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Different Vasoactive Intestinal Polypeptide Receptor Domains Are Involved in the Selective Recognition of Two $VPAC_2$-Selective Ligands

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ABSTRACT

A vasoactive intestinal polypeptide (VIP) analog, acylated on the amino-terminal histidine by hexanoic acid (C_6-VIP), behaved as a $VPAC_2$ preferring agonist in binding and functional studies on human VIP receptors, and radioiodinated C_6-VIP was a suitable ligand for binding studies on wild-type and chimeric receptors. We evaluated the properties of C_6-VIP, its analog $AcHis^1$-VIP, and the $VPAC_2$-selective agonist Ro 25-1553 on the wild-type $VPAC_1$ and $VPAC_2$ receptors and on the chimeric receptors exchanging the different domains between both receptors. VIP had a normal affinity and efficacy on the chimeras starting with the amino-terminal $VPAC_2$ receptor sequence. The binding and functional profile of these chimeric receptors suggested that the high affinity of Ro 25-1553 for $VPAC_2$ receptors is supported by the amino-terminal extracellular domain, whereas the ability to prefer C_6-VIP over VIP is supported by the $VPAC_2$ fifth transmembrane (TM5)-EC_3 receptor domain. These results further support the hypothesis that the central and carboxyl-terminal regions of the peptide (modified in RO 25-1553) recognize the extracellular amino-terminal region domain, whereas the amino-terminal VIP amino acids bind to the TM receptor core. VIP had a reduced affinity and efficacy on the N-$VPAC_1$/$VPAC_2$ and on the N→EC_2-$VPAC_1$/$VPAC_2$ chimeric receptors. C_6-VIP behaved as a high-affinity agonist on these constructions. The antagonists [$AcHis^1$,D-Phe^2,Lys^{15},Arg^{16},Leu^{27}]VIP(3-7)/GRF(8-27) and VIP(5-27) had comparable affinities for the wild-type receptors and for the two latter chimeras, supporting the hypothesis that these chimeras were properly folded but unable to reach the high-agonist-affinity, active receptor conformation in response to VIP binding.

The 28-amino-acid neuropeptide vasoactive intestinal polypeptide (VIP) acts through two distinct receptors named $VPAC_1$ and $VPAC_2$ (Harmar et al., 1998). These two G protein-coupled, seven-TM-helix receptors have only 51% similarity (Couvineau et al., 1994; Svoboda et al., 1994) but recognize with a comparable, high-affinity VIP and the pituitary adenylate cyclase-activating polypeptide (PACAP). $VPAC_1$ and $VPAC_2$ receptors are differentially distributed in tissues (Ishihara et al., 1992; Usdin et al., 1994; Vertongen et al., 1997) and cell lines (Vertongen et al., 1996).

Two selective $VPAC_2$ receptors agonists were recently discovered: Ro 25-1553 (Gourlet et al., 1997a) and Ro 25-1392 (Xia et al., 1997) are cyclic analogs that differ from VIP by acetylation of the amino terminus, some mutations in the core of the peptide, the presence of a lactam bridge, and two additional lysine residues in the carboxyl terminus. There are no known selective high-affinity antagonist for the $VPAC_2$ receptors.

Two selective $VPAC_1$ receptor agonists have also been recently developed: one of these agonists is a derivative of the chicken secretin molecule, and the second, a hybrid peptide with the VIP amino terminus and a growth hormone-releasing peptide (GRF)-like carboxyl-terminal sequence (Gourlet et al., 1997b). A selective $VPAC_1$ receptor antagonist was also obtained through the single substitution of the VIP/GRF hybrid peptide (Gourlet et al., 1997c).

The group of Gozes recently described lipophilic VIP derivatives (Gozes and Fridkin, 1992; Gozes et al., 1994) and observed that the lipophilic VIP derivative obtained through the amidation of the amino terminus of VIP with an 18-carbon fatty acid (stearyl VIP) was 100-fold more potent than VIP in promoting neuronal cell survival in dissociated spinal cord cells (Gozes et al., 1995) but did not increase cAMP levels. We observed that this compound recognizes the recombinant rat and human $VPAC_1$ and $VPAC_2$ receptors with

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ABBREVIATIONS: VIP, vasoactive intestinal polypeptide; PACAP, pituitary adenylate cyclase-activating polypeptide; GRF, growth hormone-releasing peptide; C_6-VIP, hexanoyl-VIP; CHO, Chinese hamster ovary; TM, transmembrane; $AcHis^1$, acetyl-His^1.

an affinity comparable to that of VIP and with a preference for the $VPAC_2$ over the $VPAC_1$ receptor but behaves like a partial agonist on adenylate cyclase stimulation (Gourlet et al., 1998). Related compounds like myristoyl- and palmitoyl-VIP also had a low intrinsic activity and behaved as $VPAC_2$-preferring partial agonists and/or competitive antagonists. These results suggested that acylation of the amino terminus might contribute to the $VPAC_2$ receptor selectivity of lipophilic VIP derivatives, Ro 25-1553 and Ro 25-1392, but that the length of the alkyl chain acylating the amino-terminal histidine residue may influence the intrinsic activity of the peptides.

These results prompted us to synthesize other derivatives, acylated at the amino terminus but with a shorter acyl chain. The hexanoyl-VIP (C_6-VIP) described in the present study had a 4-fold lower and 3-fold higher affinity than VIP for the human $VPAC_1$ and $VPAC_2$ receptors, respectively, and stimulated maximally the adenylate cyclase activity. The binding and functional properties of this new $VPAC_2$ preferring agonist were compared with the properties of Ro 25-1553 and acetyl-His^1- ($AcHis^1$)-VIP. By testing the ability of these agonists to recognize chimeric receptors, consisting of the interchange of the different domains of one receptor subtype grafted onto the core of the other receptor subtype, we were able to establish that the $VPAC_2$ selectivities of Ro 25-1553 and C_6-VIP were supported by different domains of the receptors.

