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A systematic comparison of intracellular cyclic AMP and calcium signalling highlights complexities in human VPAC/PAC receptor pharmacology

Louise Dickson [a], Ichiro Aramori [b], James McCulloch [a], John Sharkey [a], Keith Finlayson [a,*]

[a] *Astellas CNS Research in Edinburgh, The University of Edinburgh, The Chancellor's Building, 49 Little France Crescent, Edinburgh, EH16 4SB, UK*
[b] *Molecular Medicine Research Laboratories (II), Astellas Pharma Inc., Tsukuba, 305-8585, Japan*

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Abstract

VPAC/PAC receptor activation classically results in cyclic-AMP production, with limited reports evaluating calcium signalling. These studies systematically characterise intracellular cyclic-AMP ($[cAMP]_i$) and calcium ($[Ca^{2+}]_i$) responses in CHO-cells expressing recombinant human (h) VPAC/PAC receptors ($hVPAC_1R$, $hVPAC_2R$, $hPAC_1R$), using two simple, non-radioactive, HT-amenable assays. The rank order of potency (ROP) of the agonists VIP, PACAP-27 and PACAP-38 was similar in both assays for each individual receptor subtype, although potencies (EC_{50}) in the $[Ca^{2+}]_i$ assay were approximately 100-fold lower. Importantly, this shift was also evident in SHSY-5Y cells endogenously expressing $hPAC_1R$. Furthermore, $[Ala^{11,22,28}]$VIP and maxadilan were selective $hVPAC_1R$ and $hPAC_1R$ agonists, respectively, and although R3P65 had no demonstrable $hVPAC_2R$ selectivity, these compounds exhibited comparable reductions in $[Ca^{2+}]_i$ EC_{50} values. In contrast, PG97-269 and PG99-465, putatively selective $hVPAC_1R$ and $hVPAC_2R$ antagonists, respectively, were marginally less potent in $[cAMP]_i$ studies, whereas M65 was equipotent at $hPAC_1R$. Moreover, PG99-465 alone increased $[cAMP]_i$ at all three hVPAC/PAC receptor subtypes, with full $hVPAC_1R$ and $hPAC_1R$ agonism. With equivalent agonist ROPs generated in both assays, $[Ca^{2+}]_i$ signalling provides an alternative approach to examine hVPAC/PAC receptor pharmacology. However, these studies underscore the paucity of receptor selective compounds, complexities in comparing drug potencies across assays, and the pleiotropic nature of VPAC/PAC-receptor signalling.

Keywords: $[Ca^{2+}]_i$; $[cAMP]_i$; hVPAC/PAC-receptors; Maxadilan/M65; PG97-269/PG99-465; VIP/PACAP

1. Introduction

Vasoactive intestinal peptide (VIP) was originally isolated from porcine duodenum some 36 years ago (Said and Mutt, 1970), with subsequent studies demonstrating limited expression in other non-neuronal tissues, but a widespread distribution throughout the central and peripheral nervous systems (Delgado et al., 2004). VIP belongs to the structurally related family of polypeptide hormones which includes secretin, glucagon and pituitary adenylate cyclase-activating polypeptide (PACAP), the latter sharing 68% sequence homology with VIP (Miyata et al., 1989). PACAP-38 and its C-terminally truncated form, PACAP-27, were extracted from ovine hypothalamic tissue (Miyata et al., 1989, 1990) and as with VIP, both peptides are found throughout the body (Vaudry et al., 2000). Early reports suggested that VIP and PACAP exerted a multitude of effects on cardiovascular, circulatory and respiratory systems and on metabolic function (Said and Mutt, 1970; McCulloch and Edvinsson, 1980; Miyata et al., 1989, 1990), whereas a number of recent studies point to a plethora of putative roles in the nervous (Dickinson and Fleetwood-Walker, 1999; Harmar et al., 2002) and immune systems (Gomariz et al., 2001; Pozo and Delgado, 2004). More specifically, by regulating processes such as cell death, inflammation, microglial activation (Delgado et al., 2003), and glucose/insulin secretion (Yung et al., 2003), VIP/PACAP have been directly implicated in various clinical conditions such as

* Corresponding author. Tel.: +44 131 242 6347; fax: +44 131 242 9371.
E-mail address: keith.finlayson@ed.ac.uk (K. Finlayson).

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arthritis, neuropathic pain, Alzheimer's and Parkinson's disease, and diabetes.

The physiological actions of VIP/PACAP are produced through the activation of three distinct receptor subtypes belonging to the group B family of G-protein coupled receptors (GPCRs), namely $VPAC_1R$, $VPAC_2R$ and PAC_1R (Harmar, 2001; Harmar et al., 1998). In radioligand binding assays, VIP and the PACAP peptides have a similar sub-nanomolar affinity for $VPAC_1R$ and $VPAC_2R$, whereas both forms of PACAP have a higher affinity at the PAC_1R and induce functional responses at lower concentrations than that required for VIP (Vaudry et al., 2000). One of the main characteristics of GPCRs in the group B family, including VPAC/PAC receptors, is a ligand induced, $G\alpha_s$ dependent, increase in intracellular cAMP ($[cAMP]_i$) (Harmar, 2001). However, as is common for many GPCRs (Van Rampelbergh et al., 1997), no exclusive signalling pathway exists and VPAC/PAC receptor activation can also induce phospholipase D and intracellular calcium ($[Ca^{2+}]_i$) production in human and rat cell lines (Sreedharan et al., 1994; Xia et al., 1996; McCulloch et al., 2000; Gomariz et al., 2001). A limited number of studies have shown that the VPAC receptor mediated increase in $[Ca^{2+}]_i$ involves either $G\alpha_{i/o}$ or $G\alpha_q$ coupled mechanisms (MacKenzie et al., 1996), with observations in cells endogenously expressing hVPAC receptors (HT-29, Sreedharan et al., 1994; SUP-T1, Xia et al., 1996) and in transfected cell lines (HEK-293, Sreedharan et al., 1994; CHO, Xia et al., 1997; Langer et al., 2002a). In contrast, although an $hPAC_1$ mediated stimulation of $[Ca^{2+}]_i$ has also been reported in native (SHSY-5Y, Eggenberger et al., 1999; NB-OK-1, Delporte et al., 1993) and recombinant cell lines (CHO, McCulloch et al., 2000), transduction is exclusively mediated via $G\alpha_q$ (MacKenzie et al., 1996). In general, although $[cAMP]_i$, $[Ca^{2+}]_i$ and phospholipase D can all be produced in CHO-cells overexpressing rat VPAC/PAC receptors, production of the former second messenger occurs more readily than the other two (McCulloch et al., 2000, 2001). Indeed, for VPAC receptors even in the presence of a considerable agonist induced $[cAMP]_i$ response, sometimes no alteration in $[Ca^{2+}]_i$ is observed (McCulloch et al., 2000, 2001). Moreover, when a VPAC receptor mediated $[Ca^{2+}]_i$ signal can be detected, the EC_{50} concentration of agonist required to elicit this response varies considerably, ranging from at least one to two orders of magnitude greater than that required for $[cAMP]_i$ (Sreedharan et al., 1994; Xia et al., 1996; Langer et al., 2001; MacKenzie et al., 2001). This difference in agonist potency in stimulating $[cAMP]_i$ and $[Ca^{2+}]_i$ responses can also be seen for PAC_1 receptors (Deutsch and Sun, 1992; Spengler et al., 1993). As assay methodologies and receptor expression levels have varied considerably, it is difficult to conclude what underlies the different agonist potencies for the various VPAC/PAC receptor transduction pathways (Ciccarelli et al., 1994; Laburthe and Couvineau, 2002). In fact, the potency of VIP to induce cAMP production can vary as much as 30-fold between assays, even on switching from membranes to whole cells (Langer et al., 2002a), with the possibility of species differences further adding to the complexity (Deutsch and Sun, 1992; Spengler et al., 1993; Gourlet et al., 1997; Laburthe and Couvineau, 2002).

To date, there is still no single study that systematically investigates functional responses at all three human VPAC/PAC receptors using a range of agonists and antagonists, with the majority of previous reports focusing on the interaction between one receptor subtype and several agonists or between all three receptors and one agonist. To provide a comprehensive characterisation of human VPAC/PAC receptor pharmacology and eliminate variability arising from the use of different assay and expression systems, we have generated CHO cell lines stably expressing the three individual human receptor subtypes and established standardised, cell based, non-radioactive, high throughput (HT)-amenable, fluorescence based assays to measure $[cAMP]_i$ and $[Ca^{2+}]_i$. Agonist profiles for the transfected cell lines were generated using VIP, PACAP-27 and PACAP-38, in addition to putatively $VPAC_1$ ($[Ala^{11,22,28}]$VIP; Nicole et al., 2000), $VPAC_2$ (R3P65; Yung et al., 2003) and PAC_1 (maxadilan; Moro and Lerner, 1997) receptor selective agonists. Furthermore, the potency and selectivity of the reportedly receptor specific antagonists, PG97-269 ($VPAC_1$; Gourlet et al., 1997), PG99-465 ($VPAC_2$; Moreno et al., 2000), and M65 (PAC_1; Uchida et al., 1998) was thoroughly examined.

