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Two tyrosine residues in the first transmembrane helix of the human vasoactive intestinal peptide receptors play a role in supporting the active conformation

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1 We investigated the human vasoactive intestinal polypeptide (VIP) receptors $VPAC_1$ and $VPAC_2$ mutated at conserved tyrosine residues in the first transmembrane helix ($VPAC_1$ receptor Y146A and Y150A and $VPAC_2$ receptor Y130A and Y134A).

2 [^{125}I]-Acetyl-His^1 [D-Phe^2, K^{15}, R^{16}, L^{27}]-VIP (1–7)/GRF (8–27) (referred to as [^{125}I]-$VPAC_1$ antagonist) labelled $VPAC_1$ binding sites, that displayed high and low affinities for VIP (IC_{50} values and per cent of high affinity binding sites: wild-type, 1 nM (57±9%) and 160 nM; Y146A, 30 nM (40±8%) and 800 nM; Y150A, 4 nM (27±8%) and 300 nM). [R^{16}]-VIP behaved as a 'super agonist' at both mutated $VPAC_1$ receptors and the efficacies of VIP analogues modified in positions 1, 3 and 6 were significantly decreased.

3 VIP was less potent at the Y130A and Y134A mutated $VPAC_2$ receptors (EC_{50} 200 and 400 nM, respectively) than at the wild-type $VPAC_2$ receptor (EC_{50} 7 nM). Furthermore, [hexanoyl-His^1]-VIP behaved as a 'super agonist' at the two mutated $VPAC_2$ receptors, and VIP analogues modified in positions 1, 3 and 6 were less potent and efficient at the mutated than at wild-type $VPAC_2$ receptors. However, the Y130A and Y134A mutants could not be studied in binding assays

4 Our results suggest that the conserved tyrosine residues do not interact directly with the VIP His^1, Asp^3 or Phe^6 residues (that are necessary for receptor activation), but stabilize the correct active receptor conformation.

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Abbreviations: CHO, Chinese hamster ovary cells; EC1, first extracellular loop; EC_{50}, concentration of agonist required for half maximal response; GRF, growth hormone releasing factor; HEXA-VIP, [hexanoyl-His^1]-VIP; IC_{50}, concentration of ligand required for 50% inhibition of tracer binding; JV 1–53, Acetyl-His^1 [D-Phe^2, pCl-Phe^6, K^{15}, R^{16}, K^{20}, Y^{22}, Nle^{27}, D-Arg^{28}, Har^{29} amide]-GRF (3–26); PCR, polymerase chain reaction; Ro 25–1553, Acetyl-His^1 [E^8, K^{12}, Nle^{17}, A^{19}, D^{25}, L^{26}, K^{27}, K^{28}, G^{29}, G^{30}, T^{31}] cyclo 21–25 VIP (2–24); TM1, first transmembrane domain; VIP, vasoactive intestinal polypeptide; $VPAC_1$ antagonist, Acetyl-His^1 [D-Phe^2, K^{15}, R^{16}, L^{27}]-VIP (1–7)/GRF (8–27) amide

Introduction

The neuropeptide vasoactive intestinal peptide (VIP) activates G_S-proteins and adenylate cyclase with a high affinity through two receptors – $VPAC_1$ and $VPAC_2$ – encoded by different genes (Unson *et al*., 1996). At the protein level the $VPAC_1$ and $VPAC_2$ receptors share 62% similarity (53% identity). These receptors belong to a subfamily (termed the secretin receptor subfamily) of the seven transmembrane G-protein coupled receptor (GPCR) family. The former are characterized by conserved transmembrane helices with no significant sequence homologies to the larger 'rhodopsin-like' GPCR subfamily. The secretin receptor subfamily includes the receptors for VIP, PACAP (pituitary adenylate cyclase activating peptide), secretin, glucagon, GLP1 and GLP2 (glucagon like peptides 1 and 2), GIP (gastric inhibitory peptide), GRF (growth hormone releasing factor), CRF (corticotrophin releasing factor), parathyroid hormone and calcitonin, but also some of the latrotoxin receptors, receptors for the brain-specific angiogenesis inhibitor (BAI), a few orphan receptors, and insect diuretic hormone receptors (Horn *et al*., 1998).