Materials and Methods

All of the experiments were conducted on Chinese hamster ovary (CHO) cell membranes expressing the recombinant wild-type human $VPAC_1$, $VPAC_2$, or mutated receptors. The cell lines expressing the $VPAC_1$ and $VPAC_2$ receptors have been detailed previously (Ciccarelli et al., 1994; Svoboda et al., 1994). All chimeric receptors were constructed by the PCR overlap extension strategy. Briefly, cDNA fragments overlapping at their 5′ or 3′ extremity were generated using human $VPAC_1$ and $VPAC_2$ receptor cDNA as templates and appropriate chimeric primers. After purification of these fragments using the High Pure PCR Product Purification Kit (Boehringer-Mannheim Biochemica, Mannheim, Germany), they were used in a round of PCR overlap extension. The use of a phosphorylated forward primer surrounding the ATG initiation codon allowed us to obtain a 5′ hemiphosphorylated cDNA fragment. This particularity combined with the presence of a 3′-A overhang resulting from the terminal transferase activity of *Taq* polymerase, allowing the unidirectional cloning of the chimeric receptors in pCR 3.1-Uni (InVitrogen, San Diego, CA), suitable for both prokaryotic and eukaryotic expressions. The first series of chimeric receptor was generated by the substitution of different domains in the $VPAC_2$ receptor by the counterpart of $VPAC_1$ receptor. N-$VPAC_1$/$VPAC_2$ chimera was generated by combining codons 1 to 143 of the $VPAC_1$ receptor with codons 128 to 438 of the $VPAC_2$ receptor. The N→EC_1-$VPAC_1$/$VPAC_2$ combines codons 1 to 216 of the $VPAC_1$ receptor with codons 204 to 438 of $VPAC_2$ receptor. The receptor chimeric N→EC_2-$VPAC_1$/$VPAC_2$ combines the codons 1 to 293 of $VPAC_1$ receptor with the codons 281 to 438 of $VPAC_2$ receptor. The latest chimeric receptor of this series (N→EC_3-$VPAC_1$/$VPAC_2$) was generated by combining codons 1 to 373 of the $VPAC_1$ receptor with the codons 361 to 438 of the $VPAC_2$ receptor. The other series of chimeric receptor was generated by the substitution of the different domain in the $VPAC_1$ receptor by the counterpart of $VPAC_2$ receptor. The N-$VPAC_2$/$VPAC_1$ construction combined the $VPAC_2$ receptor codons 1 to 127 with the 144 to 457 $VPAC_1$ receptor codons. The N→EC_1-$VPAC_2$/$VPAC_1$ construction was generated by combining the codons 1 to 203 of $VPAC_2$ receptor and the codons 217 to 457 of $VPAC_1$ receptor. The N→EC_2-$VPAC_2$/$VPAC_1$ chimera combined the codons 1 to 280 of the $VPAC_2$ receptor with the codons 297 to 457 of $VPAC_1$ receptor. And the last chimeric receptor (N→EC_3-$VPAC_2$/$VPAC_1$) has the codons 1 to 360 of the $VPAC_2$ receptor and the codons 374 to 457 of the $VPAC_1$ receptor. The polymerase chain reactions were performed using the Expand Long Template system (Boehringher, Mannheim) in the Geneamp 2450 thermocycler (Perkin-Elmer Cetus, Norwalk, CT). All sequences were verified using ABI Prism By Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Norwalk, CT).

The methodology for cell transfection, as well as the cell culture medium, has been detailed previously (Van Rampelbergh et al., 1996). The cell clones expressing the different constructions were selected by testing the ability of the 10 μM VIP to stimulate the adenylate cyclase. One of the constructs (N→EC_2-$VPAC_1$/$VPAC_2$) could not be identified by this method, so we also attempted to detect its expression in binding studies with ^{125}I-VIP, ^{125}I-C_6-VIP, and ^{125}I-VIP_1 antagonist with negative results.

Membranes were prepared from scraped cells lysed in 1 mM $NaHCO_3$ solution and immediate freezing in liquid nitrogen. After thawing, the lysate was first centrifuged at 4°C for 10 min at 400*g*; the supernatant was further centrifuged at 20,000*g* for 10 min. The pellet, resuspended in 1 mM $NaHCO_3$, was used immediately as a crude membrane fraction.

Binding studies were performed as described previously (Gourlet et al., 1997a) using either ^{125}I-VIP, ^{125}I-Ro 25-1553, or ^{125}I-C_6-VIP. The three tracers were radiolabeled similarly and had comparable specific radioactivity (Gourlet et al., 1997b). In all cases, the nonspecific binding was defined as residual binding in the presence of 1 μM VIP. Binding was performed at 37°C in a total volume of 120 μl containing 20 mM Tris-maleate, 2 mM $MgCl_2$, 0.1 mg/ml bacitracin, and 1% BSA (pH 7.4) buffer. From 3 to 30 μg of protein was used per assay. Bound and free radioactivity were separated by filtration through glass-fiber GF/C filters presoaked for 24 h in 0.01% polyethyleneimine and rinsed three times with a 20 mM (pH 7.4) sodium phosphate buffer containing 1% BSA.

Adenylate cyclase activity was determined according to the procedure of Salomon et al. (1974). Membrane proteins (3–15 μg) were incubated in a total volume of 60 μl containing 0.5 mM [α-^{32}P]ATP, 10 μM GTP, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cAMP, 1 mM theophylline, 10 mM phospho(enol)pyruvate, 30 μg/ml pyruvate kinase, and 30 mM Tris · HCl at final pH 7.8.

The peptides used were synthesized in our laboratory as described previously (Gourlet et al., 1997b, 1998). The 1-hydroxybenzotriazole derivative of hexanoic acid was coupled to the amino terminus of VIP before cleavage and deprotection. The peptide purity was assessed by capillary electrophoresis, and the conformity was assessed by electrospray mass spectrometry.

All competition curves and dose-effect curves were analyzed by a nonlinear regression program (Prism; GraphPAD, San Diego, CA). The differences between the IC_{50}, EC_{50}, and maximal efficacy values were tested for statistical significance by Student's *t* test; $P < .05$ was accepted as being statistically significant.

Results

Properties of C_6-VIP on Recombinant $VPAC_1$ and $VPAC_2$ Receptors. On $VPAC_2$ receptors, the inhibition curves were identical when ^{125}I-VIP and ^{125}I-Ro 25-1553 were used as tracers (data not shown). $AcHis^1$-VIP and C_6-VIP were 6- to 4-fold less potent than VIP in binding studies on human recombinant $VPAC_1$ receptors, but 2- to 3-fold more potent than VIP in binding studies on recombinant $VPAC_2$ receptors, respectively (Table 1). Ro 25-1553 was 1000-fold less potent and 4-fold more potent than VIP on the $VPAC_1$ and $VPAC_2$ receptors, respectively.

TABLE 1

IC_{50} and EC_{50} values of tracer binding inhibition and adenylate cyclase activation on the recombinant human $VPAC_1$, $VPAC_2$, N-$VPAC_2$/$VPAC_1$, N-$VPAC_1$/$VPAC_2$, and N → EC_1 $VPAC_1$/$VPAC_2$ receptors

The values (±S.D.) were the mean of at least three determination.

