Evidence that Interaction between Conserved Residues in Transmembrane Helices 2, 3, and 7 Are Crucial for Human VPAC₁ Receptor Activation

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ABSTRACT

The VPAC₁ receptor belongs to family B of G protein-coupled receptors (GPCR-B) and is activated upon binding of the vasoactive intestinal peptide (VIP). Despite the recent determination of the structure of the N terminus of several members of this receptor family, little is known about the structure of the transmembrane (TM) region and about the molecular mechanisms leading to activation. In the present study, we designed a new structural model of the TM domain and combined it with experimental mutagenesis experiments to investigate the interaction network that governs ligand binding and receptor activation. Our results suggest that this network involves the cluster of residues Arg₁₈₈ in TM2, Gln³₈₀ in TM7, and Asn²₂₉ in TM3. This cluster is expected to be altered upon VIP binding, because Arg₁₈₈ has been shown previously to interact with Asp³ of VIP. Several point mutations at positions 188, 229, and 380 were experimentally characterized and were shown to severely affect VIP binding and/or VIP-mediated cAMP production. Double mutants built from reciprocal residue exchanges exhibit strong cooperative or anticooperative effects, thereby indicating the spatial proximity of residues Arg₁₈₈, Gln³₈₀, and Asn²₂₉. Because these residues are highly conserved in the GPCR-B family, they can moreover be expected to have a general role in mediating function.

Introduction

The human VPAC₁ receptor is expressed in liver, breast, kidney, prostate, bladder, pancreatic ducts, thyroid gland, lymphoid tissues, and gastrointestinal mucosa and in most of the tumors derived from these tissues. The VPAC₁ receptor is a member of family B of G protein-coupled receptors (GPCRs), which have seven transmembrane (TM) helices. This family includes the VPAC₂, secretin, PAC₁, glucagon, glucagon-like-peptide 1 and 2, calcitonin, corticotropin-releasing factor, and parathyroid hormone (PTH) receptors. The physiological ligands of the VPAC₁ receptor are vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (Dickson and Finlayson, 2009).

Extensive studies of the largest family of GPCRs, the GPCR-A/rhodopsin family, led to the identification of key steps frequently involved in the early signaling events of this family. These include the disruption of an ionic interaction between the cytoplasmic face of TM3 and TM6 maintaining the receptor preferentially in a ground inactive conformation in absence of agonist (ionic lock) and a “rotamer toggle switch” (modulation of the helix conformation around a proline-kink) in TM6, causing key sequences to be exposed to cytoplasmic binding partners (Ballesteros et al., 2001; Schwartz et al., 2006).

The mechanisms regulating the GPCR-B family signal transduction are less precisely understood, because no X-ray structure of the whole receptor is available, and conserved motifs of the GPCR-A family (E/DRY at TM3, NPXXY at TM7) are absent in the GPCR-B family. Although recent
studies have solved the structure of the N terminus of several family B receptors (corticotropin-releasing factor, PTH, phos-
thephase of activated cells 1, gastric inhibitory polypeptide, glucagon-like peptide-1) and clarified their role in ligand
binding (Grace et al., 2007; Parthier et al., 2007; Sun et al.,
2007; Pioszak and Xu, 2008; Runge et al., 2008), there is little
information on the events that follow ligand binding. Consid-
ering the VPAC1 receptor as a paradigm for class B, it actu-
ally seems that a large network of interactions must be
considered. Indeed, on the basis of mutagenesis studies, it
has been proposed that TM1, TM2, TM3, and TM6 and the
intracellular loop 3 and the proximal part of the C-terminal
intradacrytoplasmic tail take part in the receptor signal trans-
duction (Gaudin et al., 1998, 1999; Couvineau et al., 2003;
Langer and Robberecht, 2007).

In the present study, a network of interactions that
stabilize the VPAC1 receptor conformation in the absence
of ligand is identified by combining modeling and mu-
tageneis studies and is proposed to be involved in receptor
activation. This network includes an arginine (Arg183) lo-
cated in TM2, demonstrated previously by complementary-
paired mutagenesis to interact with the Asp8 residue of
VIP (Solano et al., 2001), an asparagine (Asn239) located in
TM3, important for VPAC1, and VPAC2-mediated G protein
activation (Nachtergaele et al., 2006), and a glutamine
(Gln380) conserved among the GPCR-B family members
and located in TM7. To our knowledge, this is the first
identification of early steps that lead to the receptor acti-
vation of a GPCR-B family member upon ligand binding.