Though, several transmembrane residues in the 'rhodopsin-like' receptors, which participate in signal transduction by stabilizing the inactive or the active receptor conformations respectively, have been identified by mutagenesis and their probable role refined by 3D modelling. Unfortunately, to date it is not possible to extend these studies to the secretin receptor family, as the amino acid 'signatures', identified in the rhodopsin receptor subfamily, are not conserved in the secretin receptor subfamily. We therefore opted to target for mutagenesis polar amino acids that are conserved in the secretin receptor family, and investigated the effect of these mutations on receptor activation. Our results suggest that the two tyrosine residues that are conserved in the first transmembrane helix of these peptide receptors are essential to stabilize the active receptor conformation, but do not

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contact the VIP His[1], Asp[3] or Phe[6] residues that are essential for full biological activity of the peptide.

Methods

Construction of the mutated receptors

The cell lines expressing the VPAC$_1$ and VPAC$_2$ wild-type (wt) receptors have been described previously (Juarranz *et al*., 1999a, Solano *et al*., 2001, Svoboda *et al*., 1994).

Two VPAC$_1$ and two VPAC$_2$ receptor mutants were generated: VPAC$_1$ receptors Y146A and Y150A, and VPAC$_2$ receptors Y130A, Y134A.

Generation of the mutated receptors was achieved using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla CA, U.S.A.) essentially according to the manufacturer's instructions. Briefly, the human VPAC$_1$ or VPAC$_2$ receptor-coding regions, inserted in the mammalian expression vector pcDNA3.1 (Invitrogen Corp. CA, U.S.A.), were submitted to 22 cycles of polymerase chain reaction (PCR) (95°C for 30 s; 54–58°C for 1 min and 68°C for 14 min) in a 50 μl reaction volume. The forward and reverse primers were complementary and contained the desired nucleotide changes, flanked on either side by 15–18 perfectly matched nucleotides (only the forward primers are shown): V1H-Y146A–GGTTCTGTGAAGACCGGCGCCACCATCGGCTACGG; V1H-Y150A–CGGCTACACCATCGGCGCCGGCCTGTCCCTCGC; V2H-Y130A–CTGGTGAAGGCCATTGCTACCCTGGGCTACAG; V2H-Y134A–CATTTATACCCTGGGCGCCAGTGTCTCTCTGATG.

Following PCR, 10 μl were removed and analysed by agarose gel electrophoresis and the remaining 40 μl were digested for at least 2 h at 37°C by 1 μl (10 u) DpnI restriction enzyme (Stratagene, La Jolla CA, U.S.A.) to remove the parental methylated DNA. The digested PCR products were transformed into TOP10 One Shot competent *E. Coli* bacterial cells (Invitrogen Corp. CA, U.S.A.). Miniprep plasmid DNA was prepared from several colonies and verified by agarose gel electrophoresis (Sambrook *et al*., 1989). Three clones were retained, and further purified on Qiaquick PCR purification spin columns (Qiagen, Hilden, Germany). The mutations were then verified on an automated DNA sequencing apparatus using the BigDye Terminator Sequencing Prism Kit from ABI (Perkin-Elmer, CA, U.S.A.). Plasmid DNA from one clone for each mutation, was prepared using the GenElute midiprep endotoxin-free kit (Sigma-Aldrich, St Louis, MO, U.S.A.), of which 20 μg were electroporated (Electroporator II, Invitrogen Corp. CA, U.S.A.) into wild-type Chinese Hamster Ovary (CHO-K1) cells. The complete nucleotide sequence of the receptor coding region was established by DNA sequencing. Selection was carried out in culture medium [50% HamF12; 50% DMEM; 10% foetal calf serum; 1% penicillin (10 mu ml^{-1}); 1% streptomycin (10 μg ml^{-1}); 1% L-glutamine (200 mM), Life Technologies Ltd., Paisley, U.K.], supplemented with 600 μg Geneticin (G418 ml^{-1}) culture medium. After 10–15 days of selection, isolated colonies were transferred to 24 well microtiter plates and grown until confluence, trypsinized and further expanded in six well microtiter plates. For each mutation, 24 recombinant cell clones were screened by testing the ability of 10 μM VIP and/or of 10 μM [R^{16}]-VIP (mutated VPAC$_1$ receptors) or 10 μM [hexanoyl-His^1]-VIP called HEXA-VIP (mutated VPAC$_2$ receptors) to stimulate membrane adenylate cyclase activity.

Membrane preparation

Membranes were prepared from scraped cells pelleted and then lysed in 1 mM $NaHCO_3$ solution followed by immediate freezing in liquid nitrogen. After thawing, the lysate was first centrifuged at 4°C for 10 min at $400 \times g$ and the supernatant then further centrifuged at $20{,}000 \times g$ for 15 min. The pellet was resuspended in 1 mM $NaHCO_3$ and used immediately as a crude membrane fraction.