2. Materials and methods

2.1. Generation of human VPAC/PAC receptor stable transfectants and cell culture

Following the design of sequence specific primers, cDNA clones encoding the $hVPAC_1R$, $hVPAC_2R$, and $hPAC_1R$ were obtained by PCR, from a human brain cDNA template (Clontech, Palo Alto, USA). The resultant PCR products were individually subcloned into pBluescript SK(−) (Stratagene, California, USA), with the identities of the resultant clones confirmed by nucleotide sequence analysis, using an automated sequence analyser (Applied Biosystems, Foster City, USA). CHO cell lines expressing the hVPAC/PAC receptors were established in a manner similar to that described previously (Aramori and Nakanishi, 1992). Briefly, individual hVPAC/PAC receptor cDNAs were sub-cloned into a eukaryotic expression vector (pMH009) containing the simian virus 40 early promoter and the mouse dihydrofolate reductase (dhfr) gene as a selective marker, with the resultant plasmids transfected into CHO(dhfr-) cells using calcium phosphate precipitation. CHO cells were chosen as it is known that phospholipase C/$[Ca^{2+}]_i$ stimulation can occur via either $G\alpha_{q/11}$ or $G\alpha_{i/o}$ proteins in this cell line (Van Rampelbergh et al., 1997) and that these cells do not constitutively express VPAC or PAC receptors (Ciccarelli et al., 1994). Cell lines expressing hVPAC/PAC receptors together with dihydrofolate reductase were selected in α-minimal essential medium (α-MEM) lacking ribonucleosides and deoxyribonucleosides, but supplemented with 10% dialysed foetal bovine serum (FBS). From the selected cell populations, clonal lines were isolated by single cell cloning, and the resultant CHO cell lines expressing $hVPAC_1R$, $hVPAC_2R$, and $hPAC_1R$ were maintained in culture, in α-MEM (supplemented with 10% dialysed FBS; L-glutamine, 2 mM; penicillin, 100 U/ml, and streptomycin, 100 μg/ml), at 37°C in a humidified atmosphere with 5% CO_2. The $hPAC_1$ cell line generated was the $hPAC_{1-null}$ variant described by Pisegna and Wank (1996). Individual stable cell lines were sub-cultured twice weekly, and used from passage 2 to 45, with no obvious deleterious effect in either assay on maximal response size or on compound potencies. The three stable cell lines examined produced equivalent maximum responses in both assays ($[cAMP]_i$: ~60 nM, ~25× basal; $[Ca^{2+}]_i$: ~7× basal), following stimulation with a saturating concentration of ligand (VIP or PACAP). Vertongen et al. (2004) have shown that this level of cAMP correlates with a receptor density of approximately 1 pmol/mg and moreover that cAMP and $[Ca^{2+}]_i$ levels are linearly correlated with a receptor density

up to 5 pmol/mg (Langer et al., 2002b; Langer and Robberecht, 2005). In an additional set of studies, human neuroblastoma cells (SHSY-5Y; ECACC, Salisbury, UK) endogenously expressing PAC_1 receptors (Vertongen et al., 1996) were maintained in culture; (Finlayson et al., 2001) and prepared for the in vitro assays in an identical manner to the hVPAC/PAC receptor stable cell lines. For the $[cAMP]_i$ and $[Ca^{2+}]_i$ assays described below, cells were rinsed with pre-warmed Hanks' balanced salt solution (HBSS), detached by trypsinisation (0.25%) and pelleted by centrifugation (150 g, 5 min). Cell pellets were re-suspended in media and seeded overnight (0.1 ml) in 96-well plates (pre-treated with poly-D-lysine; 20 μg/ml for CHO-lines), to produce a monolayer (1×10^5 and 1.25×10^5 cells/well for CHO transfectants and SHSY-5Y cells, respectively). The culture plates used were flat clear bottom clear plates ($[cAMP]_i$ assay) or flat clear bottom black plates ($[Ca^{2+}]_i$ assay). In both assays agonist addition and fluorescence measurements were performed by the FlexStation (Molecular Devices (MD), Wokingham, UK).

2.2. Intracellular cAMP assay

$[cAMP]_i$ was quantified by ELISA (CatchPoint Cyclic-AMP™ MD), with the assay performed as per manufacturers instructions. In brief, on the day following cell seeding, the media was aspirated, and wells rinsed with 0.25 ml of pre-warmed Krebs–Ringer bicarbonate buffer (KRBG; pH 7.4, D-glucose (9.1 mM), anhydrous magnesium chloride (0.49 mM), potassium chloride (4.56 mM), sodium chloride (0.12 M), anhydrous sodium phosphate dibasic (0.70 mM), anhydrous sodium phosphate monobasic (1.5 mM) and sodium bicarbonate (15 mM)). Following removal of the KRBG, 0.1 ml of stimulation buffer (KRBG plus 0.75 mM 3-isobutyl-1-methylxanthine) was added, and the plates incubated at 37 °C for 10 min. To evaluate agonist potencies (or antagonists alone), 50 μl of peptide solutions (3× final assay concentration (FAC) in phosphate buffered saline (PBS), containing 1 mg/ml bovine serum albumin (BSA)) were added to the 96-well plate, which was gently mixed and then incubated at 37 °C for 15 min. The reaction was terminated by addition of pre-warmed lysis buffer (50 μl) to individual wells and the plate shaken gently (10 min). Lysates (40 μl) were then transferred to a second 96-well plate pre-coated with Goat anti-Rabbit IgG; excess lysates were stored at −20 °C. cAMP standards were prepared (diluted in 3 parts assay buffer/1 part lysis buffer) and added to the plate (40 μl/well) prior to addition of a rabbit anti-cAMP antibody (40 μl), after which the plate was shaken for 5 min. HRP-cAMP conjugate (40 μl) was added, the plate mixed for a further 5 min and then left for 2 h at room temperature (RT). Individual wells were then rinsed (4× with wash buffer) before addition of Stoplight Red Substrate (0.1 ml; 15 ml of substrate buffer containing 0.15 ml Stoplight Red Substrate and 1 mM hydrogen peroxide), the plates protected from light (RT, 10 min), and then fluorescence levels were measured (excitation, 530 nm; emission, 590 nm; cut-off, 570 nm). For inhibition studies, the KRBG was replaced with 75 μl of stimulation buffer and the plate incubated at 37 °C for 5 min prior to addition of antagonist (25 μl; 6× FAC in PBS with 1 mg/ml BSA). The cells were then pre-incubated with antagonist for a further 10 min prior to agonist (50 μl) addition and the procedure followed as above.

2.3. Intracellular calcium assay

$[Ca^{2+}]_i$ responses were monitored fluorometrically (Calcium Plus™, MD), with the assay performed as per the manufacturer's instructions. The day after cell seeding, the media was aspirated, then cell buffer (0.1 ml; HBSS containing HEPES (20 mM) and sulfinpyrazone (0.25 mM)) and loading dye (0.1 ml: Calcium Plus™) added. Plates were left in the dark for 1 h at 37 °C, followed by 30 min at RT. To determine compound potencies, peptides (50 μl; 5× FAC in cell buffer containing 1 mg/ml BSA) or ATP (FAC 10 μM) were added, and fluorescence levels measured over a 90 s period, at excitation and emission wavelengths of 485 and 525 nM, respectively (cut-off; 515 nM). For inhibition studies, the volume of cell buffer was reduced (50 μl), and 50 μl of antagonist (5× FAC in cell buffer, plus 1 mg/ml BSA) was added 10 min prior to agonist addition, with fluorescence determined as above.

2.4. Data analysis

Data collection and analysis were performed using Softmax Pro (MD) and SigmaPlot 8.0 (SPSS Ltd, UK). For the $[cAMP]_i$ assay, standard curve and regression analysis were performed using a four parameter logistic equation to determine intracellular cAMP concentrations (nM), and ligand potencies. In the $[Ca^{2+}]_i$ assay the minimum fluorescence level was subtracted from the maximum response and compound potencies determined as above (EC_{50} values were not different if slope or area under the curve were used); the concentration of $[Ca^{2+}]_i$ produced following agonist stimulation cannot be determined using the Calcium Plus™ kit. The data from both assays are presented in the form of representative concentration effect curves; EC_{50} values for agonists and IC_{50} values for antagonists are expressed as mean ± SEM for a minimum of three separate experiments, with each point representing the mean value obtained from duplicate wells.

2.5. Materials

VIP, PACAP-27 and PACAP-38 were purchased from Synpep Corporation (Dublin, USA) or NeoMPS (Strasbourg, France), PG97-269 from Phoenix Pharmaceuticals (Belmont, CA, USA) and [Ala^{11,22,28}]VIP from Tocris (Bristol, UK). The peptides, R3P65 (Yung et al., 2003) and PG99-465 (Moreno et al., 2000) were custom synthesized by Albachem (Gladsmuir, UK). Maxadilan and M65 were a kind gift from Dr Ethan Lerner (Massachusetts General Hospital, Charlestown, MA, USA). The peptide sequences of VIP, PACAP-27, PACAP-38, [Ala^{11,22,28}]VIP, R3P65, maxadilan, PG97-269, PG99-465 and

Table 1
Amino acid sequences for peptide ligands

	1		5	10	15	20	25	30	35	40	45	50	55	60
VIP	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											
PACAP-27	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											
PACAP-38	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											
[Ala^{11,22,28}]VIP	H	S	D A V F T D N Y A R L R K Q M A V K K A L N S I L A											
R3P65	H	S	D A V F T D N Y T R L R K Q M A A K K Y L N S I K N K R											
Maxadilan	C	D	A T C Q F R K A I D D C Q K Q A H H S N V L Q T S V Q T T A T F T S M D T S Q L P G N S V F K E C M K Q K K K E F K A G K											
PG97-269	AcH	D-Phe	D A V F T N S Y R K V L K R L S A R K L L Q D I L											
PG99-465	MyrH	S	D A V F T D N Y T K L R K Q M A V K K Y L N S I K K G G T											
M65	C	D	A T C Q F R K A I D D C Q K Q A H H S N V L - - - - - - - - - - - - - - - - - - L P G N S V F K E C M K Q K K K E F K A G K											

AcH, acetyl-histidine; MyrH, myristoyl-histidine.

M65 are shown in Table 1. Peptides were stored (−20 °C) lyophilised, or as stock solutions prepared in molecular grade water (0.1 mM); neither form of storage altered peptide potencies over time (up to 1 year). Stock solutions of ATP were prepared as required in molecular grade water and serially diluted in calcium assay cell buffer. The CatchPoint Cyclic-AMP™ and Calcium Plus™ assay kits were obtained from Molecular Devices (Wokingham, UK), with clear and black walled 96-well plates from Nunc (Roskilde, Denmark) and Costar (New York, USA), respectively. All cell culture products, ATP and other standard chemicals were obtained from Sigma-Aldrich (Poole, UK).