Materials and Methods

Comparative Modeling Procedure. Comparative modeling
was carried out by Modeler 9V3 (Martí-Renom et al., 2000)
on the basis of alignments between the target and template sequences
obtained as described under Results. The modeling was constrained
to create an obligate disulfide bond between the residues Cys9135 at
the extracellular end of TM3 and Cys206 at the extracellular loop 2;
this disulfide bridge is indeed known to occur in GPCR-B members.
All models were stepwise energy-relaxed in the following way:
1) with all heavy atoms fixed; 2) with backbone atoms fixed; and
3) with Ca atoms fixed. Gromacs 3.3.1 was used for energy calcula-
tions (Lindahl et al., 2001).

Quality Assessment of the Structural Models. To evaluate
the quality of the structural models generated from different sequence
alignments, we used the membrane score approach (Chugunov et al.,
2007a,b), which was developed for the assessment of the packing
quality of α-helical TM domains of membrane proteins. In this
method, a database-derived scoring function (Sscore) is used to quan-
titatively estimate the fitness of a given amino acid residue for its
three-dimensional class of protein-membrane environment. This
scoring function was derived from the analysis of a nonredundant set of
α-helical membrane protein structures (Chugunov et al., 2007a).
The larger Sscore the model has, the better it is packed in space.
Generally, models with Sscore 0 should be considered to be mis-
folded. This method has been proven to be useful in discriminating
close-to-native structures from large decoy sets built from mislead-
ing alignments (Chugunov et al., 2007b). A second quality assess-
ment, performed on the best structural models identified by the
membrane score approach, consisted of a detailed analysis of the
variability moment vectors. In a first step, all protein sequences
homologous to the target are aligned, and the amino acid variability
at each position is computed. In a second step, a vector is assigned to
each residue in each TM helix of the 3D model of the target. The
vector is put in a plane parallel to the surface of the membrane,
using 3 to 30 μg of protein per assay. Bound and free radioactivities were separated by filtration through glass-fiber GF/C filters pre-soaked for 24 h in 0.01% polyethylenimine and rinsed three times with a 20 mM, pH 7.4, sodium phosphate buffer containing 0.8% bovine serum albumin. The binding sites density was estimated by analysis of homologous competition curves assuming that the labeled and unlabeled ligands had the same affinity for the receptors.

**Peptide Synthesis.** The peptides used were synthesized in our laboratory as described by Nachtergaele et al., (2006). Peptide purity (at least 95%) was assessed by capillary electrophoresis, and conformity was assessed by electrospray MS.

**Data Analysis.** All competition curves, dose-response curves, pIC₅₀, and pEC₅₀ values were calculated using nonlinear regression (Prism software; GraphPad Software Inc., San Diego, CA). Statistical analyses were performed with the same software.

## Results

### Molecular Modeling

Because no experimental VPAC₁ structure is available, molecular modeling of its TM domain was performed in view of identifying residues involved in VIP binding and receptor activation, selecting potentially interesting mutations to be studied experimentally and rationalizing the results of these analyses. We took advantage of a preliminary 3D model of the TM domain of VPAC₁ (Conner et al., 2005) and designed a new, carefully optimized model.

An important ingredient toward optimal modeling is the production of a correct amino acid alignment between the template and target proteins, given the almost nonsignificant level of sequence identity between the members of the GPCR-B family and the GPCR-A receptors, for which several structures have been solved (Palczewski et al., 2000; Okada et al., 2004; Cherezov et al., 2007; Jaakola et al., 2008; Warne et al., 2008). There is moreover no evidence that the receptors’ activation mechanism should be the same in GPCR-A and GPCR-B and involve similar intermediate states. Therefore, there is no clear reason for selecting any particular structural template for modeling GPCR-B proteins. Here we chose the well resolved crystallographic structure of bovine visual rhodopsin (Protein Data Bank code 1U19) (Okada et al., 2004).