	$VPAC_1$		$VPAC_2$		N-$VPAC_2$/$VPAC_1$		N-$VPAC_1$/$VPAC_2$		N → EC_1 $VPAC_1$/$VPAC_2$	
	IC_{50}	EC_{50}	IC_{50}	EC_{50}	IC_{50}	EC_{50}	IC_{50}	EC_{50}	IC_{50}	EC_{50}
					nM					
VIP	0.5 ± 0.1[a]	0.5 ± 0.1[a]	3.0 ± 0.2[b]	15 ± 2.5[b]	0.5 ± 0.1	0.5 ± 0.1	100 ± 20[a,b]	50.0 ± 12[a,b]	80 ± 22	100 ± 25
Ro 25-1553	800 ± 200[a]	1000 ± 250[a]	1.5 ± 0.4[b]	5.0 ± 1.2[b]	5.0 ± 1.0[b]	3.0 ± 0.8[b]	3000 ± 800[a]	1000 ± 200[a]	200 ± 50	1000 ± 200[a]
Ac His^1 VIP	3.0 ± 0.7	10.0 ± 2.2	1.5 ± 0.3	5.0 ± 0.1	3.0 ± 0.1	8.0 ± 0.3	100 ± 15[a,b]	30.0 ± 8[a]	80 ± 10	100 ± 25
C_6-VIP	2.0 ± 0.3[c]	3.0 ± 0.7[a,c]	1.0 ± 0.1[c]	0.3 ± 0.1[b,c]	5.0 ± 1.2[a,c]	10.0 ± 1.5[a,c]	3.0 ± 0.8[c]	1.0 ± 0.2[c]	5.0 ± 1.0[c]	5.0 ± 0.9[c]
VIP(5-28)	2000 ± 220		300 ± 90[b]		3000 ± 800[a]		30 ± 12[b]	100 ± 25[b,d]	20 ± 7[b]	100 ± 15[b,d]
$VPAC_1$ antagonist	2.0 ± 0.3[a]	2.0 ± 0.3[c]	2000 ± 300		1000 ± 200[b]		8.0 ± 3.0[a]	15.0 ± 4[a,d]	3.0 ± 0.8	5.0 ± 1.1

[a] Values significantly different from the corresponding $VPAC_2$ values.
[b] Values significantly different from the corresponding $VPAC_1$ values.
[c] C_6-VIP EC_{50} and IC_{50} values significantly different from the corresponding VIP values.
[d] These values corresponded to the K_i values of inhibition of VIP-stimulated adenylate cyclase activity.

C_6-VIP increased adenylate cyclase activity on membranes expressing the $VPAC_1$ and $VPAC_2$ receptors. The maximal stimulation through both receptors was identical to that of VIP (Figs. 1 and 2); C_6-VIP was 6-fold less potent and 20-fold more potent than VIP on the $VPAC_1$ and $VPAC_2$ receptors, respectively. As previously reported (Gourlet et al., 1997a), Ro 25-1553 was a partial agonist on the $VPAC_1$ receptors (Fig. 1). AcHis^1-VIP was comparable to C_6-VIP on the $VPAC_1$ receptors but had a lower potency than C_6-VIP on the $VPAC_2$ receptors (Table 1).

Thus, C_6-VIP, like Ro 25-1553, was a superagonist compared with VIP on the $VPAC_2$ receptors.

Contribution of Amino-Terminal Portion and TM5 to EC_3 Region of Receptor to $VPAC_2$ Receptor Preference of Ro 25-1553 and C_6-VIP, Respectively. In a first set of experiments, we evaluated the properties of a chimeric receptor consisting in the amino-terminal (extracellular) $VPAC_2$ receptor domain followed by the seven-TM-helix domain of the $VPAC_1$ receptor (N-$VPAC_2$/$VPAC_1$). On that chimeric receptor, IC_{50} values of binding inhibition were 0.5, 3.0, 5.0, and 5.0 nM for VIP, AcHis^1-VIP, C_6-VIP, and Ro 25-1553, respectively (Fig. 1B and Table 1). Thus, the IC_{50} values of VIP, AcHis^1-VIP, and C_6-VIP were comparable to their $VPAC_1$ receptor IC_{50} values, and that of Ro 25-1553 was comparable to its $VPAC_2$ receptor IC_{50} value. In addition, the maximal stimulatory effect of Ro 25-1553 was higher than those of VIP, AcHis^1-VIP, and C_6-VIP. Thus, the amino-terminal domain of the $VPAC_2$ receptor was essential for high-affinity Ro 25-1553 recognition but not for that of C_6-VIP. In an attempt to identify the receptor region that supported the preferential C_6-VIP > VIP recognition by the $VPAC_2$ receptor, we tested additional chimeric receptors increasing the contribution of the $VPAC_2$ receptor sequence (Figs. 1C and 2, A and B). As shown in Figs. 1 and 2, chimeric receptors with the amino-terminal to EC_2-$VPAC_2$ receptor sequence (Fig. 2A) had, like $VPAC_1$ receptor, a preference for VIP over C_6-VIP. In contrast, the chimeric receptor with the amino-terminal to EC_3-$VPAC_2$ receptor sequence (Fig. 2B) preferred (like the $VPAC_2$ receptor; Fig. 2C) C_6-VIP over VIP. These results suggested that the hexanoyl anchoring point is somewhere between TM5 and EC_3.

In a second set of experiments, we attempted to express the "mirror image" chimeric receptors, starting with the $VPAC_1$ and followed by the complementary $VPAC_2$ receptor sequence (Fig. 3). The binding and functional properties of the last chimeric receptor (N→EC_3-$VPAC_1$/$VPAC_2$) were identical with wild-type $VPAC_1$ receptor (Fig. 3C). This result supported the hypothesis that the TM7 and carboxyl-terminal receptor region did not participate in the recognition of either Ro 25-1553 or C_6-VIP. We were unable to identify any clone expressing the N→EC_2-$VPAC_1$/$VPAC_2$ chimeric receptor (not shown) or to perform ^{125}I-VIP binding studies on the amino-terminal $VPAC_1$/$VPAC_2$ and N→EC_1-$VPAC_1$/$VPAC_2$ chimeric receptors. The EC_{50} value of VIP on these two chimeras for adenylate cyclase stimulation was close to 50 nM, significantly higher than its EC_{50} value for both wild-type receptors (Fig. 3, D and E, and Table 1). Ro 25-1553 was a partial agonist on these chimeras with an EC_{50} value of 1 μM. AcHis^1-VIP behaved as VIP (Table 1). C_6-VIP, in contrast, had a higher affinity and efficacy than VIP on these two chimeric receptors (Fig. 3, D and E, and Table 1).