3. Results

3.1. [cAMP]$_i$ assay: agonist pharmacology of hVPAC/PAC receptor stable cell lines

VIP, PACAP-27, PACAP-38 and R3P65 (a putatively selective $VPAC_2$ agonist, Yung et al., 2003) increased $[cAMP]_i$ in a concentration dependent manner in the CHO cell lines stably expressing $hVPAC_1$ (Fig. 1a), $hVPAC_2$ (Fig. 1c) or $hPAC_1$ (Fig. 2a). In all three transfected cell lines, the maximal concentration of $[cAMP]_i$ produced by agonist stimulation was equivalent, at around 60 nM, approximately 25× basal levels, and consistent with a receptor density of <1 pmol/mg (Vertongen et al., 2004). In addition, $[Ala^{11,22,28}]$VIP (reportedly $VPAC_1$ selective, Nicole et al., 2000) was a potent sub-nanomolar $hVPAC_1$ agonist (Fig. 1a), eliciting a similar maximal response. However, $[Ala^{11,22,28}]$VIP was also a full agonist at $hPAC_1$ (Fig. 2a), albeit at micromolar concentrations, whilst having a negligible effect at $hVPAC_2$ (Fig. 1c). In contrast, maxadilan (Moro and Lerner, 1997) was a selective and full $hPAC_1$ agonist, equipotent to both PACAPs (Fig. 2a), but with no effect at either hVPAC receptor (Fig. 1). Excluding maxadilan, all agonist potencies were similar at $hVPAC_1R$, a trend also observed for $hVPAC_2R$ (except $[Ala^{11,22,28}]$VIP), albeit with about a 4-fold lower potency at the latter subtype (Table 2). In contrast, at $hPAC_1$, PACAP-27 and PACAP-38 were more potent than at $hVPAC_1$ (∼4-fold) and $hVPAC_2$ (∼10-fold) receptors, whereas VIP and R3P65 were more than 100 times less potent compared to $hVPAC_1$ (Table 2). $[Ala^{11,22,28}]$VIP, which was the most potent $hVPAC_1$ agonist, was more than four orders of magnitude less potent at $hPAC_1$ with an EC_{50} of approximately 1.5 μM, whereas maxadilan had sub-nanomolar affinity at the latter receptor and even greater selectivity over both $hVPAC_1$ and $hVPAC_2$ (Table 2).

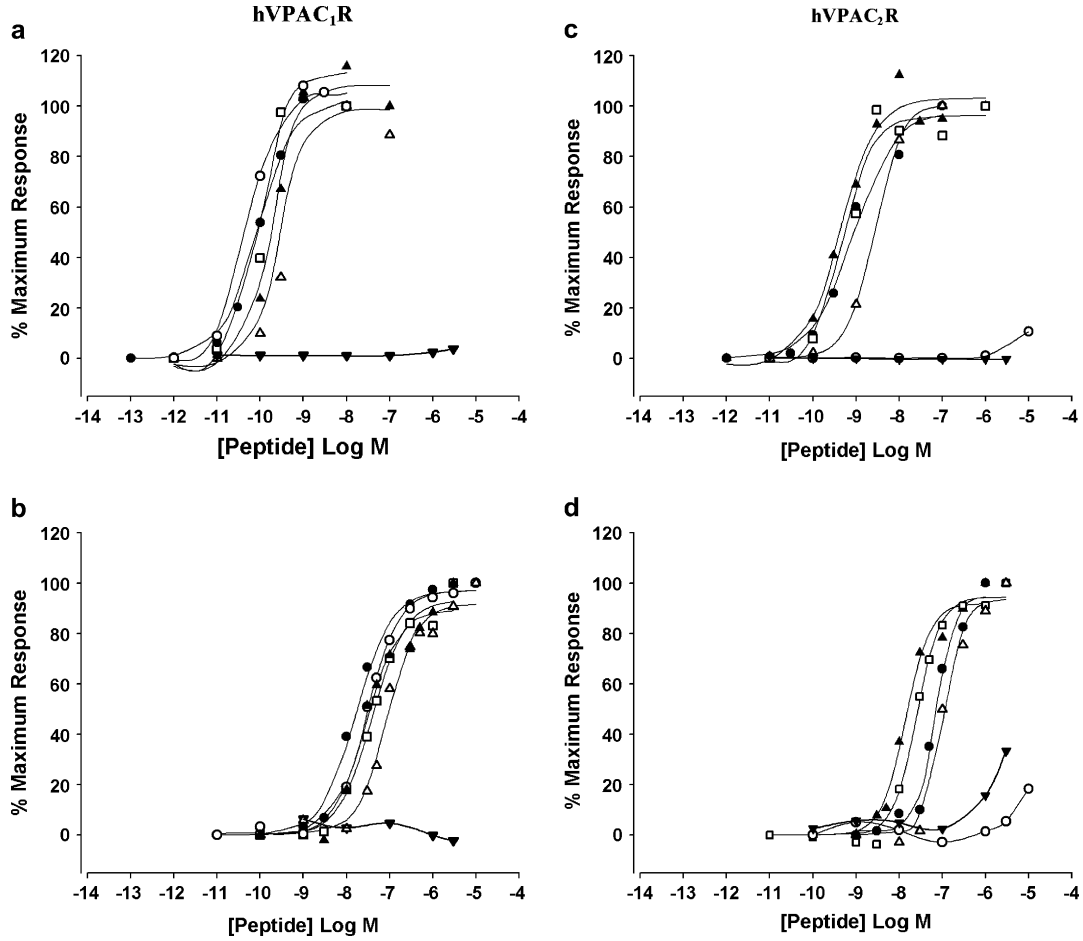

Fig. 1. VIP (●), PACAP-27 (□), PACAP-38 (▲), $[Ala^{11,22,28}]$VIP (○), R3P65 (△) and maxadilan (▼) stimulated $[cAMP]_i$ (a,c) and $[Ca^{2+}]_i$ (b,d) production in CHO cells stably expressing $hVPAC_1$ (a,b) and $hVPAC_2$ receptors (c,d). Cells were seeded and left overnight in 96-well plates (1×10^5 cells per well), with the protocol for each assay conducted as described in Section 2. Representative curves are shown, with individual points performed in duplicate; mean EC_{50} values ± SEM values are shown in Table 2.

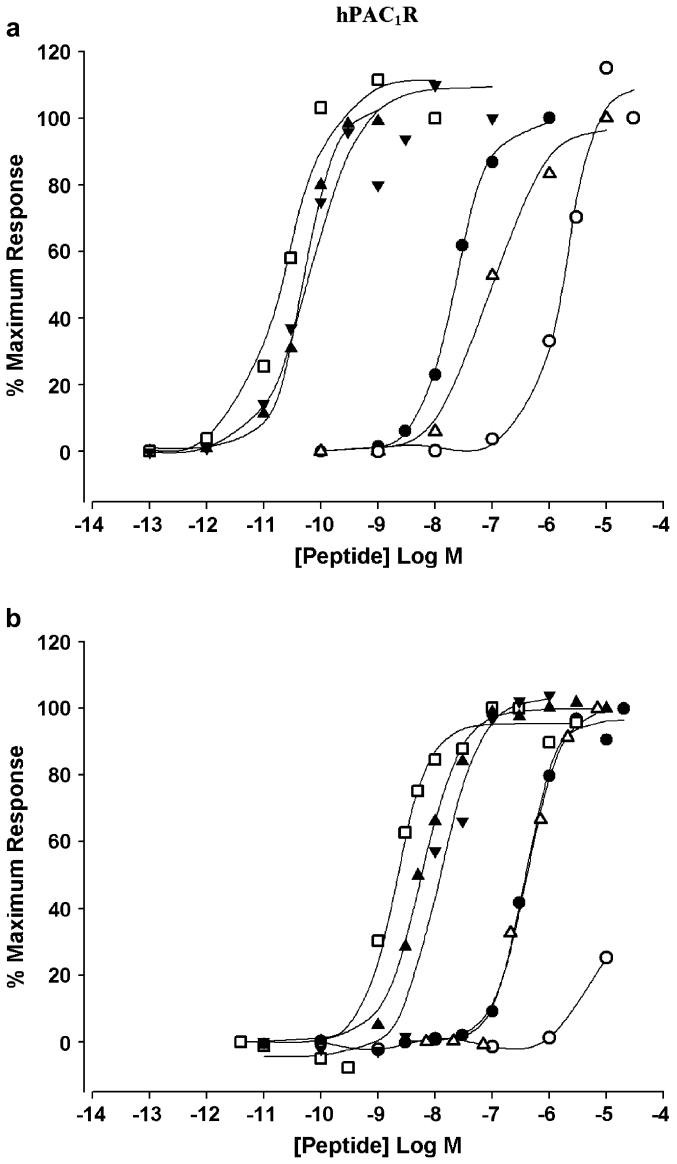

Fig. 2. Representative concentration response curves illustrating agonist induced $[cAMP]_i$ (a) and $[Ca^{2+}]_i$ (b) production in CHO-$hPAC_1R$ cells (1×10^5 cells per well). VIP (●), PACAP-27 (□), PACAP-38 (▲), [$Ala^{11,22,28}$]VIP (○), R3P65 (△) and maxadilan (▼) were tested in both assays, with average EC_{50} values ($n \geq 3$) determined for each ligand (Table 2).

