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Mutational analysis of the human vasoactive intestinal peptide receptor subtype $VPAC_2$: role of basic residues in the second transmembrane helix

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1 We investigated the role of two conserved basic residues in the second transmembrane helix arginine 172 (R172) and lysine 179 (K179) of the $VPAC_2$ receptor.

2 Vasoactive intestinal polypeptide (VIP) activated $VPAC_2$ receptors with an EC_{50} value of 7 nM, as compared to 150, 190 and 4000 nM at R172L, R172Q and K179Q-$VPAC_2$ receptors, respectively. It was inactive at K179I mutated $VPAC_2$ receptors. These results suggested that both basic residues were probably implicated in receptor recognition and activation.

3 The $VPAC_2$-selective VIP analogue, [hexanoyl-His^1]-VIP (C_6-VIP), had a higher affinity and efficacy as compared to VIP at the mutated receptors.

4 VIP, Asn^3-VIP and Gln^3-VIP activated adenylate cyclase through R172Q receptors with EC_{50} values of 190, 2 and 2 nM, respectively, and through R172L receptors with EC_{50} values of 150, 12 and 8 nM, respectively. Asn^3-VIP and Gln^3-VIP behaved as partial agonists at the wild type receptor, with E_{max} values (in per cent of VIP) of 75 and 52%, respectively. In contrast, they were more efficient than VIP (E_{max} values of 150 and 150% at the R172Q $VPAC_2$ receptors, and of 400 and 360% at the R172L receptors, respectively). These results suggested that the receptor's R172 and the ligand's aspartate 3 are brought in close proximity in the active ligand-receptor complex.

5 The K179I and K179Q mutated receptors had a lower affinity than the wild-type receptors for all the agonists tested in this work: we were unable to identify the VIP amino acid(s) that interact with K179.

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Abbreviations: C_6-VIP, [hexanoyl-His^1]-VIP; CHO, Chinese hamster ovary cells; EC1, first extracellular loops; EC_{50}, concentration of agonist required for half maximal response; GRF, Growth Hormone Releasing Factor; IC, intracellular domain; IC_{50}, concentration of ligand required for 50% inhibition of tracer binding; 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); TM2, second transmembrane domain; VIP, vasoactive intestinal polypeptide

Introduction

The neuropeptides Vasoactive Intestinal Polypeptide (VIP) and Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) contribute to the regulation of intestinal secretion and motility, of the vascular tone, of the exocrine and endocrine secretions, of immunological responses and to the development of the central nervous system (Christophe, 1993; Rawlings & Hezareh, 1996; Vaudry *et al*., 2000). Vasoactive Intestinal Peptide (VIP) acts through interaction with two receptors – $VPAC_1$ and $VPAC_2$ – encoded by different genes, that share 49% similarities (Harmar *et al*., 1998). These receptors belong to a subfamily of seven transmembrane G protein coupled receptors, characterized by a large amino-terminal domain containing highly conserved cysteine residues and by conserved transmembrane helices. This subfamily includes the receptors for VIP, PACAP (Pituitary Adenylate Cyclase Activating Peptide), secretin, glucagon, glucagon like peptides 1 and 2, GIP (Gastric Inhibitory Peptide), GRF (Growth Hormone Releasing Factor), CRF (Corticotrophin Releasing Factor), parathyroid hormone and calcitonin (Horn *et al*., 1998).

$VPAC_1$ and $VPAC_2$ receptors recognize with a comparable high affinity VIP and the related peptide PACAP. They can however be discriminated by low affinity natural ligands like secretin and GRF, and by high affinity synthetic ligands derived from VIP (Harmar *et al*., 1998; Nicole *et al*., 2000). Furthermore, mutation of extracellular amino acid residues that are conserved in both receptors subtypes but different in other members of the subfamily indicated that there are dissimilarities in the structure-function relationship of $VPAC_1$ and $VPAC_2$ receptors (Nicole *et al*., 1998).

We identified in the secretin (Di Paolo *et al*., 1999; Vilardaga *et al*., 1996) and $VPAC_1$ receptors (Solano *et al*., 2001) two basic residues within the second transmembrane helix that serve as anchorage points for the Asp^3 residue of secretin and VIP, respectively.

