Evidence for a direct interaction between the Thr¹¹ residue of vasoactive intestinal polypeptide and Tyr¹⁸⁴ located in the first extracellular loop of the VPAC₂ receptor

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We developed previously VPAC₁ [vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide (PACAP) receptor] > VPAC₂ receptor selective ligands. Replacement of the VIP-Thr¹¹ by an Arg¹¹ in these ligands contributed to their selectivity: Arg¹¹-VIP had a 200-fold lower affinity when compared with VIP at VPAC₂ receptors as opposed to 3- to 5-fold higher affinity at VPAC₁ receptors. Comparison of the binding and functional properties of related VIP analogues suggested that the VPAC₁ selectivity of Arg¹¹-VIP was due to the loss of a hydrogen bond between the hydroxy group of Thr residue and the VPAC₂ receptor, steric hindrance between the Arg side chain and the VPAC₂ receptor and charge attraction by the VPAC₁ receptor. Comparison of the ability of VIP analogues to activate adenylate cyclase through chimaeric VPAC₁/VPAC₂ and VPAC₂/VPAC₁ receptors indicated that the first extracellular

receptor loop carried most of the VPAC₂ receptors' ability to discriminate VIP from Arg¹¹-VIP. Based on results obtained for a truncated VPAC₂ receptor and the closely related PACAP-preferring receptor (PAC₁) and secretin receptors, we hypothesized that Thr¹¹ interacted with the VPAC₂ receptor Tyr¹⁸⁴ (similar to the VPAC₁ receptor Phe²⁰⁰ residue). The Y184F (Tyr¹⁸⁴ → Phe) VPAC₂ mutant lost the ability to discriminate VIP from Val¹¹-VIP, and the F200Y VPAC₁ mutant acquired the ability to discriminate the natural peptide from Val¹¹-VIP. These results support the hypothesis that the hydroxy group of the native VIP-Thr¹¹ side chain can indeed form a hydrogen bond with the Tyr side chain in the VPAC₂ receptor.

Key words: VIP binding site, VPAC₁ antagonist, VPAC₁ selective agonist, VPAC₂ receptor.

INTRODUCTION

The vasoactive intestinal polypeptide (VIP) exerts its biological activities through interaction with two receptors, now known as VPAC₁ [VIP/PACAP (pituitary adenylate cyclase-activating peptide)] and VPAC₂ [1], which are encoded by different genes [2,3] with only 49% identity. They belong to the class II G-protein-coupled receptor family [4], which also includes the calcitonin-, parathormone-, glucagon-, glucagon-like peptides 1 and 2, growth hormone-releasing factor (GRF), corticotrophin-releasing factor, secretin and PACAP receptors, as well as a few orphan receptors. The interaction surface between VIP and its receptors, investigated by Ala scan, is very extensive [5–7]: 14 and 16 of 28 amino acids were identified as important for VPAC₁ and VPAC₂ receptor recognition respectively. NMR analysis of the VIP structure (in methanol/water solutions) [8] and theoretical models [6] predict that VIP forms an α -helix, preceded and followed by random coils or by 1 or 2 type III β turns [9–11]. In aqueous solutions, VIP however does not assume a well-defined three-dimensional structure. This peptide is capable of recognizing and activating PACAP-preferring receptors (PAC₁) with a low affinity the recently described receptor-bound PACAP fragment structure (N-terminal β -coil followed by an α -helix) [12] might serve as a template to build a model of the active VIP conformation.

The receptors belonging to the secretin-receptor family do not possess the 'signature' amino acids that characterize the rhodopsin-like receptors. They nevertheless activate the same G protein families, suggesting that their intracellular structure must be

similar to that of rhodopsin [13]. It has indeed been suggested that there is a close similarity between the structural profile of the transmembrane domains of a secretin family receptor (the glucagon-like peptide 1 receptor) and those of the rhodopsin-like family [14,15]. A three-dimensional model of the receptor extracellular binding domain has also been proposed, by analogy with the subdomain of a yeast lipase [16]. Identification of VIP-receptor contact points is essential to assess critically the validity of these models.