3.2. $[Ca^{2+}]_i$ assay: agonist pharmacology of hVPAC/PAC receptor stable cell lines

In the intracellular calcium studies VIP, PACAP-27, PACAP-38 and R3P65 all produced a concentration dependent increase in $[Ca^{2+}]_i$ in the stable cell lines expressing $hVPAC_1$ (Fig. 1b), $hVPAC_2$ (Fig. 1d) or $hPAC_1$ (Fig. 2b). Although the exact concentration of $[Ca^{2+}]_i$ cannot be determined using this assay, maximal responses to saturating concentrations of ligand resulted in comparable levels of fluorescence (RFU), which were all approximately 7× basal levels. ATP (10 μM; $n = 3$), which stimulates endogenous purinergic receptors in CHO cells (Langer et al., 2001), produced a larger response than that elicited by a saturating concentration of peptide ligands at $hVPAC_1$ (72 ± 3.2%), $hVPAC_2$ (63 ± 1.2%) and $hPAC_1$ (77 ± 1.8%, data not shown), again indicating a receptor density of 1–2 pmol/mg (Langer et al., 2001, 2002b; Langer and Robberecht, 2005). In the $[Ca^{2+}]_i$ assay, four of the peptides (VIP, PACAP-27, PACAP-38 and R3P65) exhibited similar potencies in CHO cells expressing either $hVPAC_1$ or $hVPAC_2$ receptors, although these values were approximately two orders of magnitude less than that observed for $[cAMP]_i$ (Table 2). At $hPAC_1$, PACAP-27, PACAP-38 and maxadilan were again the most potent agonists despite the 100-fold reduction in potency (Table 2). As in the $[cAMP]_i$ studies, [$Ala^{11,22,28}$]VIP was a potent $hVPAC_1$ agonist in the $[Ca^{2+}]_i$ assay (Fig. 1b), however it had no effect at $hVPAC_2$ (Fig. 1d) or $hPAC_1$ (Fig. 2b) receptors. The lack of effect of [$Ala^{11,22,28}$]VIP at $hPAC_1$ ($EC_{50} = 1.5$ μM in the $[cAMP]_i$ studies; Table 2), was probably a consequence of the 100-fold reduction in potency observed for the other agonists. It was not possible to confirm this assertion as all the peptides induced a non-specific response in the $[Ca^{2+}]_i$ and $[cAMP]_i$ assays, in both the stable and untransfected CHO cell lines, at concentrations above 10 μM (data not shown). Fig. 3 shows a correlation of the agonist potencies from the $[cAMP]_i$ and $[Ca^{2+}]_i$ assays for the three human VPAC/PAC stable cell lines, with regression analysis ($r^2 = 0.86$) confirming that the agonists are in general about 100-fold less potent in the calcium assay.

3.3. Antagonist pharmacology of hVPAC/PAC receptor stable cell lines

PG97-269 (Gourlet et al., 1997) and M65 (Uchida et al., 1998) are reported to be $VPAC_1$ and PAC_1 selective antagonists, respectively. Alone, at concentrations up to 3 μM, neither peptide had any significant effect on $[cAMP]_i$ and $[Ca^{2+}]_i$ in the three stable cell lines (Fig. 4, Table 2). However, PG97-269 produced a concentration dependent inhibition of the VIP-induced $[cAMP]_i$ and $[Ca^{2+}]_i$ (Fig. 4a,b) responses in cells expressing $hVPAC_1$, being slightly less potent in the former assay (Table 2). At concentrations up to 3 μM, PG97-269 had no effect on agonist-induced responses at either $hVPAC_2$ or $hPAC_1$ (Table 2). M65 exhibited comparable selectivity, inhibiting PACAP-27 induced $[cAMP]_i$ and $[Ca^{2+}]_i$ (Fig. 4c,d) responses at $hPAC_1$ with similar potency, whilst having no effect at hVPAC receptors (Table 2).

In contrast to PG97-269 and M65, the situation with PG99-465 (Moreno et al., 2000), the supposedly $hVPAC_2$ receptor selective antagonist, was considerably more complex. In the $[cAMP]_i$ assay, PG99-465 was a full agonist at $hVPAC_1$ and $hPAC_1$ receptors, with nanomolar potency (Fig. 5). Moreover, this putative $VPAC_2$ antagonist also acted as a partial agonist (10–20% of maximum response) at the $hVPAC_2$ receptor with an EC_{50} of 4.9 nM (Fig. 6a) for $[cAMP]_i$ production. Interestingly, at concentrations up to 3 μM, PG99-465 had no effect in the $[Ca^{2+}]_i$ assay in any of the three human VPAC/PAC cell lines (Fig. 6b; Table 2). Inhibition studies were therefore complicated with PG99-465 appearing to act synergistically with the EC_{50} concentration of VIP at both $hVPAC_1$ and

Table 2
Comparison of EC_{50}/*IC_{50} values for peptide induced $[cAMP]_i$ and $[Ca^{2+}]_i$ stimulation/inhibition in CHO cells stably expressing $hVPAC_1$, $hVPAC_2$ and $hPAC_1$ receptors

	$hVPAC_1$ EC_{50}/*IC_{50} (nM)		$hVPAC_2$ EC_{50}/*IC_{50} (nM)		$hPAC_1$ EC_{50}/*IC_{50} (nM)	
	$[cAMP]_i$	$[Ca^{2+}]_i$	$[cAMP]_i$	$[Ca^{2+}]_i$	$[cAMP]_i$	$[Ca^{2+}]_i$
VIP	0.11 ± 0.03 (7)	14.1 ± 0.9 (6)	0.63 ± 0.19 (6)	50.9 ± 8.7 (9)	15.1 ± 2.76 (4)	389 ± 38 (5)
PACAP-27	0.13 ± 0.02 (3)	22.6 ± 5.3 (8)	0.38 ± 0.09 (3)	23.9 ± 2.4 (6)	0.026 ± 0.003 (7)	3.3 ± 0.5 (6)
PACAP-38	0.22 ± 0.04 (4)	37.4 ± 7.9 (10)	0.53 ± 0.14 (4)	19.4 ± 2.3 (8)	0.049 ± 0.01 (3)	5.1 ± 0.6 (7)
$[Ala^{11,22,28}]$VIP	0.057 ± 0.015 (4)	25.6 ± 2.1 (4)	>10 μM	>10 μM	1582 ± 225 (3)	>10 μM
R3P65	0.27 ± 0.006 (4)	86.1 ± 1.3 (4)	1.74 ± 0.40 (4)	102 ± 17 (7)	63.6 ± 19.5 (3)	568 ± 89 (6)
Maxadilan	NE	NE	NE	NE	0.054 ± 0.009 (3)	10.7 ± 2.3 (4)
PG97-269 with VIP	*80.0 ± 3.2 (3)	*9.9 ± 2.1 (7)	NE	NE	NE	NE
PG99-465	8.04 ± 1.88 (5) (full agonist)	NE	4.9 ± 0.38 (3) (partial agonist)	NE	71.3 ± 9.6 (6) (full agonist)	NE
PG99-465 with VIP	Additive effect	NE	*22.9 ± 5.73 (4)	*4.4 ± 0.8 (6)	Additive effect	NE
M65 with PACAP-27	NE#	NE#	NE#	NE#	*149.6 ± 13.2 (3)	*277.4 ± 71 (4)

EC_{50} and *IC_{50} values are shown as the mean ± SEM, with the number of independent experiments given in parentheses. NE, is no effect up to 3 μM and NE# is no effect against the appropriate VIP response.

$hPAC_1$ receptors in the $[cAMP]_i$ assay, whilst having no clear effect at either receptor in the $[Ca^{2+}]_i$ assay (data not shown). At the $hVPAC_2$ receptor, PG99-465 produced a concentration dependent inhibition of the VIP-induced $[cAMP]_i$ response, plateauing at 80% inhibition (Fig. 6a); this is probably a consequence of the partial agonist properties exhibited by the compound. PG99-465 did however produce complete inhibition of the VIP-induced $[Ca^{2+}]_i$ response, demonstrating a marginally higher potency, compared with its effects on $[cAMP]_i$ (Fig. 6b; Table 2).

3.4. $hPAC_1$ receptor pharmacology in SHSY-5Y human neuroblastoma cells

To confirm that the shift in potencies seen for the VPAC/PAC agonists in the two second messenger assays (Table 2) was not a consequence of receptor overexpression, we examined $[cAMP]_i$ and $[Ca^{2+}]_i$ responses in SHSY-5Y cells, a human neuroblastoma cell line that endogenously expresses PAC_1 receptors (Vertongen et al., 1996). VIP, PACAP-27 and maxadilan produced a concentration dependent increase in $[cAMP]_i$ (Fig. 7a) to the same maximum, although the total amount produced (~3 nM; 3× basal) was about 20 times lower than that observed for the stable cell lines. In the $[Ca^{2+}]_i$ assay, maxadilan and VIP, evoked changes in fluorescence of only 2-fold above the basal level, with the overall fluorescent signal being five times less than that of the stable cell lines (Fig. 7b). Interestingly, in the same experiments the PACAP-27 induced $[Ca^{2+}]_i$ response was approximately double that of the two other ligands (Fig. 7b). In general, the agonist potencies in SHSY-5Y cells for both assays (Table 3) were on average 3.7-fold lower than that observed for the $hPAC_1$ cell line (Table 2). However, and most importantly, agonist potencies were significantly lower in the $[Ca^{2+}]_i$ assay, despite low endogenous levels of $hPAC_1$ receptor expression (Fig. 7; Table 3).