Binding studies

Binding studies were performed as described below using as tracer either [^{125}I]-VIP, [^{125}I]-Acetyl-His^1 [D-Phe^2, K^{15}, R^{16}, L^{27}]-VIP (1–7)/GRF (8–27) coined [^{125}I]-VPAC$_1$ antagonist; Acetyl-His^1 [E^8, K^{12}, Nle^{17}, A^{19}, D^{25}, L^{26}, K^{27}, K^{28}, G^{29}, G^{30}, T^{31}] cyclo 21–25 VIP (2–24) termed [^{125}I]-Ro 25-1553 or [^{125}I]-HEXA-VIP. The tracers were radiolabelled similarly and had comparable specific radioactivity (Gourlet *et al*., 1997). In all cases, the non-specific binding was defined as residual binding in the presence of 1 μM of the corresponding unlabelled peptide. Binding was performed at 25°C in a total volume of 120 μl containing 20 mM Tris-maleate, 2 mM $MgCl_2$, 0.1 mg ml^{-1} bacitracin and 1% bovine serum albumin (pH 7.4) buffer, to which 3–30 μg of protein were added per assay. Bound and free radioactivity were separated 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.

Adenylate cyclase activity

Adenylate cyclase activity was determined by the procedure previously described (Salomon *et al*., 1974). Membrane proteins (3–15 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, cAMP 1, theophylline 1, phospho(enol)pyruvate 10, 30 μg ml^{-1} pyruvate kinase and 30 mM Tris-HCl at a final pH of 7.8.

Peptides synthesis

With the exception of JV-1-53 (Rekasi *et al*., 2000) that was a generous gift from Drs A. Schally and J. Varga (New Orleans, LA, U.S.A.), all the peptides used were synthesized in our laboratory as described (Gourlet *et al*., 1998a, O'Donnell *et al*., 1994). The 1-hydroxybenzotriazole derivative of hexanoic acid was coupled to the amino-terminus of VIP before cleavage and deprotection. Peptide purity was assessed by capillary electrophoresis, and conformity by electrospray mass spectrometry.

Statistics

All competition curves and dose-effect curves were analysed by non-linear regression (Graph Pad Prism). The differences

between the IC_{50}, EC_{50} and efficacy values were tested for statistical significance by the Student's *t*-test; $P<0.05$ was accepted as being significant.

Results

Interaction of VIP and analogues with the human wild-type recombinant VPAC$_1$ and VPAC$_2$ receptor expressed in CHO cells

The $VPAC_1$ and $VPAC_2$ receptor cell lines used expressed 850 ± 60 fmol [^{125}I]-VIP (K_D 2 nM) and 210 ± 40 fmol [^{125}I]-Ro 25-1553 (K_D 6 nM) binding sites per mg of protein, respectively. The [^{125}I]-$VPAC_1$ antagonist labelled concentrations of $VPAC_1$ receptor 1.2 to 2 fold higher as compared to VIP. Indeed VIP had a high affinity (1 nM) for only 57 ± 9% of the [^{125}I]-$VPAC_1$ antagonist binding sites, and a low affinity (160 nM) for the remaining sites (Figure 1). As previously published, [R^{16}]-VIP (Gourlet *et al*., 1996) and HEXA-VIP (Juarranz *et al*., 1999b) had significantly higher affinities than VIP at the $VPAC_1$ and $VPAC_2$ receptors, respectively (Table 1). In contrast, VIP analogues modified in positions 1, 3 and 6 had lower affinities at both receptors (Table 1). They were also significantly less efficient and less potent than VIP in functional studies at both receptor subtypes (Tables 2 and 3).

Analysis of the mutated Y146A and Y150A VPAC$_1$ receptors and of the Y130A and Y134A VPAC$_2$ receptors

Mutations were performed on two-conserved tyrosine residues located in the first transmembrane helix of the $VPAC_1$ and $VPAC_2$ receptors. CHO cells expressing the mutated receptors were selected by screening geneticin resistant clones for the ability of 10 μM VIP, 10 μM [R^{16}]-VIP and/or 10 μM HEXA-VIP to increase adenylate cyclase activity. [^{125}I]-VIP and [^{125}I]-$VPAC_1$ antagonist binding studies and adenylate cyclase stimulation dose-effect curves were then performed to select one of the positive clones for further studies.

However, [^{125}I]-VIP specific binding was too low to allow competition curve analysis. Indeed, [^{125}I]-VIP binding to the mutated Y146A and Y150A $VPAC_1$ receptor clones did not exceed ±200 c.p.m. specific binding as compared to the ±600 c.p.m. of non-specific binding observed. Nevertheless, it is noteworthy that a mutated $VPAC_1$ receptor Y150A clone, expressing the mutated receptor at very high levels (5000 ± 200 fmol mg^{-1} protein) did in fact exhibit significant ^{125}I-VIP labelling.