### Sequence Alignments and TM Model

Because commonly available and automatic sequence alignment methods fail to produce reliable OPSD-VPAC₁ alignments because of their very low sequence identity, we turned to an iterative and partly manual procedure of sequence alignment selection. In a first step, we compiled a set of four OPSD-VPAC₁ alignments (Aln-1 to Aln-4). Aln-1 was adapted from the approach of Frimurer and Bywater (1999) for the modeling of the glucagon-like peptide-1 receptor based on a comprehensive sequence analysis, a low-resolution structure of frog rhodopsin obtained by electron crystallography, and the so-called “cold-spot” alignment method for sequences with low similarity. Aln-2 was taken from the work of Bissantz et al. (2004), in which they defined a framework for the automated modeling ofGPCRs of the three main subfamilies. The latter approach tends to superimpose highly conserved positions, irrespective of their physicochemical nature, rather than residues viewed as similar according to substitution matrices. Aln-3 was built manually by implementing a kind of “cold-spot” approach idea. No gaps were allowed inside the TM domain; they were moved to the middle of loop regions. Aln-4 was produced by mGenThreader (McGuffin et al., 2000) via the BioInfoBank Meta Server (Ginalska et al., 2003). All of these alignments along with final variant are shown in Supplemental Fig. S1.

The four alignments were in agreement for helices TM3, TM6, and TM7, but they differed considerably for the other ones: we obtained four, two, two, and three variants for TM1, TM2, TM4, and TM5, respectively. Considering the sequence as a whole, this corresponds to 48 (4 × 2 × 2 × 3) global alignment variants of the TM region.

Each of the 48 alignments so obtained was used to generate a set of 10 structural models using the comparative modeling approach described under Materials and Methods. The packing quality of the TM helices in these 480 models was assessed using the membrane score $S_{mem}$ (see Materials and Methods). In a second step, the alignment that produced the best structural models, which display the maximum $S_{mem}$ value averaged over the 10 models ($< S_{mem} >$), was used as starting point for further exploration of the alignment space. This involved generating $3^7 = 2187$ alignment variants by shifting each of the 7 TM helices independently by −1, 0, or +1 residue. From each of these new alignments, 10 structural models were built and evaluated on the basis of the membrane score $S_{mem}$. The best alignment at this stage, referred to as ReAln (see Supplementary Fig. S1), was submitted again to the same helix shifting procedure, leading to 2187 other alignment variants. However, the models produced from these new alignments are not superior to that from ReAln (data not shown). The procedure was therefore stopped, and ReAln was considered to be the optimal alignment as measured by the $S_{mem}$ score.

Given the shortcomings of the empirical membrane score method, it is essential to consider available data on homologous proteins and check whether the model meets the general packing principles for membrane proteins such as hydrophobicity and variability organization. In particular, it is well known that the side of a given secondary structure element (here, an α-helix) that has mutated most during evolution is always exposed to the surrounding medium (here, the membrane). On the contrary, the conserved side, which is likely to play some important structural or functional roles, is buried inside the protein interior (Beuming and Weinstein, 2004). We performed a detailed analysis of the variability moment vectors in the structural model (see Experimental Procedures and Supplementary Fig S2) and corrected manually the ReAln alignment to fulfill the requirement that the most variable side of the helix should face the membrane. The final alignment, which we refer to as finalAln, is given in Fig. 1 (see also Supplementary Fig S1).

Despite a better variability and hydrophobicity organization, the models based on the final alignment finalAln (Fig. 1) demonstrated seriously impaired $S_{mem}$ values in comparison with the ReAln-based models. This may be the consequence of a known caveat of the membrane score method (i.e., an excessive sensitivity to small conformational changes of the amino acids’ side chains). To analyze whether these bad $S_{mem}$ values are indeed due to not accounting for the flexible nature of protein side chains, we performed MD runs with a fixed backbone conformation and computed the $S_{mem}$ values along the MD trajectories, as described under Materials and Methods. The comparison of the resulting $S_{mem}$ distributions...
helical interaction Arg\textsuperscript{188} to Gln\textsuperscript{380} between TM2 and TM7, analogous to the Arg\textsuperscript{233} to Gln\textsuperscript{451} interaction shown to be important for PTHR1 receptor (Gardella et al., 1996), is moreover observed in the model. This interaction can partially compensate for the unfavorable presence of the positive charge of Arg\textsuperscript{188} inside the helix bundle in the absence of ligand. These two residues belong to a chain of polar residues inside the receptor bundle: Arg\textsuperscript{188} in TM2 to Gln\textsuperscript{380} in TM7 to Asn\textsuperscript{229} in TM3 (Fig. 3c). His\textsuperscript{178} in TM2 and Thr\textsuperscript{243} in TM6, described as important for the activation and constitutive activity of some family B receptors (Hjorth et al., 1998; Gaudin et al., 1999), are also incorporated in a polar network in the cytoplasmic half of the TM domain of the receptor.