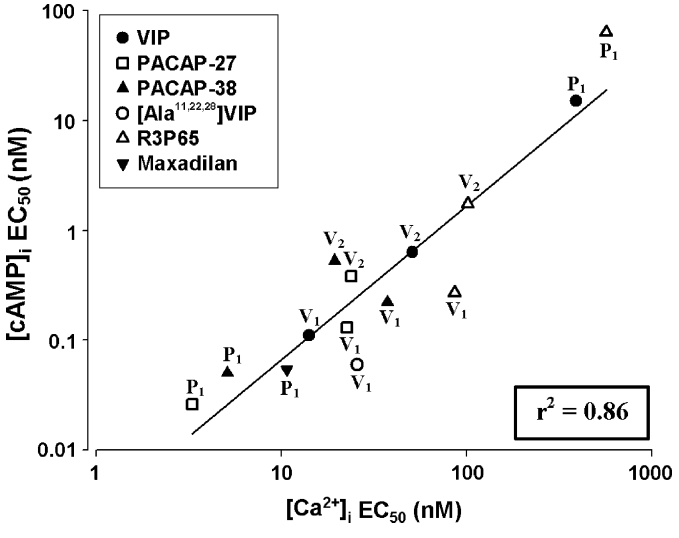

Fig. 3. Correlation of agonist EC_{50} values for stimulation of $[cAMP]_i$ and $[Ca^{2+}]_i$ responses in CHO cells expressing $hVPAC_1$ (V_1), $hVPAC_2$ (V_2), and $hPAC_1$ (P_1) receptors. EC_{50} values for the agonists VIP (●), PACAP-27 (□), PACAP-38 (▲), $[Ala^{11,22,28}]$VIP (○), R3P65 (△) and maxadilan (▼) are taken from Table 2, generating a correlation coefficient (r^2) of 0.86 using a linear regression analysis.

4. Discussion

To date, studies directly comparing the pharmacology of human VPAC/PAC receptor mediated modulation of $[cAMP]_i$ and $[Ca^{2+}]_i$ are limited, with comparison between data complicated by the variation in protocols used. To address these limitations, we generated stable cell lines expressing the three human VPAC receptor subtypes ($hVPAC_1R$, $hVPAC_2R$ and $hPAC_1R$) and established and characterised two simple, cell based, non-radioactive, HT-amenable, $[cAMP]_i$ and $[Ca^{2+}]_i$ assays, whilst assessing some new and reportedly receptor selective agonists and antagonists. For the endogenous peptides VIP, PACAP-27 and PACAP-38, the agonist ROP was identical in both assays for the three human receptor subtypes, although EC_{50} values in the $[Ca^{2+}]_i$ assay were around 100-fold less (range: 26–174-fold). In the $[cAMP]_i$ studies, the agonists $[Ala^{11,22,28}]$VIP and maxadilan showed considerable selectivity for $hVPAC_1R$ and $hPAC_1R$ (>25,000-fold), respectively,

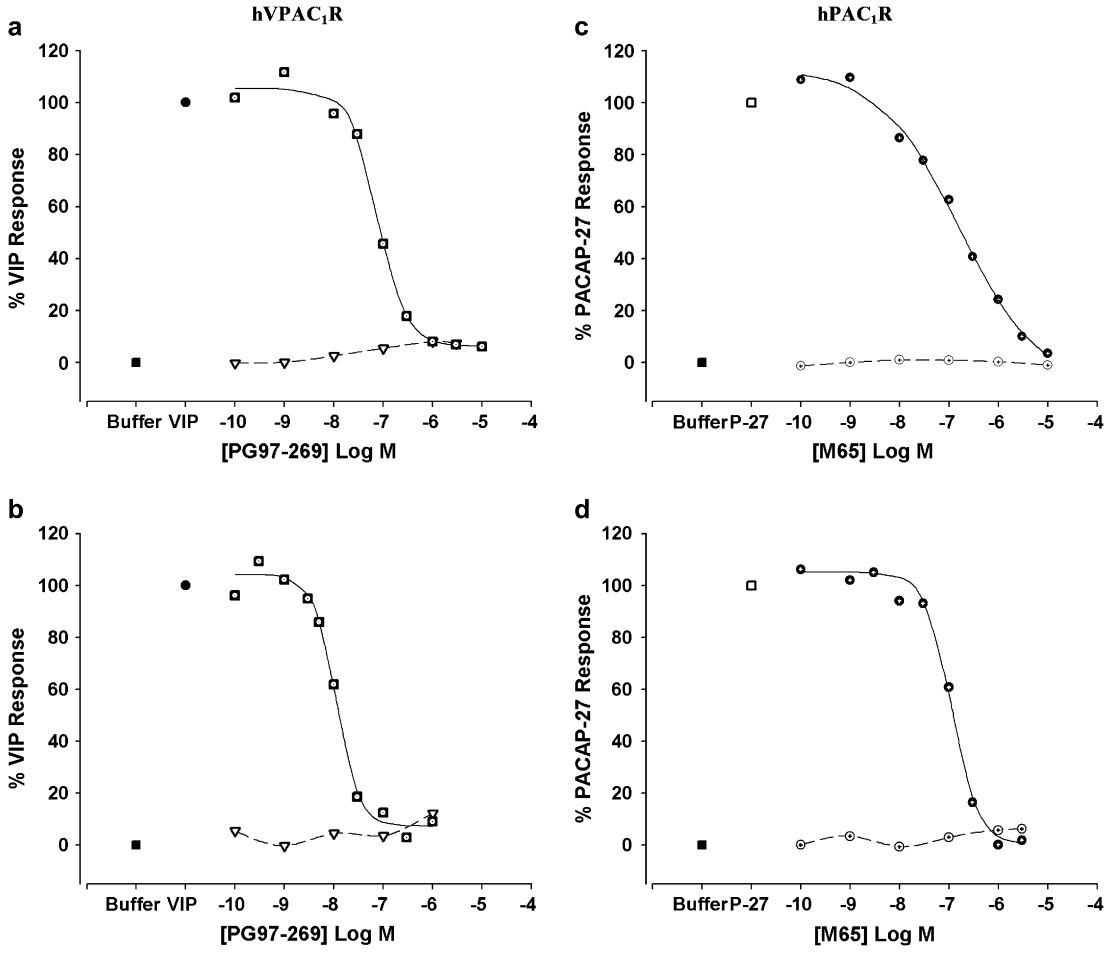

Fig. 4. Inhibition of agonist evoked $[cAMP]_i$ (a,c) and $[Ca^{2+}]_i$ (b,d) release by PG97-269 in CHO-hVPAC$_1$R cells (■; a,b) and by M65 in CHO-hPAC$_1$R cells (◉; c,d). Representative inhibition curves are shown for both assays, in which cells (1×10^5/well, seeded overnight) were pre-incubated (10 min) with PG97-269 or M65, prior to the addition of either VIP (hVPAC$_1$R) or PACAP-27 (hPAC$_1$R). Agonists were used at the following concentrations: VIP, 0.3 nM ($[cAMP]_i$) or 30 nM ($[Ca^{2+}]_i$); PACAP-27, 0.1 nM ($[cAMP]_i$) and 30 nM ($[Ca^{2+}]_i$). The dashed lines represent the effects of PG97-269 (▽) and M65 alone (◉).

and whilst R3P65 was not hVPAC$_2$R selective in our hands, all three peptides were comparably less potent in the $[Ca^{2+}]_i$ assay. With the reduction in agonist potency in the $[Ca^{2+}]_i$ assay also observed in SHSY-5Y cells, which endogenously express hPAC$_1$ receptors, this effect is unlikely to be a consequence of receptor overexpression. In contrast to the agonists, the reportedly selective VPAC$_1$R (PG97-269) and VPAC$_2$R (PG99-465) antagonists had slightly lower IC_{50} values and hence greater potency in the $[Ca^{2+}]_i$ assay at their respective receptor subtypes. In contrast, the hPAC$_1$ selective antagonist M65 was almost equipotent in both assays. With PG99-465 alone acting as a partial (hVPAC$_2$R) and full (hVPAC$_1$R and hPAC$_1$R) agonist in the $[cAMP]_i$ assay, this compound is clearly not a selective antagonist. These studies re-iterate that there remains a paucity of VPAC/PAC receptor selective drugs, in particular non-peptides, and that functional responses both in vitro and in vivo will critically depend upon ligand concentration(s) and the cellular complement of intracellular messengers.

VIP and PACAP, through stimulation of their cognate receptors produce a multitude of biological effects which are ultimately dependent upon the density and complement of both the receptors and downstream signalling molecules within a given cell type. Although VPAC/PAC receptor activation routinely results in a $G\alpha_s$-dependent increase in $[cAMP]_i$, evidence outlining a $G\alpha_i$ and/or $G\alpha_q$-dependent enhancement of $[Ca^{2+}]_i$ is increasing (Langer et al., 2001), perhaps underlying the pleiotropic nature of the effects produced by these peptides. Unlike VPAC$_1$ and VPAC$_2$, the PAC$_1$ receptor was, until now, thought to have at least four splice variants for both rat (Spengler et al., 1993) and human (Pisegna and Wank, 1996) subtypes, which can differentially activate adenylate cyclase and phospholipase C/$[Ca^{2+}]_i$. However, Lutz et al. (2005) have recently cloned up to 14 splice variants of the human PAC$_1$ receptor. In the current study, the EC_{50} values of PACAP-27, PACAP-38 and maxadilan at the hPAC$_{1-null}$ (Pisegna and Wank, 1996; equivalent to rat PACAP-R, Spengler et al., 1993) receptor were similar within assays, however a 1–200-fold loss in potency was observed when comparing $[Ca^{2+}]_i$ with $[cAMP]_i$. Although limited, previous studies have shown that the three peptides have a similar affinity/potency in radioligand binding (Moro and Lerner, 1997) and $[cAMP]_i$ assays (Eggenberger et al., 1999), with the