Additionally, radiolabelled peptides bind non-specifically to CHO cell membranes; consequently, the increase in tracer binding due to specific recognition of receptors remained non-significant even at increased membrane concentrations. On the other hand, the $VPAC_1$-selective antagonist, Acetyl-His^1 [D-Phe^2, K^{15}, R^{16}, L^{27}]-VIP (1–7)/GRF (8–27), displayed a high apparent affinity in functional studies not only for the $VPAC_1$ receptor but also for the two mutated $VPAC_1$ receptor constructs (see below). Subsequently, as expected from these data, the radioiodinated peptide labelled significantly the $VPAC_1$ receptors as well as the two mutated receptors. This tracer was therefore used to analyse the binding properties of the three $VPAC_1$-related receptors. We

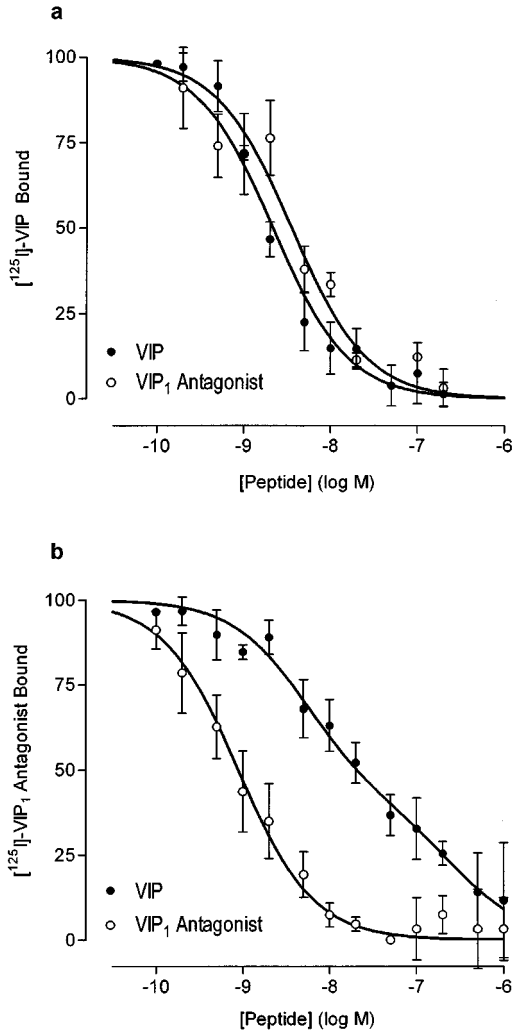

Figure 1 [^{125}I]-VIP and [^{125}I]-$VPAC_1$ antagonist binding at $VPAC_1$ receptors. Competition curves were obtained as explained under Methods, using (a) [^{125}I]-VIP or (b) [^{125}I]-$VPAC_1$ antagonist as tracer. The results are expressed as per cent ($\pm$s.e.mean) of specific tracer binding in the absence of unlabelled competitor. Average of four experiments carried out in duplicate.

also verified that the three cell lines used for functional studies expressed receptor densities comparable to the wild-type receptors: 1300 ± 100 fmol for the wild-type receptor, 1500 ± 200 fmol for the Y150A mutated $VPAC_1$ receptor and 900 ± 150 fmol for the Y146A mutated $VPAC_1$ receptors per mg protein. Moreover, the high affinities obtained by two-sites analysis of the [^{125}I]-$VPAC_1$ antagonist/VIP and [^{125}I]-$VPAC_1$ antagonist/[R^{16}]-VIP competition curves (Figure 1) were in good agreement with their IC_{50} values in [^{125}I]-VIP competition curves (Figure 1 and results not shown).

As shown in Table 1, the affinity of VIP and of most VIP analogues ([A^1]-, [F^1]-, [E^3]-, [Y^6]- and [H^6]-VIP) for the Y146A $VPAC_1$ receptor was decreased as compared to wild-type $VPAC_1$ receptor: the only compounds that had the same affinity in binding studies for the wild-type $VPAC_1$ and Y146A $VPAC_1$ receptors were the $VPAC_1$ antagonist and the very poor partial agonist, [C^6]-VIP (Table 1). Mutation of the $VPAC_1$ receptor Tyr^{150} affected only marginally this receptor's binding properties (Table 1).

