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Characterization of a novel $VPAC_1$ selective agonist and identification of the receptor domains implicated in the carboxyl-terminal peptide recognition

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1 Vasoactive Intestinal Polypeptide (VIP) interacts with a high affinity to two subclasses of G protein coupled receptors named $VPAC_1$ and $VPAC_2$, and has a 3–10 fold preference for $VPAC_1$ over $VPAC_2$ receptors. Selective ligands for each receptor subclass were recently described. [R^{16}]-PACAP (1–23) and [L^{22}]-VIP are two selective $VPAC_1$ agonists.

2 Chimaeric human $VPAC_2$-$VPAC_1$ recombinant receptors expressed in CHO cells were used to identify the receptor domains implicated in these two selective ligands recognition.

3 The $VPAC_2$ preference for [R^{16}]-PACAP (1–27) over [R^{16}]-PACAP (1–23) did not require the receptor's NH_2-terminus domain but involved the whole transmembrane domain.

4 In contrast, the selectivity of [L^{22}]-VIP depended only on the presence of the NH_2 terminus and EC_2 domains of the $VPAC_1$ receptor.

5 The present data support the idea that in the GPCR-B family of receptors the different selective ligands require different domains for their selectivity, and that the peptides carboxyl terminal sequence (amino acids 24–27) folds back on the transmembrane receptor domain, close to the peptides, aminoterminus.

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Abbreviations: CHO, Chinese hamster ovary; EC, extracellular loop; EC_{50}, concentration of agonist required for half maximal response; GPCR, G-protein coupled receptor; IC_{50}, concentration of ligand required for 50% inhibition of tracer binding; PACAP, pituitary adenylate cyclase activating polypeptide; TMD, transmembrane domain; VIP, vasoactive intestinal polypeptide

Introduction

Vasoactive intestinal polypeptide (VIP) interacts with two receptor subtypes, previously named VIP_1 and VIP_2 and currently known as $VPAC_1$ and $VPAC_2$ (Harmar *et al*., 1998), that have been cloned in rat (Lutz *et al*., 1993; Ishihara *et al*., 1992), human (Couvineau *et al*., 1994; Svoboda *et al*., 1994) and mouse (Inagaki *et al*., 1994). These receptors belong to the class B of the G-protein coupled receptors that includes the calcitonin-, the corticotrophin releasing factor-, an insect diuretic hormone-, the gastric inhibitory peptide-, the glucagon-, the glucagon-like peptide I-, the growth hormone releasing factor-, the parathyroid hormone-, the pituitary adenylate cyclase activating polypeptide (PACAP)-, the secretin-, the $VPAC_1$- and $VPAC_2$- and also orphan receptors (Donnelly, 1997).

All these receptors have significant sequence homology (especially in the transmembrane domains) and possess a large extracellular NH_2-terminal domain with six conserved cysteine residues. They are all positively coupled to adenylate cyclase but some of them may also be coupled to other effectors (Van Rampelbergh *et al*., 1997; Spengler *et al*., 1993).

In situ hybridization reveals that both VIP receptor subtypes are transcribed – and thus probably expressed – in different tissues or cells (Usdin *et al*., 1994). This is particularly obvious in brain where their localization in many areas appear mutually exclusive (Vertongen *et al*., 1997). The development of agonists and antagonists selective for each receptor subclass is therefore of great pharmacological importance.

The neuropeptides VIP and PACAP recognize both receptor subtypes with a high affinity (Couvineau *et al*., 1996; Svoboda *et al*., 1994). The peptide with a NH_2-terminal histidine and a COOH-terminal isoleucine amide (PHI) or its longer form with a COOH-terminal valine amide (PHV) – that are co-synthesized and released with VIP – have a higher affinity for the human $VPAC_2$ than for the human $VPAC_1$ receptor (Couvineau *et al*., 1996; Gourlet *et al*., 1998), which is not the case for the rat receptors (Couvineau *et al*., 1996).

Several synthetic ligands, selective for each receptor subtype have been described recently (Gourlet *et al*., 1997a,b; 1998; Xia *et al*., 1997). Amongst them, [R^{16}]-PACAP (1–23) (as example of truncated peptide) and [L^{22}]-VIP (as example of single change in the peptide sequence) are selective $VPAC_1$ receptor agonists (this study and Gourlet *et al*., 1998).

Little is known to date about the receptor determinants that are responsible for the different selectivity profiles observed for these related receptors.

In the present study, we constructed and stably expressed in Chinese hamster ovary (CHO) cells chimaeric receptors made by the replacement of parts of the human $VPAC_1$ receptor sequence by the corresponding sequence of the human $VPAC_2$ receptor to

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identify the receptor domains involved in this selectivity. The shortened original PACAP (1–27) derivative, [R^{16}]-PACAP (1–23) and a mono-substituted VIP analogue [L^{22}]-VIP were used in this study as $VPAC_1$ selective ligands and were compared to [R^{16}]-PACAP (1–27) and VIP respectively.

