British Journal of Pharmacology (1999) 128, 934–940
http://www.stockton-press.co.uk/bjp

Domains determining agonist selectivity in chimaeric VIP_2 ($VPAC_2$)/PACAP (PAC_1) receptors

*,[1]E.M.Lutz, [3]C.J. MacKenzie, [1]M. Johnson, [2]K. West, [4]J.A. Morrow, [2]A.J. Harmar & [1]R. Mitchell

[1]MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ; [2]MRC Brain Metabolism Unit, Royal Edinburgh Hospital, Morningside Park, Edinburgh EH10 5HF; [3]Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow G12 8QQ and [4]Akzo Nobel, Research and Development, Organon Laboratories Ltd, Newhouse, Lanarkshire ML1 5SH

1 The $VPAC_2$ and PAC_1 receptors are closely related members of the Group II G protein-coupled receptor family. At the $VPAC_2$ receptor, VIP is equipotent to PACAP-38 in stimulating cyclic AMP production, whereas at the PAC_1 receptor PACAP-38 is many fold more potent than VIP. In this study, domains which confer this selectivity were investigated by constructing four chimaeric receptors in which segments of the $VPAC_2$ receptor were exchanged with the corresponding segment from the PAC_1 receptor.

2 When expressed in COS 7 cells all the chimaeric receptors bound the common ligand [^{125}I]PACAP-27 and produced cyclic AMP in response to agonists.

3 Relative selectivity for agonists was determined primarily by the amino terminal extracellular domain of the PAC_1 receptor and the $VPAC_2$ receptor. The interchange of other domains had little effect on the potency of PACAP-38 or PACAP-27.

4 For chimaeric constructs with a PAC_1 receptor amino terminal domain, the substitution of increasing portions of the $VPAC_2$ receptor decreased the potency of VIP yet increased that of helodermin.

5 This suggests that the interaction of VIP/helodermin but not PACAP with the PAC_1 receptor may be influenced (and differentially so) by additional receptor domains.

Keywords: VIP; PACAP; helodermin; receptor; chimaeric construct; cyclic AMP

Abbreviations: AC, adenylate cyclase; BSA, bovine serum albumin; C terminal, carboxyl terminal; DMSO, dimethyl sulphoxide; e, extracellular loop; EBSS, Earle's Balanced Salt solution; i, intracellular loop; IBMX, isobutyl methylxanthine; MEM, Minimal Essential Medium; N terminal, amino terminal; PACAP, pituitary adenylate cyclase-activating polypeptide; PCR, polymerase chain reaction; PLC, phospholipase C; PTH, parathyroid hormone; tm, transmembrane domain; VIP, vasoactive intestinal peptide

Introduction

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) are structurally similar neuropeptides, with 68% conservation of amino acids. Receptors for VIP (which also recognise PACAP with equal affinity) are found in both the CNS and periphery (Arimura, 1992), and may mediate the peripheral actions of PACAP as well as VIP. High affinity PACAP receptors (where VIP is many fold less potent than PACAP in evoking receptor-mediated responses) are present mainly in the CNS, but also in the adrenal medulla (Arimura, 1992). Two genes encoding VIP/PACAP receptors have been cloned; formerly known as the VIP_1 receptor (Ishihara *et al*., 1992), and the VIP_2 receptor (Lutz *et al*., 1993). The gene encoding the PACAP selective receptor has been cloned by several laboratories (Hashimoto *et al*., 1993; Hosoya *et al*., 1993; Morrow *et al*., 1993; Pisegna & Wank, 1993; Spengler *et al*., 1993; Svoboda *et al*., 1993) and has been shown to undergo differential splicing, for instance five splice variants in the region encoding intracellular loop three have been reported (Spengler *et al*., 1993). Recently, a new nomenclature has been approved for these receptors, the $VPAC_1$, the $VPAC_2$ and the PAC_1 receptor respectively (Harmar *et al*., 1998).

*Author for correspondence at: Department of Bioscience & BioTechnology, Royal College, University of Strathclyde, Glasgow G1 1XW; E-mail: elutz@srv1.bmu.mrc.ac.uk

The $VPAC_2$ and PAC_1 receptor are 50% identical at the amino acid level, and have 60% identity within the transmembrane spanning domains (the receptor trunk). These receptors belong to the secretin (Group II) G protein-coupled receptor family (Segre & Goldring, 1993), which does not have the consensus amino acid motifs which have been defined for the rhodopsin/β-adrenergic (Group I) G protein-coupled receptor family (Wess, 1997). Ligands for the secretin receptor family are all relatively large peptide hormones ($\geqslant$27 amino acids); all members couple to stimulation of adenylyl cyclase (AC), apparently through the heterotrimeric G protein G_s; many also couple to phospholipase C (PLC) stimulation through the G_q family (Hezareh *et al*., 1996; Offermanns *et al*., 1996). These receptors are highly conserved at the amino acid level (Segre & Goldring, 1993) suggesting that there may be common principles to the molecular mechanisms by which these receptors transduce agonist signals from the extracellular surface to the intracellular second messenger systems. It is likely that these receptors have evolved from a common ancestral gene, with strong selection pressure to maintain certain key molecular features which are necessary for this mechanism, but diverging in ligand specificity.