Table 1 [^{125}I]-VPAC$_1$ antagonist binding studies at the wild-type and mutated VPAC$_1$ receptors: IC$_{50}$ values (in nM)

	Wild-type	*VPAC$_1$ h* *Y164A*	*Y150A*
VIP	0.8±0.4 (56±8%)	49±25 (52±6%)	2.6±1.5 (27±8%)
	160±100	3300±1600	300±160
[R^{16}]-VIP	0.9±0.3 (100%)	25±10 (100%)	0.2±0.1 (30±6%)
			12±8
[A^1]-VIP	200±80	1200±400	230±90
[F^1]-VIP	50±20	360±150	75±25
[E^3]-VIP	200±80	550±260	150±60
[N^3]-VIP	400±150	350±120	190±70
[Y^6]-VIP	180±70	450±50	120±40
[H^6]-VIP	1500±500	900±300	600±250
[C^6]-VIP	650±200	250±90	200±70
VPAC$_1$ antagonist	0.8±0.3	3±1	4±1

Inhibition of [^{125}I]-VPAC$_1$ antagonist binding to VPAC$_1$, Y146A and Y150A VPAC$_1$ receptors by the indicated peptides was evaluated at 23°C, as explained in Methods. Two IC$_{50}$ values are indicated (as well as the per cent of high affinity sites) when a two sites model gave a significantly better fit than the one site model (F test, P value >0.05). [^{125}I]-VIP binding to VPAC$_1$ and Y150A VPAC$_1$ receptor was inhibited by VIP with IC$_{50}$ values of 2 and 5 nM and by [R^{16}]-VIP with IC$_{50}$ values of 0.5 and 1 nM, respectively. The [^{125}I]-VIP IC$_{50}$ values of the other peptides (not shown) were not significantly different from their [^{125}I]-VPAC$_1$ antagonist IC$_{50}$ values. The results represent the average±s.d. of two to five experiments in duplicate.

Table 2 Functional studies at the wild-type and mutated VPAC$_1$ receptors: EC$_{50}$ values (in nM) and efficacy, in per cent of the VIP E$_{max}$

	Wild-type		*VPAC$_1$ h* *Y146A*		*Y150A*	
	EC$_{50}$	*Efficacy*	*EC$_{50}$*	*Efficacy*	*EC$_{50}$*	*Efficacy*
VIP	5±2	100	40±10	78±5	80±20	87±3
[R^{16}]-VIP	0.3±0.1	100±3	4±1.2	100	8±3	100
[A^1]-VIP	60±20	64±7	2000±600	26±8	920±150	37±5
[F^1]-VIP	20±5	77±9	500±100	51±5	200±70	70±10
[E^3]-VIP	90±20	77±8	140±20	45±2	115±25	48±6
[N^3]-VIP	45±10	35±5	60±30	9±2	170±8	52±5
[Y^6]-VIP	6±2	86±8	2000±500	16±4	290±60	57±7
[H^6]-VIP	80±15	67±5	inactive[a]	–	4000[b]	0
[C^6]-VIP	10±6	18±10	inactive[a]	–	4500[b]	0

Adenylate cyclase stimulation through VPAC$_1$, Y146A VPAC$_1$ or Y150A VPAC$_1$ receptors by the indicated peptides was evaluated at 37°C, as explained in Methods. The peptides' efficacies (maximal stimulation by the agonist) was normalized by comparison to the maximal effect of the most efficient agonist, [R^{16}]-VIP, on the same membranes. The results represent the average±s.d. of at least two experiments in duplicate. [a]No detectable modification of the [R^{16}]-VIP dose-effect curve in the presence of 4 M peptide. [b]The K_i value was calculated from [R^{16}]-VIP dose-effect curves in the absence and presence of 4 μM [H^6]-VIP or [C^6]-VIP.

Table 3 Functional studies at the wild-type and mutated VPAC$_2$ receptors: EC$_{50}$ values (in nM) and efficacy, in per cent of the VIP E$_{max}$

	Wild-type		*VPAC$_2$* h *Y130A*		*Y134A*	
	EC$_{50}$	*Efficacy*	*EC$_{50}$*	*Efficacy*	*EC$_{50}$*	*Efficacy*
VIP	7±3	100	200±90	62±9	400±160	87±5
HEXA-VIP	0.9±0.4	105±10	10±3	100	24±15	100
[A^1]-VIP	280±40	47±6	>3000±1200	14±4	–[a]	–[a]
[F^1]-VIP	150±70	72±9	3000±1500	19±9	2000±800	22±10
[E^3]-VIP	75±30	100±10	1500±600	54±5	720±300	70±12
[N^3]-VIP	1500±600	80±7	2700±150	9±3	1400±600	34±11
[Q^3]-VIP	200±80	60±8	300±200	8±4	1000±400	44±8
[Y^3]-VIP	80±10	100±10	>3000	50±4	2500±600	98±22