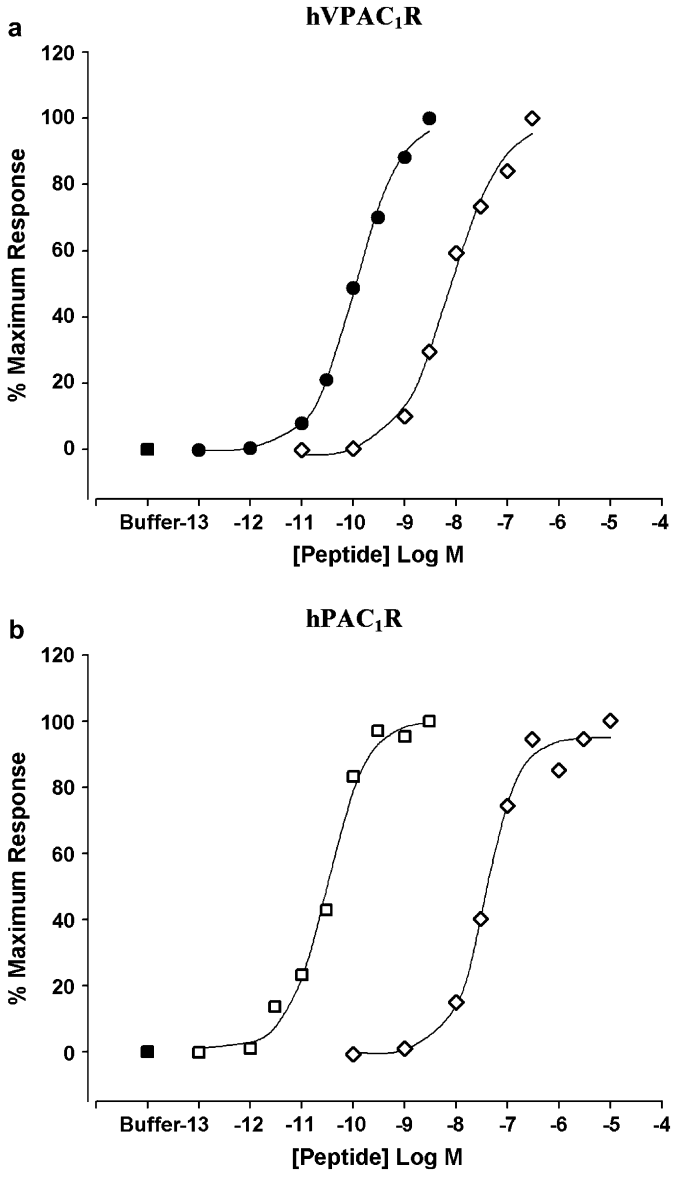

Fig. 5. Concentration response curves showing VIP (●), PG99-465 (◇) or PACAP-27 (□), induced stimulation of $[cAMP]_i$ in $hVPAC_1$ (a) and $hPAC_1$ (b) receptor expressing cell lines. Both cell lines (1×10^5 cells/well) were exposed to the three peptides for 15 min prior to stopping the reaction by the addition of lysis buffer. $[cAMP]_i$ levels were determined in duplicate for each peptide concentration and representative curves shown; mean EC_{50} values ($n \geq 3$) for each ligand are shown in Table 2.

former study also demonstrating considerable selectivity for maxadilan at PAC_1 receptors, as shown in Table 2. However, this is the first study to fully characterise the maxadilan evoked $[Ca^{2+}]_i$ response at $hPAC_1$, with the compound clearly acting as a full agonist. Consistent with these observations, Pisegna and Wank (1996) had reported PACAP-27 and PACAP-38 to be equipotent at the human PAC_{1-null} variant expressed in NIH/3T3 cells using a [^{3}H]cAMP and [^{3}H]IP assay, with both peptides being 60-fold less potent in the latter assay. Moreover, in the human neuroblastoma cell line, NB-OK-1, that endogenously expresses a PAC_1 receptor variant, Delporte et al. (1993) showed that PACAP-27 and PACAP-38 were considerably less potent in stimulating $[Ca^{2+}]_i$ release when compared to $[cAMP]_i$. Importantly, we confirmed and extended this early observation using an alternative human neuroblastoma cell line, SHSY-5Y (Table 3), which also endogenously expresses PAC_1 receptors (Vertongen et al., 1996). Both $[Ca^{2+}]_i$ and $[cAMP]_i$ were elevated in response to agonists, although PACAP-27 clearly evoked a considerably larger response than VIP and maxadilan in the former assay (Fig. 7b), extending a preliminary observation made by Eggenberger et al. (1999). The recent findings of Lutz et al. (2005) support these data, with PACAP-38 and VIP producing a similar fold increase in a [^{3}H]cAMP assay, however no [^{3}H]IP data was presented for SHSY-5Y cells, despite comparing both second messengers for some of the new, individual $hPAC_1$ splice variants. More significantly, in our SHSY-5Y studies, there was a clear reduction in agonist potency in the $[Ca^{2+}]_i$ assay, despite $[cAMP]_i$ levels in these cells being 20-fold lower than in the stable cell lines. These data support the assertion that the reduction in agonist potency is not a consequence of receptor over-expression (MacKenzie et al., 2001), but could reflect inefficient coupling (Langer et al., 2002b). These observations are not unique to human PAC receptors; in CHO cells expressing rat type I PACAP-R (Delporte et al., 1995) the potency of PACAP-27 (cAMP, 0.01 nM; $[Ca^{2+}]_i$, 1.1 nM) and the fold difference (110) was almost identical to our human data. Although Spengler et al. (1993) reported a smaller 40-fold reduction in potency for PACAP-38 at the same rat receptor (PACAP-R), these authors showed that PACAP-27 did not stimulate inositol phosphate (IP) production, despite a robust production of cAMP, suggesting species differences in PAC_1 receptor pharmacology and supporting a similar observation in PC12 cells (Deutsch and Sun, 1992). In our hands, VIP was approximately 300× less potent than PACAP-38 in the $[cAMP]_i$ assay, which is consistent with published data (Spengler et al., 1993), although Pisegna and Wank (1996) suggest that VIP at concentrations 1000× in excess of the PACAP EC_{50} had no effect on adenylate cyclase. In the present $hPAC_1$ $[Ca^{2+}]_i$ studies, VIP was only 25× less potent than in the $[cAMP]_i$ assay, resulting in this peptide only being about 75× less potent than the PACAPs (rather than 300×), and possibly why we could determine an EC_{50} value of about 400 nM for VIP. This is however consistent with the new data from Lutz et al. (2005), with EC_{50} values for VIP of 55 nM ([^{3}H]cAMP) and approximately 1.5 μM ([^{3}H]IP) giving a ratio of 27. The pharmacological properties of R3P65 (Yung et al., 2003) at $hPAC_1R$ were akin to VIP, with only a 10-fold difference in potency in the two assays (Table 2). This peptide's sequence differs from VIP by only four amino acids (two within the VIP sequence; Table 1), and it is not inconceivable that both peptides induce $[Ca^{2+}]_i$ production more readily than $[cAMP]_i$ at human PAC_1R (compared to the PACAPs), akin to that described for PACAP-38 at rPAC-R (Spengler et al., 1993).

In contrast to the data showing that PAC_1 receptor activation is associated with downstream production of both $[cAMP]_i$ and $[Ca^{2+}]_i$, studies fully characterising VPAC-mediated stimulation of the latter second messenger have been more limited and variable (DeHaven and Cuevas, 2004).

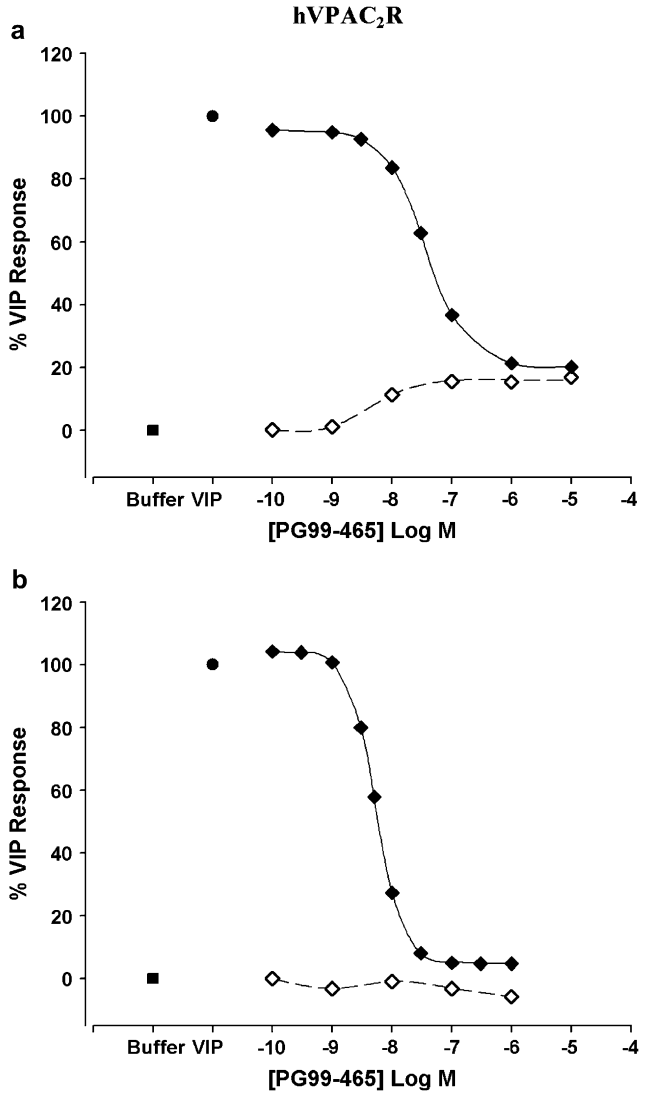

Fig. 6. PG99-465 inhibition of VIP-induced $[cAMP]_i$ (a) and $[Ca^{2+}]_i$ (b) responses in CHO-hVPAC$_2$R cells (1×10^5/well). VIP (●) was used at a concentration of 3 and 100 nM, in the $[cAMP]_i$ and $[Ca^{2+}]_i$ assays respectively. In both panels, the effect of PG99-465 alone is represented by the dashed line (◇), with the inhibition curves shown as a solid line (◆) following a 10 min pre-incubation with antagonist. Representative curves are shown with the mean IC_{50} values for PG99-465 ($n \geq 3$) shown in Table 2.