We anticipated that radioiodinated C_6-VIP might be an

appropriate ligand to study the binding properties of these two chimeric receptor. Specific ^{125}I-C_6-VIP binding to the chimeric receptors was indeed measurable, and competition curves yielded IC_{50} values of 3, 100, 100, and 3000 nM for C_6-VIP, VIP, AcHis^1-VIP, and Ro 25-1553, respectively, in N-$VPAC_1$/$VPAC_2$ chimeric receptor (Fig. 3A and Table 1) and 5, 80, 80, and 200 mM for C_6-VIP, VIP, AcHis^1-VIP, and Ro 25-1553, respectively, in N→EC_1-$VPAC_1$/$VPAC_2$ chimeric receptors (Fig. 3B and Table 1). ^{125}I-C_6-VIP was also used as a tracer to characterize the $VPAC_1$, $VPAC_2$, and N-$VPAC_2$/$VPAC_1$ receptors. The competition curves for the four unlabeled peptides were not different from those obtained with ^{125}I-VIP or ^{125}I-Ro 25-1553 (data not shown).

The observations that VIP had a lower affinity for chimeras beginning with the $VPAC_1$ receptor sequence and that it behaved as a partial agonist with respect to C_6-VIP on these constructions suggested that the chimeric receptors were either misfolded or unable to reach the active receptor conformation in response to VIP. To discriminate between these two hypotheses, we measured the affinity of two VIP receptor antagonists, anticipating that a misfolded receptor would have a lower affinity for such ligand, whereas a nonactivatable receptor should have a lower affinity for agonist only (see Discussion).

[AcHis^1,D-Phe^2,Lys^{15},Arg^{16},Leu^{27}]VIP(3-7)/GRF(8-27) and the $VPAC_2$ preferring VIP fragment VIP(5-28) antagonized the effect of VIP on adenylate cyclase stimulation through the wild-type and chimeric receptors. These values were confirmed in functional assays: the VIP(5-28) and the $VPAC_1$ antagonist K_i values are summarized in Table 1. At a concentration of 1 μM, the $VPAC_1$ antagonist did not significantly affect the VIP dose-effect curves on $VPAC_2$ and on N-$VPAC_2$/$VPAC_1$ receptors.

Discussion

The natural ligands VIP and PACAP(1-27) have a clear preference for $VPAC_1$ over $VPAC_2$ receptors. In contrast, Ro 25-1553 and Ro 25-1392 are highly selective for $VPAC_2$ receptors. They differ from VIP by the acetylation of the amino-terminal histidine, several mutations in the central part of the molecule, a lactam ring between residues 21 and 25, and a basic carboxyl-terminal tail. Each modification could a

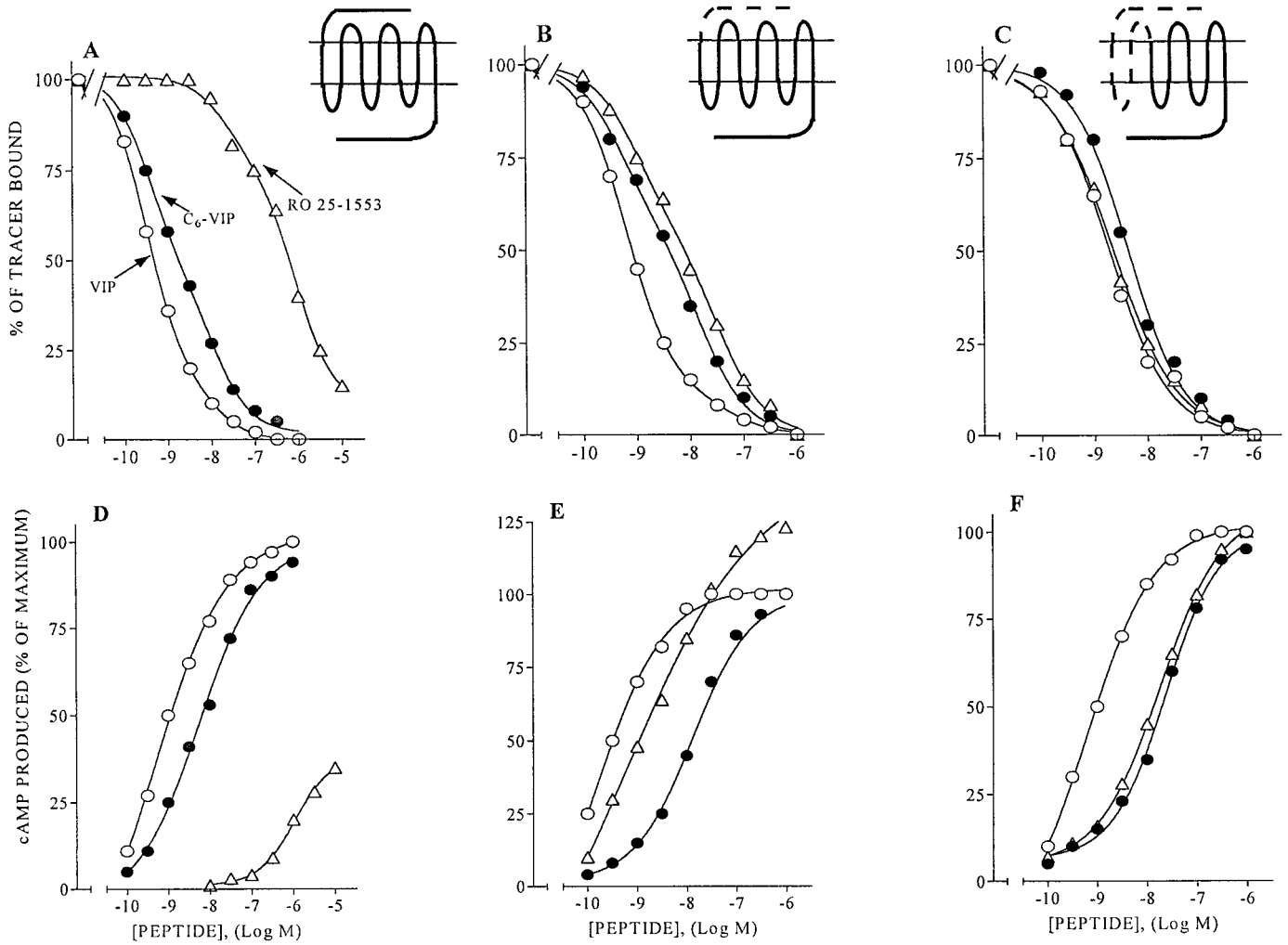

Fig. 1. Inhibition of tracer binding (top) and adenylate cyclase activation (bottoms) by VIP (○), C_6-VIP (●), and Ro 25-1553 (△) using CHO cell membranes expressing the recombinant $VPAC_1$ (A and D), N-$VPAC_2$/$VPAC_1$ chimera (B and E), and N→EC_1-$VPAC_2$/$VPAC_1$ chimera (C and F). ^{125}I-VIP and ^{125}I-Ro 25-1553 were used to characterize the $VPAC_1$ receptors and the two chimeric receptors, respectively. The curves are the mean of at least three experiments performed in duplicate.

priori contribute to the high affinity for the $VPAC_2$ receptor and the low affinity for the $VPAC_1$ receptor.