Methods

Wild-type receptors

The human $VPAC_2$ receptor cDNA was previously cloned and characterized in our laboratory (Svoboda *et al*., 1994). The human $VPAC_1$ receptor cDNA was cloned by PCR according to the sequence previously reported by Couvineau *et al*. (1994) using specific primers.

Chimaeric receptor constructs

The receptors studied in this work represented eight chimaeric constructs of wild type human $VPAC_2$ and $VPAC_1$ receptors, four of which (chimaeras C1, C4, C7 and C8) have been described previously (Juarranz *et al*., 1999a,b). Chimaeras were designed to replace portions of one wild type receptor cDNA with the corresponding portions of the other receptor. The following constructs were prepared (where the amino acids are numbered according to the human $VPAC_1$ and $VPAC_2$ receptor sequences): **VPAC1**, human wild-type $VPAC_1$ receptor (1–457); **VPAC2**, human wild-type $VPAC_2$ receptor (1–438); **C1 (N-$VPAC_2$-$VPAC_1$)**, $VPAC_2$ receptor (1–127) and $VPAC_1$ receptor (144–457); **C2 (N→IC_1 $VPAC_2$-$VPAC_1$)**, $VPAC_2$ receptor (1–158) and $VPAC_1$ receptor (175–457); **C3 (N→TMD-2 $VPAC_2$-$VPAC_1$)**, $VPAC_2$ receptor (1–178) and $VPAC_1$ receptor (195–457); **C4 (N→EC_1 $VPAC_2$-$VPAC_1$)**, $VPAC_2$ receptor (1–203) and $VPAC_1$ receptor (217–457); **C5 (N→TMD-3 $VPAC_2$-$VPAC_1$)**, $VPAC_2$ receptor (1–227) and $VPAC_1$ receptor (241–457); **C6 (N→TMD-4 $VPAC_2$-$VPAC_1$)**, $VPAC_2$ receptor (1–261) and $VPAC_1$ receptor (275–457); **C7 (N→EC_2 $VPAC_2$-$VPAC_1$)**, $VPAC_2$ receptor (1–279) and $VPAC_1$ receptor (294–457); **C8 (N→EC_3 $VPAC_2$-$VPAC_1$)**, $VPAC_2$ receptor (1–360) and $VPAC_1$ receptor (374–457).

The strategy was based on the use of PCR overlap extension. Briefly : (i) using human $VPAC_2$ and $VPAC_1$ receptor cDNA as templates and appropriate chimaeric primers, we first generated cDNA fragments overlapping at their 5′ or 3′ extremity; (ii) after purification of these fragments, using High Pure PCR Product Purification Kit (Boehringer, Mannheim), they were used in a round of PCR overlap extension. The use of a phosphorylated forward primer surrounding the ATG initiation codon produced a 5′ hemi-phosphorylated cDNA fragment. This particularity combined with the presence of a 3′-A overhang resulting from the terminal transferase activity of Taq polymerase, allowed the unidirectional cloning of the chimaeric receptors in pCR3.1-Uni (Invitrogen) that was suitable for both prokaryotic and eukaryotic expressions. All PCR reactions were performed using Expand Long Template system (Boehringer, Mannheim) in the Geneamp 2400 thermocycler (Perkin-Elmer). Successful construction of chimaeras was confirmed by nucleotide sequence determination.

Receptor expression-stable transfection

Recombinant plasmids were transfected into CHO cells by electroporation using a gene pulser (Electroporator II, Invitrogen, San Diego, CA, U.S.A.). Briefly, 4.10^6 cells were preincubated on ice for 10 min with 20 μg of plasmid DNA in 0.25 ml of F12 nutrient mixture without serum (Gibco Life Technologies, Gent, Belgium). Electroporation was performed at 330 V and 1000 μF. After electroporation, cells were kept on ice for 10 min and then transferred into Petri dishes containing 10 ml of complete culture medium (Dulbecco-F12 supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 $\mu g\ ml^{-1}$ penicillin and 100 $\mu g\ ml^{-1}$ streptomycin). After 48 h, cells were selected by addition of geneticin (400 $\mu g\ ml^{-1}$) for 2 weeks. Resistant cells were cloned and the final selection was made on the basis of their ability to express VIP stimulated adenylate cyclase activity. The CHO cells were routinely grown in Dulbecco-F12 medium enriched with 10% foetal bovine serum and geneticin was maintained in the stock cultures only.

Peptide synthesis

All the non-cyclic peptides were synthesized as carboxyl-terminal amides by solid phase methodology using the Fmoc (9-fluorenylmethoxy carbonyl) strategy on an Applied Biosystems Apparatus 431A (Foster City, CA, U.S.A.). The cleavage and the purification of the peptides have already been described (Robberecht *et al*., 1992). The purity of the material was at least 95% as judged by capillary electrophoresis and analytical reverse-phase chromatography and the peptide conformity was assessed by electrospray mass spectrometry and, when possible, by sequencing.