**Fig. 3.** 3D model of the TM domain of VPAC\textsubscript{1} receptor. Each of the TM \(\alpha\)-helices is individually colored and marked. The most important residues that are discussed in the main text are shown including Arg\textsuperscript{188}, Asn\textsuperscript{229}, and Gln\textsuperscript{380} that were mutated in this study. A, side view (from the membrane); B, top view (from the extracellular space); C, zoomed view of the mutated region (the view direction is shown by the orange arrow in A). Mutated residues form a chain: Arg\textsuperscript{188} in TM2 to Gln\textsuperscript{380} in TM7 to Asn\textsuperscript{229} in TM3. They possibly form hydrogen bonds, as shown with dashed orange lines. Note that the figure illustrates the possibility of forming H-bonds but does not imply that these two interactions should (or may) exist simultaneously.
Experimental Analyses

Gln<sup>380</sup> Located in TM7 Is Important for VPAC<sub>1</sub> Activation. Mutagenesis and functional studies identified previously an asparagine located in TM3 (Asn<sup>229</sup> and Asn<sup>216</sup> in VPAC<sub>1</sub> and VPAC<sub>2</sub> receptor, respectively) that is essential for receptor activation (Nachtergaeel et al., 2006). Indeed, as reported in Table 1, the N<sup>229</sup>A and N<sup>229</sup>Q mutations were shown to reduce the potency to stimulate adenylate cyclase by 10-fold. Furthermore, Arg<sup>188</sup> in TM2 was demonstrated previously to establish a salt bridge interaction with Asp<sup>5</sup> of VIP, which is essential for VPAC<sub>1</sub> activation (Solano et al., 2001). Indeed, the R<sup>188</sup>A and R<sup>188</sup>Q mutations drastically impair both VIP binding and adenylate cyclase stimulation (Table 1), whereas the double mutant R<sup>188</sup>Q in VPAC<sub>1</sub> and D<sup>2</sup>N in VIP is fully functional. These results led us to postulate that both Asn<sup>229</sup> and Arg<sup>188</sup> residues could be involved in an interaction network between TM helices, stabilizing the active receptor conformation, as often observed in GPCR-A family receptors.

To identify other residues likely to take part in the network, we took advantage of the 3D model presented here and searched for amino acids located in vicinity of Asn<sup>229</sup> and Arg<sup>188</sup>. As shown in Fig. 3, the ideal candidate is Gln<sup>380</sup> in TM7, which seems to be sandwiched between Asn<sup>229</sup> and Arg<sup>188</sup>. To evaluate the potential role of Gln<sup>380</sup>, we substituted this residue into alanine, arginine, or asparagine and performed binding competition curves and dose-response curves of adenylate cyclase stimulation. As shown in Table 1 and Fig. 4, the Q<sup>380</sup>A and Q<sup>380</sup>N mutants preserve the affinity for VIP, whereas Q<sup>380</sup>R shows a 20-fold decrease in affinity (ΔpIC<sub>50</sub> = −1.35). The three mutants moreover display a decrease in the maximal cAMP stimulation for a comparable receptor density and a decrease in the pEC<sub>50</sub> value of adenylate cyclase activation. This effect is stronger for Q<sup>380</sup>A and Q<sup>380</sup>R and relatively weak for Q<sup>380</sup>N. These results indicate that Q<sup>380</sup> is important for VPAC<sub>1</sub> activation, but probably is not directly involved in VIP binding. Note that the reason why Q<sup>380</sup>R displays a decreased affinity for VIP is probably related to the proximity of Arg<sup>188</sup> and the repulsion between positive charges.

