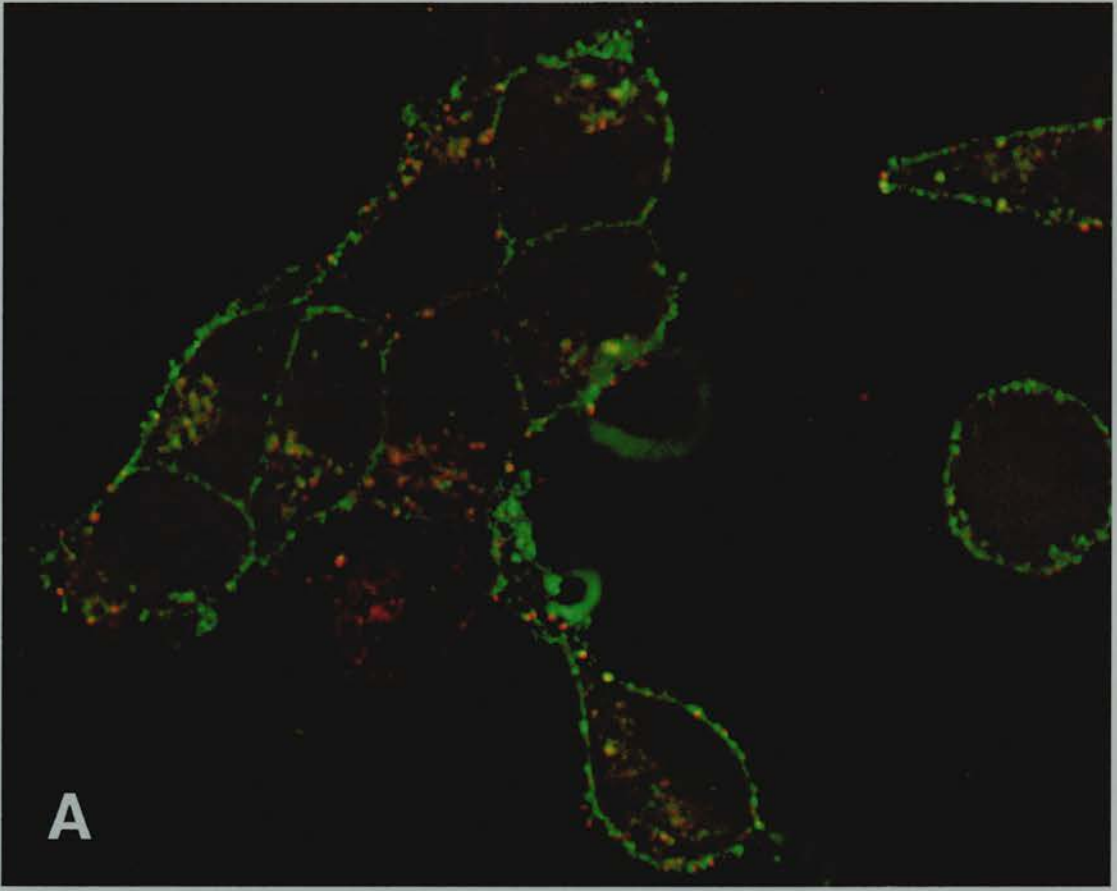
Figure 6.11. Effect of bafilomycin A_1 on recycling of the $VPAC_2$ receptor. HEK293 cells stably transfected with the $VPAC_2$-HA receptor were treated with 1 μM bafilomycin A_1 (in 0.3% DMSO) for 30 mins at 37°C. The cells were then incubated in serum free medium (**A**) or 10 μM VIP (**B,C,D**) washed three times with PBS and fixed (**C**) or incubated for a further 2 hrs at 37°C in serum free medium in the presence of baf A_1 (**A,B,D**). Cells were fixed and viewed at x100 magnification under appropriate fluorescence conditions.
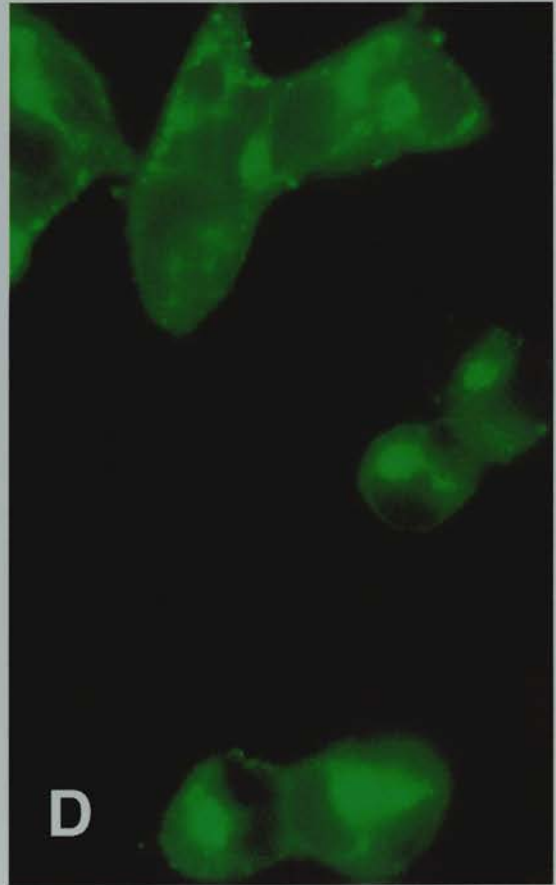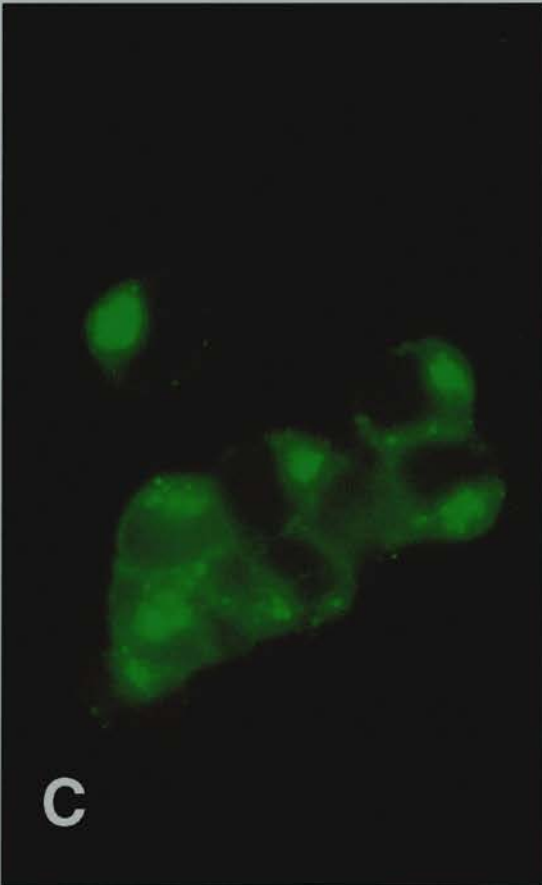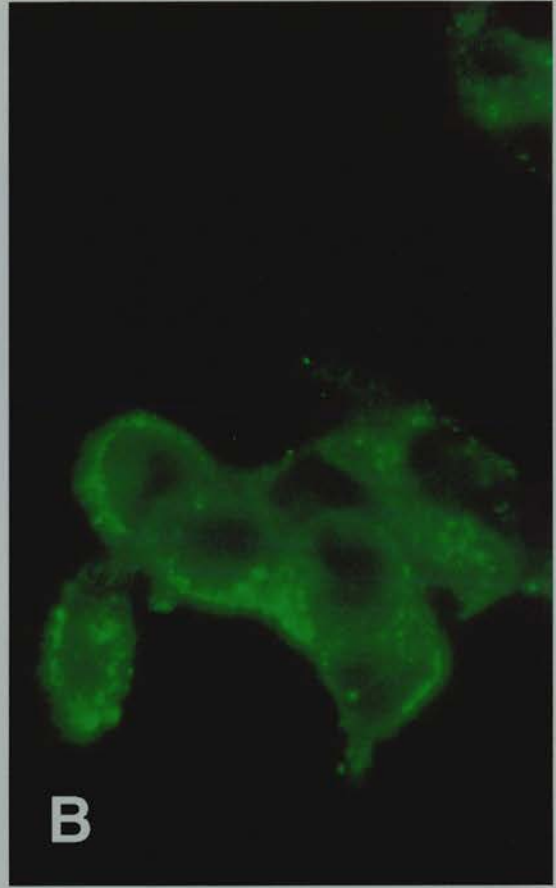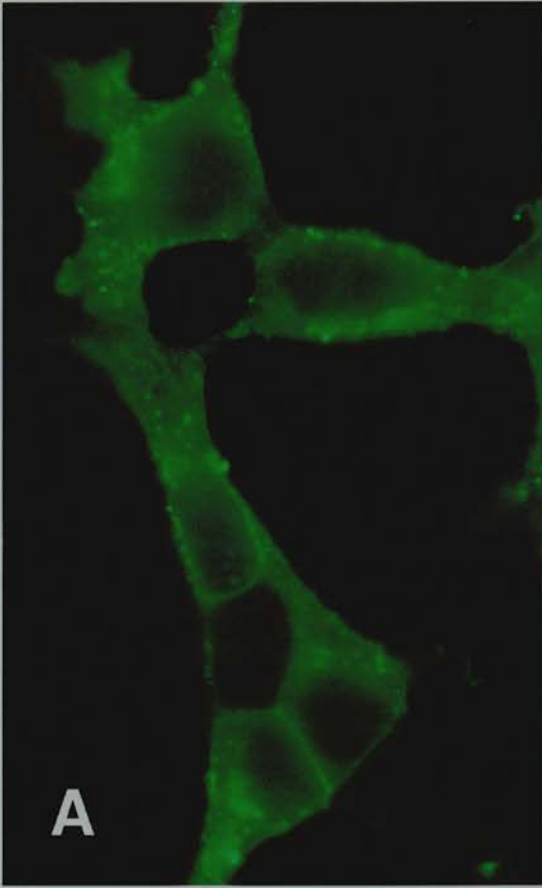
Figure 6.13 Schematic representation of the Golgi complex. This model divides the Golgi apparatus into three compartments. Each compartment is thought to have a different function; (1) *cis*-Golgi network (CGN) recieves and sorts membrane and soluble cargo arriving from the endoplasmic reticulum; (2) *medial*-Golgi is involved in glycosylation and processing of glycoproteins and glycolipid; (3) *trans*-Golgi network (TGN) sorts membrane and soluble cargo arriving and exiting at the *trans* face of the Golgi apparatus. Reproduced from Mellman and Simmons (1992).
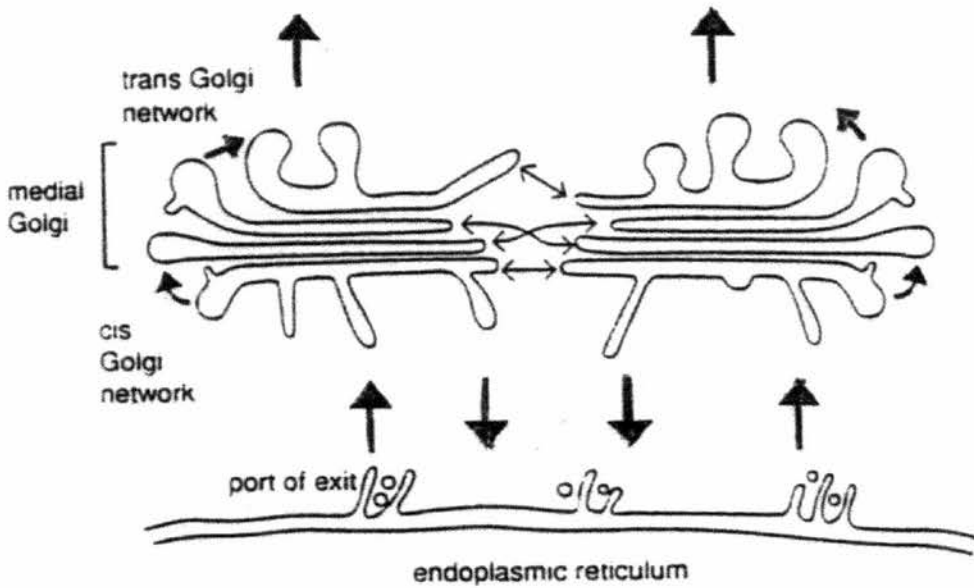
Figure 6.14   General structure of the trans-Golgi network (TGN) integral membrane glycoproteins. TGN38 and TGN46 are rat and human homologues, respectively, of an integral membrane glycoprotein. These proteins recycle from the TGN to the cell surface, however they are predominantly located in the TGN and antibodies against these glycoproteins are used as markers for the TGN. Each protein contains five domains: (I) the signal sequence; (II) the unique repeat region; (III) the conserved region of the lumenal domain; (IV) the membrane spanning domain; (V) the cytoplasmic tail. Both the membrane spanning domain and the cytoplasmic tail are required for localisation of this protein to the TGN. The number of amino acid residues that constitute each domain are indicated below. Reproduced from Ponnambalam et al. (1996).
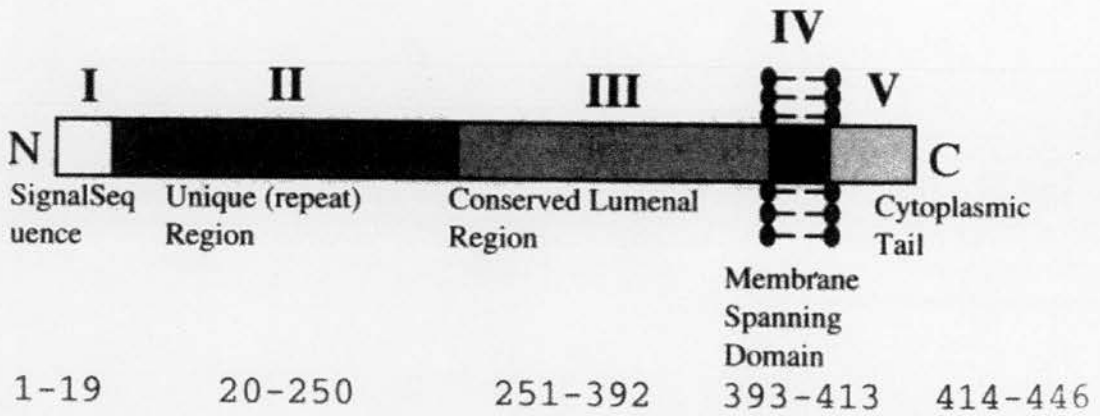
6.3 Discussion