Membrane preparation and receptor identification

Stably transfected CHO cells were harvested with a rubber policeman and pelleted by low speed centrifugation; the supernatant was discarded and the cells lysed in 1 mM $NaHCO_3$ solution and immediately frozen in liquid nitrogen. After thawing, the lysate was first centrifuged at 4°C for 10 min at $400 \times g$ and the supernatant was further centrifuged at $20{,}000 \times g$ for 10 min. The pellet, resuspended in 1 mM $NaHCO_3$ was used immediately as a crude membrane fraction. Binding was performed as described (Ciccarelli *et al*., 1994; Vertongen *et al*., 1997). In all cases, non-specific binding was defined as the residual binding in the presence of 1 μM VIP. Binding was performed at 37°C in a 20 mM (pH 7.4) Tris-maleate buffer with 2 mM $MgCl_2$, 0.1 mg ml^{-1} bacitracin and 1% bovine serum albumin. Bound radioactivity was separated from free by filtration through glass-fibre GF/C filters pre-soaked for 24 h in 0.01% polyethyleneimine and rinsed three times with a 20 mM (pH 7.4) sodium phosphate buffer containing 1% bovine serum albumin.

Tracer choice

We confirmed in the present work that ^{125}I-RO 25-1553 had a 3 fold higher affinity than ^{125}I-VIP for the $VPAC_2$ receptor (Vertongen *et al*., 1997), as well as for chimaeras C7 and C8. Consequently, the results obtained with the former tracer were technically more satisfactory (higher total over non-specific binding ratio). We therefore used [^{125}I]-VIP for characterization of the $VPAC_1$, C1 and C4 receptors, and [^{125}I]-RO 25-1553 (Ac^1-[Glu^8, Lys^{12}, Nle^{17}, Ala^{19}, Asp^{25}, Leu^6, $Lys^{27,28}$, $Gly^{29,30}$, Thr^{31}]-NH_2(cyclo 21–25)) for characterization of the $VPAC_2$, C7 and C8 receptors. At the tracer concentrations used in these experiments (0.2–0.3 fold their K_d values), the unlabelled peptides IC_{50} values are very close to their inhibition constants, K_i, and should therefore be independent of the nature of the tracer used. We verified that the unlabelled peptides IC_{50}

values were indeed independent of the tracer (^{125}I-RO 25-1553 or ^{125}I-VIP) chosen (not shown).

Specific tracer binding is proportional to the B_{max} over K_D ratio: it should theoretically be possible to compensate for low tracer affinities by increasing the membrane (and receptor) concentration. Unfortunately, non-specific ^{125}I-RO 25-1553 or ^{125}I-VIP binding also increased with increasing protein concentrations. We were therefore unable to obtain usable competition curves on CHO cell membranes expressing chimaeras C2, C3, C5 and C6, probably because their affinities for ^{125}I-VIP and ^{125}I-RO 25-1553 were too low.

Measurement of adenylate cyclase activity

Adenylate cyclase activity was determined according to the procedure of Salomon *et al.* (1974) on membranes from CHO cells stably transfected with the recombinant receptors. Membrane proteins (3–10 μg) were incubated in a total volume of 60 μl containing (mM): [α^{32}P]-ATP 0.5, GTP 10, $MgCl_2$ 5, EGTA 0.5, cyclic AMP 1, theophylline 1, phospho(enol)pyruvate 10, pyruvate kinase 30 μg ml^{-1} and Tris-HCl 30 mM at a final pH of 7.8. The reaction was initiated by adding membranes and was terminated after a 15 min incubation at 37°C by adding 0.5 ml of a 0.5% sodium dodecyl-sulphate solution containing ATP 0.5 mM, cyclic AMP 0.5 mM and 20,000 c.p.m. [8-^{3}H]-cyclic AMP. Cyclic AMP was separated from ATP by two successive chromatographies on Dowex 50 W $\times$ 8 and neutral alumina.

Receptor density: absence of spare receptors?

The adenylate cyclase EC_{50} value may underestimate the full agonists K_{act} if the receptor is expressed at a very high density (spare receptors). The maximal responses to VIP and PACAP (1–27) were comparable for all the constructs studied in this work, suggesting that, like wild type receptors, the chimaeric receptors could be expressed at high levels by the CHO cells. It is difficult to estimate correctly the density of VPAC receptors, because our tracers (labelled agonists) discriminate at least two receptor states in transfected CHO cells (Busto *et al.*, 1999). We therefore used functional criteria to ensure that the results were not affected by the presence of spare receptors. Whenever binding studies were possible ($VPAC_1$, $VPAC_2$, C1, C4, C7 and C8 receptors), we verified that the IC_{50} and EC_{50} values were in reasonable agreement ($EC_{50} \geqslant IC_{50}$). RO 25-1553 behaves as a good partial agonist on clones expressing low human $VPAC_1$ receptor densities, and fully activates the adenylate cyclase at high $VPAC_1$ receptor expression levels (Gourlet *et al.*, 1997b). The observation that it behaved as a partial agonist on chimaeras C2 to C6 therefore supported the hypothesis that the clones selected for this study did not express spare receptors.