We have found marked functional differences in respect to ligand selectivity, levels of expression and second messenger coupling for the $VPAC_2$ and PAC_1 receptors when transiently expressed in COS 7 cells. The $VPAC_2$ and PAC_1 receptors not only stimulate AC (Lutz *et al*., 1993; Morrow *et al*., 1993) but

couple to other signal transduction pathways as well (MacKenzie *et al*., 1996; McCulloch *et al*., 1995; Spengler *et al*., 1993). In the present study we have begun to characterize receptor domains which confer the characteristics of agonist recognition for the PAC_1 and the $VPAC_2$ receptors by exchanging equivalent regions between the two types of receptor (see Figure 1), with divisions at the extracellular boundary of transmembrane region 1 (tm1) and within transmembrane region 5 (tm5). Chimaeric receptors were designated according to a scheme describing amino N-terminal portion/junction site/Carboxyl C-terminal portion (for example, the construct consisting of NH_2-PAC_1 receptor/junction site in TM5/$VPAC_2$–COOH was designated P_5V). Four chimaeric receptors thus were constructed between the rat $VPAC_2$ and the rat PAC_1 receptor. Wild-type and chimaeric receptors were transiently expressed in COS 7 cells. Levels of expression at the plasma membrane were determined by whole cell ligand binding. The ability of PACAP-38, PACAP-27, VIP and helodermin to activate cyclic AMP production at each receptor was then compared. All chimaeric receptors were functionally expressed at levels lower than those found for the PAC_1 receptor, but were 2–5 fold higher than that of the $VPAC_2$ receptor. Some of these results have been published in preliminary form (Lutz *et al*., 1994; 1996).

Methods

Drugs and chemicals

Tissue culture media were obtained from Life Technologies, Paisley, UK; DEAE dextran was from Pharmacia Biotech Ltd., St Albans, UK. [^{125}I]PACAP-27 (2200 Cimmol^{-1}) and [^{125}I]VIP (2200 Cimmol^{-1}) were obtained from DuPont (UK) Ltd, Stevenage, UK. Peptides were supplied by Calbiochem-Novabiochem (UK) Ltd, Nottingham, UK. Standard laboratory chemicals of Analar grade were obtained from Sigma-Aldrich Chemical Company, Poole, UK. Oligonucleotide primers were synthesized by Cruachem Ltd, Glasgow, UK or Oswel DNA Service, Southampton, UK.

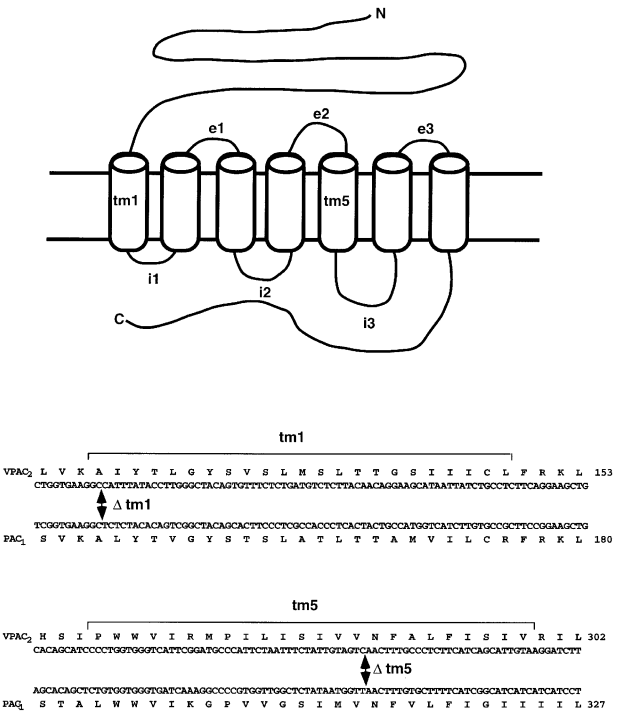

Figure 1 Exchange sites for construction of the tm1 and tm5 $VPAC_2$/PAC_1 chimaeric receptors. Schematic diagram of the seven transmembrane-spanning receptor. Junction sites for making the chimaeric receptor constructs are shown below and are labelled with the transmembrane domain in which they occur.

Construction of chimaeric receptors

Chimaeric receptors were made by exchanging the equivalent regions between the $VPAC_2$ receptor and the short intracellular loop 3 (i3) splice variant of the PAC_1 receptor, at exchange sites within tm1 and tm5 (Figure 1). The tm5 chimaeric receptors V_5P: $VPAC_2$(1–294)PAC_1(319–467) and P_5V: PAC_1(1–318)$VPAC_2$(295–437) were made by utilizing a conserved restriction (*Hinc*II) site in the region encoding the fifth transmembrane region within the $VPAC_2$ and PAC_1 receptor cDNA sequences. The N-terminal V_1P: $VPAC_2$ (1–127)PAC_1(155–467) and P_1V: PAC_1(1–154)$VPAC_2$(128–437) chimaeric receptors were made by overlap extension polymerase chain reaction (PCR) mutagenesis (Huang *et al*., 1995). For each junction site a set of four oligonucleotide primers was used, two external primers derived from flanking sequences of the vector and two internal primers one of which (the overlap primer) contained sequences derived from both receptor encoding cDNAs and which spanned the junction site of the chimaeric construct [PAC_1(155–467): 5′-CG TTTT A-TAT TCTG GTGA AG GCTC TCTA CACAG TC; $VPAC_2$ (128–437) 5′-GAT TATT ACTA CCT GTC GGT GAAGG C-CATT TATACC TTG G). The second internal primer (cDNA specific primer) contained sequences complementary to the 5′ end of the overlap primer and corresponding to the cDNA encoding the receptor portion 5′ of the junction site [$VPAC_2$ (1–127): 5′-CA CCA GA ATAT AAA ACGTG ATCTT AC); PAC_1 (1–154); 5′-CACCGACAGGTAGTAATAATCCTG). In the first round of PCR amplification the 5′ region encoding the N-terminal end of the chimaeric receptor was amplified with the cDNA specific primer along with the corresponding flanking external primer while the 3′ region encoding the C-terminal end was amplified with the overlap primer and corresponding flanking external primer. PCR reactions were set up in 100 μl volumes containing 15 ng cDNA, 15 pmol of 5′ and 3′ primer, in PCR buffer with 2 mM $MgCl_2$, 100 nM dNTPs and 10% DMSO and overlaid with mineral oil. The reaction was heated to 95°C for 5 min, then maintained at 80°C while adding 2.5U Pfu polymerase (Stratagene), after which the reaction was put through 30 cycles with denaturing at 94°C (1 min), annealing at 57°C (1 min) and extension at 72°C (3 min). After the first round of PCR, 10 μl samples were analysed by electrophoresis. The remaining PCR reactions were purified by extracting with Wizard cDNA purification system (Promega), then in the second round of PRC amplification 1 μl of each appropriate extract were mixed and amplified using the flanking pBluescript primers under the same conditions as the first round of amplification. The polymerase enzyme was removed by Wizard cDNA purification system and the reaction digested with either *Eco*RI or *Eco*RI + *Xho*I, then run on agarose gels for size selection. These were ligated into pBluescript for selection of appropriate clones by sequence analysis, then inserted into the expression vector pcDNA 1 (InVitrogen, R&D Systems Europe Ltd., Abingdon, UK) for functional expression in COS 7 cells.