Recycling of the VPAC2 receptor

VPAC$_2$ receptors internalise to a single juxtanuclear site after incubation with VIP. Experiments presented in this chapter show that this process is reversible, as evidenced by the reappearance of specific staining for the VPAC$_2$ receptor back at the plasma membrane following removal of agonist. This process did not appear to be complete after a 2 hr incubation in the absence of agonist, however the effect of longer incubations in the absence of stimulating peptide was not investigated. Further experiments to determine the complete time course of receptor recycling back to the membrane would be of interest. Cycloheximide had no effect on the reappearance of VPAC$_2$ receptors at the cell surface indicating that newly synthesised receptors do not constitute a major part of the recycling population of receptors. Therefore, it seems likely that following acute stimulation with VIP the majority of internalised VPAC$_2$ receptors are able to recycle back to the cell surface.

Indirect evidence for VPAC$_1$ receptor recycling has been determined using [^{125}I]-VIP binding to HT-29 cells which express an endogenous VPAC$_1$ receptor. This technique allows receptor numbers at the cell surface during and after removal of the stimulating peptide to be measured. The recovery of [^{125}I]-VIP binding sites (after a 3 hr incubation with 10 nM VIP) in HT-29 cells was found to be rapid: with a half life of 10 mins at 37°C. [^{125}I]-VIP binding approached ~70% of that observed in control (untreated cells) after prolonged incubation (3 hrs) in the absence of peptide (Boissard et al., 1986). A similar study found that recovery of [^{125}I]-VIP binding (after a 10 min incubation with 10 nM VIP) in HT-29 cells was almost complete within 1 hr of removing VIP (Luis et al., 1986). Again the reappearance of VIP binding sites was rapid with a half-life of 15 mins (Luis et al., 1986). Two phases of recovery were identified by Luis et al. (1987): an initially rapid phase followed by a slow phase in the reappearance of [^{125}I]-VIP binding. This group postulated that the rapid recovery was due to recycling from an intracellular pool of VIP receptors, whilst the slow phase, which was inhibited by cycloheximide, corresponded to maturation of existing receptors and *de novo* synthesis (Luis et al., 1987).

Similar evidence for VPAC$_2$ receptor recycling has been obtained in SUP-T1 lymphoblasts. [^{125}I]-helodermin was used to assess the recovery of binding sites in these cells after preincubation with VIP. Incubation of SUP-T1 cells with agonist (30

nM VIP for 16 hrs) caused an ~80% reduction in the binding of $[^{125}I]$-helodermin (Robberecht et al., 1989a). In common with studies of HT-29 cells the recovery of binding was biphasic, with both rapid (0-2 hrs) and slow rates (2-8 hrs), only 80% of binding was recovered after 8 hrs. The rapid phase of recovery was unaffected by pretreatment with cycloheximide, whereas, the slow phase was significantly inhibited (Robberecht et al., 1989a). Thus both $VPAC_1$ and $VPAC_2$ receptors appear to share a similar route for receptor trafficking. Recycling of $VPAC_2$ receptors at the plasma membrane has been confirmed by immunofluorescence experiments presented in this chapter. In addition we found that the recycling of $VPAC_2$ receptors during a 2 hr recovery time was unaffected by cycloheximide.

Long term treatment with VIP may be expected to cause down regulation of VIP/PACAP receptors, where the receptors are degraded and new synthesis is reduced. The relationship between VIP/PACAP receptor endocytosis and down regulation remains to be defined, although a few studies have suggested a role for internalisation in down regulation of VIP/PACAP receptors (Pozo et al., 1995; Rosselin et al., 1988). For future experiments it would be interesting to determine how chronic treatment with VIP effects receptor recycling and whether new protein synthesis becomes a more important source of receptors under these circumstances.

Koenig and Edwardson (1997) have derived mathematical models to account for receptor recycling. The most simple, two-compartment model, reveals that the amount of cell surface and internalised receptors is determined by the rate constants for endocytosis and recycling (Koenig and Edwardson, 1997). The endocytosis rate constant is related to the agonist concentration (although the precise nature of this relationship is unknown), whereas, the recycling rate constant is believed to be constitutive and thus independent of external stimulation. In practice this model is over simplistic and a multi-compartment model has also been proposed (Koenig and Edwardson, 1997). This model is difficult to define as the precise routing of receptors through intracellular compartments remains to be clarified. In addition estimates of receptor internalisation and recycling are likely to be complicated by new receptor synthesis and degradation.
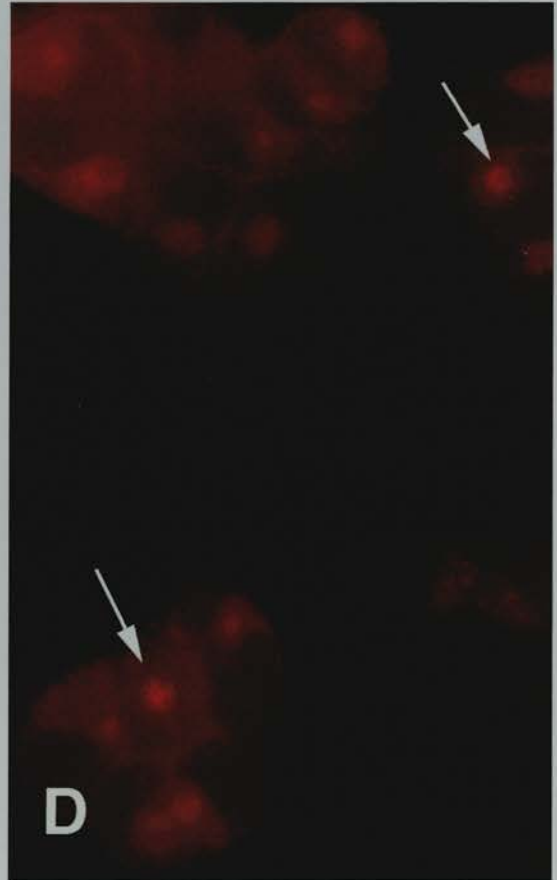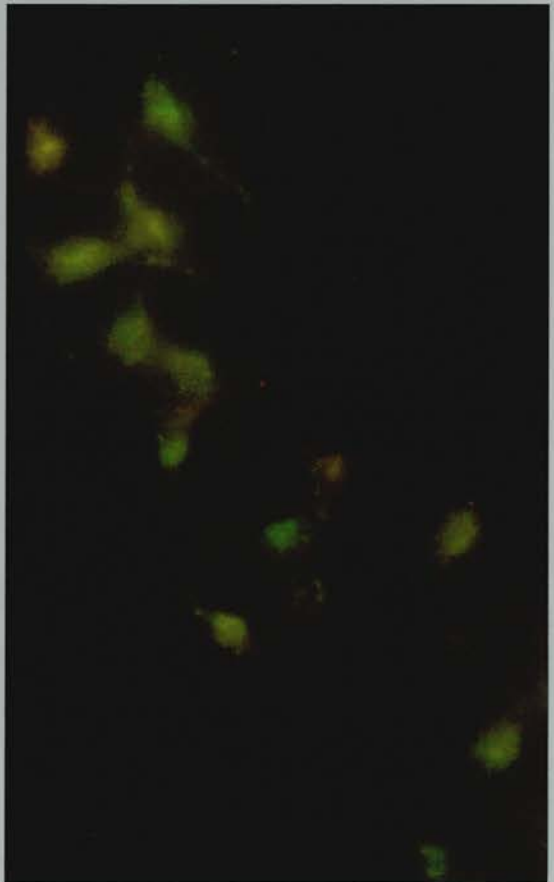
Localisation of the $VPAC_2$ receptor and a marker for the trans-Golgi network
Several cell surface proteins have been shown to be transported to the Golgi apparatus, including the low and high density lipoprotein receptors and mannose 6-phosphate receptor (Duncan and Kornfeld, 1988; Green and Kelly, 1992;

Takahashi et al., 1989). For a review see (Gruenberg and Maxfield, 1995). The simplest model of the Golgi apparatus divides it into three distinct compartments based on functional, immunological and morphological differences; these include the *cis* (CGN), *medial* and *trans*-Golgi network (TGN) (for a review see Mellman and Simons, 1992) **(Figure 6.13)**. Antibodies against integral membrane glycoproteins of the TGN have provided the first markers for this region. The rat glycoprotein, TGN38 was first identified in 1990 (Luzio et al., 1990). TGN46, the human homologue of TGN38, was cloned from a foetal liver cDNA library (Ponnambalam et al., 1996). TGN38 and TGN46 share significant sequence identity, particularly in the membrane spanning and cytoplasmic domains **(Figure 6.14)**. Although primarily located in the TGN both TGN38 and TGN46 constitutively cycle between the cell surface and TGN. TGN38 contains two motifs which act to target it to the TGN, a tyrosine-based motif (YXXØ) in its cytoplasmic tail and a second unidentified region in the membrane spanning domain (Ponnambalam et al., 1994). The YXXØ motif is homologous to endocytic motifs found in the cytoplasmic domains of cell surface receptors (Bos et al., 1993), including the TfnR and insulin receptor, for a review see (Ohno et al., 1998). In addition the VPAC$_2$ receptor contains a YXXØ motif in its third intracellular domain (discussed in section 5.3). The function of TGN38 and TGN46 remains to be clarified, they have been postulated to act as receptors for transporting ligand through endocytic or exocytic compartments (Ponnambalam et al., 1996) or may have a role in the packaging of materials for export from the TGN (Jones et al., 1993). Electron microscopy revealed that TGN38 is localised to a tubulo-vesicular network at the *trans* side of the Golgi apparatus (Luzio et al., 1990). Localisation of TGN46 using rabbit polyclonal antibodies directed against two short peptide sequences in its extracellular domain reveal a juxtanuclear staining pattern in HeLa and COS cells (Ponnambalam et al., 1996). To date no reports of the distribution of TGN46 with the sheep polyclonal antibody used in this study have been published, however, its distribution is similar to that seen with rabbit polyclonal antibodies described in a study by Prescott et al. (1997) (Dr S.Ponnambalam; personal communication). The VPAC$_2$ receptor did not colocalise with TGN46, indicating that this receptor does not recycle through the TGN under the conditions used in these experiments.

Localisation of the VPAC$_2$ receptor and a marker for sorting and recycling endosomes

The transferrin receptor (TfnR) is a well studied constitutively recycling receptor. It is comprised of a disulphide-linked dimer of two 90K transmembrane glycoproteins.

Transferrin transports iron atoms into the cell via receptor-mediated endocytosis, the iron atoms are released intracellularly and the receptor-ligand complex is recycled back to the cell surface (Willingham et al., 1984). Labelled transferrin or its receptor can be used as markers for sorting and recycling endosomes and have also been used to demonstrate that endocytosis occurs via clathrin-coated pits (Clague, 1998). Several GPCRs have been shown to colocalise with the TfnR, including the α_{1B}-adrenergic receptor (Fonseca et al., 1995), gonadotrophin-releasing hormone receptor (Vrecl et al., 1998), μ-opioid receptor (Keith et al., 1998). Other receptors have been reported to colocalise with fluorescently-labelled transferrin, including the β_2AR (Kallal et al., 1998) and TRH receptor (Ashworth et al., 1995).