Table 1 Comparative amino acid sequences of the peptide ligands

Non selective ligands	
VIP	HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH$_2$
PACAP (1–27)	HSDGIFTDSYSRYRKQMAVKKYLAAVL-NH$_2$
[R^{16}]-PACAP (1–27)	HSDGIFTDSYSRYRKRMAVKKYLAAVL-NH$_2$
$VPAC_1$ selective ligands	
[R^{16}]-PACAP (1–23)	HSDGIFTDSYSRYRKRMAVKKYL-NH$_2$
[L^{22}]-VIP	HSDAVFTDNYTRLRKQMAVKKLLNSILN-NH$_2$
VIP_1 agonist	HSDAVFTNSYRKVLKRLSARKLLQDIL-NH$_2$

Data analysis

All competition- and dose-effect curves were analysed by a non-linear regression program (Graph Pad Prism). They were compatible with recognition of a single binding site. IC_{50} and EC_{50} values were the ligand concentrations required for half maximal inhibition of tracer binding and half maximal adenylate cyclase activation, respectively. In order to calculate the peptide-receptor interaction free energy, we used the van't Hoff relationship: $\Delta G^0 = -RT \ln(EC_{50} \text{ or } IC_{50})$, where R is the gas constant and T, the absolute temperature. $\Delta\Delta G^0$ represents the difference between the control and modified peptide binding or activation free energies. Differences between mean IC_{50}, EC_{50} values were tested by Student's *t*-test. $P<0.05$ was accepted as being significant. The IC_{50} and EC_{50} standard deviations were always below 0.1 log units (typically, 0.05–0.08 log units). We therefore assumed that the standard deviations of the $\Delta\Delta G^0$ values (corresponding to an IC_{50} or EC_{50} ratios s.d. <0.2 log unit) were below 1.17 kJ mol^{-1} K^{-1}.

Results

VIP, PACAP and analogues were tested on recombinant human $VPAC_1$, $VPAC_2$, and chimaeric receptors for their ability to bind to the receptors and to activate adenylate cyclase in membranes from transfected CHO cells. The ligands and a schematic representation of the chimaeric receptors are shown in Table 1 and in the Figures, respectively.

VIP, PACAP (1–27) and [R^{16}]-PACAP (1–27) are considered as non-selective reference ligands. As previously published, the $VPAC_1$ receptor had 5–10 fold lower IC_{50} and EC_{50} values for the three agonists than the $VPAC_2$ receptor. Surprisingly, four chimaeric receptors (C2, C3, C5 and C6) showed much higher EC_{50} values for adenylate cyclase activation than the two wild-type receptors, and ^{125}I-VIP or ^{125}I-RO 25–1553 binding could not be valuably studied on these receptors (see Methods). If we consider only the chimaeric receptors on which binding studies were possible, the $VPAC_1$ receptor sequence 294–373 (TMD-5 to EC_3) seemed responsible for the higher affinity of the $VPAC_1$ receptor for these three agonists.

In order to identify the receptor region(s) that interact with the C-terminal peptide sequences, we compared a mono-substituted VIP analogue ([L^{22}]-VIP) with VIP, and the carboxyl-terminally truncated ligand ([R^{16}]-PACAP (1–23)) with [R^{16}]-PACAP (1–27).

[L^{22}]-VIP had a 10 fold lower affinity than VIP for $VPAC_1$ receptors, and a 120 fold lower affinity than VIP for $VPAC_2$ receptors. The effect of the [Y]→[L^{22}]-VIP substitution was 3–4 fold greater on chimaeras C1 and C4 than on $VPAC_1$ receptor, and 10 to 20 fold greater on chimaeras C7 and C8 (Figure 1 and Table 2). Representing the differences between the VIP-receptor interaction free energy and the [L^{22}]-VIP-receptor interaction free energy ($\Delta\Delta G^0$) in the wild type and the different chimaeric receptors, we observed two 'steps' in the binding affinity loss: one between $VPAC_1$ and C1 and the other between C6 and C7 (Figure 2, top). This suggested that the VIP tyrosine 22 recognized the $VPAC_2$ receptor N-terminal and EC_2 region. Comparison of the VIP and [L^{22}]-VIP EC_{50} values (adenylate cyclase activation) further supported this hypothesis.