Transfection of COS cells

COS 7 cells were transfected using DEAE dextran as described previously (Morrow *et al*., 1993) and allowed to recover for

24 h. Transfected cells were then trypsinized and plated into 12 well plates (for whole cell binding) or 24 well plates (for cyclic AMP assays). Assays were performed 48 h after plating.

Receptor binding assay

Transfected cells in 12 well plates were washed twice and incubated at 0°C for 1 h in ice-cold medium 199 containing 0.2% BSA with [^{125}I]PACAP-27 (14,000 c.p.m. per well) in the absence or presence of increasing concentrations of unlabelled PACAP-27 or with [^{125}I]VIP (30,000 c.p.m. per well) in the absence or presence of increasing concentrations of unlabelled VIP. Non-specific binding was defined with 300 nM PACAP-27 or 3 μM VIP respectively as preliminary experiments showed that higher concentrations began to displace ligand binding non-specifically from COS 7 cells transfected with empty vector. Unbound radioactivity was removed by washing cells three times with Earle's Balanced Salt solution (EBSS) containing 0.1% BSA. Bound radioactivity was removed by acid wash (0.2 M acetic acid, 0.5 M NaCl) for 5 min on ice, and measured by γ-counting.

Cyclic AMP formation assay

Transfected cells were treated essentially as described previously (Morrow *et al*., 1993). Forty-eight hours after trypsinisation cells were washed twice in MEM containing 0.25% BSA, and preincubated at 37°C for 15 min in the presence of 0.5 mM isobutyl methylxanthine (IBMX). Peptides were directly added, and incubations were continued at 37°C for 5 min. The reaction was stopped by adding an equal volume of ice-cold 0.2 M HCl and frozen. Cyclic AMP levels were measured by radioimmunosassay with antibodies to cyclic AMP kindly provided by Dr Ian Gow, Department of Physiology, University of Edinburgh, UK.

Data analysis

Curve fitting and standard error calculation was performed using a non-linear regression program, P-fit (Elsevier Biosoft, Cambridge, UK).

Results

Cell surface expression of wild-type and chimaeric receptors in COS 7 cells

To determine cell surface receptor levels and binding characteristics for the wild-type and chimaeric $VPAC_2$ and PAC_1 receptors, B_{max} and IC_{50} values were measured by homologous displacement of [^{125}I]PACAP-27 binding (at 0°C) to whole cells expressing $VPAC_2$ or PAC_1 receptors (Table 1). Ligand affinities measured by this *in vivo* method for cell surface binding are routinely lower than those found *in vitro* membrane binding (presumably as a result of different assay constituents and conditions). Nevertheless, this approach gives information on cell surface expression of receptors, rather than the entire cellular complement, and allows direct internal comparisons between the different constructs in this study. Receptor expression levels monitored in this way varied between 59 fmol10^{-5} cells (the $VPAC_2$ receptor) and 494 fmol10^{-5} cells (the PAC_1 receptor). The chimaeric receptors displayed levels 130–215 fmol10^{-5} cells (between 26 and 44% of the levels found for the PAC_1 receptor). Table 1 also shows the IC_{50} values for PACAP 27 displacement of [^{125}I]PACAP-27 binding in whole cells expressing wild-type and chimaeric receptors. Values for wild-type receptors were 19 ± 1 nM for $VPAC_2$ and 30 ± 1 nM for PAC_1. The affinity of wild-type receptors and chimaeric constructs for PACAP-27 was very similar in all cases with IC_{50} values at the chimaeric receptors differing by less than 2 fold from their corresponding wild-type controls (Table 1). This indicates, in general terms, that the ability of the chimaeras to recognise an appropriate agonist ligand is not grossly perturbed by the presence of exchanged domains. Both the best fit slope values from curve fitting (ranging from 0.81–1.22) and Scatchard-type plots of the data gave no cause to suggest the presence of multiple components in [^{125}I]PACAP-27 binding under these conditions. Pilot experiments were carried out using [^{125}I]VIP as a ligand in a similar protocol. Binding that was displaceable with high affinity by unlabelled VIP was observed in each case, but the computed B_{max} values varied considerably from those obtained with the broad specificity ligand [^{125}I]PACAP-27 probably as a result of the heterogeneous affinity of [^{125}I]VIP for $VPAC_2$/PAC_1 receptors. Since results would not be directly comparable with those obtained using [^{125}I]PACAP-27 (Table 1), these studies were not pursued any further. It was possible to confirm however that [^{125}I]VIP can label, with relatively high affinity (39 ± 9 nM), a subpopulation of the PAC_1 receptors identified by [^{125}I]PACAP-27 binding (approximately 44% in our hands compared to 32% in the previous report of Hashimoto *et al*., (1993). The nature of this subpopulation is unclear.