Evidence presented in this chapter demonstrates that the internalised $VPAC_2$ receptor colocalises with the TfnR, indicating that both receptors share a common endocytic pathway. Previous experiments have compared the trafficking of $[^{125}I]$-VIP with horseradish peroxidase (HRP)-labelled transferrin in HT-29 cells (which express the $VPAC_1$ receptor by quantitative autoradiography (Lefevre et al., 1990; Phan et al., 1992). Electron micrographs reveal that VIP and transferrin share a common endocytic pathway, although transferrin is released intact into the surrounding medium, whilst VIP is degraded (Lefevre et al., 1990; Marvaldi et al., 1986). Another study using sucrose density fractionation to examine $[^{125}I]$-VIP and HRP-transferrin trafficking confirmed these results, showing that about 60% of VIP colocalises with transferrin throughout the endocytic pathway (Phan et al., 1992). The rapid breakdown of VIP is believed to be caused by its vulnerability to enzymatic degradation (Phan et al., 1992). Colocalisation of transferrin with other ligands which undergo receptor-mediated endocytosis, for example EGF and asialoglycoprotein (ASGP), have shown a divergence in trafficking as these ligands are targeted towards lysosomes (Lefevre et al., 1990). Surprisingly, this was not found for VIP even though it is known to undergo degradation in lysosomes. Lefevre et al. (1990) propose that VIP may undergo partial degradation in endosomes shared with transferrin. This is possible as VIP gradually dissociates from its receptor at low pH (Rosselin et al., 1988), whereas, the transferrin ligand-receptor complex is stable in acidic conditions (Shepherd, 1989). Examination of the trafficking of both VIP and its receptor may be a useful means to determine the point at which ligand and receptor are dissociated during endocytosis.

Colocalisation with the TfnR has also been interpreted as evidence for clathrin-mediated endocytosis of GPCRs. However, the studies presented in this

chapter involved relatively long incubations with peptide, such that the early stages of endocytosis were not observed. Shorter incubation times with VIP would be necessary to establish whether VPAC$_2$ receptors and TfnRs colocalise at the plasma membrane prior to endocytosis. One would predict that these receptors will colocalise, as evidence presented in chapter 4 indicates that the VPAC$_2$ receptor is internalised through clathrin-coated pits. A thorough examination of the location of both these receptors is needed, especially in view of a recent study which provided evidence for the existence of distinct subpopulations of clathrin-coated pits (Cao et al., 1998). This study demonstrated that endocytosis of the β_2AR was mediated partly by temperature sensitive clathrin-coated pits, which could be inhibited at 16°C, whereas all of the clathrin-coated pits involved in TfnR internalisation were unaffected by incubation at 16°C. Detailed examination revealed that ~50% of coated pits in HEK293 cells contained either β_2ARs or TfnRs but not both; these differences were associated with different protein components in the clathrin-coated pits. Ultimately both receptors are extensively colocalised in a common population of endocytic vesicles. Cao et al. (1998) proposed that the involvement of select clathrin-coated pits may form the basis for activation of an alternative signalling pathway by internalised β_2ARs (Cao et al., 1998). This has been confirmed by work from the same group who found that endocytosed β_2ARs were able to stimulate mitogen-activated protein (MAP) kinase activity (Daaka et al., 1998). Hence, experiments using different incubation temperatures and detailed comparisons of VPAC$_2$ receptor localisation with the TfnR could help to determine whether the VPAC$_2$ receptor internalises through a select group of clathrin-coated pits. It would also be interesting to determine whether internalised VPAC$_2$ receptors are able to activate other second messenger systems.

Inhibition of endosome acidification prevents VPAC$_2$ receptor recycling
Considerable evidence has accumulated for a role for endosomal acidification in efficient receptor recycling. These findings have been derived from studies using lysomotropic agents which inhibit acidification and mutant CHO cell lines which are defective in acidification (Robbins et al., 1983). Receptor recycling can be inhibited by a number of chemical agents, including chloroquine, ammonium chloride (NH$_4$Cl), monensin, nigericin and baf A$_1$. These compounds share a common mechanism of action; they are all able to raise the pH in endosomes. Chloroquine and NH$_4$Cl are weak bases which readily penetrate the cell and endosomal membranes, once within an acidic environment they become protonated and are no longer able to traverse the membrane (Mellman et al., 1986). Carboxylic ionophores,

such as monensin and nigericin, also cause a rise in vacuolar pH, they do this by integrating into vacuolar membranes and exchanging protons for potassium ions. These compounds have been invaluable for studies of the importance of endosome acidification in receptor trafficking. Treatment with weak bases and ionophores causes a rapid decrease in cell surface receptor numbers due to a disruption in receptor recycling (for a review see Mellman et al., 1986). Treatment with ammonium chloride (NH_4Cl) slowed the rate of internalisation and caused rapid release of nondegraded [^{125}I]-VIP into the incubation medium (Muller et al., 1985). Nonetheless these agents lack specificity and tend to dissipate pH gradients throughout the cell leading to osmotic swelling of vacuolar compartments.

Bafilomycin A_1 (baf A_1) is one of a family of macrolide antibiotics first isolated from *Streptomyces* (Werner et al., 1984). It is an extremely potent and specific inhibitor of the vacuolar ATPases (vATPases). vATPases generate proton gradients using energy gained from ATP hydrolysis (Bowman et al., 1988) and are necessary for the acidification of endocytic compartments (Harada et al., 1996). Baf A_1 has been shown *in vitro* to raise the pH of endosomes, lysosomes and phagosomes, without causing the morphological changes elicited by weak bases and ionophores (Umata et al., 1990). The specific action of this agent provides a useful tool for investigating the role of endosome acidification in receptor trafficking. Treatment with baf A_1 did not influence internalisation of receptors for transferrin, EGF, LDL and ASGPs but caused a reduction in the rate of receptor recycling in a number of different cell types (Johnson et al., 1993). Baf A_1 has also been shown to inhibit trafficking from endosomes to lysosomes (van Weert et al., 1995). Several reports of the effect of baf A_1 on internalisation of GPCRs have been published, examples include the β_2AR (Moore et al., 1999), α_{1B}-adrenoreceptor (Hirasawa et al., 1998), TRH receptor (Petrou and Tashjian Jr, 1995) and somatostatin receptor (SSTR) (Roosterman et al., 1997). In all of these studies baf A_1 has been shown to have little effect on receptor internalisation but inhibits receptor recycling and in some cases delivery of receptors to lysosomes. This effect is thought to result from the ability of this agent to inhibit endosomal acidification.

The mechanism by which perturbed acidification interferes with receptor recycling remains to be clarified. A study of trafficking of the TfnR in CHO cells found that inhibition of receptor recycling by baf A_1 required the presence of its cytoplasmic domain (Johnson et al., 1993). A YXXØ internalisation motif in this region was of particular importance for this effect suggesting that endosomal acidification may

regulate receptor interactions involved in receptor trafficking (Johnson et al., 1993). Similar results were obtained by Presley et al. (1997). This group found that baf A_1 reduces the rate of exit of TfnRs from the recycling compartment and identified two underlying components: firstly a general reduction in the rate of bulk membrane flow from recycling compartments and secondly an active retention of the receptor. The latter process being dependent upon the presence of a YXXØ internalisation signal in the cytoplasmic domain (Presley et al., 1997). In addition van Weert et al. (1995) showed that, after treatment with baf A_1, the iron atoms (Fe^{3+}) carried by the transferrin ligand were not released intracellularly (van Weert et al., 1995). Interestingly, a recent study of the insulin receptor found that baf A_1 inhibition of endosomal acidification prevented termination of receptor signalling, thus, acidification was postulated as a mechanism for inducing a conformational change of the insulin receptor which stops its kinase activity (Contreres et al., 1998). Studies of the β_2AR have found that conformational changes in receptors at low pH may be important for dephosphorylation and resensitisation of receptors (Krueger et al., 1997).

In the work presented here baf A_1 inhibited recycling of the $VPAC_2$ receptor. Extrapolating from studies of the TfnR it seems likely that this effect is due to endosomal acidification and a reduction in the rate of recycling. Whether, like the TfnR, the underlying mechanism requires the presence of an internalisation signal remains to be determined. It is possible that altered receptor trafficking may simply be caused by effects on the receptor-ligand interaction as the pH is neutralised, as proposed by Clague (1998). Inhibiting endosomal acidification may prevent dissociation of ligand from receptor in sorting endosomes and confine the receptor-ligand complex to this compartment. Indeed, $VPAC_1$ receptors have been shown to require an acidic environment for ligand to dissociate (Boissard et al., 1986). Experiments to establish the role of endosomal acidification in $VPAC_2$ receptor recycling and resensitisation would be of interest.

Intracellular markers and immunofluorescence techniques
Many markers are now available which allow different intracellular compartments to be visualised. Some, like transferrin and its receptor are constitutively recycling receptors with well characterised routes of internalisation and recycling. In addition a family of small GTPases, known as rabs, have proven effective at distinguishing different compartments involved in endocytosis; rab11, rab5, and rab7 or rab9 are now established as markers for recycling, sorting and late endosomes, respectively

(for a review see Clague, 1998). Immunofluorescence of the VPAC$_2$ receptor could be used in combination with labelled VIP or related peptides to compare their trafficking. Techniques are now available to quantitate fluorescence data and allow comparisons of the kinetics of trafficking between different intracellular compartments. In addition green fluorescent protein (GFP)-tagged receptors allow receptor trafficking to be observed in live cells which provides an excellent method of gaining both temporal and spatial data on receptor-mediated endocytosis and trafficking. Recent studies have demonstrated internalisation of β$_2$AR-GFP (Barak et al., 1997; Kallal et al., 1998) and TRH receptor-GFP constructs (Drmota et al., 1998a). These techniques could be applied to the internalisation and trafficking of the VPAC$_2$ receptor to further define the mechanism, intracellular route and relevance of endocytosis for regulating receptor dephosphorylation and resensitisation.

Summary

Data presented in this chapter reveal that agonist-induced VPAC$_2$ receptor internalisation is reversible. Although it is likely that following chronic treatment with VIP new protein synthesis may be involved in recycling, over the time scale studied here, it does not have a primary role. Colocalisation with the TfnR indicates that the VPAC$_2$ receptor traverses both sorting and recycling endosome compartments. Further experiments to determine whether VPAC$_2$ and TfnRs endocytose via the same clathrin-coated pits and to determine when the receptor-ligand complex dissociates and follow divergent endocytic pathways would be of interest.

CHAPTER 7

Overview

Agonist-induced internalisation of the VPAC$_2$ receptor

The data presented in this thesis provide the first direct demonstration of agonist-induced internalisation of the VPAC$_2$ receptor. VPAC$_2$ receptor endocytosis requires specific peptide agonists and is dependent upon agonist concentration, incubation time and temperature. Internalisation was inhibited by pretreatment with hypertonic sucrose or mild acid indicating that these receptors, in common with other GPCRs, internalise through clathrin-coated pits. PMA an inhibitor of calveolae had no effect on agonist-induced internalisation of the VPAC$_2$ receptor in HEK293 cells. However additional, or alternative, mechanisms for VPAC$_2$ receptor endocytosis cannot be ruled out, as other GPCRs have been shown to internalise by both clathrin-dependent and -independent pathways depending on the cell line used for expression. Once internalised VPAC$_2$ receptors appear to follow a classical endocytic pathway as they colocalise with the constitutively recycling TfnR, a marker for sorting and recycling endosomes. Previous studies have shown that VIP is degraded following internalisation, whilst its receptor is recycled, accordingly VPAC$_2$ receptor recycling was observed when agonist was removed from the cell medium. Many accessory proteins involved in receptor endocytosis have been identified over the last decade, their mechanism of action and involvement in GPCR internalisation is still being resolved. The β_2AR has been the prototype for many of these studies; indeed the mechanism of β_2AR internalisation seems to be applicable to many other members of the class I GPCRs, and may be relevant for class II GPCRs. Accordingly the VPAC$_2$ receptor appears to follow a similar route of internalisation as this receptor.