The other selective ligand tested, [R^{16}]-PACAP (1–23) had a similar (3 fold lower) affinity as the related non selective peptide [R^{16}]-PACAP (1–27) for $VPAC_1$ and C1 receptors, but had a 25 fold lower affinity for C4, and was 100–150 fold less

potent on chimaeras C7, C8 and $VPAC_2$ receptors (Table 3 and Figure 3 for binding data). It is possible to estimate the contribution of aminoacids 24–27 to $[R^{16}]$-PACAP (1–27) recognition by calculating the difference between the binding free energy of $[R^{16}]$-PACAP (1–27) and $[R^{16}]$-PACAP (1–23) ($\Delta\Delta G^{\circ}$). As shown in Figure 2 (bottom), the receptor amino terminus did not contribute to the discrimination between $[R^{16}]$-PACAP (1–23) and $[R^{16}]$-PACAP (1–27) ($VPAC_1 \rightarrow$ C1). The chimaeric receptor's ability to discriminate the two peptides increased progressively, from C1 to C8; the last chimaera behaved like $VPAC_2$ receptors in this respect. Functional data (adenylate cyclase activation) on the eight chimaeric receptors did not allow us to pinpoint more accurately the receptor regions(s) involved in the C-terminal PACAP recognition.

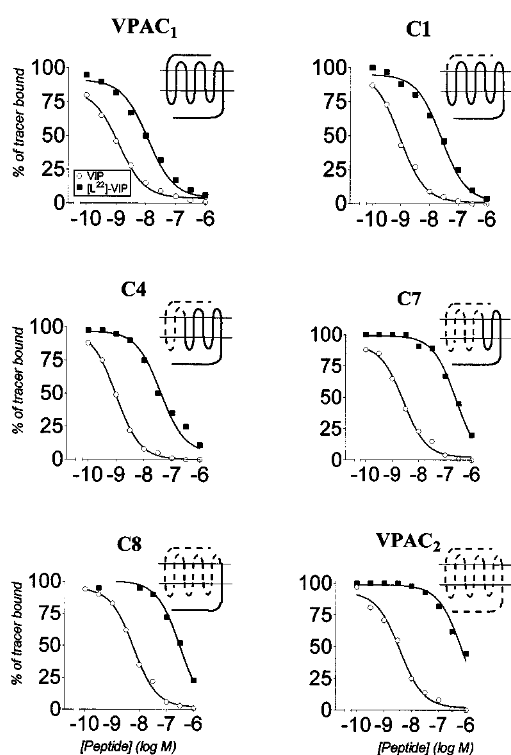

Figure 1 Effect of VIP and $[L^{22}]$-VIP on tracer binding to membranes from CHO cells expressing the $VPAC_1$, $VPAC_2$ and $VPAC_2$-$VPAC_1$ chimaeric receptors C1, C4, C7 and C8. The values were the means of at least three experiments made in duplicate. The data are expressed as per cent of specific tracer binding in the absence of added ligand.

Discussion

It has been established, before the cloning of the receptors, that VIP acts through at least two functionally distinct receptors: the 'classical' VIP receptor, exemplified by the receptor of the intestinal epithelial cells, now known as the $VPAC_1$ receptor and the helodermin-preferring VIP receptor, exemplified by the receptor of the lymphoblastic cell line SUP T_1 and now named $VPAC_2$ receptor.

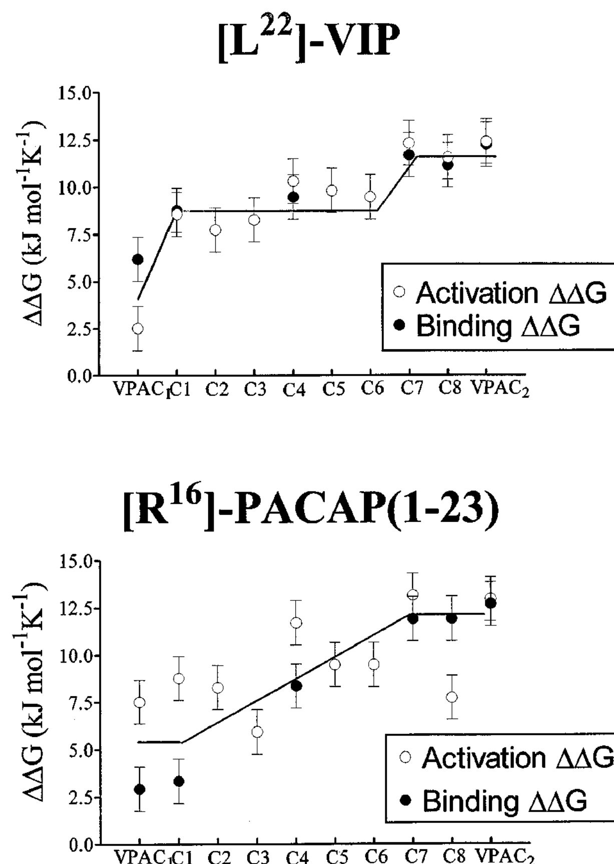

Figure 2 Top panel: The differences between the free energy of binding or activation (±s.d.) of the different constructs by VIP and $[L^{22}]$-VIP were calculated from at least three experiments. The following binding $\Delta\Delta G^0$ differences were ($\neq$) or were not ($=$) significantly different: (1): $VPAC_1$, $\neq C1 = C4 \neq C7 = C8 = VPAC_2$. Bottom panel: The differences between the free energy of binding or activation (±s.d.) of the different constructs by $[R^{16}]$-PACAP (1–27) and $[R^{16}]$-PACAP (1–23) were calculated from at least three experiments. The following binding $\Delta\Delta G^0$ differences were ($\neq$) or were not ($=$) significantly different: $VPAC_1 = C1 \neq C4 \neq C7 = C8 = VPAC_2$.