Adenylate cyclase stimulation through VPAC$_2$, Y130A VPAC$_2$ or Y134A VPAC$_2$ receptors by the indicated peptides was evaluated at 37°C, as explained in Methods. The peptides' efficacies (maximal stimulation by the agonist) was normalized by comparison to the maximal effect of the most efficient agonist HEXA-VIP, on the same membranes. The results represent the average±s.d. of at least two experiments in duplicate. [a]Peptides that did not stimulate the adenylate cyclase at 10 μM.

In terms of functional studies, mutation of the VPAC$_1$ receptor tyrosine residues reduced at least 10 fold the potency of most of the agonists tested, and reduced the efficacy of the VIP analogues [A^1]-, [E^3]-, [H^6]- and [C^6]-VIP (Table 2, Figure

2). The Y150A $VPAC_1$ receptor, in contrast to the wild-type and Y146A $VPAC_1$ receptors, that appeared to stimulate the adenylate cyclase through the high affinity receptor state (compare Tables 1 and 2), apparently activated adenylate cyclase through low affinity receptors.

^{125}I-VIP labelled significantly the wild-type $VPAC_2$ but not the Y130A and Y134A mutated $VPAC_2$ receptors. The $VPAC_2$ selective agonists, Ro 25-1553 and HEXA-VIP were significantly more potent than VIP at these three receptors (Table 1 and Figure 3): we therefore attempted to use [^{125}I]-Ro 25-1553 or [^{125}I]-HEXA-VIP to label the mutated receptors. The increase in tracer binding due to specific recognition of the mutated $VPAC_2$ receptors was not significant. Furthermore, we attempted to use in binding studies at the mutated receptors the novel antagonist JV 1–53 (that we radioiodinated) and reported to have a high affinity for $VPAC_2$ receptors (Rekasi *et al*., 2000). Non-specific binding of [^{125}I]-JV 1–53 was even greater than observed with [^{125}I]-VIP, and tracer binding due to specific recognition of the mutated $VPAC_2$ receptors was not significantly increased (Data not shown).

Mutation of the $VPAC_2$ receptor tyrosine residues reduced at least 10 fold the potency of all the agonists tested, and reduced the efficacy of the VIP analogues [A^1]-, [F^1]-, [N^3]- and [E^3]-VIP (Table 3, Figure 3).

Discussion

Radiolabelled agonists and antagonists binding to G-protein coupled receptor typically have different binding properties: indeed, in contrast to antagonists, the affinity of agonists for their receptors is markedly affected by interaction of the receptor with their cognate G-proteins. Consequently, agonists selectively label the high affinity G-protein coupled receptors state, while antagonists label indifferently coupled and uncoupled receptors. As put forward in this work,

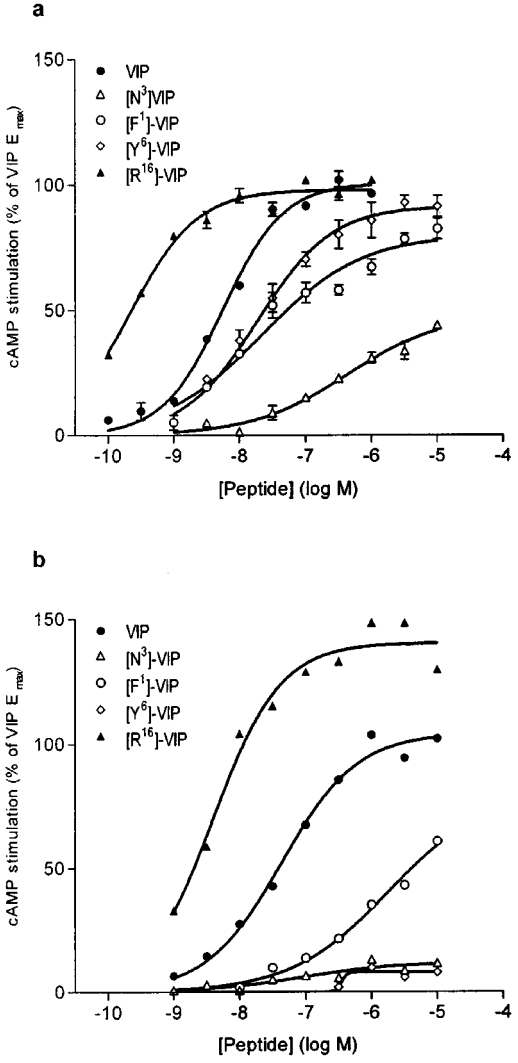

Figure 2 Adenylate cyclase stimulation through (a) $VPAC_1$ and (b) Y146A $VPAC_1$ receptors. In contrast to the Tables, the peptides' effect (over basal stimulation of cAMP synthesis by the agonist ± s.e.mean) is normalized in the Figure by comparison to the maximal effect of the natural ligand, VIP, on the same membranes. Representative of at least three experiments carried out in duplicate.