A study of the functional properties of chimaeric receptors suggested that VIP interacts at least with the N-terminal domain of the receptors, the three extracellular loops (ECL) and the first ECL (ECL₁)-proximal regions of transmembrane helix 2 (TM2) and TM3 [17–23]. In previous studies, we attempted to understand the orientation of the peptide relative to its receptor by studying the interaction of hybrid or point-mutated peptides with chimaeric and point-mutated receptors. Some of our results suggested that, as for parathyroid hormone [24], the central and C-terminal peptide region anchor the peptide to the N-terminal, extracellular receptor domain, and that the N-terminal VIP sequence activates the receptor through recognition of its transmembrane domain. Indeed, (i) the VIP Asp³ side chain recognized an arginine residue in TM2 [25], and preferential recognition of a VIP analogue hexanoylated on the His¹ α -amino group (known as C₆-VIP) required an additional proline residue in the VPAC₂ receptor ECL₂ region [26] that probably altered the conformation of adjacent VPAC₂ receptor regions. (ii) Hybrid PACAP/secretin peptides were potent agonists at secretin/VPAC₁ chimaeric receptors, provided that the central portion (amino acids 8–15)

Abbreviations used: ECL, extracellular loop; GRF, growth hormone-releasing factor; PACAP, pituitary adenylate cyclase-activating peptide; PAC, PACAP-preferring receptor; TM, transmembrane helix; VIP, vasoactive intestinal peptide; VPAC, VIP/PACAP receptor; Y184F, Tyr¹⁸⁴ → Phe.

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of the peptide was cognate to the N-terminal extracellular receptor domain [27]. (iii) The selectivity pattern of VIP and secretin fragments suggested that the C-terminal ends of secretin and VIP also interact with the N-terminal domains of the receptors [28].

Other results, however, suggest that the seven-helix receptor domain also participates in the recognition of central and C-terminal peptide regions: the discrimination of VPAC₁ and VPAC₂ receptors by Arg¹⁶-PACAP (amino acids 1–23) was shared by their three ECLs [23], and the lower affinity of VPAC₂ receptors for Leu²²-VIP as compared with VIP (with a Tyr²²) is supported not only by the N-terminal domain but also by the ECL₂ of the receptor [23].

Although VPAC₁ and VPAC₂ receptors have a comparably high affinity for VIP, they can be distinguished pharmacologically by their affinity for the related peptides, secretin and GRF [29]. This observation served for the synthesis of selective agonists and antagonists of the VPAC₁ receptor [29,30].

The preference of GRF for VPAC₁ over VPAC₂ receptors is probably explained by two mutations: as shown previously [5–7,31] and in the present study, the VIP Thr¹¹ and Tyr²², which are mutated to Arg¹¹ and Leu²² in GRF, are significantly more important for recognition of the VPAC₂ than VPAC₁ receptor. We used in the present study previously characterized VPAC₁/VPAC₂ receptor chimaeras [22,23,32] to identify the receptor regions that are responsible for the high affinity of Arg¹¹-VIP for VPAC₁ and its low affinity for the VPAC₂ receptor, and subsequently used point mutations to identify the VPAC₂ receptor tyrosine that forms a hydrogen bond with the VIP Thr¹¹ side chain.

MATERIALS AND METHODS

The cell lines expressing the VPAC₁ and the VPAC₂ receptors have been detailed in previous publications [3,22,23,32]. Chimaeric receptors were constructed by the PCR overlap extension strategy as described previously [22,23,32].