The following experimental data support the hypothesis that acylation of the amino terminus of these peptides probably does contribute to their $VPAC_2$ selectivity. 1) $AcHis^1$-VIP was reported to have a 2-fold higher affinity than VIP on the "helodermin preferring receptor" from the SUP-T1 lymphoblastic cell line (Svoboda et al., 1994), a receptor subsequently identified as the human $VPAC_2$ receptor (Robberecht et al., 1996), and a 5-to 10-fold lower affinity than VIP on the cloned rat $VPAC_1$ receptor (Gourlet et al., 1996a) (these data were confirmed in the present report on recombinant human $VPAC_1$ and $VPAC_2$ receptors: Table 1). 2) Three fatty acyl derivatives of VIP (myristoyl-, palmitoyl-, and stearyl-[N,Leu^{17}]VIP), obtained through the conjugation of a fatty acid to the amino terminus of the peptide chain by an amide bond, also had a lower and higher affinity than VIP for the human $VPAC_1$ and $VPAC_2$ receptors, respectively. These three modified peptides were, however, partial agonists on the $VPAC_1$ and $VPAC_2$ receptors. Their intrinsic activity was in fact extremely low on the $VPAC_2$ receptors, where they behaved like partial agonists (Gourlet et al., 1998). These results prompted us to test the properties of an amino-terminally acylated VIP derivative modified with hexanoic acid. The C_6-VIP derivative had, compared with VIP, a 3-fold higher and a 4-fold lower IC_{50} value in binding studies on $VPAC_1$ and $VPAC_2$ receptors, respectively. It behaved as a full and selective $VPAC_2$ agonist on adenylate cyclase stimulation.

We then attempted to identify the domains of the receptor implicated in the high- and low-affinity interaction of Ro 25-1553 and C_6-VIP for the $VPAC_2$ and $VPAC_1$ receptors, respectively. The results obtained with the first N-$VPAC_2$/$VPAC_1$ chimera, in the amino-terminal part of the $VPAC_2$ receptor grafted onto the $VPAC_1$ receptor TM domain, indicated clearly that the VPAC receptor amino-terminal domain is responsible for selective Ro 25-1553 recognition. This is not the case for $AcHis^1$-VIP and C_6-VIP, which had an identical potency (lower than that of VIP) on both $VPAC_1$ and N-$VPAC_2$/$VPAC_1$ receptors. These results supported the hypothesis that the amino-terminal sequence of the ligands interacted with the core of the receptor, whereas the remainder of the molecule, probably starting around amino acid 9 (the first mutated residue in Ro 25-1553) interacts with the extracellular amino-terminal domain of the receptor. These conclusions are in line with several observations on closely