Table 1 Relative receptor expression levels and IC_{50} values for PACAP-27 displacement of [^{125}I]PACAP-27 binding at wild-type and chimaeric receptors

Expressed receptor		*Bmax (fmol 10^{-5} cells)*	*IC_{50} (nM) PACAP-27*
	PAC_1	494	30 ± 1
	P_5V	202	30 ± 6
	P_1V	130	48 ± 16
	$VPAC_2$	59	19 ± 1
	V_5P	163	32 ± 5
	V_1P	215	16 ± 2

Receptor expression levels (B_{max}) were measured for intact cells and are expressed as fmol 10^{-5} cells. Receptor affinity was measured by homologous displacement of [^{125}I]PACAP-27 from whole cells at 0°C. Values are the means ± s.e.mean ($n = 3-6$).

Agonist activation of cyclic AMP production mediated by wild-type and chimaeric receptors

We have previously shown that the wild-type $VPAC_2$ and PAC_1 receptors expressed in COS 7 cells show clear differences in agonist specificity. VIP and PACAP-38 are equipotent in stimulating cyclic AMP production at the $VPAC_2$ receptor (Lutz *et al*., 1993), whereas VIP is 50 fold less potent than PACAP-38 at the PAC_1 receptor (Morrow *et al*., 1993). In order to determine which receptor domains were involved in

agonist recognition, transiently-transfected COS 7 cells were stimulated with VIP, PACAP-38, PACAP-27 and helodermin before cyclic AMP levels were measured (Table 2). All peptides caused a concentration-dependent increase in cyclic AMP levels in COS 7 cells transfected with the wild-type and chimaeric receptors. As predicted from the [^{125}I]PACAP-27 binding data, both PACAP-27 and PACAP-38 showed very similar EC_{50} values for cyclic AMP production at wild-type PAC_1 and $VPAC_2$ receptors and at all the chimaeric constructs (Table 2). This confirms that viable coupling to second messenger production is not in itself impaired by the presence of non-matching domains in the construct. Responses to VIP and helodermin however showed a number of differences between the receptors. The most striking difference correlated closely with the presence of particular N-terminal domains. All receptors with the $VPAC_2$ N-terminal domain (wild-type $VPAC_2$, V_5P and V_1P) displayed high affinities for VIP and helodermin at sub-nM concentrations similar to those shown for PACAP-38 and PACAP-27. All receptors with the PAC_1 N-terminal domain (wild-type PAC_1, P_5V and P_1V) showed lower affinities for VIP and helodermin. The clear segregation of pharmacological characteristics according to the presence of $VPAC_2$ or PAC_1 receptor N-terminal domain strongly suggests that this domain acts as the primary determinant of agonist recognition in these receptors. In general terms, $VPAC_2$-like or PAC_1-like characteristics are conferred by the presence of a $VPAC_2$- or PAC_1-receptor N-terminal domain respectively.

However, the $VPAC_2$ and PAC_1 receptors clearly differ in the extent to which additional receptor elements affect agonist potency. The potency of VIP and helodermin at receptors with the $VPAC_2$ N-terminal domain was unaltered (no more than 2 fold changes) by replacement of the tm5–C-terminal domain (in V_5P) or the tm1–C-terminal domain (in V_1P). In receptors with the PAC_1 N-terminal domain however, the progressive replacement of tm5–C-terminal (in P_5V) and tm1–C-terminal (in P_1V) with corresponding $VPAC_2$ receptor domains led to a decline in VIP potency by 3.6 fold and 6.7 fold respectively.

In contrast, the potency of helodermin was increased when the tm1–C-terminal segment of the PAC_1 receptor were replaced with homologous $VPAC_2$ receptor domains (in P_5V and P_1V). In the case of the tm5–C-terminal substitution the effect was marginal (1.6 fold) but when the tm1–tm5 segments were additionally replaced, a very marked (16 fold) increase in helodermin potency was apparent. The concentration-response data for the key shifts in potency of VIP and helodermin (but not PACAP-38) between wild-type PAC_1 receptors and the P_1V chimaeric construct are shown in Figure 2. There was no evidence that any agonist at any receptor investigated produced a maximal response less than that of PACAP-38.

Table 2 EC_{50} values for PACAP-38, PACAP-27, VIP and helodermin-stimulated cAMP production at wild-type and chimaeric receptors

Expressed receptor	*EC_{50} cAMP production* (nM) *PCAP-38*	*PACAP-27*	*VIP*	*helodermin*
PAC_1	0.4±0.1	0.9±0.1	23±2	39±3
P_5V	0.3±0.1	0.6±0.1	82±14	24±3
P_1V	0.3±0.1	1.5±0.4	155±13	2.4±0.5
$VPAC_2$	0.8±0.1	0.4±0.1	0.5±0.2	0.4±0.2
V_5P	0.8±0.1	0.7±0.1	0.6±0.1	0.6±0.1
V_1P	0.7±0.2	0.7±0.1	0.4±0.1	0.8±0.2

EC_{50} values for cyclic AMP production were determined from concentration response curves by non-linear curve fitting and are expressed as the means±s.e.mean (n=4–12).