The chimaeric receptors investigated were: C1, VPAC₂ (N₁ → Val¹²⁶)/VPAC₁ (Lys¹⁴³ → C₁); C2, VPAC₂ (N₁ → Arg¹⁵⁸)/VPAC₁ (Ala¹⁷⁵ → C₁); C3, VPAC₂ (N₁ → Lys²⁰³)/VPAC₁ (Ala²¹⁷ → C₁); C4, VPAC₂ (N₁ → Leu²²⁷)/VPAC₁ (Tyr²⁴¹ → C₁); C5, VPAC₂ (N₁ → Val²⁸⁰)/VPAC₁ (Leu²⁹³ → C₁); C6, VPAC₂ (N₁ → Glu³⁶⁰)/VPAC₁ (Leu³⁷⁴ → C₁) and C7, VPAC₁ (N₁ → Val¹⁴²)/VPAC₂ (Lys¹²⁷ → C₁); C8, VPAC₁ (N₁ → Lys²¹⁶)/VPAC₂ (Leu²⁰⁴ → C₁); C9, VPAC₁ (N₁ → Leu²⁹³)/VPAC₂ (Trp²⁸¹ → C₁).

Generation of the truncated and point-mutated receptors was achieved using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Briefly, the human VPAC₁ or VPAC₂ receptor coding region, inserted into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, U.S.A.), was submitted to 25 cycles of PCR (95 °C for 30 s, 55 °C for 1 min and 68 °C for 14 min) in a reaction volume of 50 µl. The forward and reverse primers were complementary and contained the required nucleotide changes flanked on either side by 15 perfectly matched nucleotides. The forward primers used were the following. VPAC₂ΔSSW: 5'-CACTGCCCTGACCCAGTGGCTGCAAGCTGAG-3'; F200Y VPAC₁ receptors: 5'-CAAGACTTGGCCCTCTaCGACAGCGGGGAGTCGGA-CC-3'; Tyr¹⁸⁴ → Phe (Y184F) VPAC₂ receptors: 5'-GTCAAGGACGACGTTCTCTtCTCCAGCTCTGGCAGCTTG-3'.

After PCR, the parental methylated DNA was removed by *DpnI* restriction enzyme digestion. The digested PCR products were transformed into TOP10 One Shot competent *Escherichia coli* bacterial cells (Invitrogen). DNA extracts from several

Figure 1 VIP and Arg¹¹-VIP binding and functional properties: wild-type receptors

Binding and functional properties of VIP (□) and Arg¹¹-VIP ([R¹¹]-VIP) (△) were investigated at VPAC₁ (left panels) and VPAC₂ (right panels) receptors. The tracers used were ¹²⁵I-labelled VIP for binding studies at VPAC₁ receptors and ¹²⁵I-labelled C₆-VIP for binding studies at VPAC₂ receptors respectively. The adenylate cyclase activity was evaluated as pmol of cAMP produced per min per mg protein. Results are representative of at least three experiments performed in duplicate.

colonies were verified by agarose-gel electrophoresis; three were checked for the presence of the correct mutation by automated DNA sequencing (ABI Division of PerkinElmer, CA, U.S.A.). Plasmid DNA from one clone, containing the correct nucleotide substitutions, was prepared using a midiprep endotoxin-free kit (Stratagene); 20 µg was electroporated (Electroporator II; Invitrogen) into wild-type Chinese-hamster ovary-K1 cells, and the complete nucleotide sequence of the receptor coding region was verified by DNA sequencing. Selection was performed in typical culture medium [Ham's F12/Dulbecco's modified Eagle's medium (1:1), containing 10% foetal calf serum, 1% penicillin (10 m-units/ml), 1% streptomycin (10 µg/ml) and 1% L-glutamine (200 mM)] (Life Technologies, Paisley, Scotland, U.K.), supplemented with 600 µg Geneticin (G418)/ml of culture medium. After 10–15 days of selection, isolated colonies were transferred to 24-well microtitre plates and further expanded into 6-well microtitre plates, from which cells were scraped and membranes prepared for screening by an adenylate cyclase activity assay.