**Effect of Double Mutations of Arg<sup>188</sup>, Asn<sup>229</sup>, and Gln<sup>380</sup>.** To evaluate whether Arg<sup>188</sup>, Asn<sup>229</sup>, and Gln<sup>380</sup> are functionally interdependent, as proposed by the model, we next introduced in VPAC<sub>1</sub> double mutations and compared the results with the effects obtained with the corresponding single mutants. Indeed, functionally independent residues should exhibit additive effects, whereas some synergy, either positive or negative, may appear for functionally interdependent residues. We thus constructed mutant receptors containing double substitutions of the three residues of interest and the double mutation in which both Asn<sup>229</sup> and Gln<sup>380</sup> are replaced by an alanine. Note that we did not test double mutants containing mutation of Arg<sup>188</sup> into alanine because the R<sup>188</sup>A mutant was so much affected that we were unable to characterize it (Solano et al., 2001). Cell surface expression of all mutants tested was evaluated by fluorescence-activated cell sorting analysis using specific monoclonal anti-VPAC<sub>1</sub> antibody to ensure that the effect observed is not due to receptor misfolding or altered cell surface targeting (data not shown).

As shown in Table 1, the capability to stimulate adenylate cyclase activity of the N<sup>229</sup>A/Q<sup>380</sup>A mutant is lower than that of the wt and similar to that of the individual single-site mutants. Furthermore, the double substitution N<sup>229</sup>A/Q<sup>380</sup>N only slightly reduces VIP potency and efficacy to activate adenylate cyclase and displayed a pharmacological profile intermediary between the N<sup>229</sup>A and the Q<sup>380</sup>N mutants (Fig. 4). The Q<sup>380</sup>N mutation thus partly restores the loss of activity caused by the N<sup>229</sup>A mutation. These results tend to confirm the model, in particular the proximity of Asn<sup>229</sup> and

**TABLE 1**

<table>
<thead>
<tr>
<th>Binding Studies</th>
<th>Adenylate Cyclase Assay</th>
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<tr>
<td></td>
<td>pIC&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>VPAC&lt;sub&gt;1&lt;/sub&gt;</td>
<td>8.53 ± 0.07</td>
</tr>
<tr>
<td>N&lt;sup&gt;229&lt;/sup&gt;A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N.D.</td>
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<tr>
<td>R&lt;sup&gt;188&lt;/sup&gt;N</td>
<td>7.08 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>N&lt;sup&gt;229&lt;/sup&gt;A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.52 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>N&lt;sup&gt;229&lt;/sup&gt;A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.42 ± 0.08</td>
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<tr>
<td>N&lt;sup&gt;229&lt;/sup&gt;A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.57 ± 0.10</td>
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<tr>
<td>N&lt;sup&gt;229&lt;/sup&gt;R</td>
<td>8.65 ± 0.06</td>
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<tr>
<td>Q&lt;sup&gt;380&lt;/sup&gt;A</td>
<td>8.61 ± 0.08</td>
</tr>
<tr>
<td>Q&lt;sup&gt;380&lt;/sup&gt;N</td>
<td>8.34 ± 0.08</td>
</tr>
<tr>
<td>Q&lt;sup&gt;380&lt;/sup&gt;R</td>
<td>7.18 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>pnmol/mg protein</td>
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<td>Single mutants</td>
<td>VPAC&lt;sub&gt;1&lt;/sub&gt;</td>
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<tr>
<td>N&lt;sup&gt;229&lt;/sup&gt;A/Q&lt;sup&gt;380&lt;/sup&gt;A</td>
<td>7.61 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>R&lt;sup&gt;188&lt;/sup&gt;N/N&lt;sup&gt;229&lt;/sup&gt;R</td>
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<td>N&lt;sup&gt;229&lt;/sup&gt;A/Q&lt;sup&gt;380&lt;/sup&gt;N</td>
<td>7.87 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Double mutants</td>
<td>N.D., not detectable.</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.05 evaluated by Mann-Whitney test.

<sup>b</sup>From Solano et al. (2001).

<sup>c</sup>From Nachtergaeel et al. (2006).
Gln\(^{380}\) and the importance of their interaction for VPAC\(_1\) activation. Note that both double mutants present some decreased affinity for VIP, whereas no such effect is observed for the single mutants. This may result from packing defects that indirectly affect the surrounding ligand-binding residues. This is consistent with the larger decrease in affinity caused by N229A/Q380A, compared with N229Q/Q380N.