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In the present work we extended these observations to the $VPAC_2$ receptor. The replacement of these two basic residues by either hydrophobic or polar uncharged residues markedly reduced the affinity of VIP for the receptor and its capacity to stimulate adenylate cyclase activity. Acylation of the amino-terminus or replacement of the VIP Asp^3 by an Asn or a Gln residue partially or completely restored VIP activity at the mutated $VPAC_2$ receptors.

Methods

Construction of the mutated receptors

The cell line expressing the $VPAC_2$ wild-type (wt) receptor has already been described (Svoboda *et al*., 1994). Four $VPAC_2$ receptor mutants were obtained: R172L, R172Q, K179I, K179Q.

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 $VPAC_2$ receptor-coding region, inserted into the mammalian expression vector pcDNA3.1 (Invitrogen Corp. CA, U.S.A.), was submitted to 22 cycles of polymerase chain reaction (PCR) (95°C for 30 s; 54°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 perfectly matched nucleotides (only the forward primers are shown): V2H-R172L CTGTCCTTCATCCTGTTAGCCATCTCAGTGCTG; V2H-R172Q CTGTCCTTCATCCTGCAAGCCATCTCAGTGCTG; V2H-K179I CATCTCAGTGCTGGTCATTGACGACGTTCTCTAC; V2H-K179Q CATCTCAGTGCTGGTCCAGGACGACGTTCTCTAC.

Following PCR, 10 μl were analysed by agarose gel electrophoresis and the remaining 40 μl were digested for at least 2 h at 37°C by 1 μl *Dpn*I 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), and three were retained, further purified on Qiaquick PCR purification spin columns (Qiagen, Hilden, Germany) and the mutations verified by DNA sequencing on an ABI automated sequencing apparatus (using BigDye Terminator Sequencing Prism Kit also from ABI (Perkin-Elmer, CA, U.S.A.)). Plasmid DNA from one clone for each mutation, was prepared using a midiprep endotoxin-free kit (Stratagene, La Jolla CA, U.S.A.). The complete nucleotide sequence of the receptor coding region was verified by DNA sequencing. Twenty μg plasmid DNA were electroporated (Electroporator II, Invitrogen Corp. CA, U.S.A.) into wild-type Chinese Hamster Ovary (CHO-K1) cells. Selection was carried out in culture medium HamF12 50%, DMEM 50%, Foetal Calf Serum 10%, 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 ml^{-1} Geneticin (G418) medium. After 10 to 15 days of selection, isolated colonies were transferred to 24 well microtiter plates and grown until confluence, trypsinized and further expanded in 6-well microtiter plates. The cell clones expressing the different constructions were selected by testing the ability of 10 μM VIP or 10 μM C_6-VIP to stimulate the membrane adenylate cyclase activity.

Membrane preparation

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 $400 \times g$ and the supernatant 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 studies

Binding studies were performed as described using either [^{125}I]-VIP, [^{125}I]-C_6-VIP, 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) ([^{125}I]-Ro 25-1553) and [^{125}I]-Gln^3-VIP as tracer. The tracers were radiolabelled similarly and had comparable specific radioactivity (Gourlet *et al*., 1997). In all cases, the non-specific binding as defined as residual binding in the presence of 1 μM VIP. 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. Ten to 20 μg of protein were used per assay in the experiments shown; the membrane concentration was increased up to 50 μg protein per assay in an attempt to observe tracer binding to R172L, K179I and K179Q $VPAC_2$ receptors. 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 of Salomon *et al*. (1974) as described previously. 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 cyclic AMP, 1 mM theophylline, 10 mM phospho(enol)pyruvate, 30 μg ml^{-1} pyruvate kinase and 30 mM Tris-HCl at a final pH of 7.8.