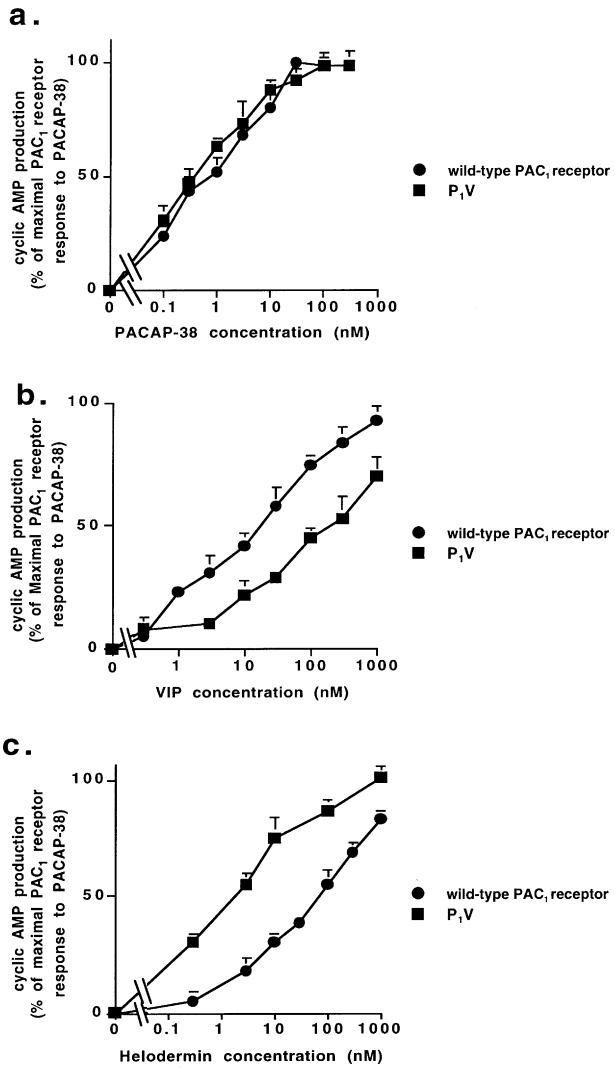

Figure 2 Comparison of agonist-evoked cyclic AMP responses at the wild-type PAC_1 receptor and the P_1V chimaeric receptor construct. COS 7 cells transiently expressing the wild-type PAC_1 receptor or the P_1V chimaeric construct were preincubated with IBMX for 15 min before stimulation for 5 min with agonists: (a) PACAP-38; (b) VIP; (c) helodermin. All values are means ±s.e.mean (n=4–12). Error bars not shown fall within the dimensions of the symbol.

Discussion

The present data accord closely with the general idea that the main determinant of agonist response pharmacology at the $VPAC_2$ and PAC_1 receptors is within the N-terminal extracellular domain of the receptors. This broadly matches conclusions on the influence of the PAC_1 receptor N-terminal domain derived from experiments with PAC_1/$VPAC_1$ receptor chimaeric constructs (Hashimoto *et al*., 1997; Van Rampelbergh *et al*., 1996). This issue has not been previously addressed in the $VPAC_2$ receptor, but chimaeric and mutant

constructs of the $VPAC_1$ receptor suggest that elements in the N-terminal domain are also key determinants of its agonist selectivity (Couvineau *et al*., 1995; Hashimoto *et al*., 1997; Holtmann *et al*., 1995; Van Rampelbergh *et al*., 1996).

There is increasing evidence that the effective potency of agonists at some receptors in the family is not always a simple reflection of docking affinity (Hjorth *et al*., 1996; Stroop *et al*., 1995). For example, it has been recently demonstrated that VIP binds with high affinity to the rat secretin (Holtmann *et al*., 1996) and PAC_1 receptors (Hashimoto *et al*., 1997), although it has much lower potency in activating cyclic AMP production at these receptors. Similarly, a report on a chimaeric construct between the N-terminal domain of the PAC_1 receptor and the body of the $VPAC_1$ receptor described its high affinity binding of [^{125}I]VIP although binding to the wild-type PAC_1 receptor was not noted (Van Rampelbergh *et al*., 1996). This suggests the idea that some agonists, for example VIP at the PAC_1 receptor, may bind strongly (at least to a subpopulation of sites) but display a reduced molecular efficiency in causing the conformational changes that elicit signal transduction. Together with results from the literature, our data suggest that at the $VPAC_2$ receptor, PACAP-38, PACAP-27 and VIP dock strongly and efficiently activate cyclic AMP production. In contrast at the PAC_1 receptor, while PACAP-38 and PACAP-27 both dock and activate strongly, VIP appears to show rather low potency of cyclic AMP production (25 fold less than PACAP-27) despite labelling almost half of the PAC_1 receptors identified by [^{125}I]PACAP-27 binding with a very similar affinity (39 ± 9 nM for VIP; 30 ± 1 μM for PACAP-27). This is consistent with the hypothesis that VIP may show a reduced activation efficiency at the PAC_1 receptor compared to PACAP.