The double substitution R\(^{188}\)N/N\(^{229}\)R resulted in a marked synergistic effect on the decrease in binding compared with R\(^{188}\)N and N\(^{229}\)R mutants. Indeed, we were unable to detect any VIP-specific binding for the R\(^{188}\)N/N\(^{229}\)R mutant, suggesting that VIP affinity is much more affected than for the R\(^{188}\)N mutant despite the fact that it was preserved for the N\(^{229}\)R mutant. Likewise, VIP potency to stimulate adenylate cyclase was reduced by 100-fold for the R\(^{188}\)N/N\(^{229}\)R mutant, by 10-fold for R\(^{188}\)N mutant, but was preserved for N\(^{229}\)R; the VIP efficacy was reduced by 25\% for both R\(^{188}\)N/N\(^{229}\)R and N\(^{229}\)R mutants (Table 1 and Fig. 4).

Moreover, R\(^{188}\)Q/Q\(^{380}\)R showed synergy in reducing the potency of VIP on cAMP production and a less-than-additive effect on binding compared with R\(^{188}\)Q and Q\(^{380}\)R mutants. Indeed, VIP affinity was reduced by 100-, 20-, and 100-fold for R\(^{188}\)Q, Q\(^{380}\)R, and R\(^{188}\)Q/Q\(^{380}\)R mutants, respectively. VIP potency to stimulate adenylate cyclase was reduced by 7- and 6-fold for R\(^{188}\)Q and Q\(^{380}\)R mutants, respectively, but by 150-fold for the R\(^{188}\)Q/Q\(^{380}\)R mutant. VIP efficacy was dramatically impaired for the Q\(^{380}\)R and R\(^{188}\)Q/Q\(^{380}\)R mutants but preserved for the R\(^{188}\)Q mutant (Table 1 and Fig. 4). Altogether, these data suggest that Arg\(^{188}\), Asn\(^{229}\), and Gln\(^{380}\) are functionally interdependent and important for both VIP affinity and VPAC\(_1\) activation.

**Discussion**

G protein-coupled receptors, also referred to as seven-transmembrane domain receptors, represent the largest family of signal transducers for extracellular stimuli. The determination of the high-resolution structure of members of family A of GPCRs (Palczewski et al., 2000; Okada et al., 2004; Cherezov et al., 2007; Jaakola et al., 2008; Warne et al., 2008) has confirmed that receptor activation is mediated by relative movements among the seven transmembrane helices that are stabilized by different network of interactions. However, because these key residues are not conserved in family B GPCRs, and structural data are only available for the N-terminal extracellular domain, little is known about the precise mechanisms involved in the activation of this family of receptors.

The commonly accepted model for agonist action uses the PTH receptor as template and suggests that the N-terminal domain is the principal binding site for the C-terminal region of the exogenous ligand, whereas binding of residues 1 to 3 of the ligand to the extracellular loops and TM helices are believed to drive the receptor activation and subsequent G protein coupling. After agonist binding, subsequent conformational changes are expected within the TM domain of the receptor. This is illustrated by the fact that a Zn\(^{2+}\) bridge between helices 3 and 6 of the PTH receptor constrains the receptor in a conformation unable to promote PTH-mediated G-protein activation, whereas agonist-induced internalization or phosphorylation was preserved (Vilardaga et al., 2001; Castro et al., 2005).

In the present study, by combining pharmacological and in silico approaches, we have identified a network of interactions between residues located in helices 2, 3, and 7 of the VPAC\(_1\) receptor, which are involved in the stabilization of the receptor in absence of agonist and in early steps of receptor activation. We propose that, in the absence of VIP, the Gln\(^{380}\) residue of TM7 interacts with Arg\(^{188}\), located in TM2 and identified previously as essential for recognition of the Asp\(^3\) side chain of VIP and subsequent receptor activation. Upon VIP binding, the interaction between Arg\(^{188}\) and Gln\(^{380}\) is broken and a stronger interaction (salt bridge) is established between Arg\(^{188}\) and the Asp\(^3\) side chain of VIP. This necessarily has an affect on the network of interactions essential for G protein activation, in which Gln\(^{380}\) and Asn\(^{229}\) are proposed to play an important role. This view is supported by several experimental and modeling results.