Peptide synthesis

The peptides used were synthesized in our laboratory as described (Gourlet *et al*., 1998; O'Donnell *et al*., 1994). The 1-hydroxybenzotriazole derivative of hexanoic acid was coupled to the amino-terminus of VIP before cleavage and deprotection. The purity of peptides was assessed by capillary electrophoresis, and their conformity to the expected sequence, 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 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$_2$ receptor expressed in CHO cells

Three tracers could be used to characterize binding to $VPAC_2$ receptors, [^{125}I]-VIP, [^{125}I]-Ro 25-1553 and [^{125}I]-C_6-VIP. The unlabelled ligands IC_{50} values did not depend on the tracer used. The cell line used expressed at least 210 ± 40 fmol of $VPAC_2$ receptors·(mg protein)$^{-1}$. As previously shown (Juaranz *et al*., 1999) VIP, Ro 25-1553 and C_6-VIP recognized $VPAC_2$ receptors with a high affinity, and stimulated maximally the adenylate cyclase activity (Figure 1, Table 1). Three VIP analogues in which the Asp^3 residue was replaced by a Glu, an Asn or a Gln residue (Glu^3-VIP, Asn^3-VIP, Gln^3-VIP) had significantly higher IC_{50} values in competition curves, and higher EC_{50} values for adenylate cyclase activation (Figure 1, Table 1). Asn^3-VIP and Gln^3-VIP were partial agonists, as the maximal adenylate cyclase stimulation by these analogues reached only 50% of the value obtained with VIP.

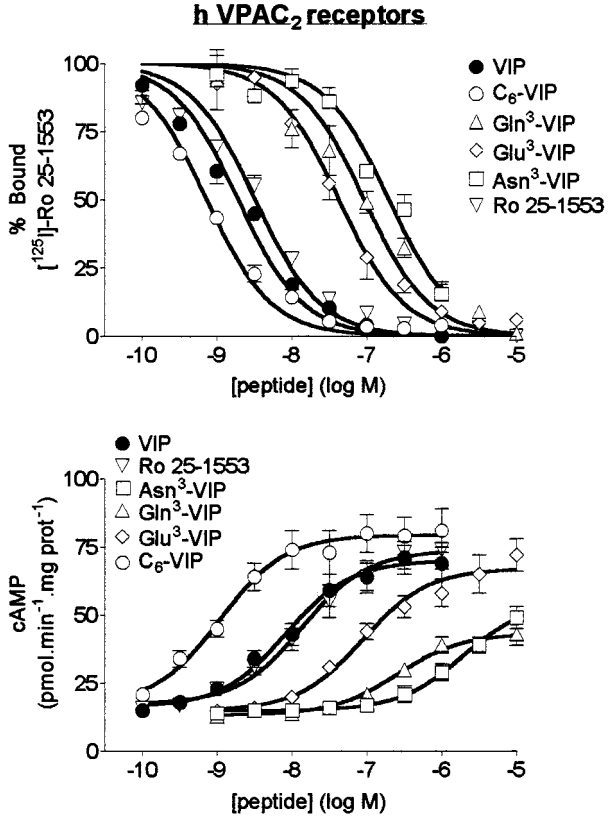

Figure 1 Binding to and adenylate cyclase stimulation through human $VPAC_2$ receptors. VIP (closed circles), C_6-VIP (open circles), Asn^3-VIP (open squares), Gln^3-VIP (open triangles), Glu^3-VIP (open diamonds) and Ro 25-1553 (inverted triangles) competition curves (top panel) and dose-effect curves (bottom panel) were obtained on membranes from cloned CHO cells expressing human $VPAC_2$ receptors (mean$\pm$s.e.mean of four experiments in duplicate).

Analysis of the mutated R172Q, R172L, K179Q and K179I VPAC$_2$ receptors

Mutations were performed on residues located in the second transmembrane helix. The CHO cells expressing the mutated receptors were selected by screening geneticin resistant clones for the ability of 10 μM VIP or 10 μM of C_6-VIP to increase adenylate cyclase activity. We then attempted to measure [^{125}I]-VIP, or [^{125}I]-Ro 25-1553 binding, and performed dose-effects curves of adenylate cyclase in presence of VIP and the various analogues.