Structural or sequence requirements for VPAC$_2$ receptor internalisation remain to be defined. The results presented here suggest that the C-terminal intracellular domain of the VPAC$_2$ receptor was not an absolute requirement for agonist-induced internalisation. Almost complete removal of the C-terminal tail appeared to have no effect on the ability of the receptor to internalise. Surprisingly, a moderate increase in the initial rate of internalisation was observed in HEK293 cells expressing severely truncated VPAC$_2$ receptors, indicating that the C-terminal tail may be able to influence the rate of internalisation. Currently no consensus motifs for GPCR internalisation have been identified. However, several endocytic signals are located in the second and third intracellular loops of the VPAC$_2$ receptor, including a tyrosine (YXXØ) and two dileucine (LL) which could potentially be involved in internalisation. The YXXØ motif has been identified as an internalisation signal for several receptors (for a review see (Ohno et al., 1998), whereas dileucine motifs are

normally associated with lysosomal trafficking, but have also been shown to mediate receptor internalisation from the cell surface (Gabilondo et al., 1997; Hamer et al., 1997). Site-directed mutagenesis of these residues would be necessary to determine whether either of these motifs are involved in agonist-induced internalisation of the $VPAC_2$ receptor.

The function of internalisation of $VPAC_2$ receptors remains to be clarified. For other GPCRs internalisation has been postulated to have a role in receptor desensitisation (1), down regulation (2), dephosphorylation and resensitisation (3) and/or coupling to alternative second messenger pathways (4). These possible functions are considered below:

(1) Two lines of evidence presented here indicate that agonist-induced internalisation of the $VPAC_2$ receptors is not involved in desensitisation: firstly, inhibiting internalisation, with hypertonic sucrose, did not effect desensitisation, secondly, a severely truncated $VPAC_2$ receptor which was unable to desensitise still internalised. Similar reports have been published for both class I and class II GPCR families, there are some exceptions where internalisation is required for desensitisation but these generally involve receptors which are irreversibly activated by enzyme cleavage.

(2) VIP/PACAP receptors undergo down regulation (Pozo et al., 1995). Down regulation occurs after chronic agonist treatment, it has a longer time course (hrs/days) and is characterised by a reduction in receptor density at the cell surface. The mechanism of down regulation is not well understood and may be due to accelerated degradation of receptors, impaired delivery of new receptors to the cell surface, or a combination of both. For some GPCRs internalisation is a prerequisite for down regulation (Gagnon et al., 1998; Heck and Bylund, 1998), whereas for other receptors this relationship is not well defined. Early reports of agonist-induced internalisation of VIP/PACAP receptor suggested that it may have a role in down regulation (Pozo et al., 1995; Wiik, 1988). Chronic treatment of cells expressing the $VPAC_2$ receptor was not investigated in the work presented here. Nonetheless, some of the techniques used in these studies could also be applied to investigations of long term regulation of receptor function.

(3) Over the past decade evidence has accumulated to support a role for agonist-induced internalisation in receptor dephosphorylation and resensitisation. The β_2AR is one of the best characterised GPCRs. For this receptor endocytosis is a prerequisite for receptor dephosphorylation and resensitisation (Barak et al., 1994; Pippig et al., 1995; Yu et al., 1993; Zhang et al., 1997). Similar findings have been

reported for other members of both class I and class II GPCRs. Acidification of endosomes may permit dissociation of ligand from receptor, so that the ligand can be targeted for degradation whilst the receptor recycles back to the cell surface (Mellman et al., 1986). In addition low pH has been postulated to induce changes in receptor conformation that permit receptor dephosphorylation and resensitisation (Krueger et al., 1997). Whether internalisation of the VPAC$_2$ receptor is necessary for dissociation and/or dephosphorylation and resensitisation remains to be determined. Interestingly treatment with baf A$_1$ an inhibitor of endosomal acidification, prevented receptor recycling.

(4) The majority of GPCRs are able to signal through several different classes of G-protein. The prototypic β_2AR is able to couple through G$_s$ and G$_i$ proteins. The latter pathway has been shown to require PKA phosphorylation of the β_2AR and thus uncoupling from G$_s$ and desensitisation (Daaka et al., 1997). Recent studies have found that internalisation of the β_2AR is necessary for Ras-dependent activation of mitogen-activated protein (MAP) kinase signalling cascade in HEK293 cells (Daaka et al., 1998). Thus receptor internalisation may support coupling to alternative second messengers enabling signalling to be directed intracellularly.

VIP/PACAP receptors are also able to couple to G$_s$ and G$_i$, and evidence is accumulating for their ability to signal via PLC and mitogen-activated protein (MAP) kinase (see section 1.3.6). To date there are few published reports investigating the role of internalisation in activating alternate second messenger pathways. Interestingly some of the prolonged effects of treatment with VIP and related peptides cannot be accounted for by coupling to G$_s$. For example VIP, PACAP and GIP all stimulate insulin secretion in the HIT-T15 clonal β-cell line: this action is biphasic, resulting from acute activation of AC and a more prolonged effect via a wortmannin-sensitive pathway (Straub and Sharp, 1996b; Straub and Sharp, 1996a). The precise nature of the latter pathway remains to be defined, however the action of wortmannin indicates that it is likely to involve a PI3-kinase. PI3-kinases are heterodimers consisting of 85 kDa and 110 kDa regulatory and catalytic subunits, respectively, the catalytic subunit can be stimulated by G-protein $\beta\gamma$-subunits (without requiring the 85 kDa subunit). The wortmannin-sensitive pathway was found to be involved in the sustained stimulation of insulin secretion (Straub and Sharp, 1996a), the delayed onset of this activity could result from signalling by internalised receptors. Interestingly a recent study reported that GIP activated MAP kinase through both wortmannin-sensitive and -insensitive pathways in CHO cells (Kubota et al., 1997). Consideration of all this evidence indicates a potential

mechanism for internalisation in VIP/PACAP activation of MAP kinase, in common with the β_2AR. PACAP has been shown to stimulate MAP kinase in the insulin-secreting β-cell line, INS-1 (Frodin et al., 1995). In addition recent reports indicate that the neuroprotective actions of PACAP on primary cultures of rat cerebellar granule neurones (Journot et al., 1998; Villalba et al., 1997) and its mitogenic action on tumour cells (Lelievre et al., 1998; Schafer et al., 1996) are mediated through MAP kinase activation, although some of these actions may be regulated by a cAMP-dependent pathway (Journot et al., 1998).

Future perspectives

Ultimately studies of agonist-induced internalisation of VIP/PACAP receptors *in vivo* to determine the physiological relevance of $VPAC_2$ receptor endocytosis would be of interest. Current work in our laboratory has established transgenic mice which express the C-terminal epitope-tagged $VPAC_2$ receptor ($VPAC_2$-HA). The presence of the tag could be used for investigating receptor mediated endocytosis of VIP *in vivo*. To date there are few studies which have examined GPCR internalisation in whole animals. One example is the receptor for substance P (neurokinin-1), this receptor has been shown *in vitro* to internalise in response to agonist treatment (Garland et al., 1994). This phenomenon occurs *in vivo* when substance P is released in dorsal root ganglia in response to noxious stimuli in the periphery (Mantyh et al., 1995). The extent of neurokinin-1 receptor internalisation shows a direct correlation with the intensity of noxious stimuli, thus providing a pharmacologically specific image of neurones activated by substance P (Allen et al., 1997). This method could be applicable to other peptides released in response to noxious stimuli and, as such, would help to discern the peptides involved in transmitting sensory information about particular modalities and intensities of pain. Expression of VIP in the dorsal horn appears to be increased in animal models of chronic pain states and a role for the $VPAC_2$ receptor under these circumstances is emerging (Dickinson and Fleetwood-Walker, 1998). Therefore, this type of investigation could potentially be used to define the nature of VIP involvement in transmitting somatosensory information in the dorsal root ganglia.

Agonist-induced phosphorylation and desensitisation of the $VPAC_2$ receptor

Second messenger signalling of the $VPAC_2$ receptor desensitises upon repeated or prolonged stimulation with VIP; observed in these studies as a reduction in maximal cAMP stimulation. Phosphorylation is a key mechanism in the desensitisation of many GPCRs (Lefkowitz et al., 1998). Work from our laboratory has provided the

first evidence for agonist-induced phosphorylation of the $VPAC_2$ receptor (McDonald et al., 1998). Protein kinase inhibitors have been shown to enhance cAMP signalling of the $VPAC_2$ receptor (McDonald et al., 1998), thus it seems likely that phosphorylation is required for $VPAC_2$ receptor desensitisation. The C-terminal intracellular domain of the $VPAC_2$ receptor is a major site for phosphorylation (McDonald et al., 1998). In accordance with this result, data presented here revealed that removal of the C-terminal intracellular domain prevented desensitisation. These studies indicate a role for phosphorylation of the C-terminal tail in desensitisation of the $VPAC_2$ receptor, however further work is necessary to clarify this relationship. The $VPAC_2$ receptor is phosphorylated by PKA, however the action of this kinase alone cannot account for the observed level of agonist-induced phosphorylation (McDonald et al., 1998). GRKs, which specifically phosphorylate agonist-occupied receptors, are likely candidates for agonist-induced $VPAC_2$ receptor phosphorylation. Indeed some members of this kinase family have already been shown to phosphorylate other class II GPCRs (Fukayama et al., 1997; Shetzline et al., 1998).

Overall it appears that determinants of agonist-induced desensitisation and internalisation for the $VPAC_2$ receptor are located on distinct receptor domains: the former requiring the C-terminal intracellular domain whilst motifs for the latter remain to be identified. The majority of GPCRs exhibit both of these phenomenon, the β_2AR receptor is one of the best characterised receptors and remains the prototype for class I GPCR regulation. Nonetheless there are still many unresolved issues in research into GPCR desensitisation and internalisation and the mechanisms which underlie these phenomenon. It is perhaps surprising that members of the class II GPCRs show such a similar route of regulation to the class I GPCRs, particularly in view of their lack of sequence identity with this family. However the relatively recently cloning of class II GPCRs has precluded a thorough examination of the sequence motifs and regulatory mechanisms that may be specific to this family.

Future perspectives

It is important to understand the mechanisms underlying GPCR desensitisation as efficient regulation of receptor signalling is fundamental to maintaining physiological conditions. Thus defects in GPCR desensitisation could lead to the development of clinical problems in man. One example of a potential clinical application from work on receptor desensitisation involves the β_2AR system. The β_2AR has been shown to be down-regulated in cases of heart failure. In man this has

been associated with rising levels of GRK2 in the heart, which result in desensitisation and down regulation of the receptor. Whether this is a primary cause of heart failure remains to be determined. These studies have been advanced *in vivo* using a transgenic approach; where animals overexpressing GRK2 displayed a reduction in heart contractility which could be counteracted by the addition of GRK2 inhibitors. Thus evidence is accumulating for GRK2 as a key regulator of cardiac contractility, ultimately this could form the basis for the development of new agents to treat heart failure (for review articles see Lefkowitz, 1996; Rockman, 1997). One would predict that any defects in regulation of signalling by VIP or PACAP could have widespread implications as VIP/PACAP receptors have numerous functions in the CNS, PNS and peripheral organs.

Summary

The demonstration of agonist-induced internalisation of the $VPAC_2$ receptor has confirmed reports from several groups indicating that VIP undergoes receptor-mediated endocytosis. The immunofluorescence technique is an excellent qualitative means of demonstrating internalisation, in combination with current technical developments this method could be applied to visualising receptor internalisation and trafficking in live cells, quantitation of receptor movement and colocalisation with a number of markers. Further experiments to delineate the mechanism and function of $VPAC_2$ receptor endocytosis are required. Evidence is accumulating for a phosphorylation-dependent mechanism of $VPAC_2$ receptor desensitisation. A better understanding of the mechanism of internalisation and desensitisation may ultimately be of therapeutic value.

APPENDIX

APPENDIX

I Methods of binding data analysis

The simplest model of ligand-receptor interaction obeys the law of mass action:

$$L + R \underset{k_2}{\overset{k_1}{\rightleftarrows}} RL \tag{1}$$

Ligand (L) binds to the receptor (R), with an association constant (k_1), this model assumes the process is reversible and the LR complex will dissociate to form free ligand and receptor with a dissociation constant (k_2). When binding reaches equilibrium the association and dissociation constants must be equal, therefore:

$$\frac{[L][R]}{[RL]} = \frac{k_2}{k_1} = K_D \tag{2}$$

K_D is the equilibrium dissociation constant, it is the concentration of ligand which occupies 50% of the receptors.

The total receptor number (R_T) or maximal binding (B_{max}) for an assay is equal to the sum of the bound and free receptor populations, thus an increase in the amount of bound receptor (RL) will reduce the amount of free receptor (R):

$$R_T = B_{max} = R + RL \tag{3}$$

At equilibrium the amount of bound receptor (RL) and free receptor (R) will be equal, thus from equation (2), the K_D will be equivalent to the ligand concentration ([L]).

Ligand binding to a receptor can be characterised according to the equilibrium dissociation constant (K_D) for the receptor and the maximal binding (B_{max}) or total receptor number (R_T). Saturation assay or competition binding assays can be used to determine these parameters. In saturation experiments the amount of receptor in the assay is fixed, whilst the concentration of radioligand is increased, levels of specifically bound radiolabel are calculated by subtracting the non-specific from the total binding. Nonspecific binding increases linearly with radioligand concentration, whereas specific binding is saturable.