Table 2 Interaction of the VIP and $[L^{22}]$-VIP with the $VPAC_1$, $VPAC_2$ and $VPAC_2$-$VPAC_1$ chimaeric receptors (C1 to C8)

	Receptors									
Ligands	*$VPAC_1$*	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*	*C7*	*C8*	*$VPAC_2$*
VIP										
IC_{50}	1.0	0.9	–	–	1.0	–	–	2.6	4.0	6.0
EC_{50}	3.0	1.0	50.0	60.0	1.8	22.0	50.0	25.0	11.0	8.0
$[L^{22}]$-VIP										
IC_{50}	11.0	27.0	–	–	40	–	–	250	300	700
EC_{50}	8.0	30.0	1000	1500	100	1000	2000	3000	1000	1000

The IC_{50} and EC_{50} were expressed in nM. Data were obtained on stably transfected CHO cell membranes. ^{125}I-VIP was the tracer used for the $VPAC_1$, C1 and C4 receptors. ^{125}I-RO 25-1553 was used for C7, C8 and the $VPAC_2$ receptors. '–' when not determined due to absence of tracer binding. The standard error for the IC_{50} and EC_{50} values (on a logarithmic scale), were always lower than 0.1 log unit.

The importance of the receptor NH_2-terminus for selective ligand recognition has already been shown for other related receptors: chimaeric constructions between the rat secretin and the rat $VPAC_1$ receptors, between the rat $VPAC_1$ and rat PACAP (PAC_1) receptors, and between human $VPAC_1$ and $VPAC_2$ receptors (Van Rampelbergh *et al*., 1996; Gourlet *et al*., 1996c,d; Vilardaga *et al*., 1996; Cao *et al*., 1995; Hashimoto *et al*., 1997; Holtmann *et al*., 1995; 1996a,b; Juarranz *et al*., 1999a,b) indicated that in all cases, the NH_2-terminal domain is a major contributor to the ligand selectivity.

The 'Ala-Scan' of VIP performed by O'Donnell *et al*. (1994) focused our attention on the importance of amino acid [Y^{22}]. By substituting the tyrosine residue present in VIP by a leucine or an alanine residue (Gourlet *et al*., 1998), single mutated VIP analogues selective for the $VPAC_1$ receptors were obtained. The search for the shortest VIP or PACAP sequence that binds to the VIP and PACAP receptors led to the discovery that the carboxyl-terminally shortened peptides also have a preference for the $VPAC_1$ receptor (Gourlet *et al*., 1996b), and that PACAP (1–23) retained a relatively high affinity for the $VPAC_1$ receptor but not for the $VPAC_2$ receptor. Furthermore, the introduction in VIP and PACAP analogues of an arginine residue in position 16 increased their affinities for both VIP receptors (Gourlet *et al*., 1996a). [R^{16}]-PACAP (1–23) was synthesized and behaved as a selective, high affinity $VPAC_1$ receptor ligand (this study).

The aim of the present work was to identify the receptor region(s) recognized by the C-terminal ligand sequence. To achieve this goal, we measured the ability of chimaeric receptors to discriminate two selective ligands, [L^{22}]-VIP and [R^{16}]-PACAP (1–23) from the corresponding non-selective ligands VIP and [R^{16}]-PACAP (1–27), respectively. Chimaeras C1, C4, C7 and C8 had a good affinity for VIP, PACAP (1–27) and [R^{16}]-PACAP (1–27) in binding and functional studies. The chimaeric receptors C2, C3, C5 and C6, in contrast, had surprisingly low affinities for VIP and all the ligands tested: specific binding of the radioiodinated tracers was poor to undetectable, and the peptides potencies (EC_{50}^{-1}) for adenylate cyclase activation were very low (Tables 2 and 3).