Membrane preparation

Membranes were prepared from scraped cells, pelleted and then lysed in 1 mM NaHCO₃ solution followed by immediate freezing in liquid nitrogen. After thawing, the lysate was first centrifuged at 4 °C for 10 min at 400 g and then the supernatant was centrifuged for a further 15 min at 20000 g. The pellet was

Figure 2 VIP and Arg¹¹-VIP functional properties: wild-type and chimaeric receptors

Activation of adenylate cyclase by VIP (□) and by Arg¹¹-VIP ([R¹¹]-VIP) (△), acting through VPAC₁ (top left panel), VPAC₂ (top right panel) and chimaeric receptors C3: VPAC₂ (N₁ → Lys²⁰³)/VPAC₁ (Ala²¹⁷ → C₁) (bottom left panel) and C4: VPAC₂ (N₁ → Leu²²⁷)/VPAC₁ (Tyr²⁴¹ → C₁) (bottom right panel). The adenylate cyclase activity was evaluated as pmol of cAMP produced per min per mg protein. Results are representative of at least three experiments performed in duplicate.

resuspended in 1 mM NaHCO₃ and used immediately as a crude membrane fraction.

Binding studies

Binding studies were performed as described previously [22,32] using either ¹²⁵I-labelled VIP or ¹²⁵I-labelled hexanoyl-His¹-VIP (called ¹²⁵I-labelled C₆-VIP) as tracer. The tracers were radio-labelled similarly and had comparable specific radioactivity [22,32]. 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 of buffer, containing 20 mM Tris/maleate, 2 mM MgCl₂, 0.1 mg/ml of bacitracin and 0.5 % BSA (pH 7.4), to which 3–30 μg of protein was added per assay. Bound and free radioactivity was 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 % BSA.

Receptor densities

The receptor densities were evaluated by analysis of 'homologous' competition curves, using ¹²⁵I-labelled VIP/VIP at wild-type and mutated VPAC₁ receptors and ¹²⁵I-labelled C₆-VIP/C₆-VIP at wild-type and mutated VPAC₂ receptors, under the assumption that radiolabelling did not affect the affinity of the peptide for the receptors. The density (described in [22,23,26, 32]) of the wild-type and chimaeric receptors varied between 1.5 and 3.0 nmol/mg of protein. The truncated (VPAC₂ΔSSW) receptors were expressed at 11.5 ± 3.0 nmol/mg of protein, the F200Y VPAC₁ receptors at 1.92 ± 0.4 nmol/mg of protein and the Y184F VPAC₂ receptors at 4.95 ± 0.7 nmol/mg of protein.

Adenylate cyclase activity

Adenylate cyclase activity was determined by the procedure of Salomon et al. [33]. Membrane proteins (3–15 μg) were incubated in a total volume of 60 μl, containing 0.5 mM [α-³²P]ATP, 10 μM GTP, 5 mM MgCl₂, 0.5 mM EGTA, 1 mM cAMP, 1 mM theophylline, 10 mM phosphoenolpyruvate, 30 μg/ml pyruvate kinase and 30 mM Tris/HCl at a final pH of 7.8.

Table 1 Binding and functional studies

Affinity (pK_i), potency [–log(EC₅₀)] and efficacy (in % of the maximal over basal effect of VIP) ± S.D. of VIP and VIP analogues, at VPAC₁, VPAC₂, F200Y VPAC₁ and Y184F VPAC₂ receptors.

	VPAC ₁		F200Y VPAC ₁		VPAC ₂		Y184F VPAC ₂	
	Binding	Cyclase	Binding	Cyclase	Binding	Cyclase	Binding	Cyclase
VIP	8.57 ± 0.19*	8.71 ± 0.22*† (100%)	8.50 ± 0.27*†	9.66 ± 0.25*† (100%)	8.08 ± 0.22*†	8.34 ± 0.19† (100%)	7.37 ± 0.21*	8.40 ± 0.17* (100%)
Arg ¹¹ -VIP	9.45 ± 0.18†‡	9.15 ± 0.27‡ (105 ± 7%)	7.80 ± 0.20‡	8.78 ± 0.18†‡ (105 ± 13%)	5.84 ± 0.18†‡	5.90§ (20 ± 18%)	6.75 ± 0.29†‡	6.67 ± 0.38†‡ (45 ± 15%†‡)
Val ¹¹ -VIP	8.63 ± 0.27*	9.18 ± 0.21‡ (102 ± 10%)	7.60 ± 0.24‡	8.38 ± 0.24‡ (103 ± 13%)	6.45 ± 0.26*‡	7.12 ± 0.15‡ (92 ± 8%)	7.55 ± 0.21*	8.26 ± 0.28* (85 ± 8%)
Citrulline ¹¹ -VIP	8.55 ± 0.18*	8.45 ± 0.17*† (104 ± 7%)	7.45 ± 0.21‡	8.08 ± 0.12*‡ (105 ± 9%)	5.75 ± 0.26†‡	5.95 ± 0.35†‡ (40 ± 15%†‡)	6.92 ± 0.25†	7.13 ± 0.19†‡ (77 ± 8%†‡)