These data can also be transformed to give a linear relationship by the method of Scatchard (Scatchard, 1949) where bound/free radioligand is plotted against bound. The receptor density (B_{max}) is determined from the intercept on the horizontal axis and the plot slope is equal to $-1/K_D$:

$$\frac{Bound}{Free} = -\frac{1}{K_D} Bound - \frac{B_{max}}{K_D} \tag{4}$$

The Scatchard method has now been superseded by computer-aided nonlinear regression analyisis, several commercially available graph fitting packages have been developed to analyse this type of data. By substituting equations (2) and (3) a measure of bound receptor [RL] at any given concentration of ligand can be derived, from the logistic expression:

$$b = \frac{B_{max}L^p}{L^p + K_D} \tag{5}$$

Where b=bound ligand or [RL], B_{max}=maximum binding, L=ligand concentration and P= the slope of the curve.

Saturation analysis requires large amounts of radioligand and is not always a feasible means of determining receptor number and binding affinity. Nevertheless, these parameters can also be determined using competitive binding experiments, when the radioligand is in competition with its unlabelled counterpart, a method know as homologous competition. This method is commonly used for peptide receptors because of the lack of specific peptide antagonists. Thus an homologous competition assay was used to determine the receptor number and binding affinity of the VPAC$_2$ receptor and truncated/tagged mutants stably transfected in HEK293 cells. Helodermin in both radiolabelled and unlabelled forms was used for this assay. This type of experiment is essentially a saturation experiment with the specific activity of the radioligand being lowered by the addition of unlabelled ligand.

There are several reports in the literature which outline methods for performing and interpreting data from homologous competition experiments (DeBlasi et al., 1989; Swillens, 1995a) and several computer based programmes that are able to analyse this type of data (e.g. GraphPad, LIGAND and Sigmaplot). This analysis is based on several assumptions; the unlabelled and labelled ligand bind to the same site with the same affinity, the concentration of free radioligand remains constant and only a

single binding site is involved. If these criteria are fulfilled the K_D and Bmax can be determined accordingly (DeBlasi et al., 1989). However for the membrane binding data presented here, this was often not the case and high levels of [^{125}I]-helodermin binding were unavoidable. Therefore the data is presented as a function of the free ligand concentration. Without accounting for radioligand depletion the homologous competition curves will be steeper than predicted and K_D values may be overestimated. Swillens (1995) presented a methodology for analysing homologous competition binding data with respect to the free ligand concentration (Swillens, 1995a).

II Generation of antibodies against the rat VPAC$_2$ receptor

a) Materials

Glutathione S-transferase (GST) gene fusion system plasmids were purchased from Pharmacia Biotech, Uppsala, Sweden. Components of bacterial growth media were obtained from Difco Laboratories, Detroit, MI. Urea was supplied by Gibco Life Technologies Ltd., Paisley, UK and isopropyl β-D-thiogalactoside (IPTG) by Promega Corporation, Southampton, UK. Solvents and alcohols were obtained from BDH, Merck Ltd., Lutterworth, Leics, UK. All other reagents for fusion protein purification were obtained from Sigma, Dorset, Poole, UK, unless otherwise stated.

b) Bacterial growth media/agar

Luria bertani (LB) broth agar plates

> LB and 1% bactoagar was melted in a microwave for 10 mins on defrost. The solution was allowed to cool and supplemented with 100 µg/ml ampicillin and aseptically poured into 10 ml dishes and left to set at room temperature.

2x YTA medium

> Disssolve 16 g/l tryptone, 10 g/l yeast extraction and 5 g/l NaCl in 900 mls H$_2$O. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 l. Sterlise by autoclaving for 20 mins. Cool the medium to ~50°C then aseptically add 100 µg/ml ampicillin.

c) Buffer and gel recipes

Glutathione agarose

> Invert mix 2.5 mls S-linked glutathione agarose with 2.5 mls PBS, leave for 1 hr at room temp. Add 20 mls PBS, invert mix, centrifuge for 5 mins at 162 g and discard supernatant, repeat for a total of two washes. Store pellet as a slurry at 50% (v/v) with PBS for up to one month at 4°C.

Glutathione elution buffer (GEB)

> Make up 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). Dispense in 1-10 ml aliqots and store at -20°C until needed. Avoid more than 5 freeze/thaw cycles.

Citrate phosphate buffer

> To 100 mls dH$_2$O add 1.013 g citric acid, 1.815 g sodium phospate (Na$_2$PO$_4$.2H$_2$O) and 0.01% hydrogen peroxide (H$_2$0$_2$) (pH 5.0).

4x resolving gel buffer/4x stacking gel buffer

Make up 18% Tris and 0.4% SDS for 4x resolving gel buffer (RGB) and 5.8% Tris and 0.4% SDS for 4x stacking gel buffer (SGB) in 200 mls deionised H_2O, adjust pH with conc HCl to 8.9 and 6.7 respectively. Filter through fluted filter paper and store at -20°C.

12% resolving gel

4 mls 30% acrylamide (2.6% cross-linker), 2.5 mls 4x RGB, 3.5 mls distilled H_2O, 100 µl ammonium persulphate (APS) and 10 µl N,N,N',N'-tetramethylethylenediamine (temed)

5% stacking gel

670 µl 30% acrylamide (5.0% cross-linker), 1 ml 4x SGB, 2.33 mls distilled H_2O, 40 µl APS, 4 µl temed.

Gel loading buffer

1 ml 4x SGB, 0.8 ml 25% SDS, 0.5 ml b-mercaptoethanol, 1.0 ml glycerol and 0.01% bromophenol blue.

Tank buffer

For 1 l of tank buffer, add 6.32 g Tris base, 4 g glycine and 1 g SDS in dH2O, stir slowly until dissolved.

Blotting transfer buffer

Add 1.1 g SDS, 9 g Tris base, 43.4 g glycine and 600 ml methanol to make a final volume of 3 l with dH2O.

d) Generation of VPAC$_2$ receptor/GST fusion protein plasmids

The glutathione S-transferase (GST) gene fusion system is a commercially available system for the expression and purification of fusion proteins produced in *E. coli*. Protein expression from pGEX vectors is under the control of a *tac* promotor which can be chemically induced by isopropyl β-D-thiogalactoside (IPTG). The expressed protein is fused to GST and which allows it to be affinity purified with glutathione agarose and isolated from the agarose matrix under mild elution conditions. Two pGEX-2T vector (Pharmacia Ltd Biotech, Uppsala, Sweden) constructs were generated: one containing the first 62 amino acids of the rat VPAC$_2$ receptor N-terminus (excluding the signal sequence) and the other corresponding to the last 46 amino acid of the rat VPAC$_2$ receptor C-terminus. The fusion protein contructs were generated in our laboratory by Dr E.M.Lutz.

e) Cell transformation

Competent *E. coli* strain BL21 were transformed with either uncut pGEX-2T vector or pGEX DNA containing the rat VPAC$_2$ receptor inserts, as follows; 200 ng of

cDNA was mixed with 100 µl BL21 cells and left on ice for 30 mins. The reaction mix was heat shocked at 42°C in a water bath for 90 secs and chilled briefly for a further 2 mins on ice. The transformed cells were then plated onto separate luria bertani (LB) agar plates supplemented with ampicillin and incubated overnight at 37°C.

f) Large scale GST fusion protein preparation

GST fusion protein purification protocol was adapted from the manufacturers instructions (Pharmacia Biotech, Uppsala, Sweden). Single bacterial colonies were selected from the BL21 transformed plates, used to inoculate 25 mls 2 x yeast tryptone ampicillin (YTA) medium and incubated overnight at 37°C, whilst shaking vigorously. This culture was diluted 1:100 in 2 x YTA and incubated at 37°C until an OD_{600} of ~0.4 was reached. GST fusion protein expression was induced by the addition of 100 mM IPTG to give a final concentration of 0.1 mM and the culture was incubated at 37°C for a further 2 hrs. The culture was spun at 10 000g for 10 mins at 4°C in a Sorvall® centrifuge (DuPont Instruments). The supernatant was discarded and each pellet resuspended in 50 µl PBS/ml of culture. The cell suspension was flash frozen and stored at -70°C. The solution was thawed slowly at room temp and 10 mg/ml lysosyme added to lyse the cells. Each 5 ml aliquot was sonicated twice for 10 secs on ice. The aliquots were pooled in 50 ml tubes and mixed gently with 1% Triton X-100 for 30 mins at 4°C. The solubilised fusion protein was then centrifuged at 11 950 g for 10 mins at 4°C in a Sorvall® centrifuge (DuPont Instruments), SS-34 rotor, and the supernatant decanted into a fresh container.

For affinity purification the GST fusion protein was bound to 0.5 mls S-linked glutathione agarose for 30 mins at room temperature, whilst mixing gently. The suspension was centrifuged at 500x g for 5 mins in a Megafuge 2.0R (Heraeus instruments Ltd., Brentwood, Essex, UK) to sediment the matrix and the supernatant discarded. The glutathione agarose pellet was washed with 2.5 mls of 1x PBS and centrifuged at 500x g for 5 mins, the wash was discarded. This wash step was repeated twice more for a total of three washes. The fusion protein was eluted from the matrix by mixing gently with 0.5 mls glutathione elution buffer (GEB) at room temperature for 30 mins. The solution was centrifuged at 500x g for 5 mins to sediment the matrix and the supernatant containing eluted protein saved in a fresh tube. Elution and centrifugation steps were repeated twice more and the three eluates

pooled. A sample of each elute was then run on an SDS-page gel to confirm the size of the protein.

The VPAC$_2$ C-terminus/GST fusion protein was successfully purified by following this protocol and this protocol was repeated to obtain sufficient amounts necessary for rabbit immunisation (see section 2.7.9). However, only small amounts of the VPAC$_2$ receptor N-terminal/GST fusion protein were obtained; samples taken during the procedure revealed that the majority of protein was insoluble and located in inclusion bodies. To counteract this problem we varied experimental conditions to improve solubility; these adaptions included lowering the bacterial growth temperature, decreasing IPTG concentration, altering timing of induction and increasing sonication time. None of these techniques proved successful so a different approach using high urea concentrations to denature and solubilise the protein was used (see section 2.7.7).

g) Solubilisation of VPAC$_2$ N-terminal/GST fusion protein

To aid solubilisation of the N-terminal/GST fusion protein 8 M urea was used instead of Triton X-100 before the glutathione agarose step in the purification procedure previously described. The postsonicate pellet was resuspended in 5 ml 8 M urea containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and left to solubilise for 1 hr at room temperature. The pH was raised to 10.7 with 1 M NaCO$_3$ and the solution incubated for 30 mins. Next the pH was lowered to 8.0 by dropwise addition of 1.0 N HCl and incubated for a further 30 mins. The solubilised mixture was centrifuged at 12000 g for 15 mins at room temperature in a microcentrifuge (Eppendorf, 5415 C) and the supernatant decanted and placed into sterile dialysis tubing (boiled in PBS prior to use). The solubilised mixture was dialysed sequentially with 4 M, 2 M, 1 M urea and finally in PBS (to remove all of the urea) for 1 hr at 4°C. A 50 µl sample was taken after each dialysis step, spun at full speed in a microcentrifuge (Eppendorf, 5415 C) for 5 mins at room temperature and the pellet and supernatant retained for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, see section 2.7.8. The final dialyate containing the solubilised VPAC$_2$ N-terminal/GST fusion protein which was then purified as described above.

h) SDS-PAGE analysis

Protein samples were separated on denatured sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) minis gels. A

commercially available mini-gel system was used for all gels (Bio-rad Laboratories, Hercules, CA). Glass plates (10 x 7 cm) were cleaned and sealed into the gel apparatus, the plates were filled with approximately 4 mls resolving gel buffer, overlaid with a couple of drops of isobutanol and left to set. The isobutanol was removed and the gel surface washed with dH_2O. Stacking gel was gently pipetted on top of the resolving gel and a 10 lane comb inserted into it. Once set the gel was removed from the pouring container and placed into a mini-gel tank filled with tank buffer. The samples were mixed with gel loading buffer, vortexed and heated for 5 mins at 90-100°C before loading onto the gel. 2 µl of a rainbow molecular weight marker (Amersham Life Science Ltd., Little Chalfont, Bucks, UK) was added to the first lane of each gel. The samples were run for approximately 1 hr at ~120 V, until the dye front had reached the bottom of the gel. After which the gel was carefully removed from the plates and stained overnight in 0.25% Coomassie blue, 50% methanol and 10% acetic acid solution whilst shaking gently. The staining solution was removed and the gel destained in 5% methanol plus 7.5% acetic acid, this solution was changed regularly to facilitate destaining. The destained gel was dried onto filter paper (Whatman International Ltd., Kent, UK) for 1 hr at 80°C in a Bio-rad gel drier (Bio-rad Laboratories, Hercules, CA).