The non selective peptides VIP, PACAP (1–27) and [R^{16}]-PACAP (1–27) had a slightly weaker affinity and potency for $VPAC_2$ receptor as compared to $VPAC_1$ receptor. The binding determinant(s) involved appeared to belong to the receptor's transmembrane domain, between TMD-5 and EC_3, as chimaeras C1, C4 and C7 had binding and functional properties equivalent to $VPAC_1$, and chimaera C8 to the $VPAC_2$ receptor. We are unfortunately not able to be more precise: the intermediate chimaeras, C5 and C6, had a very weak affinity for the three ligands, suggesting that one of their common anchoring points was unavailable in these constructions. This might be due to incompatibilities between the

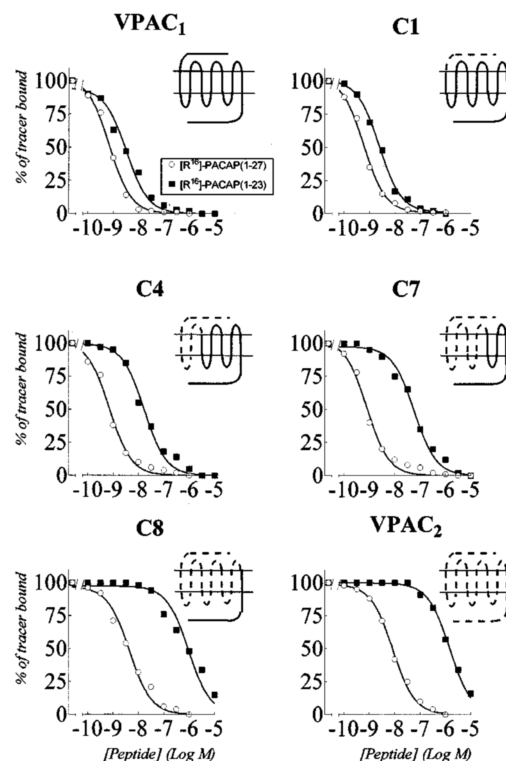

Figure 3 Effect of [R^{16}]-PACAP (1–27) and [R^{16}]-PACAP (1–23) on tracer binding to membranes from CHO cells expressing the $VPAC_1$, $VPAC_2$ and $VPAC_2$-$VPAC_1$ chimaeric receptors C1, C4, C7 and C8. The values were the means of at least three experiments made in duplicate. The data are expressed as per cent of specific tracer binding in the absence of added ligand.

Table 3 Interaction of PACAP (1–27), [R^{16}]-PACAP (1–27) and [R^{16}]-PACAP (1–23) with the $VPAC_1$, $VPAC_2$ and $VPAC_2$-$VPAC_1$ chimaeric receptors (C1 to C10)

	Receptors									
Ligands	*$VPAC_1$*	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*	*C7*	*C8*	*$VPAC_2$*
PACAP (1–27)										
IC_{50}	0.6	0.5	–	–	5.0	–	–	5.0	15.0	15.0
EC_{50}	0.8	0.5	40.0	30.0	0.8	8.0	8.0	10.0	10.0	8.0
[R^{16}]-PACAP (1–27)										
IC_{50}	0.8	0.6	–	–	0.6	–	–	1.0	4.0	8.0
EC_{50}	0.1	0.2	20.0	20.0	0.2	5.0	5.0	3.0	5.0	5.0
[R^{16}]-PACAP (1–23)										
IC_{50}	2.5	2.3	–	–	15	–	–	100	400	1200
EC_{50}	2.0	5.7	500	200	18.0	200	200	500	100	700

The IC_{50} and EC_{50} were expressed in nM. Data were obtained on stably transfected CHO cell membranes. ^{125}I-VIP was the tracer used for the $VPAC_1$, C1 and C4 receptors. ^{125}I-RO 25-1553 was used for C7, C8 and the $VPAC_2$ receptors. '–' when not determined due to absence of tracer binding. The standard error for the IC_{50} and EC_{50} values (on a logarithmic scale), were always lower than 0.1 log unit.

$VPAC_2$ extracellular loops and $VPAC_1$ transmembrane domain: the extracellular loops EC_1 and EC_2, that are probably involved in two or three disulphide bridges (Vilardaga *et al*., 1997), have different lengths and sequences in the two receptors.

$[L^{22}]$-VIP, with a single (tyrosine to leucine) mutation in position 22, had an 11 fold lower affinity than VIP on $VPAC_1$ receptors and 120 fold lower affinity than VIP on $VPAC_2$ receptors. As shown in Table 2, the introduction of the N-terminal $VPAC_2$ receptor domain was sufficient to decrease the $[L^{22}]$-VIP affinity, as compared to VIP. This observation is in line with the results of Dong *et al*. (1999) indicating that the $[L^{22}]$ of secretin is close to the receptor amino-terminal tail in the secretin-receptor complex. A further decrease of $[L^{22}]$-VIP affinity and potency was observed between chimaeras C4 and C7, the latter chimaeric receptor having an affinity ratio comparable to $VPAC_2$ receptors: Leu^{22} probably interacted, in addition, with a region found between EC_1 and TMD-5, that is, with TMD-3, TMD-4 or EC_2. We made the assumption that, as suggested above, chimaeras C5 and C6 had lost a 'common contact' with all the peptides (contributing approximately 6.3 kJ mol^{-1} to the peptide-receptor interaction). We calculated the $[L^{22}]$-VIP over VIP EC_{50} ratios to estimate the additional contribution of Tyr^{22} to VIP recognition by the different chimaeras (Figure 2, top). Our results supported the hypothesis that Tyr^{22} was in contact with EC_2 (C6→C7), rather than with TMD-3 or TMD-4.