* Values significantly different from the Arg¹¹-VIP EC₅₀, EC₅₀ or efficacy.

† Values significantly different from the Val¹¹-VIP EC₅₀, EC₅₀ or efficacy.

‡ Values significantly different from the VIP EC₅₀, EC₅₀ or efficacy.

§ The activation was too weak to allow a precise estimate of the EC₅₀ and efficacy values (see Figures 1, 2 and 4): Arg¹¹-VIP was almost unable to activate adenylate cyclase through VPAC₂ receptors. We nevertheless believe that the VPAC₂ receptor efficacy and potency values reported in Table 1 (computer-extrapolated from the experimental data) are approximately correct for the following reasons: (1) The 'best-fit' agonist EC₅₀ values were close to their IC₅₀ values in binding studies. (2) Since Arg¹¹-VIP displaced ¹²⁵I-labelled VIP almost completely at 10 μM, it probably achieved almost its maximal effect at that concentration. (3) In two experiments using a VPAC₂ clone with spare receptors (> 10 pmol/mg of protein), Arg¹¹-VIP achieved 50% of the maximal (overbasal) effect of VIP with an EC₅₀ value of 2 μM (pEC₅₀ = 5.70 ± 0.15), very close to the extrapolated value (pEC₅₀ = 5.90).

Figure 3 Discrimination of Arg¹¹-VIP from VIP by the active state of VPAC₁, VPAC₂ and chimaeric receptors

Arg¹¹-VIP ([R¹¹]-VIP) behaved as a full agonist on VPAC₁ receptors as well as on chimaeras C1 to C3; it was unable to activate chimaera C7 and behaved as a very weak antagonist ($K_i > 1 \mu\text{M}$) at this receptor; its efficacy at all the other chimaeras was $> 60\%$, allowing valid estimates of the EC_{50} values. The difference between the contributions of Arg¹¹ and Thr¹¹ to receptor activation was evaluated as explained in the Materials and methods section. The chimaeric receptors used are schematized in the lower part of the graph. *Significantly different from VPAC₁ receptors. The data were confirmed by binding studies at the two wild-type receptors and at chimaeras C1, C4, C5 and C6 (results not shown). Binding to the other chimaeric receptors was too low to allow a valid analysis. Results are average of at least three experiments performed in duplicate.

Peptide synthesis

All the peptides used were synthesized in our laboratory as described previously [34]. The 1-hydroxybenzotriazole derivative of hexanoic acid was coupled to the N-terminus of VIP before cleavage and deprotection. Peptide purity was assessed by capillary electrophoresis and conformity by electrospray MS.