i) Immunisation

New Zealand White rabbits were immunised with 300 µg/ml of either the rat $VPAC_2$ receptor N-terminal or C-terminal/GST fusion proteins. The proteins were dissolved in 0.9% NaCl (w/v), an equal volume of Freund's complete adjuvant was added and the resulting emulsion vortexed vigorously to mix. The primary injection was administered by subcutaneous injection of 250 µl at each of four sites on the back of each rabbit. Booster doses of 300 µg/ml antigen in Freund's incomplete adjuvant were given monthly. One week after the third booster dose, ~5 mls of blood was taken from each rabbit to assess the antibody response. Two further booster injections were given and the blood sampled as above. One week after the last booster injection, the rabbits were exsanguinated according to Home Office procedures. The presence of specific anti-rat $VPAC_2$ receptor antibodies in the rabbit serum was assessed using two immunological techniques described below.

j) Enzyme-linked immunosorbent assay (ELISA)

Purified $VPAC_2$ N-terminus/GST or $VPAC_2$ C-terminal/GST fusion proteins, at a concentration of 20 µg/ml, were preadsorbed onto polyvinylchloride (PVC) 96 well dishes (Greiner, UK) over a 2 hr period at room temp. The wells were then washed

twice in PBS. Any remaining sites for protein binding to the PVC were saturated by incubation overnight with PBS containing 3% BSA. Rabbit antisera was preincubated overnight with GST (200 µg/ml) or the original antigen VPAC$_2$ N-terminus/GST or VPAC$_2$ C-terminal/GST (200 µg/ml). The plates were washed twice in PBS before the addition of the preadsorbed antisera at; 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000 and 1:32000 dilutions in PBS containing 1% normal rabbit serum (Scottish Antibody Production Unit, Carluke, Lanarkshire, UK) and incubated for 2 hrs at room temperature. Free antibody was removed by four washes with PBS. The secondary reagent, protein A-horseradish peroxidase (HRP), was added to each well at 0.5 µg/ml and incubated for 2 hrs at room temperature. The wells were washed a further seven times in PBS to remove free antibody. The reaction mix of 0.5 mg/ml 2,2,azino-bis (3-ethylbensthiasoline-6-sulfonic acid) (ABTS) in citrate phosphate buffer was added to each well and the plates were incubated for 20 mins under dark conditions. A colour change was observed when significant amounts of antibody was bound to the wells. The colour change was measured at 410 nm wavelength on a microplate reader (Dynatech instrument, inc., Torrance, CA) and the levels of antibody bound were plotted. No specific antibody binding was observed for the VPAC$_2$ C-terminal/GST fusion protein rabbit antisera, however specific binding was found for the VPAC$_2$ N-terminal/GST fusion, this experiment was performed twice to confirm this result. In order to further characterise the specificity of the VPAC$_2$ N-terminal/GST fusion protein Western blots were performed on membrane preparations from a Chinese hamster ovary (CHO) cell line stably transfected with the rat VPAC$_2$ receptor.

k) Western blots using rabbit polyclonal N-terminal/GST fusion protein antibodies

Untransfected and stably transfected Chinese hamster ovary cells were revived from existing frozen stocks (as described in section 2.2.3) and maintained in Nutrient Mixture Ham's F-12 culture media supplemented with 10% heat inactivated new calf serum and 100 U/ml each of penicillin and streptomycin, stably transfected cell media also contained 200 µg/ml geneticin. Cells were passaged once every 6-7 days. Cell extracts were prepared from 75 cm^2 flasks. Each flask was washed once in ice cold PBS and sterile cell scrapers were used to remove the monolayer into 8 mls PBS. Cells were spun down at 210 g for 5 mins at 4°C in a Megafuge 2.0R (Heraeus instruments Ltd, Brentwood, Essex, UK) and the pellet resuspended in 180 µl 150 mM NaCl and 50 mM Tris (pH 8.0). This solution was left on ice with 1% NP-40 (BDH, Merck Ltd, Lutterworth, Leics, UK) for 30 mins and agitated every 10 mins

by flicking. The solution was microcentrifuged at 8500 g for 30 mins at 4°C and the supernatant was retained for Western blotting and a 5 μl sample removed for protein assay. The samples were flash frozen and stored at -70°C. 10 μg samples of untransfected CHO cell extract and rat $VPAC_2$ receptor transfected CHO cell extract were loaded onto two SDS-PAGE mini-gels (see section.2.7.8), however the samples were not boiled before loading. One gel underwent SDS-PAGE analysis to determine the size of proteins present, whereas Western blotting was used to identify specific rat $VPAC_2$ receptor antibodies, see below.

A piece of 0.2 μM Trans-Blot® transfer membrane (Bio-rad Laboratories, Hercules, CA) was cut to the same size as the SDS-PAGE gel, soaked in methanol and then blotting buffer. The gel was removed carefully from the plates onto the transfer membrane, ensuring that no bubbles were allowed to form. The proteins were transferred overnight using 20 mA current at 4°C on a Bio-rad trans-blot® apparatus. The membrane was washed in PBS and incubated for 2 hrs at room temp in 5% dried milk powder containing 0.1% Tween-20. Further washes in the fresh blocking solution were carried out 5 times over a 25 min period. After which the membrane was cut into strips and incubated with titred rabbit antisera against the $VPAC_2$ N-termini/GST fusion protein for 2 hrs at room temperature. The rabbit antisera was added alone or preincubated overnight with either GST (200 μg/ml) or the original antigen ($VPAC_2$ N-terminus/GST) (200 μg/ml). All antibodies were diluted in PBS containing 3% BSA. The membrane was washed again, 5 times over a 25 min period in blocking solution. The secondary antibody, anti rabbit IgG conjugated to horseradish peroxidase (HRP) (Sigma, Poole, Dorset, UK) was added at a 1:15000 dilution and incubated at room temp for 1 hr. The wash step was repeated and an enhanced chemiluminescence (ECL) kit (Amersham, Little Chalfont, Bucks, UK) was used to detect the presence of any primary antibody. The ECL immunodetection procedure was performed according to the manufacturers instructions, the kit contains luminol and phenol solutions. Luminol is oxidised in the presence of HRP, as the luminol decays from its excited state to a ground state light is emitted, providing the basis for antibody binding detection. This process is enhanced in the presence of phenols. Transfer membranes were incubated in the presence of luminol and enhancer for 1 min at room temp, following which they were wrapped in cling film and left for 10-20 mins. Light emission from the membrane was detected by short exposure to autoradiography film (Hyperfilm ECL: Amersham, Little Chalfont, Bucks, UK) which was then developed and fixed under dark room conditions. Western blotting resulted in an identical pattern of bands of immunoreactivity in

control (untransfected) and rat VPAC$_2$ receptor transfected CHO cells. These experiments indicated that antibodies present in the rabbit antisera (following immunisation with the rat VPAC$_2$ receptor/GST fusion protein) were not able to recognise the denatured receptor on a SDS-PAGE gel. No further experiments with these antisera were performed.

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70 Evidence for protein kinase A (PKA) dependent and independent desensitisation and phosphorylation of the human vasoactive intestinal peptide receptor (VIP2).

TERENCE P. MCDONALD, CHRISTINE F. MORRISON, DIANE M. DINNIS and ANTHONY J. HARMAR

MRC Brain Metabolism Unit, University Department of Pharmacology, 1 George Square, Edinburgh EH8 9JZ, UK

The neuropeptide vasoactive intestinal peptide (VIP) belongs to a family of regulatory peptides including secretin, glucagon and growth hormone-releasing hormone. Receptors for these peptide hormones constitute a distinct family of seven transmembrane spanning proteins that are all coupled, via G-proteins, to cyclic AMP (cAMP) production. Two distinct receptors for VIP (VIP$_1$ and VIP$_2$) have been cloned [1, 2] that display similar pharmacological properties. However, the receptors are differentially distributed within the brain [3].

The desensitisation of membrane-bound receptors by phosphorylation provides a means by which agonist stimulated signal can be regulated. Subtypes of a given receptor family can differ markedly in their desensitisation properties and this may be one of the main reasons for the existence of receptor subtypes. We decided to investigate phosphorylation of the VIP$_2$ receptor and its role in mediating receptor desensitisation.

In the presence of the specific PKA inhibitor H-89, the maximum level of cAMP production in response to VIP in COS7 cells transiently expressing VIP$_2$ receptor was increased by 31.1 $\pm$ 5 % (n=3) although there was little variation in EC$_{50}$ values (Fig. 1). The protein kinase C (PKC) inhibitor bisindolymaleimide caused a small reduction in cAMP levels at high concentrations. These data suggest that PKA but not PKC plays a role in the desensitisation of VIP$_2$ receptors.

In order to investigate phosphorylation of the VIP$_2$ receptor, the hemagglutinin epitope (HA: YPYDVPDYASL) was fused to the C-terminus and the cDNA transfected into COS7 and CHO cells. Cyclic AMP production in response to VIP was unaffected by the presence of the epitope tagg. Western blot analysis (Fig. 2) revealed that two major immunoreactive proteins of 44kDa and 66kDa were expressed in COS7 cells and a single 66kDa protein in CHO cells. No immunoreactive proteins were detected in cells not expressing the tagged receptor. The relationship between the two bands was demonstrated by endoglycosidase treatments. Digestion with N-glycosidase F (PNGase F), which cleaves all of the N-linked sugars, revealed a single immunoreactive protein of 44kDa in both cell types, indicating that both forms of receptor were identical polypeptide chains.
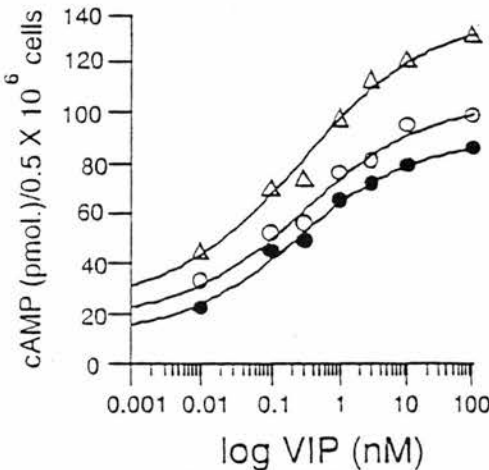

Fig. 1 The PKA inhibitor H-89 enhances VIP stimulated cAMP production in COS7 cells expressing VIP$_2$ receptor. Cells transfected with VIP$_2$ receptor cDNA were exposed to H-89 ($\bullet$ 0μM, O 5μM, $\triangle$ 20μM) for five minutes before stimulation with VIP for 20 minutes. Cyclic AMP assays were carried out as described previously [4].

However, exposure to endoglycosidase H (Endo H) did not modify the electrophoretic pattern suggesting the absence of significant amounts of mannose-rich, N-linked carbohydrates.
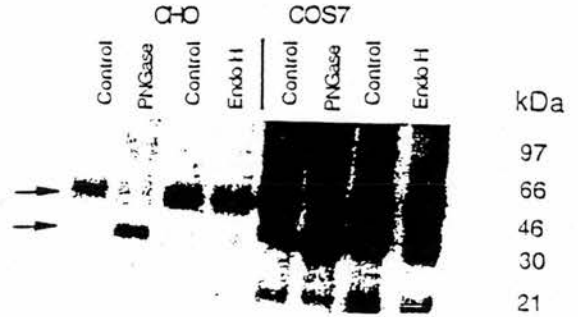

Fig. 2 Characterisation of VIP$_2$ by Western blot analysis. COS7 and CHO cell membranes containing HA tagged VIP$_2$ receptor were solubilised and treated with the PNGase F or Endo H. The receptor was then detected after SDS-PAGE and Western blotting by antibodies specific to the HA epitope. The upper arrow indicates the mature VIP$_2$ receptor and the lower arrow the deglycosylated receptor.