Tracer binding is directly proportional to its affinity constant. At a receptor concentration of 1000 fmol mg protein^{-1}, using 50 μg protein per assay, the predicted specific binding varies between 0.5 and 0.05% of the added radioactivity if the tracer K_D value varies between 100 and 1000 nM. Radiolabelled peptides bind non-specifically to CHO cell membranes: non-specific binding represented up to 8% of the added tracer at this protein concentration. The tracer binding increment due to specific recognition of receptors was, therefore, non-significant when the receptors' affinity for the radiolabelled peptide was too low. This probably explains why we were unable to demonstrate significant specific binding of [^{125}I]-VIP or [^{125}I]-Ro 25-1553 to the mutated receptors. We therefore attempted to identify another high affinity ligand to allow binding studies at the mutated receptors.

VIP, Ro 25-1553 and Glu^3-VIP had very high EC_{50} values at the R172Q and R172L mutants (Figure 2, Table 1). C_6-VIP, Asn^3-VIP and Gln^3-VIP had a lower EC_{50} values and stimulated more efficiently adenylate cyclase activity. Based on these data, we attempted to use [^{125}I]-Gln^3-VIP and [^{125}I]-C_6-VIP in binding studies. Binding of both tracers was sufficient to analyse competition curves at the R172Q mutant: the receptor concentration was 400 ± 90 fmol mg protein^{-1}. Competition curves are shown in Figure 3, and the IC_{50} values in Table 1.

[^{125}I]-Gln^3-VIP binding to the R172L $VPAC_2$ receptors was detectable, but too low to allow competition curves analysis, as non-specific binding represented >50% of the total tracer binding. Assuming that the [^{125}I]-Gln^3-VIP K_D value was close to its EC_{50} value, the R172L $VPAC_2$ receptor concentration was estimated between 250 and 1000 fmol mg protein^{-1}.

The EC_{50} values of all analogues tested on the K179Q mutant receptor were in the μM range. C_6-VIP had the lowest EC_{50} value, and was also the most efficient analogue – giving much higher maximal stimulation than VIP itself (Figure 4 and Table 1). On the K179I $VPAC_2$ mutant, C_6-VIP was the only peptide able to significantly stimulate the adenylate cyclase activity (Figure 4 and Table 1). We were unable to observe significant specific binding of either [^{125}I]-C_6-VIP or [^{125}I]-Gln^3-VIP to membranes from CHO cells expressing the K179Q or K179I $VPAC_2$ receptor mutants.

Discussion

Most members of the secretin-receptor family share two basic residues in the second transmembrane domain (TM2) or at

Table 1 Peptide concentrations necessary to activate the adenylate cyclase activity (EC_{50}) or inhibit tracer binding (IC_{50}) at membranes from CHO cells expressing the wild-type or mutated $VPAC_2$ receptors

	Wild-type VPAC$_2$		*R172L VPAC$_2$*	*R172Q VPAC$_2$*		*K179I VPAC$_2$*	*K179Q VPAC$_2$*
	EC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)	EC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)	EC_{50} (nM)
VIP	7	3	150*	190*	150	not detectable†	4000*
C_6-VIP	0.9	1	50*	17	13	1000	900
Ro 25-1553	10	5	not detectable†	>10,000	150	not detectable†	5000*
Asn^3-VIP	2000*	300	12	2	3	not detectable†	2000*
Gln^3-VIP	200*	200	8*	2	3	not detectable†	5000*
Glu^3-VIP	70	50	180*	700*	900	not detectable†	2500*

All results represent the average of at least three experiments in duplicate. The standard deviation of the log (IC_{50}) and log (EC_{50}) values was always below 0.15 log units. †'not detectable' indicates that the compound displayed neither agonist nor antagonist activity at 10 μM. *(Partial agonist as compared to VIP, C_6-VIP or Asn^3-VIP).