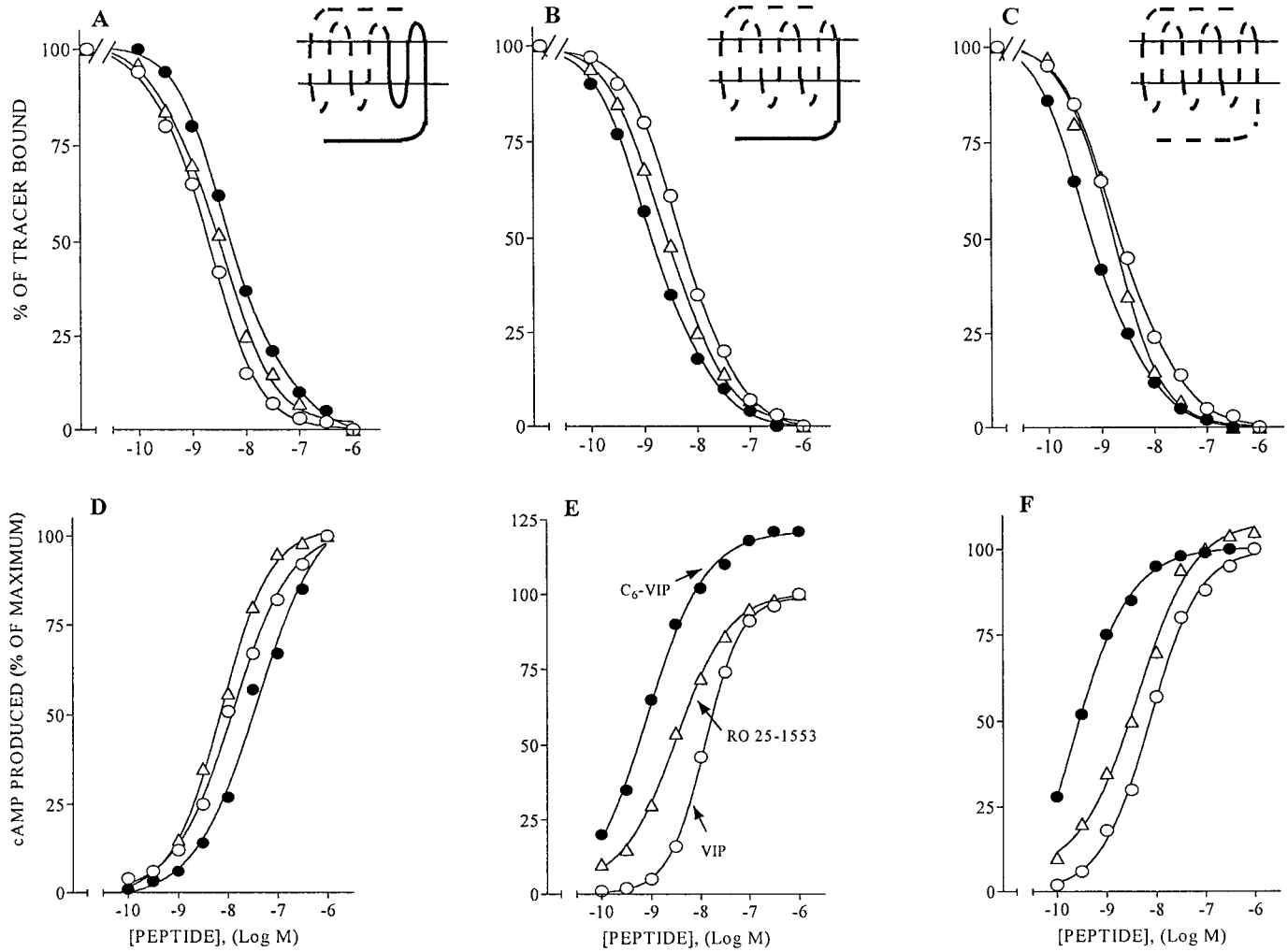

Fig. 2. Inhibition of tracer binding (top) and adenylate cyclase activation (bottom) by VIP (○), C_6-VIP (●), and Ro 25-1553 (△) using CHO cell membranes expressing the N→EC_2-$VPAC_2$/$VPAC_1$ chimera (A and D), N→EC_3-$VPAC_2$/$VPAC_1$ chimera (B and E), and the recombinant $VPAC_2$ receptor (C and F). ^{125}I-Ro 25-1553 were used to characterize these receptors. The curves are the mean of at least three experiments performed in duplicate.

related receptors. 1) The amino-terminal domain of the secretin receptor is essential for the high-affinity interaction of secretin with its receptor (Vilardaga et al., 1995), and recognizes amino acids 8 to 15 (Gourlet et al., 1996b) and 22 (Dong et al., 1999) of secretin; the amino-terminal portions of the PACAP receptor (Van Rampelbergh et al., 1996; Hashimoto et al., 1997) and of the parathyroid hormone/parathyroid hormone-related peptide receptor (Zhou et al., 1997) also participate in the selective recognition of their respective ligands. 2) The amino terminus of secretin, and particularly the aspartate residue in position 3, interacts with several receptor residues located in the first and second TM domains (Vilardaga et al., 1996; Di Paolo et al., 1998, 1999).

We should very much like to identify the receptor region that recognizes the amino-terminal VIP histidine because this interaction is very important for receptor activation. Unfortunately, this histidine residue is likely also involved in stabilizing the active VIP conformation: conceiving VIP analogs that retain the right conformation but interact differentially with the receptor histidine anchoring site is by no means a trivial problem. We were therefore extremely interested by the observation that C_6-VIP is a full agonist, with a greater affinity than VIP for $VPAC_2$ receptor and only slightly lower affinity than VIP for $VPAC_1$ receptor. The VPAC receptor region that is responsible for the differential VIP/C_6-VIP recognition must be very close to the histidine anchoring site.

VIP was more potent than C_6-VIP on the N→EC_2-$VPAC_2$/$VPAC_1$ chimeric receptor but less potent than VIP on the N→EC_3-$VPAC_2$/$VPAC_1$ chimeric receptor and on the $VPAC_2$ receptor: the hexanoyl group probably recognized a binding site situated somewhere between TM5 and EC_3, and the amino-terminal histidine binding site (which is essential for $VPAC_1$ receptor activation) should be sought in the same region or in its immediate vicinity.

To further support this hypothesis, we attempted to express and study the binding and functional properties of the "mirror image" chimeras. Our results confirmed that the $VPAC_2$ TM7 sequence is not sufficient to support preferential C_6-VIP > VIP recognition (Fig. 3, C and F). We were, unfortunately, unable to detect the expression of one of these chimeras cloned in CHO cells (N→EC_2-$VPAC_1$/$VPAC_2$) by functional or binding studies, and VIP had a surprisingly low affinity and efficacy on the two remaining chimeras (Fig. 2, A, B, D, and E). We subsequently observed that C_6-VIP had a higher affinity and efficacy than VIP on these chimeras (Table 1). Taken together, these results suggested that they could not be fully activated by the natural agonist, VIP, and