The three endogenous peptide ligands, VIP, PACAP-27 and PACAP-38 all exhibited similar, sub-nanomolar potencies in the $[cAMP]_i$ assay in the cell lines stably expressing either hVPAC$_1$ or hVPAC$_2$ (Table 2). The maximal amount of cAMP produced (~60 nM) and the fold stimulation above basal levels (~25-fold) was the same for all three ligands at both hVPAC receptor subtypes (and hPAC$_1$). A recent study by Vertongen et al. (2004), correlating hVPAC receptor density with cAMP production would suggest that the stable cell lines used in the current study have a receptor density of ~1 pmol/mg. However, radioligand binding studies would be required to confirm this assertion. Sreedharan et al. (1994), reported similar EC_{50} values for VIP at hVPAC$_1$ expressed stably in either HEK-293 or CHO cells, although the same group reported a considerably lower potency (70-fold) for VIP at hVPAC$_2$ in both SUP-T1 and transfected cells (Xia et al., 1996, 1997). In studying cAMP responses at hVPAC$_1$R, Langer et al. (2002a) demonstrated that the potencies of VIP (30×) and PACAP-27 (10×), were higher in intact cells when compared to membranes, with the former values similar to those described in Table 2. Similarly, the EC_{50} values of VIP and PACAP-38 for recombinantly expressed rat VPAC/PAC receptors in a whole cell cAMP assay (MacKenzie et al., 2001; McCulloch et al., 2001), were consistent with the present data using human receptors (Table 2). Therefore it is clear that for the endogenous peptide ligands like VIP and the PACAPs, the use of membrane preparations may result in an underestimate of their potencies, and that the variation in use of whole cell and/or membrane preparations could contribute to the inconsistencies seen in the literature.

In the $[Ca^{2+}]_i$ assay, VIP, PACAP-27 and PACAP-38 were again almost equipotent at hVPAC$_1$ and hVPAC$_2$ receptors, although the agonist EC_{50} values were approximately 100× greater than those in the $[cAMP]_i$ studies (Table 2). The maximal response induced by agonists was about 7× that of basal fluorescence readings for all three VPAC/PAC stable cell lines, being equivalent to ~70% of the endogenous response elicited by ATP (10 μM). This larger response elicited by ATP would again be consistent with the cell lines used in the current study having a receptor density of 1–2 pmol/mg (Langer et al., 2001, 2002b; Langer and Robberecht, 2005). Although EC_{50} values were not determined, Sreedharan et al. (1994) had shown in an earlier study, that in cells stably or endogenously (HT-29) expressing hVPAC$_1$R, VIP could elicit an increase in $[Ca^{2+}]_i$, albeit at higher concentrations than that required for $[cAMP]_i$. Using a luminescence based calcium assay, Langer et al. (2001, 2002a) have reported similar EC_{50} values for VIP and PACAP-27 in CHO cells expressing hVPAC$_1$R. In further studies, the same authors (Langer et al., 2002b; Langer and Robberecht, 2005) have since shown that the enhanced VPAC$_1$ coupling is due to a small number of residues in the third intracellular loop, whilst also suggesting that their absence underlies the inefficient VIP induced $[Ca^{2+}]_i$ response at VPAC$_2$ receptors (Langer et al., 2001, 2002a). In human T-lymphoblast cells (SUP-T1) that endogenously express hVPAC$_2$ receptors (Xia et al., 1996), the concentration of VIP ($EC_{50} = 30$ nM) required to induce $[Ca^{2+}]_i$ production was very similar to the current study (Table 2). Xia et al. (1997) also reported, but did not fully characterise a $[Ca^{2+}]_i$ response in CHO-cells expressing hVPAC$_2$, although the present data are consistent with that of Langer et al. (2002b), who reported a comparable EC_{50} value for VIP at hVPAC$_2$. In COS-7 cells expressing rat VPAC$_2$, the EC_{50} of PACAP-38 was also similar (37 nM) in an $[^3H]$IP assay (MacKenzie et al., 2001), however it must be noted that when the same authors expressed either rat VPAC$_1$ or VPAC$_2$ in CHO cells, no stimulation of IP was observed, despite similar data for rat PAC$_1$ (McCulloch et al., 2000, 2001). The rightward shift in potency that we observed for VIP, PACAP-27 and PACAP-38 between the cAMP and calcium assays for all three human VPAC/PAC receptors (Fig. 3), is therefore consistent with other studies on cloned human VPAC/PAC receptors, when

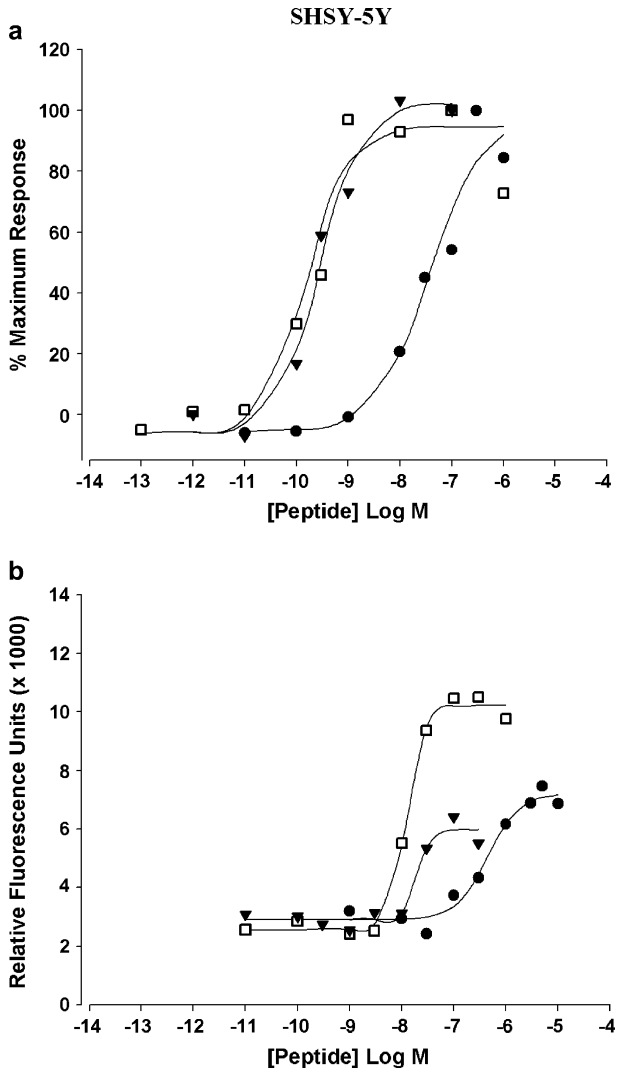

Fig. 7. Stimulation of $[cAMP]_i$ (a) and $[Ca^{2+}]_i$ (b) responses in SHSY-5Y cells with PACAP-27 (□), maxadilan (▼) and VIP (●). In both assays, cells were seeded overnight (1.25×10^5 cells/well), with peptide addition and fluorescent readings performed using the FlexStation (MD), as detailed in Section 2. Raw data is displayed for the $[Ca^{2+}]_i$ assay (RFU (×1000)), with representative curves shown for all three agonists. Mean EC_{50} values ($n \geq 3$) are shown in Table 3.

Table 3
EC_{50} values for peptide induced $[cAMP]_i$ and $[Ca^{2+}]_i$ stimulation in SHSY-5Y cells

	SHSY-5Y EC_{50} (nM)	
	$[cAMP]_i$	$[Ca^{2+}]_i$
VIP	26.2 ± 6.64 (5)	471 ± 43 (5)
PACAP-27	0.13 ± 0.015 (3)	13.6 ± 1.9 (6)
Maxadilan	0.37 ± 0.052 (3)	13.9 ± 4.3 (6)

EC_{50} values are shown are the mean ± SEM, with the number of independent experiments shown in parentheses.

cell-based assays are compared (Langer et al., 2002a), with the ratios lower when $[cAMP]_i$ responses are determined in a membrane based assay (Langer et al., 2001, 2002b; Langer and Robberecht, 2005). A similar ratio has also been shown for rat $VPAC_2$ (MacKenzie et al., 2001) and PAC_1 (McCulloch et al., 2001) receptors. In contrast, Xia et al. (1996) only reported a 2-fold difference in potency for VIP between cAMP and calcium assays, using SUP-T1 cells, perhaps as a consequence of the low potency of VIP in their cAMP assay, as mentioned above. Although the $[Ca^{2+}]_i/[cAMP]_i$, and phospholipase D/$[cAMP]_i$, ratios were also very similar for rat PAC_1 (McCulloch et al., 2000, 2001), no $[Ca^{2+}]_i$ response was found for $VPAC_1$ and $VPAC_2$, despite a similar phospholipase D/$[cAMP]_i$ ratio for these two subtypes. It remains unclear whether these variations are due to expression level (Delporte et al., 1993; Ciccarelli et al., 1994; Van Rampelbergh et al., 1997; Langer et al., 2002b), as MacKenzie et al. (2001) clearly showed there was no difference in the $[Ca^{2+}]_i/[cAMP]_i$ potency ratio for PACAP-38 on comparing transfected and endogenously expressed $VPAC_2$ receptors. In contrast, Langer et al. (2002b) do show that the magnitude of the cAMP and calcium responses may be dependent upon the level of receptor expression, however they do not discuss what impact this has upon agonist EC_{50} values. These authors suggest (Langer et al., 2002b) that the lower potencies observed in calcium assays, relative to cAMP, are a consequence of the former EC_{50} values being representative of the association rate constant of the ligand rather than the equilibrium constant. Whether it is possible to verify this assertion given the complexities of GPCR signalling remains to be determined (Colquhoun, 1998, 2006), however, it is important to stress that the VPAC/PAC induced increase in calcium is of physiological importance (Laburthe and Couvineau, 2002; Delgado et al., 2003; DeHaven and Cuevas, 2004).