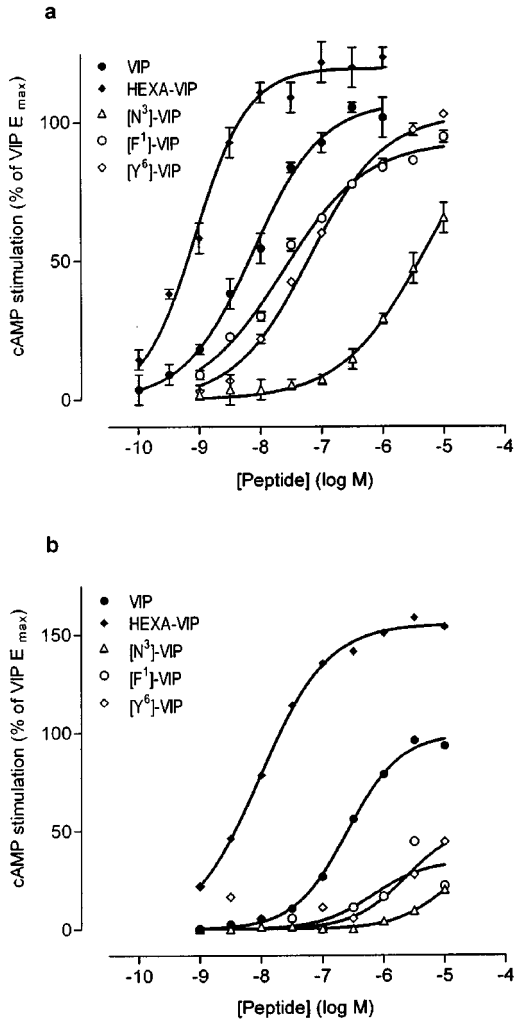

Figure 3 Adenylate cyclase stimulation through (a) $VPAC_2$ and (b) Y130A $VPAC_2$ receptors. In contrast to the Tables, the peptides' effect (over basal stimulation of cAMP synthesis by the agonist ± s.e.mean) is normalized in the Figure by comparison to the maximal effect of the natural ligand, VIP, on the same membranes. Representative of at least three experiments carried out in duplicate.

VPAC$_1$ receptors are no exception to this rule: [^{125}I]-VIP and the [^{125}I]-VPAC$_1$ antagonist labelled the same high affinity receptor population, however the [^{125}I]-VPAC$_1$ antagonist labelled additionally a second receptor population with low affinity for VIP. This second set of receptors are indeed VPAC$_1$ receptors, as established by the following observations: (1) [^{125}I]-VPAC$_1$ antagonist did not bind significantly to non-transfected cells or to CHO cells transfected with other receptors (including the VPAC$_2$ receptors); (2) [^{125}I]-VPAC$_1$ antagonist binding was not affected by addition of GTP to the incubation medium; and (3) [^{125}I]-VIP labelled two rate VPAC$_1$ receptor populations with high and low affinities (Busto *et al*., 1999); Therefore, taken together, our results suggest that, in contrast with [^{125}I]-VIP, the [^{125}I]-VPAC$_1$ antagonist labelled indifferently G-protein coupled and uncoupled human VPAC$_1$ receptors.

It may be argued that different ligands recognise different binding sites on the same receptor, and that one should therefore always use the native ligand for binding studies. However, it must be stressed that 10 of the 12 positions known to be essential for high affinity VPAC$_1$ receptor recognition (Nicole *et al*., 2000) are identical in the VPAC$_1$ antagonist sequence; and that of the two 'significant' mutations (R14L, K20R), one is conservative. Moreover, the VPAC$_1$ over VPAC$_2$ selectivity of this antagonist can be accounted for by mutation of two positions (Thr^{11} and Tyr^{22}) that are essential for VPAC$_2$ but not VPAC$_1$ recognition (Gourlet *et al*., 1998b). Altogether, we are convinced that the data substantiate that the VPAC$_1$ antagonist, like HEXA-VIP (that differs from VIP only by a modification of His^1) does indeed recognise the same binding sites as VIP on VPAC$_1$ and VPAC$_2$ receptors, respectively.