We investigated the ability of VIP to induce phosphorylation of the epitope tagged VIP$_2$ receptor in COS7 cells. Cells were incubated in media containing 200μCi/ml [β-^{32}P] orthophosphate and receptor phosphorylation was analysed by immunoprecipitation and autoradiography (Fig. 3). A small amount of basal phosphorylation was observed. Treatment with 100nM VIP for 20 minutes resulted in a significant increase in the incorporation of radioactivity into the 44kDa band. Maximal levels of receptor phosphorylation were observed even with low levels of VIP (0.01nM) (data not shown). Receptor-independent
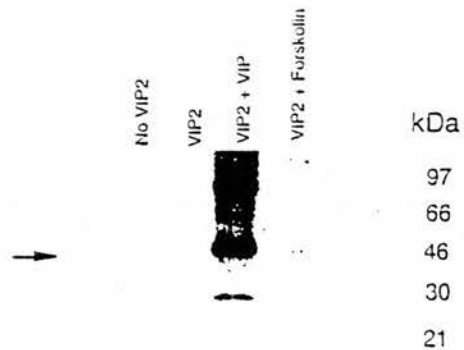

Fig. 3 VIP and forskolin induced phosphorylation of VIP$_2$. COS7 cells transfected with HA tagged VIP$_2$ receptor cDNA were labelled with [^{32}P]orthophosphate. The labelled receptor was immunoprecipitated with antibodies specific to the HA epitope and analysed by SDS-PAGE. Western blotting and autoradiography.

activation of PKA, via forskolin (100μM), induced phosphorylation but to a lesser degree than VIP. These data indicate that receptor occupancy promotes phosphorylation and gives rise to the possibility that receptor phosphorylation involves a G-protein-coupled receptor kinase as well as PKA. Further studies will focus on investigating this possibility and identifying the receptor phosphorylation sites.

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Desensitization of the Human Vasoactive Intestinal Peptide Receptor (hVIP₂/PACAP R): Evidence for Agonist Induced Receptor Phosphorylation and Internalization

TERENCE P. MCDONALD,* DIANE M. DINNIS, CHRISTINE F. MORRISON, AND ANTHONY J. HARMAR

MRC Brain Metabolism Unit, Royal Edinburgh Hospital, Morningside Park, Morningside, Edinburgh EH10 5HF.

ABSTRACT: To investigate the role of phosphorylation and internalization in the desensitization of the hVIP₂/PACAP receptor we expressed a C-terminal epitope–tagged (hemagglutinin; YPYDVPDYASL) receptor in COS7 and HEK293 cell lines. Radiolabeling experiments demonstrated that exposure to agonist induced receptor phosphorylation significantly above basal levels. This receptor phosphorylation was greater than that induced by receptor-independent activation of PKA with forskolin and then that induced by co-application of forskolin and agonist. This suggests that receptor occupancy promotes phosphorylation and also that receptor phosphorylation may involve a specific G protein–coupled receptor kinase in addition to PKA. Immunocytochemical analysis showed that the receptor was internalized in response to agonist to a single site of accumulation within the cell and this was dependent on temperature, agonist concentration, and time. Further studies will focus on identifying phosphorylation sites and endocytic signals within the hVIP₂/PACAP R.

The neuropeptide vasoactive intestinal peptide (VIP) is a member of a family of regulatory peptides that includes pituitary cyclase-activating polypeptide, secretin, glucagon and growth hormone-releasing hormone. Receptors for these peptides belong to a family of seven transmembrane-spanning G protein–coupled receptors (GPCRs) and are distinct from the rhodopsin superfamily.[1] All members of this family couple to adenylyl cyclase, probably mediated by G_s. Many are also capable of stimulating inositol phosphate production.[2-6] Two distinct receptors for VIP (VIP₁/PACAP R and VIP₂/PACAP R) have been cloned[7-8] that display similar pharmacological properties but are differentially distributed within the brain.[9] Exposure of GPCRs to ligands triggers not only their activation but also a chain of events, termed desensitization, that results in a reduction of cellular response to the agonist. This desensitization provides a means by which agonist-stimulated signal can be regulated. Two major components have been identified for G protein–coupled receptor desensitization: internalization of the occupied receptor (probably into vesicles physically separated from the plasma membrane) and phosphorylation of the intracellular domains of the receptor. Subtypes of a given receptor family can differ markedly in their desensitization properties

*Corresponding author: Tel.: 44 131 537 6530; Fax: 44 131 537 6110; e-mail: tmcdonald@srv1.bmu.mrc.ac.uk

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and this may be one of the main reasons for the existence of receptor subtypes. We decided therefore to characterize the relative contribution of phosphorylation and internalization in mediating desensitization of the human VIP./PACAP R (hVIP./PACAP R). We have used a hemagglutinin epitope tag fused to the C-terminus of the hVIP./PACAP R to immunoprecipitate the receptor from cell lysates and localize it using immunofluorescence. To carry out these studies we expressed the tagged receptor (hVIP./PACAP R-HA) in both COS7 and HEK293 cells.

MATERIALS AND METHODS

Construction of Hemagglutinin Tagged hVIP./PACAP R

The last codon of the hVIP./PACAP R cDNA contained in the expression vector pcDNA3 was changed by polymerase chain reaction from ATC to CTC thus creating a *XhoI* restriction site. This restriction site together with that of *XbaI* in the plasmid multicloning site were digested with *XhoI* and *XbaI* and the 6.99-kb fragment isolated. The oligonucleotides 5'-TCGAGTACCCATACGATGTTCCAGATTACGCCTCCCTCTAGT-3' and 5'-CTAGACTAGAGGGAGGCGTAATCTGGAACATCGTATGGGTAC-3' were annealed together and ligated with the plasmid DNA recreating the *XhoI* and *XbaI* restriction sites. The resultant plasmid contained hVIP./PACAP R cDNA with the last C-terminal amino acid changed from Leu to Ileu followed directly by Glu and the 11-amino acid hemagglutinin epitope (HA: YPYDVPDYASL) and a translational stop signal (TAC).

Cell Culture and Transfection

Cells were transfected by way of electroporation; details of transfection are described elsewhere.[11] HEK293 and COS7 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. CHO cells were grown in nutrient mixture Ham's F-12 supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Stable clones were selected with 400 µg/ml geneticin and maintained in 200 µg/ml geneticin. Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Phosphorylation of the hVIP./PACAP R

Cells (60–80% confluent) were washed in phosphate-buffered saline (PBS) and incubated for 2 h in phosphate-free minimal essential medium containing 20 mM HEPES, pH 7.4; 0.5% BSA and 200 µCi/ml[β-^{32}P] orthophosphate. After 2 h at 37°C, the cells were exposed to VIP or other agents for the times or concentrations described in the text. Following treatment, cells were washed once in PBS at room temperature and twice at 4°C. The cells were then resuspended into 50 mM Tris-HCl, 1 mM EGTA, pH 7.4 with protease inhibitors (2 µg/ml pepstatin A, 2 µg/ml aprotinin, and 4 µg/ml leupeptin) and phosphatase inhibitors (10 mM $Na_4P_2O_7$, 10 mM NaF, and 0.1 mM Na_3VO_4). The cells were then disrupted by homogenization and the membranes were collected by centrifugation. The membranes were solubilized in Nonidet buffer (0.5% Nonidet P-40, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM EGTA pH 7.4) containing protease and phosphatase inhibitors as above. A mouse anti-HA epitope monoclonal antibody (Autogen Bioclear) was added to the lysate at 1/1,000-fold dilution for 1 h (4°C) and unsolubilized

membranes then removed by centrifugation. The receptor-antibody complex was precipitated (1 h/4°C) using protein G-sepharose (Autogen Bioclear) then washed five times in Nonidet buffer. De-glycosylation was carried out with PNGase (New England Bio-labs). The proteins were separated by SDS-PAGE and the ^{32}P incorporated into the proteins detected by autoradiography. Precipitated receptor was detected using the anti HA-epitope antibody.

Immunocytochemistry

Cells grown overnight on glass coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and non-specific binding was blocked with 10% goat serum. Fixed cells were then incubated sequentially (1 h at room temp) with each of the following; anti-HA antibody, anti-mouse IgG biotinylated antibody, and avidin-fluorescein isothiocyanate. Coverslips were mounted in PBS/glycerol and viewed on a Zeiss inverted microscope (Axicvert 135 M).

RESULTS

Characterization of hVIP₂/PACAP R-HA Bearing HEK293 Cell Lines

To facilitate the investigation of phosphorylation and internalization of the hVIP₂/PACAP receptor, the hemagglutinin epitope (HA; YPYDVPDYASL) was fused to the C-terminus (see *Methods*). The hVIP₂/PACAP R-HA protein elicited VIP-stimulated cAMP production when expressed stably in HEK293 cells. No differences were detected in the extent of stimulation of adenylyl cyclase activity or in the EC_{50} values between the tagged (hVIP₂/PACAP-HA) and untagged receptors [EC_{50} for untagged, 0.97 ± 0.17 ($N=7$) and for tagged, 1.00 ± 0.2 ($N=8$)].

Desensitization

To explore desensitization of the hVIP₂/PACAP R, HEK293 cells stably expressing the receptor were exposed to 10 nM VIP for 20 min (FIG 1). After such exposure and extensive washing, stimulation with increasing concentrations of VIP elicited a reduction in the extent of cAMP production to $74.5 \pm 6.0\%$ ($N = 4$) of the maximal responses observed in controls not preincubated with VIP. In contrast, the EC_{50} values were very similar [0 nM, 0.76 ± 0.26nM ($N = 5$) and 10 nM, 0.75 ± 0.38 nM ($N = 4$)].

Effects of Phosphokinase Inhibitors

The role of the effector kinases PKA and PKC in desensitization of the hVIP2/PACAP R was investigated. COS7 cells expressing the receptor were pretreated with the specific inhibitors H-89 (PKA) and bisindolymaleimide (PKC) and cAMP production in response to VIP was measured. cAMP production in response to a range of VIP concentrations was enhanced to a maximum of 31.1% after pretreatment with 20 µM H-89, although there was little variation in EC_{50} values (FIG. 2). Pretreatment with bisindolymaleimide caused a small reduction in cAMP levels at high concentrations. These data suggest that PKA (but not PKC) plays a role in the desensitization of the hVIP₂/PACAP R.
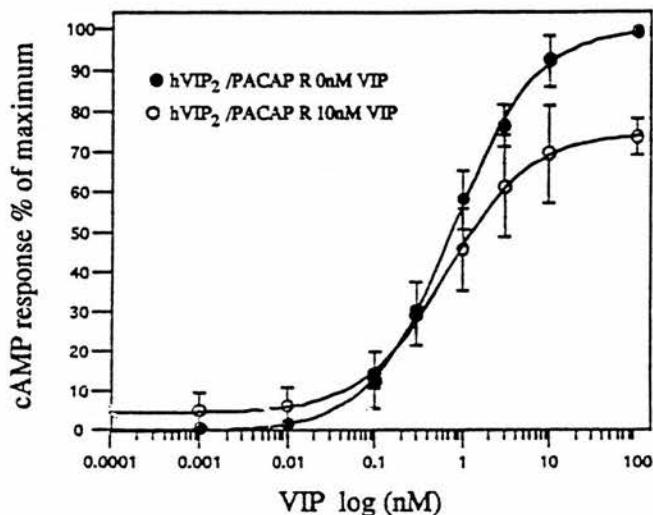
FIGURE 1. Desensitization of a hVIP₂/PACAP receptor bearing HEK293 cell line. The graph shows cAMP responses to increasing concentrations of VIP in the presence of phophodiesterase inhibitor isobutylmethylxanthine (IBMX) with or without the pretreatment with 10 nM VIP for 20 min. Values are expressed as means (standard deviations from these means are indicated by the error bars) of five and four experiments in the untreated and treated experiments respectively. The values are expressed as a percentage of the maximum response of the control and fitted by least-squares analysis to an equation for an asymmetric sigmoid. The maximum cAMP response in the VIP treated is $74.5 \pm 6.0\%$ ($N=4$) of that of the untreated. The maximum cAMP responses were $1{,}070 \pm 233$ pmol/0.5×10^6 cells for the VIP treated and 687 ± 410 pmol/0.5×10^6 for the untreated. Cellular cAMP levels were measured by radioimmunoassay as described elsewhere.[10]
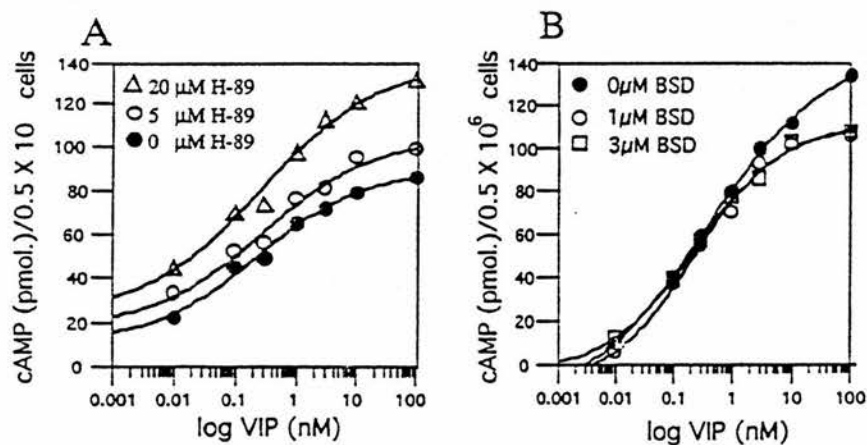

FIGURE 2. The PKA inhibitor H-89 but not the PKC inhibitor BSD enhanced VIP-stimulated cAMP production in COS7 cells expressing the hVIP₂/PACAP receptor. COS7 cells transiently transfected with hVIP₂/PACAP R cDNA were exposed to either H-89 (A) or to BSD (B) for 5 min before exposure to increasing amounts of VIP for 20 min in the presence of IBMX. Cellular cAMP levels were measured by radioimmunoassay as described elsewhere.[10]

### Characterization of the hVIP/PACAP R by Western Blot Analysis

Western blot analysis of membranes from COS7 and CHO cells stably expressing the hVIP/PACAP R-HA revealed that two major immunoreactive proteins of 44 and 66 kD were expressed in COS7 cells and a single 66kDa protein in CHO cells (FIG. 3). No immunoreactive proteins were detected in cells not expressing the tagged receptor. Digestion with *N*-glycosidase F (PNGase F), which cleaves all of the *N*-linked sugars, revealed a single immunoreactive protein of 44 kD in both cell types, indicating that both forms of receptor have identical polypeptide chains. However, exposure to endoglycosidase H (Endo H) did not modify the electrophoretic pattern, suggesting the absence of significant amounts of mannose-rich, *N*-linked carbohydrates.