It is interesting to compare the present results with our previous work on the VIP_1 agonist (Table 1) (Juarranz *et al*., 1999a). The $VPAC_1 > VPAC_2$ selectivity of the agonist peptide was due for the most part to preferential recognition of the $VPAC_1$ N-terminal and EC_1 domains, with a (smaller) contribution of a more distal receptor domain (probably EC_2: unpublished data). The Tyr→Leu replacement in position 22 explained the importance of EC_2, and part of the contribution of the N-terminal domain for the VIP_1-agonist selectivity. Further studies are needed to identify the additional positions that contribute to the $VPAC_1 > VPAC_2$ selectivity of the VIP_1-agonist.

In contrast with $[L^{22}]$-VIP, $[R^{16}]$-PACAP (1–23) had the same high affinities for the $VPAC_1$ receptor and chimaera C1. It therefore seems likely that the 3-dimensional structure of $[R^{16}]$-PACAP (1–23) was conserved, allowing the correct positioning of Tyr^{22} with respect to the N-terminal receptor domain. The region involved in the $[R^{16}]$-PACAP (1–23) selectivity (Table 3 and Figure 2, bottom) was the TMD-1 to EC_3 region. This result supported the hypothesis that the receptor core (TMD helices + EC loops) may participate to the recognition of the C-terminal sequence (amino acids 22–27 or 28) of the ligand.

The implication of both the NH_2-terminal domain and of TMD-1 to EC_2 in the selectivity of the $VPAC_1$-preferring peptides studied in this work suggests that the ligands might be 'sandwiched' between the amino-terminal domain and the upper extracellular part of the core of the receptor. Alternatively, it is also possible that the extracellular loops play an important role in the folding of the N-terminal domain and in its positioning with respect to the peptide ligand.

The present results, taken together with published experimental data, allowed us to propose a general model of the peptide-receptor interaction, as follows: (1) N-terminal peptide binding. VIP, PACAP and secretin have identical sequences in positions 1–3, 6 and 7. We previously suggested that the aspartic acid in position 3 of secretin recognizes highly conserved amino acids in the secretin receptor TMD-2 (Vilardaga *et al*., 1996), corresponding to Lysine 173 and arginine 188 in the $VPAC_1$ receptor and to lysine 179 and arginine 172 in the $VPAC_2$ receptor. The hypothesis that the VIP amino terminal region (like secretin's) interacts with the transmembrane receptor domain is further supported by the observation that acylation of $Histidine^1$, that increases VIP's affinity for $VPAC_2$ receptor but not $VPAC_1$ receptors, did not increase the peptides' affinity for the $VPAC_2$-$VPAC_1$ chimaeric receptor (C1) but did increase its affinity for the 'mirror image' chimaera (Juarranz *et al*., 1999a); (2) Aminoacids 8–15. We previously demonstrated, using a series of hybrid PACAP and secretin peptides and rat $VPAC_1$ and secretin chimaeric receptors that the positions 8 to 15 of secretin and PACAP interacted preferentially with the secretin and $VPAC_1$ receptors N-terminal domains, respectively (Gourlet *et al*., 1996b,c); (3) Aminoacids 22–27. Our present results suggest that the VIP carboxyl terminal region folds back towards the transmembrane region: Tyrosine 22 recognition was affected by the N-terminal domain and EC_2, and the C-terminal PACAP sequence (Ala^{24}, Ala^{25}, Val^{26}, Leu^{27}), necessary for high affinity recognition of the $VPAC_2$ transmembrane receptor domain (Gourlet *et al*., 1996b,d); (4) C-terminal extended peptides. The $VPAC_2$-selective peptide, RO 25-1553, differs from [acetyl-His^1]VIP in positions 9, 12, 19, 21 and 25–31. In contrast with $[R^{16}]$-PACAP (1–23) recognition: the transmembrane receptor domain did not contribute to the selective RO 25-1553-$VPAC_2$ receptor interaction (Juarranz *et al*., 1999b). This suggests that Ala^{12}, Lys^{19} and the C-terminal extension of RO 25-1553, that markedly contribute to its selectivity interact with the extracellular N-terminal domain (in preparation).

Taken together, our results suggest that the different peptides that discriminate $VPAC_1$ from $VPAC_2$ receptors do so for different reasons, and can be used to visualise the relative receptor-ligand positioning. They are compatible with the hypothesis that the N-terminal peptide sequence forms a turn that is buried in the transmembrane domain; that amino acids 8–22 interact with the extracellular domain but fold back towards the extracellular loops, in order to allow amino acids 24–27 to interact with the transmembrane domain.

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