Chimaeric $VPAC_1$/PAC_1 receptor studies have shown that the $VPAC_1$ receptor contains auxiliary sites within the extracellular loops (el–3) of the receptor trunk which facilitate VIP activation of cyclic AMP production (Hashimoto *et al*., 1997; Van Rampelbergh *et al*., 1997). Unlike chimaeric $VPAC_1$/secretin receptors where the N-terminal domain was largely sufficient to confer $VPAC_1$-like pharmacology to the secretin receptor trunk (Holtmann *et al*., 1995; 1996), chimaeric $VPAC_1$/PAC_1 receptors also required a segment encompassing e3 (Hashimoto *et al*., 1997). When either the $VPAC_1$ N-terminal–tm1 segment or the e3 segment (tm5–tm7) was inserted into the corresponding region of the PAC_1 receptor, VIP potency in activating cyclic AMP production was increased 21 or 16 fold respectively, compared to wild-type PAC_1 receptors (Hashimoto *et al*., 1997). The combination of both regions caused an additional 11 fold increment in VIP potency. Conversely the replacement of segments containing e1 and then e2 plus e3 domains in the $VPAC_1$ receptor with those of the PAC_1 receptor, progressively reduced the ability of VIP to activate cyclic AMP production for chimaeric $VPAC_1$/PAC_1 receptors compared to the wild-type $VPAC_1$ receptor (Hashimoto *et al*., 1997). Site-directed mutagenesis of the $VPAC_1$ receptor has also indicated that, in addition to elements within the N-terminal domain, sites within e1 and tm3 are important in ligand recognition and selectivity (Couvineau *et al*., 1996; Du *et al*., 1997). Differences in the structure–function relationships of agonist recognition by $VPAC_1$ and $VPAC_2$ receptors have recently been emphasised in experiments to mutate corresponding residues in the two receptors (Nicole *et al*., 1998). In the present study with $VPAC_2$/PAC_1 chimaeric constructs, we found no evidence that the ability of the $VPAC_2$ receptor to respond to PACAP-38, PACAP-27, VIP or helodermin was reduced by the replacement of tm5–C-terminal or tm1–C-terminal segments by equivalent domains of the PAC_1 receptor. This could mean either that agonist recognition and activation of the cyclic AMP production response by the $VPAC_2$ receptor is little influenced by sequence motifs outwith the N-terminal domain, or that any requisite motifs were perfectly replaced by the homologous domains of the PAC_1 receptor.

Although receptors with a $VPAC_2$ N-terminal domain were insensitive to changes in other regions of the receptor, the same was not true of those with a PAC_1 N-terminal domain. For these receptors, recognition and action of PACAP-38 and PACAP-27 was unaltered by substitutions from tm1 to the C-terminal, indicating that the PAC_1 N-terminal domain alone is fully sufficient for recognition of PACAP-38 and PACAP-27 and their activation of cyclic AMP production. It is possible that the $VPAC_2$ receptor sequences fully replace the requisite motifs in the non-N-terminal extracellular domains. The ligands VIP and helodermin however were differentially recognised by wild-type and chimaeric PAC_1-N-terminal receptors. The reduction in [^{125}I]VIP binding affinity and in VIP potency of cyclic AMP production as tm5–C-terminal and tm1–C-terminal of the PAC_1 receptor were progressively replaced with $VPAC_2$ sequences suggests that recognition of VIP by the PAC_1 receptor (and consequent cyclic AMP responses) optimally requires elements in the tm1–C-terminal segment as well as the N-terminal domain. The presence of equivalent domains substituted from the $VPAC_2$ receptor does not enhance VIP potency even though the wild-type $VPAC_2$ receptor is potently activated by VIP. This indicates that the substitutions do not make the chimaeric constructs more $VPAC_2$-like but instead remove the supportive influence provided by tm1–C-terminal tail segment in the PACAP receptor. The lack of alteration in PACAP-27 and PACAP-38 recognition by the same chimaerics shows that a generalized disruption of recognition and function is not the reason for reduced VIP potency in the receptors. Although directly comparable constructs were not made in the study of chimaeric $VPAC_1$/PAC_1 receptors (Hashimoto *et al*., 1997) PAC_1 receptor constructs with the tm3–tm5 segment and tm5–tm7 segment of the $VPAC_1$ receptor showed 1.6 fold and 16 fold increases in VIP potency at cyclic AMP in the face of minimal changes in [^{125}I]VIP binding affinity. Thus, while substitutions of $VPAC_1$ receptor sequences in these segments can enhance VIP action but not docking, substitution of $VPAC_2$ receptor sequences results in reduced binding affinity for VIP and corresponding reduced potency in cyclic AMP production. This implies that the tm1–C-terminal tail segments of $VPAC_1$ and $VPAC_2$ receptors make very different contributions to the recognition of VIP (at least in the context of a PAC_1 receptor N-terminal domain).

A different mode of action is revealed in the same receptors by the use of helodermin as agonist rather than VIP or PACAP. In this case, substitution of the tm5–C-terminal and tm1–C-terminal segments of the PAC_1 receptor by equivalent $VPAC_2$ receptor domains lead to increased potency of helodermin in cyclic AMP production. This might suggest that elements in the tm1–C-terminal segment of the $VPAC_2$ receptor facilitate helodermin recognition and action. However, this is unlikely, since in $VPAC_2$ N-terminal receptors, the replacement of tm5–C-terminal and tm1–C-terminal segments with PAC_1 receptor sequences had no detectable effect on helodermin potency. It is possible that influences are context-specific in that with a $VPAC_2$ N-terminal domain, no further contributions are needed for helodermin recognition, whereas with a PAC_1 N-terminal domain auxiliary sites are supportive and can be supplied by a $VPAC_2$ but not PAC_1 receptor body. Instead it seems most likely that elements in the

PAC_1 receptor tm1–C-terminal segment exert a negative effect on the recognition of helodermin by the PAC_1 N-terminal domain or its subsequent cyclic AMP response. Such negative regulatory elements have been described in the PTH, secretin and $VPAC_1$ receptors (Couvineau *et al*., 1996; Holtmann *et al*., 1996; Turner *et al*., 1996). Corresponding elements from the $VPAC_2$ receptor would appear to lack this influence. It is not yet clear whether this negative gating influence is exerted at the level of helodermin binding affinity or its subsequent effectiveness in signal transduction.