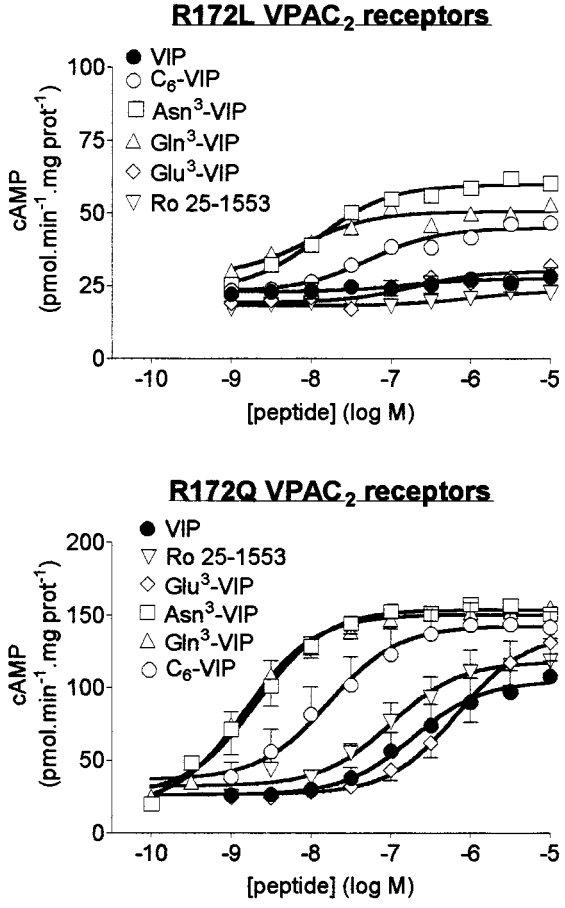

Figure 2 Adenylate cyclase stimulation through R172L and R172Q $VPAC_2$ receptors. VIP (closed circles), C_6-VIP (open circles), Asn^3-VIP (open squares), Gln^3-VIP (open triangles), Glu^3-VIP (open diamonds) and Ro 25-1553 (inverted triangles) dose-effect curves were obtained on membranes from cloned CHO cells expressing human R172L (top panel) and R172Q (bottom panel) $VPAC_2$ receptors (mean$\pm$s.e.mean of 2–4 experiments in duplicate).

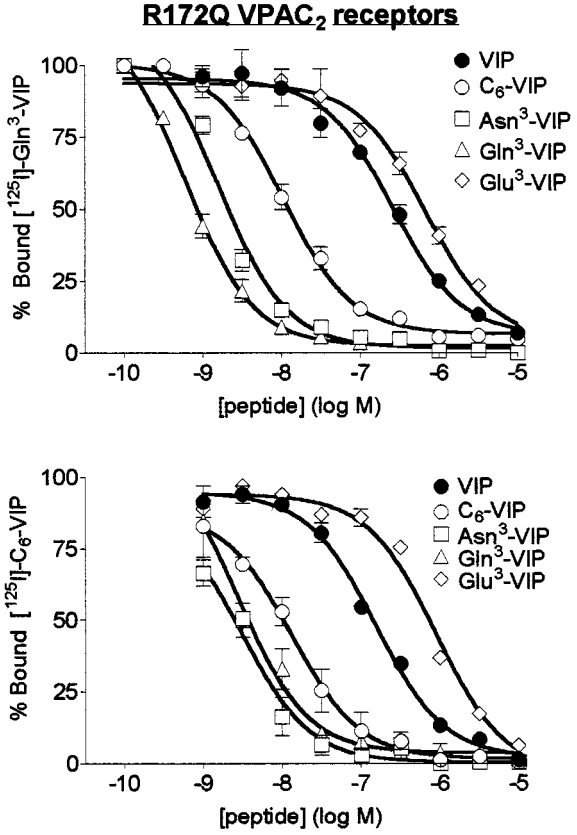

Figure 3 Binding to R172Q $VPAC_2$ receptors. VIP (closed circles), C_6-VIP (open circles), Asn^3-VIP (open squares), Gln^3-VIP (open triangles) and Glu^3-VIP (open diamonds) competition curves were obtained at R172Q $VPAC_2$ receptors using [^{125}I]-Gln^3-VIP (top panel) or [^{125}I]-C_6-VIP (bottom panel) (mean$\pm$s.e.mean of 2–4 experiments in duplicate).