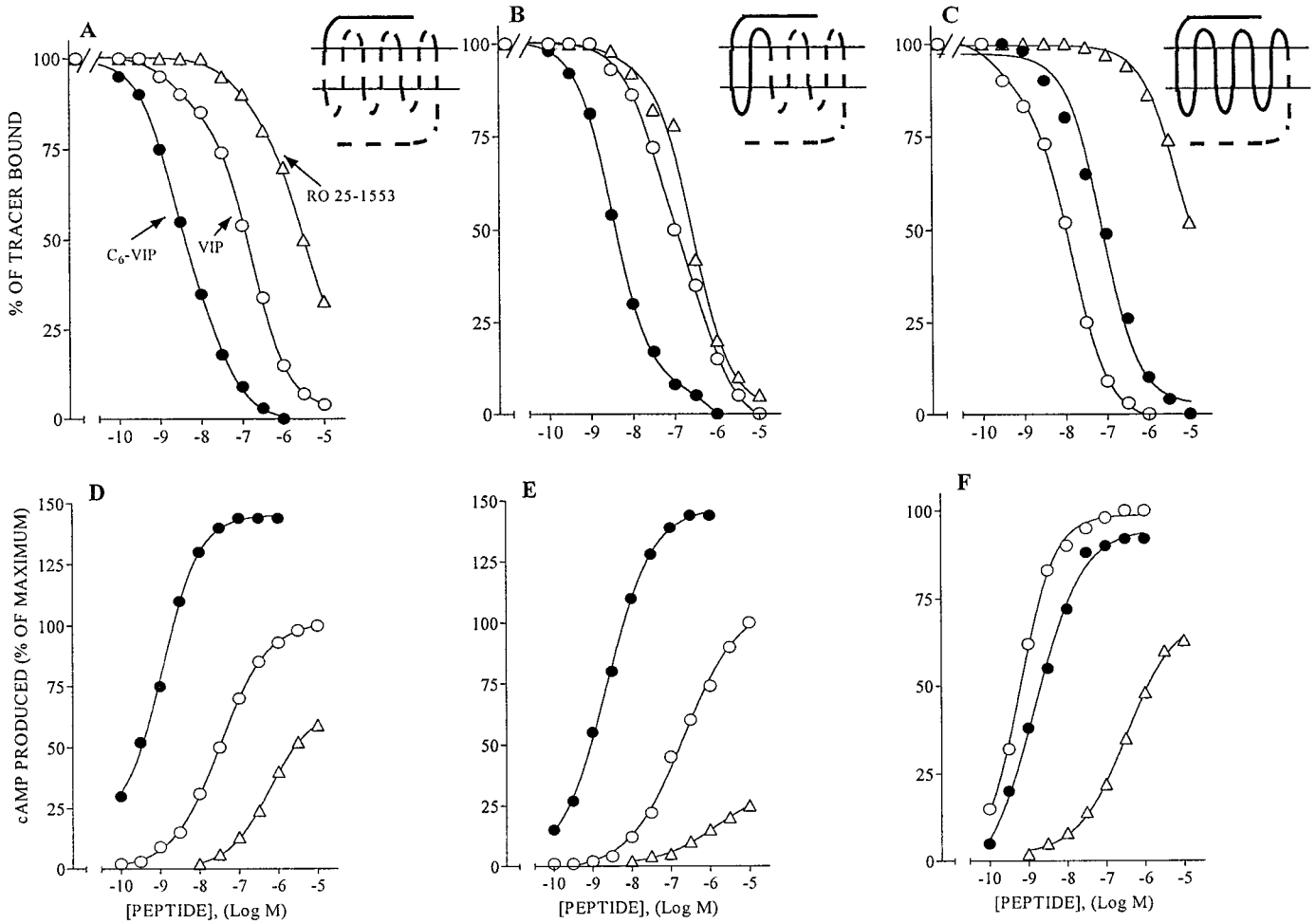

Fig. 3. Inhibition of tracer binding (top) and adenylate cyclase activation (bottom) by VIP (○), C_6-VIP (●), and Ro 25-1553 (△) using CHO cell membranes expressing the recombinant chimeric receptors N-$VPAC_1$/$VPAC_2$ (A and D), N→EC_1-$VPAC_1$/$VPAC_2$ chimera (B and E), and N→EC_3-$VPAC_1$/$VPAC_2$ chimera (C and F) receptors. ^{125}I-C_6-VIP was used to characterize these chimeric receptors. The curves are the mean of at least three experiments performed in duplicate.

that acylation with a 6-carbon acyl chain was sufficient to rescue the ability of the peptides to stabilize the active receptor conformation.

As pointed out recently by Colquhoun (1999), it is not possible to evaluate separately the affinity of agonists for the resting inactive receptors and their effect on receptor activation. If we assume that the agonist initially recognizes inactive receptors and then induces a conformational change to the active receptor conformation:

$$Ago + R \overset{K_1}{\longleftrightarrow} Ago \cdot R \overset{K_2}{\longleftrightarrow} Ago \cdot R*$$

where K_1 and K_2 represent the affinity constant for the agonists for R and the agonist-receptor complex activation constant, respectively, then the affinity of the agonists measured in binding and functional studies is $K_a = K_1(1 + K_2)$. In contrast, because antagonists recognize the inactive receptor conformation but do not induce receptor activation:

$$Ant + R \overset{K_1}{\longleftrightarrow} Ant \cdot R$$

their affinity ($K_a = K_1$) does reflect their ability to recognize the receptor.

Removal of the amino-terminal VIP amino acids, acylation of the His^1 by long-chain fatty acids, or replacement of amino acids 2, 4, or 6 by D-amino acids markedly reduced not only the affinities of the peptides but also their intrinsic efficacies at $VPAC_1$ and $VPAC_2$ receptors: in the case of VIP related peptides, K_1 probably reflects the peptide anchoring through the central and carboxyl-terminal amino acids, and K_2, additional interactions between the amino-terminal VIP amino acids and the receptor in its active conformation.

To test whether the two $VPAC_1$/$VPAC_2$ chimeric receptors had a normal conformation in the resting state, we measured their affinities (K_1) for two antagonists: the $VPAC_1$ antagonist ([$AcHis^1$,D-Phe^2,Lys^{15},Arg^{16},Leu^{27}]VIP(3-7)/GRF(8-27)] and VIP(5-28). The selectivity of the $VPAC_1$ antagonist is conferred mainly by its the carboxyl-terminal (8-27) region, that is, a peptide region thought to interact with the extracellular amino-terminal receptor domain. Our results, indicating that it had a high affinity for the N-$VPAC_1$/$VPAC_2$ chimeric receptor and low affinity for the N-$VPAC_2$/$VPAC_1$ chimeric receptor, further supported this hypothesis. They also suggested that the resting conformations of the amino-terminal domains of the $VPAC_1$/$VPAC_2$ chimeric receptors were normal or almost normal, because the antagonist affinities (K_1) were only slightly lower than those of the $VPAC_1$ receptor. In contrast, the ability of the TM chimeric receptors domain to recognize the amino-terminal VIP sequence and to change conformation in response to agonist recognition (K_2) was reduced, so we cannot exclude the hypothesis that the chimeric receptor TM domain had an altered conformation or was misoriented with respect to the amino-terminal domain. Merely acylating the amino-terminal histidine residue with an hexanoyl group (equivalent in size to a Leu or an Ile) was sufficient to "rescue" the affinity of the agonists and their ability to activate the receptor (K_2), suggesting that the energy input necessary to activate the receptor increased only slightly in the chimeric receptors compared with $VPAC_1$ and $VPAC_2$ receptors.

To conclude, our results suggested that 1) that the selectivity of the selective $VPAC_2$ agonist, Ro 25-1553, and of the selective $VPAC_1$ antagonist was supported by the extracellular amino-terminal receptor domain. The ability of the receptor to recognize preferentially either VIP or the C_6-VIP analog, in contrast, depended on the sequence of the carboxyl-terminal TM5 to EC_3 receptor domain. 2) The low affinity of the chimeric N-$VPAC_1$/$VPAC_2$ and N→EC_1-$VPAC_2$/$VPAC_1$ receptors for VIP and $AcHis^1$-VIP reflected the reduced ability of these peptides to activate the chimeras rather than their inability to recognize the resting receptor conformation. 3) Acylation of the amino-terminal VIP histidine by a hexanoyl chain restored the ability of the peptides to activate these chimeric receptors. 4) Radioiodinated C_6-VIP was a good ligand for $VPAC_1$, $VPAC_2$, and chimeric receptor identification.

References

Ciccarelli E, Vilardaga JP, De Neef P, Di Paolo E, Waelbroeck M, Bollen A and Robberecht P (1994) Properties of the VIP-PACAP type II receptor stably expressed in CHO cells. *Regul Pept* **54:**397–407.

Colquhoun D (1999) Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br J Pharmacol* **125:**924–947.

Couvineau A, Rouyer-Fessard C, Darmoul D, Maoret JJ, Carrero I, Ogier-Denis E and Laburthe M (1994) Human intestinal VIP receptor: Cloning and functional expression of two cDNA encoding proteins with different N-terminal domains. *Biochem Biophys Res Commun* **200:**769–776.