Evaluation of the importance of Thr¹¹ for receptor recognition

The agonist–receptor complex activation free energy, which reflected the receptor's interaction with the whole peptide, was calculated as $\Delta G^{\circ} = -RT(\ln \text{EC}_{50})$, in $\text{kcal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. We then evaluated the difference between the ΔG° values obtained

Figure 4 Discrimination of Arg¹¹-VIP from VIP by VPAC₁, VPAC₂, VPAC₂ΔSSW, secretin and PAC₁ receptors

Adenylate cyclase activation by VIP (□) or Arg¹¹-VIP ([R¹¹]-VIP) (Δ) through VPAC₁ (top left panel), VPAC₂ (top right panel) and VPAC₂ΔSSW (centre right panel), and by VIP (□), Arg¹¹-VIP ([R¹¹]-VIP) (Δ), secretin (■) or PACAP (1–27) (●) through secretin (bottom left panel) or PAC₁ (bottom right panel) receptors. The adenylate cyclase activity was evaluated as pmol of cAMP produced per min per mg of protein. Results are representative of at least two experiments performed in duplicate. The ECL₁ sequences are aligned in the centre left panel.

with VIP and Arg¹¹-VIP to focus on the differential interaction between the receptor and Arg¹¹ versus Thr¹¹. Arg¹¹-VIP was a very poor agonist at VPAC₂ receptors; to evaluate its activation free energy, we used dose–effect curves at a clone expressing a very high receptor density (and spare receptors for VIP) to achieve a significant, although partial, stimulation of adenylate cyclase.

Statistics

All competition curves and dose–effect curves were analysed by non-linear regression (Graph Pad Prism 3.00 for Windows 95, Graph Pad Software, San Diego, CA, U.S.A.). Constraining the curves to one-site analysis (Hill slope = 1) affected the IC_{50} , EC_{50} and relative maximal stimulation estimates by less than 5%; the best-fit curves are shown in each Figure. The differences between the IC_{50} , EC_{50} and efficacy values were tested for statistical significance by Newman–Keuls' test; $P < 0.05$ was accepted as being significant.

RESULTS AND DISCUSSION

The presence of a Leu rather than Tyr side chain in position 22 of GRF derivatives explained only in part their selectivity for the VPAC₁ receptor [31]. Indeed, replacing the VIP-Thr¹¹ residue by the Arg residue found in GRF also contributed significantly to the selectivity of the VPAC₁ agonist and antagonist (Figure 1

Figure 5 Adenylate cyclase stimulation by VIP, Arg¹¹-VIP and Val¹¹-VIP through wild-type and mutant receptors

Adenylate cyclase stimulation by VIP (□), Arg¹¹-VIP ([R¹¹]-VIP) (△) and Val¹¹-VIP ([V¹¹]-VIP) (○) acting through F200Y VPAC₁ receptors (bottom left panel) and Y184F VPAC₂ receptors (bottom right panel) was measured as indicated in the Materials and methods section. Data obtained at VPAC₁ (top left panel) and VPAC₂ receptors (top right panel) (from Figure 2) are shown for comparison. Adenylate cyclase activity was evaluated as pmol of cAMP produced per min per mg of protein. Results are representative of at least three experiments performed in duplicate.

and Table 1). Arg¹¹-VIP had a 5–8-fold higher affinity than VIP for VPAC₁ receptors, but had a very low affinity for VPAC₂ receptors in binding studies, and activated them very poorly if at all.

To obtain a low-resolution positioning of the Thr¹¹ > Arg¹¹ residue discrimination by VPAC₂ receptors, we used chimaeric VPAC₁/VPAC₂ receptors. The constructions used have been described in previous publications [22,23,26,32], and already allowed us to identify the receptor domains involved in the recognition of other selective VPAC₁ and VPAC₂ ligands [22,32]. As shown in Figures 2 and 3, two receptor regions contributed to the Arg¹¹-VIP selectivity: the N-terminal VPAC₁ receptor domain supported the (barely significant) preferential recognition of Arg¹¹-VIP and ECL₁, the significant discrimination of VIP from Arg¹¹-VIP by VPAC₂ receptors.

We then investigated whether the Arg > Thr preference by VPAC₁ receptors and Thr > Arg preference by VPAC₂ receptors were due to attraction versus repulsion of the positive charge on the Arg residue, to steric hindrance between the (large) arginine side chain and the VPAC₂ receptor, or to loss of a hydrogen bond formed between the hydroxy group of Thr¹¹ and VPAC₂ receptors. To achieve that goal, we synthesized and tested Val¹¹-VIP (i.e. replaced the hydroxy group of Thr by a methyl group) and citrulline¹¹-VIP (citrulline is isosteric with arginine, but uncharged).