In conclusion, we have provided evidence that ligand recognition and action at the rat $VPAC_2$ receptor is largely determined by the N-terminal segment of the receptor. Additional motifs in other parts of the receptor appear to make relatively little contribution to modulation of this, in contrast to observations with the $VPAC_1$ receptor (Hashimoto *et al*., 1997). While agonist recognition and activation of the PAC_1 receptor is primarily influenced by the N-terminal domain, this receptor also shows important auxiliary influences of other domains in the recognition of and activation by the agonists VIP and helodermin (but not PACAP). Hashimoto *et al*., (1997) indicated that a subpopulation of PAC_1 receptor sites can bind [^{125}I]VIP with high affinity but only weakly activate cyclic AMP production in response to VIP. Our data (Tables 1 and 2) are consistent with this although the differing usage of *in vivo*/*in vitro* binding assays means that the ligand affinity values are not directly comparable between the two studies. The findings suggest the presence of elements that normally restrict effectiveness of, but not affinity for, VIP. However, VIP appears to benefit from the influence of auxiliary sites in the tm1–C-terminal of the PAC_1 receptor, in its activation of cyclic AMP production. Replacement of these domains with corresponding $VPAC_2$ receptor sequences further reduces the potency of VIP at cyclic AMP production and reduces the affinity with which this ligand binds to the receptor. The actions of helodermin at the PAC_1 receptor appear to be quite differently regulated. Replacement of the tm1–C-terminal segment with $VPAC_2$ receptor sequences appears to remove a selective inhibitory influence which normally suppresses the potency of helodermin. These observations strongly emphasise that the structure–activity relationships for agonist docking and effectiveness in the PAC_1 receptor but perhaps not the $VPAC_2$ receptor are agonist-dependent and complex.

We wish to thank Marianne Eastwood for helping prepare the manuscript, Elma Clark for help with sequencing the constructs, and John Bennie and Sheena Carroll for help with the radioimmunoassays. R Mitchell and M Johnson are members of the Membrane Biology Group of the University of Edinburgh.

References

ARIMURA, A. (1992). Receptors for pituitary adenylate cyclase activating polypeptide. Comparison with vasoactive intestinal peptide receptors. *Trends Endocrinol. Metab.,* **3,** 288–294.

COUVINEAU, A., GAUDIN, P., MAORET, J-J., ROUYER-FESSARD, C., NICOLE, P. & LABURTHE, M. (1995). Highly conserved aspartate 68, tryptophan 73 and glycine 109 in the N-terminal extracellular domain of the human VIP receptor are essential for its ability to bind VIP. *Biochem. Biophys. Res. Commun.,* **206,** 246–252.

COUVINEAU, A., ROUYER-FESSARD, C., MAORET, J.J., GAUDIN, P., NICOLE, P. & LABURTHE, M. (1996). Vasoactive intestinal peptide (VIP)1 receptor. Three nonadjacent amino acids are responsible for species selectivity with respect to recognition of peptide histidine isoleucineamide. *J. Biol. Chem.,* **271,** 12795–12800.

DU, K., NICOLE, P., COUVINEAU, A. & LABURTHE, M. (1997). Aspartate 196 in the first extracellular loop of the human VIP1 receptor is essential for VIP binding and VIP-stimulated cAMP production. *Biochem. Biophys. Res. Commun.,* **230,** 289–292.

HARMAR, A.J., ARIMURA, A., GOZES, I., JOURNOT, L., LABURTHE, M., PISEGNA, J.R., RAWLINGS, S.R., ROBBERECHT, P., SAID, S.I., SREEDHARAN, S.P., WANK, S.A. & WASCHEK, J.A. (1998). International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol. Rev.,* **50,** 265–270.

HASHIMOTO, H., ISHIHARA, T., SHIGEMOTO, R., MORI, K. & NAGATA, S. (1993). Molecular cloning and tissue distribution of a receptor for pituitary adenylate cyclase-activating polypeptide. *Neuron,* **11,** 333–342.

HASHIMOTO, H., OGAWA, N., HAGIHARA, N., YAMAMOTO, K., IMANISHI, K., NOGI, H., NISHINO, A., FUJITA, T., MATSUDA, T., NAGATA, S. & BABA, A. (1997). Vasoactive intestinal polypeptide and pituitary adenylate cyclase- activating polypeptide receptor chimeras reveal domains that determine specificity of vasoactive intestinal polypeptide binding and activation. *Mol. Pharmacol.,* **52,** 128–135.

HEZAREH, M., SCHLEGEL, W. & RAWLINGS, S.R. (1996). PACAP and VIP stimulate Ca^{2+} oscillations in rat gonadotrophs through the PACAP/VIP type 1 receptor (PVR1) linked to a pertussin toxin-insensitive G-protein and activation of phospholipase C-b. *J. Neuroendocrinol.,* **8,** 367–374.

HJORTH, S.A., ØRSKOV, C. & SCHWARTZ, T.W. (1996). Two-domain structure of the glucagon receptor: peptide binding and signal transduction. *Regul. Pept.,* **64,** 70.

HOLTMANN, M.H., GANGULI, S., HADAC, E.M., DOLU, V. & MILLER, L.J. (1996). Multiple extracellular loop domains contribute critical determinants for agonist binding and activation of the secretin receptor. *J. Biol. Chem.,* **271,** 14944–14949.

HOLTMANN, M.H., HADAC, E.M. & MILLER, L.J. (1995). Critical contributions of amino-terminal extracellular domains in agonist binding and activation of secretin and vasoactive intestinal polypeptide receptors. *J. Biol. Chem.,* **270,** 14394–14398.

HOSOYA, M., ONDA, H., OGI, K., MASUDA, Y., MIYAMOTO, Y., OHTAKI, T., OKAZAKI, H., ARIMURA, A. & FUJINO, M. (1993). Molecular cloning and functional expression of rat cDNAs encoding the receptor for pituitary adenylate cyclase activating polypeptide (PACAP). *Biochem. Biophys. Res. Commun.,* **194,** 133–143.