First, we showed that the substitution of Gln\(^{380}\) to alanine or asparagine significantly reduced the VIP efficacy to stimulate adenylate activation, similarly to what happens for the Asn\(^{229}\) substitutions (Nachtergaele et al., 2006), and that the substitution of both residues to alanine had a less than additive effect. The altered activation observed with these mutants cannot be attributed to the disruption of the binding pocket because the affinities for VIP were not affected for N\(^{229}\)A, N\(^{229}\)Q, Q\(^{380}\)A, and Q\(^{380}\)N mutants. As suggested previously for Asn\(^{229}\), it is likely that Q\(^{380}\)A and Q\(^{380}\)N mutants still bind the G protein but fail to activate it properly. As shown for several GPCRs, it is expected that reciprocal exchange of two residues involved in a direct interaction should restore the activity of the receptor. We found that the N\(^{229}\)Q/Q\(^{380}\)N substitution partially restored the receptor activity. The N\(^{229}\)A/Q\(^{380}\)A shows a similar anticooperativity because the loss in activity of the double mutant is only slightly larger than that of each single-site mutation. Note that both double mutants present a loss in binding affinity, which contributes to the loss in activity. As a consequence, the actual anticooperative effect is even stronger than suggested by the compar-
ison of the measured and expected ΔpEC50 values (Table 1). Thus altogether the results suggest that the interaction between Asn229 and Gln380 is important for VPAC1-mediated G protein activation.

The 3D model can be taken to suggest that Gln380 functions as a floating "ferry boat," switching between Arg188 and Asn229 residues' side chains. This triad lines up in the model almost perfectly (Fig. 3c), so disruption of the Arg188 to Gln380 interaction upon VIP binding probably modifies the Asn229 to Gln380 interaction, hence contributing to signal transduction propagation and activation of G protein. However, the exact mechanism by which this occurs cannot be determined at this stage, because this would require a model of the activated receptor in complex with VIP. In particular the two N-terminal residues of VIP, His3 and Ser2, are likely to affect, directly or indirectly, the interaction network surrounding Asn229 and Gln380.

It is also interesting to note that none of the tested single-site mutations of Asn229 or Gln380 affects the affinity for VIP, except for Q380R. This exception can be explained by the proximity in the mutant of two arginine residues at positions 188 and 380, which will create repulsive interactions modifying the relative position of the two side chains compared with the wt receptor. These results indicate that Asn229 or Gln380 are probably not directly involved in VIP binding.

Reciprocal substitution mutants are in agreement with the importance of Arg188 for the high-affinity binding of VIP. Indeed, no specific VIP binding was detected for the R188N/ N229R mutant, and VIP affinity was reduced by 150-fold for R188Q/Q380R. In those mutants, the localization of the arginine could actually be much deeper into the helices, thus preventing interaction with the Asp3 side chain of VIP.

To our knowledge, this is the first study that identified, in a member of family B GPCRs, interactions between residues located in transmembrane helices that are involved in the stabilization of the receptor conformation. Interestingly, Arg172, in the closely related VPAC2 receptor and Arg166 in the secretin receptor (these positions correspond to Arg188 in VPAC1), also interact with the Asp3 side chain of VIP and secretin, respectively (Di Paolo et al., 1998; Langer and Robberecht, 2007). Some of us also demonstrated previously that Asn216 in VPAC2, corresponding to Asn229 in VPAC1, was rounding Asn229 and Gln380.

The importance of Arg188 for the high-affinity binding of VIP led to a constitutively activated receptor (Gaudin et al., 1998). Upon interaction of the N-terminal tail of VIP with the TM domain of VPAC1, which maintains TM7 in a conformation necessary for proper activation of G protein, mediated through interaction with Glu384.

Note that the importance of Arg188, Asn229, Gln380 and Glu384 residues in VPAC1 activity is further supported by their high degree of conservation among all members of GPCR-B family (see Supplemental Fig. S4 in for sequence alignment of TM2, 3, and 7 of GPCR-B family members). These residues may therefore be involved in a binding and activation mechanism that is common to the whole GPCR-B family. However, additional experiments on other family members should be performed to support this view.

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