The VPAC₁ receptors preferred Arg¹¹-VIP to citrulline¹¹-VIP: the positive charge on the arginine side chain contributed to its selectivity. The affinity difference was however rather small (5–8-fold only), suggesting that it did not reflect an ionic bond formation.

Citrulline¹¹- and Arg¹¹-VIP were equipotent in binding studies at VPAC₂ receptors (Table 1): the positive charge on arginine did not affect their recognition. Both peptides activated adenylate cyclase very poorly, even when compared with Val¹¹-VIP (Table 1): steric hindrance due to the large size of the arginine (or isosteric citrulline) side chain impaired VPAC₂ receptor activation by Arg¹¹- and citrulline¹¹-VIP.

Val¹¹-VIP had a low affinity for the VPAC₂ receptor: the hydroxy group of the VIP-Thr¹¹ (replaced by a methyl group in this analogue) was necessary for high-affinity recognition of the VPAC₂ receptors.

We were unable to identify the receptor region responsible for the poor affinity and efficacy of Arg¹¹-VIP at VPAC₂ receptors. The affinity loss due to the difference in size between valine and arginine/citrulline in binding studies at VPAC₂ receptors was small (< 10-fold) and barely significant. Comparison of chimaeras C7 and C8 suggested that the VPAC₁ receptor TM1 to ECL₁ region was necessary for efficient cyclase activation by Arg¹¹-VIP; however, the same peptide activated efficiently the cyclase (efficacy: 67 ± 8 % of VIP) with chimaera C4 that had the VPAC₂ N-terminus to ECL₁ sequence. The poor affinity and efficacy of Arg¹¹-VIP at VPAC₂ and C7 receptors probably reflected steric hindrance due to the presence of several side chains occupying the space necessary to fit the very long arginine/citrulline side chain in these two receptors: we did not make further attempts to identify the responsible receptor side chain(s).

Val¹¹-VIP had a > 10-fold lower affinity than VIP for VPAC₂ receptors in binding and functional studies. This indicated that a hydrogen bond formed between the VIP-Thr¹¹ and the VPAC₂ receptors contributed to VIP recognition. Hydrogen bonds with a Thr side chain necessitate the presence of hydrogen donor or acceptor atoms in precise positions relative to the threonine oxygen atom: we attempted to identify this group in the VPAC₂ receptor.

Alignment of the VPAC₁ and VPAC₂ ECL₁ sequences was difficult due to their different lengths (Figure 4). We therefore decided to shorten the VPAC₂ receptor by deletion of three amino acids (Ser¹⁹⁷, Ser¹⁹⁸ and Trp¹⁹⁹) in ECL₁. The shortened VPAC₂ mutant (VPAC₂ΔSSW) had a lower affinity than the wild-type receptor in binding studies (pIC₅₀ = 7.34 ± 0.22 for VIP, as compared with 8.08 ± 0.22 at VPAC₂ receptors; *P* < 0.05): the length of the VPAC₂ ECL₁ and/or the presence of the serine or tryptophan side chains were important for high-affinity receptor recognition. It was expressed at a high receptor density (8–15 nmol/mg of protein) in all the clones we obtained: the presence of spare receptors probably explained why the agonist pEC₅₀ values (8.01 ± 0.31 for VIP and 6.22 ± 0.25 for Arg¹¹-VIP) at this receptor were significantly larger than their pIC₅₀ values (7.34 ± 0.22 for VIP and 5.57 ± 0.21 for Arg¹¹-VIP). The mutated receptor retained a VIP > Arg¹¹-VIP selectivity comparable with that of the wild-type VPAC₂ receptor in functional as well as in binding studies (Figure 4; results not shown): the C-terminal region of the ECL₁ loop was probably not involved in preferential Thr¹¹ recognition.