Using a HT-mutagenesis approach, Yung et al. (2003) generated a comprehensive range of VIP-related peptides that resulted in a series of putatively receptor selective $VPAC_2$ agonists, with R3P65 (Table 1) reported to exhibit at least 80- (radioligand binding) and 500-fold (cAMP) selectivity over human $VPAC_1$ and PAC_1. Such ligands would be advantageous, as the existing $VPAC_2$ peptide agonists, e.g. Ro25-1392 and Ro25-1553 (Moreno et al., 2000), are difficult to synthesise (Yung et al., 2003) and can act as partial agonists at $VPAC_1$ and PAC_1 (Akesson et al., 2005). Although the EC_{50} values of VIP, PACAP-27 and PACAP-38 for $[cAMP]_i$ production, at the three human VPAC/PAC (Table 2) receptors were very similar to those reported by Yung et al. (2003), we could provide no evidence to support their assertion that R3P65 is a selective $hVPAC_2$ antagonist. Although our EC_{50} value for R3P65 at $hVPAC_2$ receptors in the $[cAMP]_i$ assay (Table 2) was consistent (Yung et al., 2003), we found this peptide to be slightly more potent at $hVPAC_1$, in addition to acting as a full $hPAC_1$ agonist, albeit with lower potency. In our $[Ca^{2+}]_i$ studies, the pharmacological profile of R3P65 was as observed for $[cAMP]_i$, with the compound again having a lower potency in the former assay, as described for the other peptides (Table 2). These data contrast with the published 80–500-fold selectivity for R3P65 over $hVPAC_1$ receptors and with its negligible effect at $hPAC_1$ (Yung et al., 2003). As both studies used a CHO-based cell line to express human VPAC/PAC receptors, the differences between the two studies are intriguing, although these authors did introduce the promiscuous G-protein $G\alpha_{16}$ into

their cell lines (Yung et al., 2003), which can alter receptor pharmacology (Langer et al., 2001). In both our assays, for all three human VPAC/PAC receptor subtypes, R3P65 was on average 3× less potent than VIP. Compared to the $VPAC_2R$ agonist Ro25-1553, the amino acid sequence of R3P65 is remarkably similar to VIP (Table 1), and it is interesting to note that of the four key residues identified by Yung et al. (2003), neither V19A (R3P47) or L27K (R3P42) alone, which lie within the VIP sequence, alter selectivity in the binding or functional assays; even the double (R3P49) mutant is only functionally selective (30-fold). Although the further addition of K29 and R30 resulted in R3P65, the authors comment that any reduction in the number of mutations did not produce peptides with comparable selectivity, with no data shown even for the triple mutant without the R30 addition (Yung et al., 2003). Whether issues surrounding the stability and expression of VIP, PACAP and related peptides, and the exquisite sequence dependence of VPAC receptor selectivity contribute to the differences observed, remains to be determined.

Using a combined approach of alanine scanning and molecular modelling Nicole et al. (2000), identified [$Ala^{11,22,28}$]VIP as the first, highly selective, human $VPAC_1$ receptor agonist. In agreement with these observations, our $[cAMP]_i$ data also confirm [$Ala^{11,22,28}$]VIP to be as potent as VIP at the $hVPAC_1$ receptor, whilst having no demonstrable agonist effect at $hVPAC_2$, at concentrations up to 3 μM. [$Ala^{11,22,28}$]VIP also induced a concentration dependent increase in $[Ca^{2+}]_i$ in the $hVPAC_1$ cell line, with an EC_{50} value which was in excess of two orders of magnitude greater than that observed for $[cAMP]_i$. To our knowledge this is the first report to shown that [$Ala^{11,22,28}$]VIP stimulates the production of $[cAMP]_i$ in cells expressing $hPAC_1$, albeit at micromolar concentrations, approximately 30,000× that required to activate $hVPAC_1$. The general reduction in agonist potencies in the $[Ca^{2+}]_i$ assay, meant we were unable to attain a sufficient concentration of [$Ala^{11,22,28}$]VIP to evaluate whether this peptide stimulates $[Ca^{2+}]_i$ production at $hPAC_1$.

Although a range of approaches have resulted in some moderately selective VPAC/PAC agonists, to date there is still a paucity of suitable receptor antagonists (Laburthe and Couvineau, 2002), with some recent evidence even highlighting the possibility of inverse agonism (Vertongen et al., 2004). One notable exception is PG97-269, which was reported by Gourlet et al. (1997) to be a $VPAC_1R$ selective antagonist. Alone, PG-97-269 had no effect on $[Ca^{2+}]_i$ in any of the three human VPAC/PACR cell lines (Table 2; Fig. 4b), however, there was a small (up to 5% of the maximal VIP response), but non-quantifiable, increase in $[cAMP]_i$ in the $hVPAC_1R$ cell line. Although PG97-269 produced a concentration dependent inhibition of both the $[cAMP]_i$ and $[Ca^{2+}]_i$ responses in $hVPAC_1R$ expressing cells, its potency was slightly reduced in the $[cAMP]_i$ assay, perhaps as a consequence of the small agonist response. However, the IC_{50} value in the $[Ca^{2+}]_i$ assay is consistent with that observed by Gourlet et al. (1997). Robberecht and et al. also developed PG99-465, which was reported to be the first potent and selective $VPAC_2R$ antagonist (Moreno et al., 2000). In the present investigation, PG99-465 was indeed a potent $hVPAC_2$ receptor antagonist in both the $[cAMP]_i$ and $[Ca^{2+}]_i$ assays, with the low nanomolar potency observed (Table 2), consistent with that of Moreno et al. ($IC_{50} = 2$ nM; 2000). Alone, PG99-465 had no effect on the $hVPAC_2R$ $[Ca^{2+}]_i$ response, however it did display partial agonistic activity in the $[cAMP]_i$ assay, perhaps accounting for the 5-fold reduction in potency between assays when added prior to VIP (Table 2). This finding contrasts with the original publication which showed that PG99-465 had no effect alone at the $hVPAC_2$ receptor (Moreno et al., 2000), although in two more recent studies there is a suggestion of a small increase in cAMP for both human (Langlet et al., 2004) and rat $VPAC_2$ receptors (Vanneste et al., 2004); the latter study reporting an EC_{50} similar to that in our present study. At both $hVPAC_1$ and $hPAC_1$, PG99-465 was a full agonist in the $[cAMP]_i$ assay (Fig. 5; Table 2), with again no effect on the $[Ca^{2+}]_i$ response. Moreno et al. (2000), and more recently Vanneste et al. (2004), have reported PG99-465 to be a partial agonist of human and rat $VPAC_1$ receptors respectively; however this is the first description of an agonist effect of PG99-465 at human PAC_1 receptors. As our EC_{50} value for PG99-465 at $hVPAC_1$, was more than 25× lower than that of Moreno et al. (2000), the increased sensitivity in the current and other cell based assays (Langer et al., 2002a) may account for the further agonist observations at $hVPAC_2$ and $hPAC_1$. Furthermore and in agreement with our observations for PG99-465, Langer et al. (2002a), showed that the putative antagonist VIP(4-28) (Ciccarelli et al., 1994) had no detectable effect alone in an adenylate cyclase assay using CHO-$hVPAC_1$ membranes, however it was a full agonist in a whole cell based cAMP assay. These findings suggest that the full agonism observed for PG99-465 may only be detectable in whole cells (rather than membranes) with an appropriate G-protein compliment. Indeed in preliminary studies using HT-29 cells, which endogenously express $hVPAC_1$ (Sreedharan et al., 1994), we have continued to examine the properties of PG99-465. Although cAMP production was higher in these cells at an equivalent cell number (∼80 nM), implying a higher receptor density than our stable cell lines, agonist potencies (VIP, PACAP-27 and PACAP-38) were identical to those seen for the $hVPAC_1$ stable cell line (data not shown). However, and most importantly, PG99-465 had no agonist effect despite the higher levels of cAMP, which would again suggest the agonist effects observed for PG99-465 in the CHO-stable cell lines are a consequence of their particular overall receptor/G-protein complement and the use of a cell based assay rather than just simply receptor density. These findings do however add further credence to the assertion of Laburthe and Couvineau (2002), who suggest that PG99-465 is not a satisfactory $hVPAC_2$ receptor antagonist, with these observations perhaps complicating the interpretation of some recent in vitro studies that use this peptide (Itri and Colwell, 2003; Piggins and Cutler, 2003; Akesson et al., 2005). Studies assessing the PAC_1 receptor antagonist M65 (Uchida et al., 1998), are even more limited than those examining the putative VPAC antagonists. M65 alone had no effect in either assay at any of the VPAC/PAC receptors, nor did it exhibit any antagonism of agonist

evoked responses at $hVPAC_1$ or $hVPAC_2$. It did however produce a concentration dependent inhibition of agonist evoked responses at the $hPAC_1$ receptor, exhibiting similar potencies (~210 nM) in both of the in vitro assays. These IC_{50} values (Table 2) are lower than those reported in a radioligand binding assay for rat PAC_1, however they are close to the potency of the closely related antagonist max.d.4 (Moro et al., 1999).

The generation of cell lines stably expressing human VPAC/PAC receptors, combined with the establishment of two simple, cell based, non-radioactive, HT-amenable, $[cAMP]_i$ and $[Ca^{2+}]_i$ assays, facilitated a systematic characterisation of receptor coupling, clearly demonstrating that all three receptor subtypes activate multiple downstream messengers in a single cell type. However, with agonist potencies being approximately 100-fold different in the two assays, despite a similar ROP, and with antagonist effects being complex, it is clear that the effects of any new compound should be examined on more than one signal transduction pathway as its pharmacological characteristics will be dependent upon the species of receptor used and the receptor/second messenger complement of the cell/tissue being examined. This may be of considerable physiological importance in vivo as a range of VIP/PACAP cAMP-independent effects have been noted, e.g. PAC_1 is known to function differently in the nervous system when compared with macrophages/monocytes (Laburthe and Couvineau, 2002; Delgado et al., 2003; DeHaven and Cuevas, 2004). Finally, there still remains a distinct lack of truly selective non-peptide agonists and antagonists to examine the functional role of VPAC/PAC receptors both in vitro and in vivo.

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