the beginning of the first extracellular loop (EC1) region: an arginine or lysine residue (R172 in the $VPAC_2$ receptor) is found in the second transmembrane helix of all but calcitonin receptors and with the exception of the glucagon, GRF and Corticotrophin Releasing Factor (CRF) receptors, all the receptors from the secretin-receptor family (Horn *et al*., 1998) have a second basic residue (lysine, arginine or histidine) (K179 in the $VPAC_2$ receptor) close to or inside EC1. As shown in this work, mutation of either residues in the $VPAC_2$ receptor resulted in marked alterations of the receptors' pharmacological properties. Receptor mutations that affect their binding or functional properties can be subdivided into three (non-exclusive) categories: (1) mutations that affect ligand recognition, (2) mutations that affect the receptors' biological activity (for instance by preventing G protein recognition), and (3) mutations that affect the isomerization constant between receptor conformations (Colquhoun, 1998; Galzi *et al*., 1996). If the receptor activation is described by

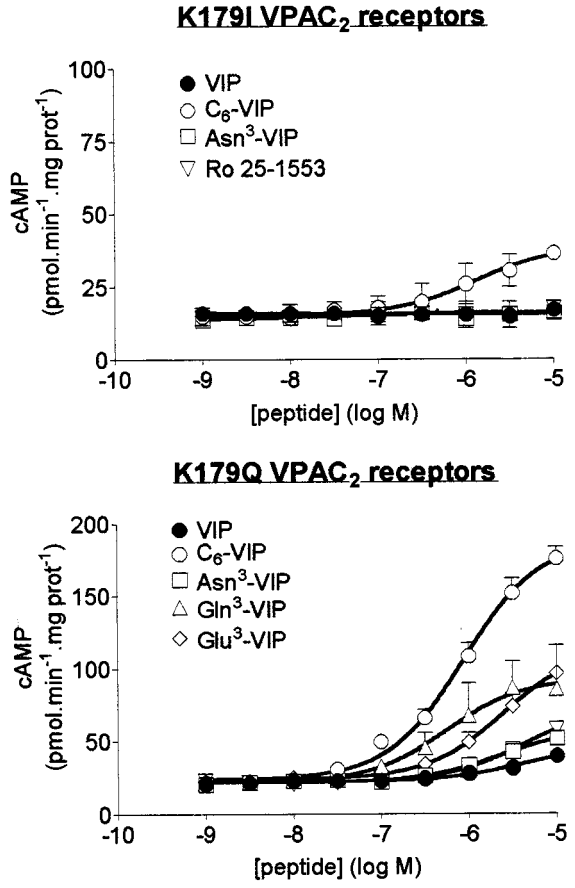

Figure 4 Adenylate cyclase stimulation through K179I and K179Q VPAC$_2$ receptors. VIP (closed circles), C_6-VIP (open circles), Asn^3-VIP (open squares), Gln^3-VIP (open triangles), Glu^3-VIP (open diamonds) and Ro 25-1553 (inverted triangles) dose-effect curves were obtained on membranes from cloned CHO cells expressing human K179I (top panel) or K179Q (bottom panel) VPAC$_2$ receptors (mean $\pm$ s.e.mean of 2–4 experiments in duplicate).

$R \overset{K_{act}}{\leftrightarrows} R^*$ in the absence of agonist and by $H + R \overset{K}{\longleftrightarrow} HR \overset{\alpha K_{act}}{\leftrightarrows} HR^*$ in the presence of agonist (where K represents the agonists' affinity constant for the resting receptor, K_{act}, the receptor constitutive activation constant and α, the effect of agonist recognition on receptor activation), it is possible to define three important parameters: K, the agonists' ability to recognize the receptor, α, that measures the agonist ability to activate the receptor, and K_{act}, the activated receptor's stability. The agonist affinity constant ($[HR + HR^*]/[H(R + R^*)]$) is defined as: $K(1 + \alpha K_{act})$, and its' efficacy ($HR^*/(HR + HR^*)$ as $\alpha K_{act}/(1 + \alpha K_{act})$. Mutations that affect the agonist binding site and modify K affects the agonists' affinities, but not their relative efficacies. If the αK_{act} product is not too large, receptor mutations that affect K_{act}, like agonist modifications that affect α, affect simultaneously the agonists affinities and efficacies. If the activated receptor stability (K_{act}) is sufficient, all compounds characterized by large α values (sufficient to achieve $\alpha K_{act} > 10$) behave as full agonists (activate $> 90\%$ of the occupied receptors) – independently of the actual values of α, K_{act}, or of the receptor density. Receptor mutations that destabilize the active receptor conformation (decrease K_{act}) decrease the affinity of all agonists, but affect their efficacy only if the agonist α value is not sufficient to compensate the decreased receptor K_{act} (Colquhoun, 1998). As discussed below, our results suggested that R172 facilitates VIP recognition (increases K) by forming an ionic bond with the ligand Asp^3 residue, and that both basic residues stabilize the activated receptor conformation (increase K_{act}).