Di Paolo E, De Neef P, Moguilevsky N, Petry H, Bollen A, Waelbroeck M and Robberecht P (1998) Contribution of the second transmembrane helix of the secretin receptor to the positioning of secretin. *FEBS Lett* **424:**207–210.

Di Paolo E, Petry H, Moguilevsky N, Bollen A, De Neef P, Waelbroeck M and Robberecht P (1999) Mutations of aromatic residues in the first transmembrane helix impair signalling by the secretin receptor. *Receptors Channels* **6:**309–315.

Dong M, Wang Y, Pinon DI, Hadac EM and Miller LJ (1999) Demonstration of a direct interaction between residue 22 in the carboxyl-terminal half of secretin and the amino-terminal tail of the secretin receptor using photoaffinity labeling. *J Biol Chem* **274:**903–909.

Gourlet P, De Neef P, Cnudde J, Waelbroeck M and Robberecht P (1997c) In vitro properties of a high affinity selective antagonist of the VIP_1 receptor. *Peptides* **18:**1555–1560.

Gourlet P, Rathé J, De Neef P, Cnudde J, Vandermeers-Piret MC, Waelbroeck M and Robberecht P (1998) Interaction of lipophilic VIP derivatives with recombinant VIP_1/PACAP and VIP_2/PACAP receptors. *Eur J Pharmacol* **354:**105–111.

Gourlet P, Vandermeers A, Vandermeers-Piret MC, Rathé J, De Neef P and Robberecht P (1996a) C-Terminally shortened pituitary adenylate cyclase-activating peptides (PACAP) discriminate PACAP I, PACAP II-VIP_1 and PACAP II-VIP_2 recombinant receptors. *Regul Pept* **62:**125–130.

Gourlet P, Vandermeers A, Vertongen P, Rathé J, De Neef P, Cnudde J, Waelbroeck M and Robberecht P (1997b) Development of high affinity selective VIP_1 receptor agonist. *Peptides* **18:**1539–1545.

Gourlet P, Vertongen P, Vandermeers A, Vandermeers-Piret MC, Rathé J, De Neef P, Waelbroeck M and Robberecht P (1997a) The long-acting vasoactive intestinal polypeptide agonist Ro 25-1553 is highly selective of the VIP_2 receptor subclass. *Peptides* **18:**403–408.

Gourlet P, Vilardaga JP, De Neef P, Vandermeers A, Waelbroeck M, Bollen A and Robberecht P (1996b) Interaction of amino acid residues at positions 8–15 of secretin with the N-terminal domain of the secretin receptor. *Eur J Biochem* **239:**349–355.

Gozes I and Fridkin M (1992) A fatty neuropeptide: Potential drug for noninvasive impotence treatment in a rat model. *J Clin Invest* **90:**810–814.

Gozes I, Lilling G, Glazer R, Ticher A, Ashkenazi IE, Davidson A, Rubinraut S, Fridkin M and Brenneman DE (1995) Superactive lipophilic peptides discriminate multiple vasoactive intestinal peptide receptor. *J Pharmacol Exp Ther* **273:**161–167.

Gozes I, Reshef A, Salah D, Rubinraut S and Fridkin M (1994) Stearyl-norleucine-vasoactive intestinal peptide (VIP): A novel analog for noninvasive impotence treatment. *Endocrinology* **134:**2121–2125.

Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA and Waschek JA (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, Ogawa N, Hagihara N, Yamamoto K, Imanishi K, Nogi H, Nishino A, Fujita T, Matsuda T, Nagata S and 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.

Ishihara T, Shigemoto R, Mori K, Takahashi K and Nagata S (1992) Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron* **8:**811–819.

Robberecht P, Gourlet P, Vertongen P and Svoboda M (1996) Characterization of the VIP receptor from SUP-T1 lymphoblasts. *Adv Neuroimmunol* **6:**49–57.

Salomon Y, Londos C and Rodbell M (1974) A highly sensitive adenylate cyclase assay. *Anal Biochem* **58:**541–548.

Svoboda M, Tastenoy M, Van Rampelbergh J, Goossens JF, De Neef P, Waelbroeck M and Robberecht P (1994) Molecular cloning and functional characterization of a VIP receptor from SUP-T1 lymphoblasts. *Biochem Biophys Res Commun* **205:** 1617–1627.

Usdin TB, Bonner TI and Mezey E (1994) Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. *Endocrinology* **135:**2662–2680.

Van Rampelbergh J, Gourlet P, De Neef P, Robberecht P and Waelbroeck M (1996) Properties of the pituitary adenylate cyclase-activating polypeptide I and II receptors, vasoactive intestinal peptide$_1$, and chimeric amino-terminal pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal peptide$_1$ receptors: Evidence for multiple receptor states. *Mol Pharmacol* **50:**1596–1604.

Vertongen P, Delhase M, Rajas F, Trouillas J, Hooghe-Peters E, Svoboda M and Robberecht P (1996) Pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide receptor subtypes are differently expressed in rat transplanted pituitary tumors (SWtTW) and in normal gland. *J Mol Endocrinol* **16:** 239–248.

Vertongen P, Schiffmann SN, Gourlet P and Robberecht P (1997) Autoradiographic visualization of the receptor subclasses for vasoactive intestinal polypeptide (VIP) in rat brain. *Peptides* **18:**1547–1554.

Vilardaga JP, De Neef P, Di Paolo E, Bollen A, Waelbroeck M and Robberecht P (1995) Properties of chimeric secretin and VIP receptor proteins indicate the importance of the N-terminal domain for ligand discrimination. *Biochem Biophys Res Commun* **211:**885–891.

Vilardaga JP, Di Paolo E, De Neef P, Waelbroeck M, Bollen A and Robberecht P (1996) Lysine 173 residues within the first exoloop of rat secretin receptor is involved in carboxylate moiety recognition of Asp 3 in secretin. *Biochem Biophys Res Commun* **218:**842–846.

Xia M, Sreedharan SP, Bolin DR, Gaufo GO and Goetzl EJ (1997) Novel cyclic peptide agonist of high potency and selectivity for the type II vasoactive intestinal peptide receptor. *J Pharmacol Exp Ther* **281:**629–633.

Zhou AT, Bessalle R, Bisello A, Nakamoto C, Rosenblatt M, Suva LJ and Chorev M (1997) Direct mapping of an agonist-binding domain within the parathyroid hormone/parathyroid hormone-related protein receptor by photoaffinity crosslinking. *Proc Natl Acad Sci USA* **94:**3644–3649.

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