Phosphorylation of the hVIP/PACAP Receptor

We investigated the ability of VIP to induce phosphorylation of the hVIP/PACAP R-HA expressed in both COS7 and HEK293 cells. Cells were incubated in media containing 200 µCi/ml[β-³²P] orthophosphate at 37°C before treatment with 100 nM VIP for 20 min. The receptor was immunoprecipitated and deglycosylated as described in *Methods*. Western blotting and autoradiography were used to analyze receptor phosphorylation. A small amount of basal phosphorylation of the receptor was observed. Treatment with VIP resulted in a significant increase in the incorporation of radioactivity into the 44 kD band identified as the deglycosylated receptor (FIG. 4). The VIP-induced incorporation of
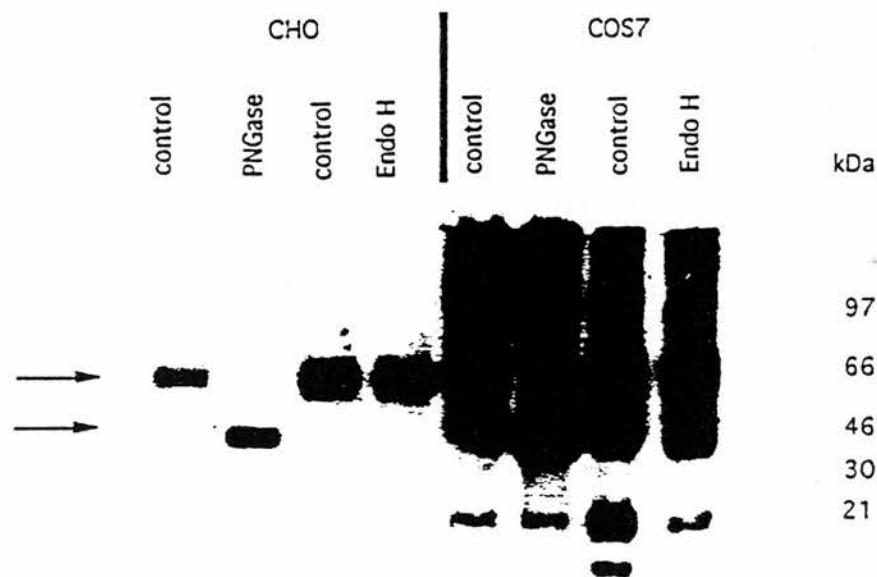
FIGURE 3. Characterization of the hVIP/PACAP R by western blot analysis. Cos7 and CHO membranes containing hVIP/PACAP R-HA were solubilized and treated with PNGase F or Endo H. The receptor was then detected after SDS-PAGE and Western blotting by antibodies specific to the HA epitope. The upper arrow indicates the mature hVIP/PACAP R-HA and the lower arrow the deglycosylated receptor.
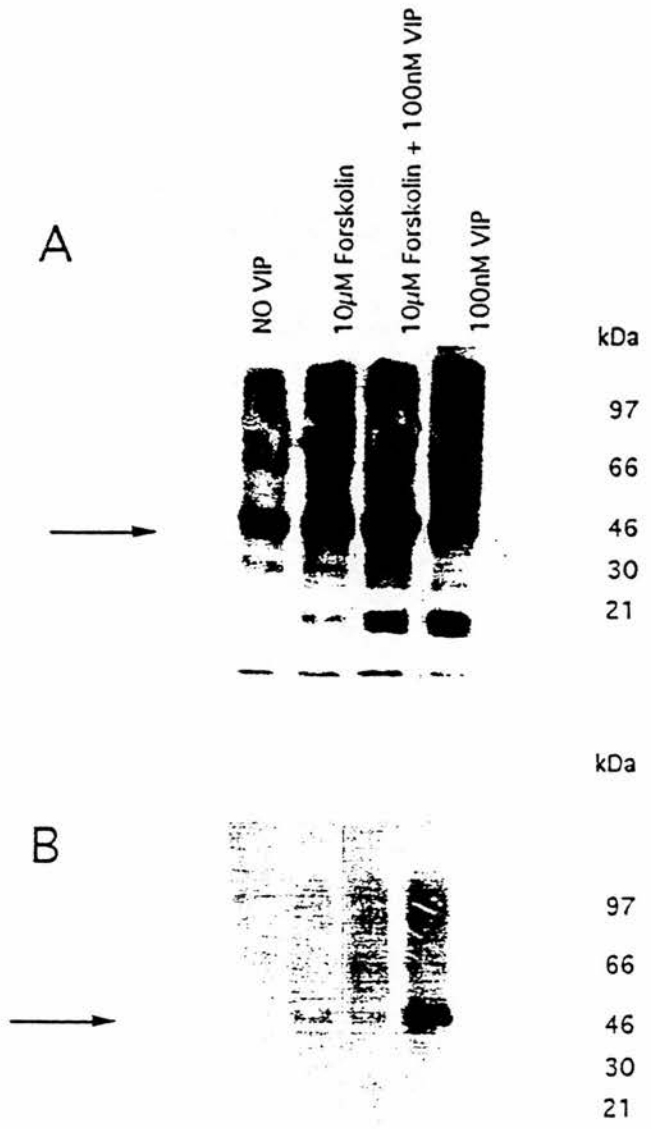
FIGURE 4. Phosphorylation of the human VIP$_2$ receptor is induced by agonist occupancy. HEK293 cells stably transfected with hVIP$_2$/PACAP R-HA cDNA were labeled in media containing 200 μCi/ml [β-^{32}P]orthophosphate for two h. Forskolin and VIP were added 20 min before the end of this incubation. The labeled receptor was immunoprecipitated with antibodies specific to the HA epitope and deglycosylated. The precipitated receptor was then detected after SDS-PAGE by immunoblotting (A) and the phosphorylated receptor by autoradiography (B). The arrows indicated the position of the immunoprecipitated and phosphorylated receptor. The results shown are representative of three independent experiments. Similar results were obtained with COS7 cells transiently transfected with hVIP$_2$/PACAP R-HA cDNA.
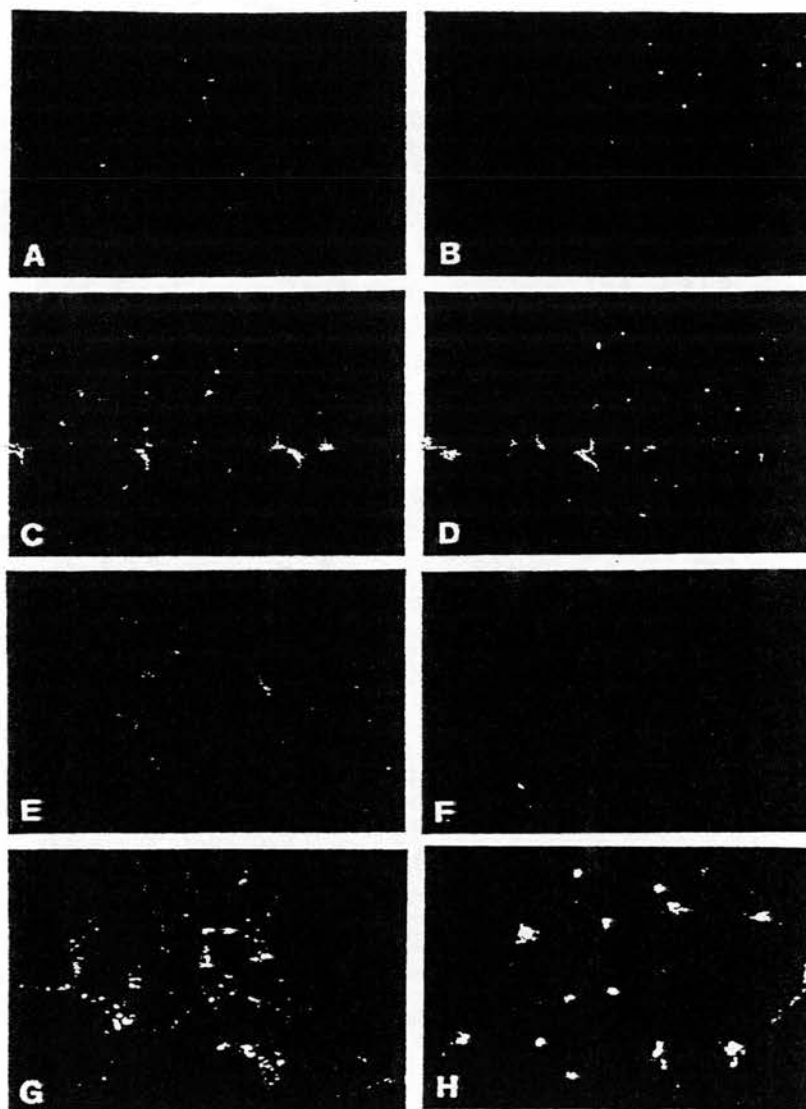
FIGURE 5. Internalization of the hVIP/PACAP R. Localization of the hVIP/PACAP R-HA in untreated HEK293 cells at $\times$ 40 magnification (A) and $\times$ 100 magnification (E). Cells were pretreated for 30 min at 37°C at with VIP at 0.1 μM (B), 1 μM (C), and 10 μM (D) ($\times$ 40 magnification). The time course of agonist-induced internalization was investigated by pretreatment of cells at 37°C with 10 μM VIP for 5 min (F), 10 min (G), and 30 min (H) ($\times$ 100 magnification).

radioactive phosphate into the receptor protein was detectable after 2 min, the shortest time analyzed, and reached apparent saturation in less than 10 min (data not shown). Receptor-independent activation of PKA via stimulation of adenylyl cyclase with forskolin (100 μM), induced phosphorylation above basal levels but to a lesser degree than VIP. These

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data indicate that receptor occupancy promotes phosphorylation. The addition of both VIP and forskolin together induced receptor phosphorylation at a level lower than that of VIP alone. These data suggest that there is more than one mechanism of phosphorylation. This gives rise to the possibility that receptor phosphorylation involves a specific G protein–coupled receptor kinase as well as PKA. G protein–coupled receptor kinases phosphorylate only agonist-occupied, active receptors and therefore promote homologous desensitization in contrast to the heterologous desensitization demonstrated by effector kinases such as PKA.

Internalization of the hVIP₂/PACAP Receptor

Internalization of the hVIP₂/PACAP R was investigated in HEK293 cells using immunocytochemistry and conventional fluorescence microscopy. In HEK293 cells stably expressing the hVIP₂/PACAP R-HA, the anti-HA antibody revealed intense staining at the cell membrane. This was reduced following exposure to VIP for 30 min at 37°C and fluorescent staining was predominant at an intracellular perinuclear site (FIG. 5). This internalization of receptor was dependent on the concentration of VIP, incubation time, and temperature (data not shown). Staining was not observed in untransfected HEK293 cells or cells expressing the untagged hVIP₂/PACAP receptor.

CONCLUSIONS

Our data show that the *hVIP₂/PACAP* receptor is desensitized in response to exposure to VIP. Furthermore, we have shown that the receptor undergoes both agonist-induced phosphorylation and internalization and it is likely that both these processes are involved in its desensitization. The observation that agonist-induced phosphorylation is inhibited by the receptor-independent activation of PKA, via forskolin, also suggests the possible involvement of a G protein–coupled receptor kinase as well as PKA. Further studies are being carried out to delineate the roles and mechanisms of phosphorylation and internalization in the desensitization of the hVIP₂/PACAP R and to ascertain their relative contribution to the process. Experiments using a series of C-terminal truncated receptors will focus on identifying phosphorylation sites and endocytic signals. Other experiments are being carried out to determine the precise intracellular location of the internalized receptor.

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