The VPAC₁ and VPAC₂ ECL₁ sequences, even after deletion of the VPAC₂ 'SSW' sequence, are poorly conserved: only 7 of the remaining 20 residues (shown in Figure 4) are identical. We reasoned that if the threonine-like Ser¹¹ side chains in PACAP and secretin were important for the recognition of their respective

Figure 6 VIP, Arg¹¹-VIP and Val¹¹-VIP binding: wild-type and mutant receptors

Binding of VIP (□), Arg¹¹-VIP ([R¹¹]-VIP) (△) and Val¹¹-VIP ([V¹¹]-VIP) (○) to VPAC₁ (top left panel), VPAC₂ (top right panel), F200Y VPAC₁ receptors (bottom left panel) and Y184F VPAC₂ receptors (bottom right panel). Results are average of at least three experiments performed in duplicate.

receptors, they would probably recognize the equivalent binding site as Thr¹¹ in the VPAC₂ receptor. The secretin and PAC₁ receptors are able to recognize VIP with a low affinity: we tested their abilities to discriminate VIP from Arg¹¹-VIP (Figure 4). Arg¹¹-VIP had the same apparent affinity as VIP for secretin receptors, but a 10-fold lower affinity than VIP for PAC₁ receptors (Figure 4). Thus VPAC₂ and PAC₁ receptors on the one hand and VPAC₁ and secretin receptors on the other behaved similarly with respect to recognition of Thr¹¹ (Figure 4).

The VPAC₂ and PAC₁ receptors possess an aligned tyrosine residue (at positions 184 and 210 respectively), which is replaced by a Phe²⁰⁰ residue in the secretin and VPAC₁ receptors (Figure 4, centre left panel): this tyrosine seemed a good candidate for interaction with the VIP-Thr¹¹. To test this hypothesis, we built and tested two receptor mutants, the F200Y VPAC₁ and Y184F VPAC₂ receptors. The IC₅₀ and EC₅₀ values were in good agreement at both clones, supporting the hypothesis that the adenylate cyclase response was proportional to receptor occupancy. As shown in Figures 5 and 6, like VPAC₂ receptors, the F200Y VPAC₁ receptors recognized preferentially VIP over Val¹¹-VIP, but the Y184F VPAC₂ receptors, like VPAC₁ receptors, did not. These results supported the hypothesis that the VPAC₂ receptor Tyr¹⁸⁴ side chain formed a hydrogen bond with the hydroxy group of VIP-Thr¹¹, but interfered with the Val¹¹-CH₃ group positioning.

As expected, the absence or presence of the tyrosine side chain was not sufficient to 'mimic' completely the VPAC₁- or VPAC₂-binding profiles. Indeed, Arg¹¹-VIP (Figure 5) and Citrulline¹¹-VIP (results not shown) were significantly less efficient than Val¹¹-VIP at the Y184F VPAC₂ receptors. This confirmed that additional VPAC₂ receptor side chains in the vicinity of Tyr¹⁸⁴

residue prevented the optimal positioning of Arg¹¹-containing VPAC₁-selective ligands.

Our present results, identifying the role of the VPAC₂ receptor ECL₁ in the recognition of the VIP-Thr¹¹ side chain, appear to contradict our previous suggestion that in the VIP/secretin peptide family, the central peptide region (residues 8–15) interacts with the N-terminal receptor domain [27]. To understand this discrepancy, it is important to remember that the discrimination of PACAP and secretin by the secretin receptor was due to sequence differences at positions 8, 13 and 15 but not at position 11 [27]: position 11 is identical in secretin and PACAP.

To conclude, we have identified the VPAC₂ receptor amino acid that forms a hydrogen bond with the VIP-Thr¹¹ side chain. Taken together with our previous results, it seems increasingly plausible that in the active peptide–receptor complex, the peptide is embraced between the receptor extracellular N-terminal domain and the transmembrane domains: some of the central and C-terminal residues (including amino acids 13, 15 and 27) faced the N-terminus of receptor, whereas others (1, 3, 11, 22, etc.) interacted with the ECLs.

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