The VIP Asp^3 residue was important for high affinity binding and activation of the VPAC$_2$ receptors: replacement by glutamic acid reduced 10 fold the VIP affinity, and replacement by the uncharged asparagine or glutamine residues reduced the peptide affinity 300 and 100 fold, respectively. Glu^3-VIP behaved as a full agonist (adenylate cyclase activation), but Asn^3-VIP and Gln^3-VIP were partial agonists: as in the VPAC$_1$ (Hoare *et al*., 1999; Nicole *et al*., 2000) and secretin receptor (Di Paolo *et al*., 1999; Vilardaga *et al*., 1996), replacement of the VIP Asp^3 residue decreased the agonist 'α' value. The following results suggested that the VIP Asp^3 interacted with the VPAC$_2$ receptor R172. Mutation of this residue to hydrophobic (L) or polar uncharged (Q) residues markedly reduced the VPAC$_2$ receptor's affinity for its natural ligand. The mutated VPAC$_2$ receptors did not discriminate VIP from Glu^3-VIP, but recognized Asn^3-VIP and Gln^3-VIP with a higher affinity than the wild-type receptor. The observation that Gln^3-VIP and Asn^3-VIP were more efficient than VIP at the mutated receptors suggested that the VIP aspartate and receptor arginine residue must be brought into close contact to allow receptor activation. Indeed, dehydration of ionic residues is a very unfavourable process: it is compensated for in the wild-type agonist-receptor complex by the interaction between the two opposite charges. We previously demonstrated that VIP acylation by hexanoic acid increased its affinity (but not its efficacy) at VPAC$_2$ receptors, in binding and functional studies (Juarranz *et al*., 1999). The ability of the Asn^3-VIP-bound R172Q-VPAC$_2$ receptor to activate adenylate cyclase was normal (compared to VIP-bound VPAC$_2$ receptors), but VIP behaved as a partial agonist at the mutated receptors, when compared to either C_6-VIP or Asn^3-VIP: the α value of C_6-VIP and Asn^3-VIP were larger than VIP's at the mutated receptor. Our results thus suggested that, like in the VPAC$_1$ receptor, the VPAC$_2$ receptor R172 and the VIP Asp^3 must be buried in close contact in the agonist-receptor complex for optimal receptor activation, and that hexanoylation of the VIP α-amino residue increased the value of α.

We were unable to measure the K179I and K179Q receptor densities. This means that we could not verify that the ability of agonist-bound mutant VPAC$_2$ receptor mutants to activate adenylate cyclase was normal, and that the agonist EC_{50} values were not underestimated due to the presence of spare receptors. Mutation of the basic residue K179 to hydrophobic (I) or uncharged (Q) residues increased at least 400 fold the VIP EC_{50} value, suggesting that its' affinity was markedly decreased. We were unable to identify the VIP amino acid (if any) that contact this lysine residue: all the VIP analogues we tested had greater EC_{50} values at the mutated VPAC$_2$ receptor as compared to wild-type receptors. C_6-VIP had a higher affinity and efficacy than VIP at the four mutated VPAC$_2$ receptors: it is likely that this compound had a greater ability than VIP to stabilize the active receptor conformation (greater 'α' value), and therefore compensated more efficiently the deleterious effect of the receptor

mutations on the stability of the active VPAC$_2$ receptor conformation (K_{act}).

Taken together, our results supported the hypothesis that the conserved arginine residue interacted with the VIP Asp^3 residue, and that the arginine and lysine residues were important to stabilize the active receptor conformation.

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