HUANG, Z., CHEN, Y. & NISSENSON, R.A. (1995). The cytoplasmic tail of the G-protein-coupled receptor for parathyroid hormone and parathyroid hormone-related protein contains positive and negative signals for endocytosis. *J. Biol. Chem.,* **270,** 151–156.

ISHIHARA, T., SHIGEMOTO, R., MORI, K., TAKAHASHI, K. & NAGATA, S. (1992). Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron,* **8,** 811–819.

LUTZ, E.M., MACKENZIE, C.J., MORROW, J., MITCHELL, R., BENNIE, J., CARROLL, S., CLARK, E. & HARMAR, A.J. (1996). Chimaeric VIP_2/PACAP receptors reveal that agonist pharmacology but not signal transduction is determined by extracellular domain 1. *Ann. N.Y. Acad. Sci.,* **805,** 574–578.

LUTZ, E.M., MENDELSON, S., WEST, K., MITCHELL, R. & HARMAR, A.J. (1994). Molecular characterisation of novel receptors for PACAP and VIP. *Biochem. Soc. Trans.,* **22,** 88S.

LUTZ, E.M., SHEWARD, W.J., WEST, K.M., MORROW, J.A., FINK, G. & HARMAR, A.J. (1993). The VIP_2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Lett.,* **334,** 3–8.

MACKENZIE, C.J., LUTZ, E.M., MCCULLOCH, D.A., MITCHELL, R. & HARMAR, A.J. (1996). Phospholipase C activation by VIP_1 and VIP_2 receptors expressed in COS 7 cells involves a pertussis toxin-sensitive mechanism. *Ann. N.Y. Acad. Sci.,* **805,** 579–584.

MCCULLOCH, D., GRIEVE, S., JOHNSON, M., LUTZ, E. & MITCHELL, R. (1995). Activation of phospholipase D by the VIP_2 receptor and evidence for its attenuation by protein kinase A. *Proc. Intnl Conf Second Messengers Phosphoproteins,* **9,** 414P.

MORROW, J.A., LUTZ, E.M., WEST, K.M., FINK, G. & HARMAR, A.J. (1993). Molecular cloning and expression of a cDNA encoding a receptor for pituitary adenylate cyclase activating polypeptide (PACAP). *FEBS Lett.,* **329,** 99–105.

NICOLE, P., DU, K., COUVINEAU, A. & LABURTHE, M. (1998). Site-directed mutagenesis of human vasoactive intestinal peptide receptor subtypes VIP1 and VIP2: evidence for difference in the structure-function relationship. *J. Pharmacol. Exp. Ther.,* **284,** 744–750.

OFFERMANNS, S., IIDA-KLEIN, A., SEGRE, G.V. & SIMON, M.I. (1996). $G\alpha_q$ family members couple parathyroid hormone (PTH)/PTH-related peptide and calcitonin receptors to phospholipase C in COS-7 cells. *Mol. Endocrinol.,* **10,** 566–574.

PISEGNA, J.R. & WANK, S.A. (1993). Molecular cloning and functional expression of the pituitary adenylate cyclase-activating polypeptide type I receptor. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 6345–6349.

SEGRE, V. & GOLDRING, S.R. (1993). Receptors for secretin, calcitonin, parathyroid hormone (PTH)/PTH-related peptide, vasoactive intestinal peptide, glucagon-like peptide 1, growth hormone-releasing hormone, and glucagon belong to a newly discovered G-protein-linked receptor family. *Trends Endocrinol. Metab.,* **4,** 309–314.

SPENGLER, D., WAEBER, C., PANTALONI, C., HOLSBOER, F., BOCKAERT, J., SEEBURG, P.H. & JOURNOT. L. (1993). Differential signal transduction by 5 splice variants of the PACAP receptor. *Nature,* **365,** 170–175.

STROOP, S.D., KUESTNER, R.E., SERWOLD, T.F., CHEN, L. & MOORE, E.E. (1995). Chimaeric human calcitonin and glucagon receptors reveal two dissociable calcitonin interaction sites. *Biochemistry,* **34,** 1050–1057.

SVOBODA, M., TASTENOY, M., CICCARELLI, E., STIEVENART, M. & CHRISTOPHE, J. (1993). Cloning of a splice variant of the pituitary adenylate cyclase- activating polypeptide (PACAP) type I receptor. *Biochem. Biophys. Res. Commun.,* **195,** 881–888.

TURNER, P.R., BAMBINO, T. & NISSENSON, R.A. (1996). A putative selectivity filter in the G-protein-coupled receptors for parathyroid hormone and secretin. *J. Biol. Chem.,* **271,** 9205–9208.

VAN RAMPELBERGH, J., GOURLET, P., DE NEEF, P., ROBBERECHT, P. & WAELBROECK, M. (1996). Properties of the pituitary adenylate cyclase-activating polypeptide I and II receptors, vasoactive intestinal peptide 1, and chimaeric amino-terminal pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal peptide 1 receptors: evidence for multiple receptor states. *Mol. Pharmacol.,* **50,** 1596–1604.

VAN RAMPELBERGH, J., POLOCZEK, P., FRANCOYS, I., DELPORTE, C., WINAND, J., ROBBERECHT, P. & WAELBROECK, M. (1997). The pituitary adenylate cyclase activating polypeptide (PACAP I) and VIP (PACAP II VIP_1) receptors stimulate inositol phosphate synthesis in transfected CHO cells through interaction with different G proteins. *Biochim. Biophys. Acta,* **1357,** 249–255.

WESS, J. (1997). G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J.,* **11,** 346–354.

(Received June 7, 1999
Revised July 19, 1999
Accepted August 6, 1999)