The preceding conclusions allowed us therefore to test the hypothesis at the heart of this work. Indeed, most members of the secretin-receptor family share two tyrosine residues in the first transmembrane domain (TM1): one (Y146 or Y130 in the VPAC$_1$ and VPAC$_2$ receptors, respectively) is found at the beginning of TM1 in the GIP, GLP1, GLP2, glucagon, GRF, PACAP, VIP and secretin, PTH (parathyroid hormone) and PTH related peptide receptors; the second (Y150 or Y134 in the VPAC$_1$ and VPAC$_2$ receptors, respectively), one helix turn further, is found in all except for the GRF, CRF, calcitonin and CGRP (calcitonin gene related peptide) receptors (Horn *et al*., 1998).

We previously observed that the corresponding two tyrosine residues (Y124 and Y128) in the secretin receptor (Di Paolo *et al*., 1999), were important for recognition of their natural ligands by the secretin receptor, and suggested that they might participate in the recognition of the Secretin Asp^3. In this work, we tested the hypothesis that this might also be true for the VIP-selective receptors, VPAC$_1$ and VPAC$_2$.

Replacement of the conserved VPAC$_1$ and VPAC$_2$ receptors Tyr by an Ala reduced the receptors' ability to discriminate VIP from [E^3]- and [N^3]-VIP, suggesting that they were indeed necessary for recognition of the VIP Asp^3 side chain. Both analogues, however, behaved as partial agonists at the two mutant receptors, indicating that additional contacts between the VIP Asp^3 and the mutated receptors were necessary for full receptor activation.

We previously demonstrated that the VIP Asp^3 interacted with the receptors' R168 only upon receptor activation (Solano *et al*., 2001): recognition of the VPAC$_1$ antagonist by the wild-type VPAC$_1$ receptor did not require an acidic side chain in position 3 or R168 in the receptor. We therefore speculated whether the effect of the mutation of the two tyrosine residues on VIP recognition might be indirect (destabilisation of the active receptor conformation that recognises preferentially agonist peptides), rather than direct (hydrogen bonds between the tyrosine's hydroxyl groups and the VIP Asp^3 side chain). In order to test this hypothesis, we synthesised several VIP analogues, mutated in positions 1 and 6, that were expected to behave as partial agonists based on the results of the VIP 'Ala scan' (Nicole *et al*., 2000). In order to avoid as far as possible modifications of the peptides' 3-dimensional structure (Nicole *et al*., 2000), we made conservative (H1F, F6Y and F6H) and nonconservative (H1A, F6C) substitutions. The modification of the VPAC$_1$ receptor's binding profile was not limited to VIP analogues modified in position 3: the mutated receptors' ability to discriminate VIP from [A^1]-, [F^1]-, [H^6]-, and/or [C^6]-VIP also decreased, suggesting that the two receptor tyrosine residues did not interact directly with the VIP Asp^3 but generally facilitated the recognition of VIP agonists. This hypothesis was further supported by our other observations, as outlined below: (1) the 'super agonists', [R^{16}]-VIP (for the VPAC$_1$ receptors) and HEXA-VIP (for the VPAC$_2$ receptors) were not only more potent but also more efficient than VIP at the mutated receptors, suggesting that the natural agonist, VIP, was unable to fully activate the mutated receptors; (2) the efficacy of most if not all the partial VIP agonists tested was reduced with respect to VIP, even more when compared to [R^{16}]-VIP (for the VPAC$_1$ receptors) or HEXA-VIP (for the VPAC$_2$ receptors). [H^6]- and [C^6]-VIP in fact behaved as (low affinity) antagonists at the Y150A receptors; and (3) in contrast with the agonist ligands, the affinity of the VPAC$_1$ receptor for its antagonist (a ligand that does not need the activated receptor conformation for high affinity binding) was unchanged.

Taken together, the alteration of the receptors binding and functional properties suggested that the ability of the mutated receptors to remain in the active conformation in the presence of VIP agonists was decreased: indeed mutations that destabilise the 'active' receptor conformation are expected to decrease the agonists but not the antagonists affinities (Colquhoun, 1998).

In conclusion, our data strongly suggest that the two tyrosine residues found in TM1 are involved in intra-receptor interactions required for receptor activation by stabilising receptor conformations necessary for agonist binding and stabilisation of an active receptor conformation. Modelling of the receptors structures will therefore require identifying the partner residues interacting with these two tyrosine side chains.

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