Molecular Basis and Species Specificity of High Affinity Binding of Vasoactive Intestinal Polypeptide by the Rat Secretin Receptor

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Accepted for publication July 8, 1996

ABSTRACT

The affinity and specificity of the binding interaction between ligands and their receptors are key for appropriate hormonal regulation of target tissues. However, it is now apparent that vasoactive intestinal polypeptide (VIP) binds to the rat secretin receptor with similar affinity to that for its natural ligand, secretin (Holtmann et al., 1995). In this report, we establish that this is not a characteristic of the human secretin receptor, and use rat-human secretin receptor chimeras, site mutants and truncated receptor constructs to establish the molecular basis for this unusual binding interaction. Of note, isolated N-terminal domains of the rat secretin and the VIP receptors are capable of high affinity binding of VIP. In the recently recognized secretin family of receptors, this domain has six conserved cysteine residues and disulfide bonds that are likely important to achieve the complex conformation critical for this binding. A single acidic residue (Asp98) present in the rat secretin receptor appears to be critical, because a site-mutant changing this to the polar, but uncharged residue present in that position in the human receptor (Asn) eliminates the high affinity binding of VIP. Of interest, a previously identified critical basic residue in VIP (Lys15) provides a candidate for charge-pairing with this residue, potentially aligning the peptide ligand in a nonproductive orientation within this receptor.

The high affinity and exquisite specificity of the binding interaction between many ligands and their receptors is key for the appropriate hormonal regulation of target tissues. There are now multiple examples of structurally homologous ligands recognizing the same receptor, and of the same ligand recognizing structurally homologous receptors. Almost invariably, however, the binding affinity decreases as structures become more divergent. The focus of our work is a unique observation in which two different, yet structurally related peptide hormones, secretin and VIP, bind to a single receptor, the rat secretin receptor, with the same high affinity (Holtmann et al., 1995).

The secretin receptor is a member of a recently recognized family of G protein-coupled receptors that includes receptors for moderate-sized peptides with large pharmacophoric domains, including VIP, calcitonin, parathyroid hormone, glucagon and pituitary adenylate cyclase-activating peptide (Segre and Goldring, 1993). Although these receptors are predicted to have the heptahelical topology typical of this superfamily, they do not share the signature sequences of the β-adrenergic receptor family and, instead, have a long extracellular N-terminus containing six highly conserved cysteine residues (Segre and Goldring, 1993). In our recent report (Holtmann et al., 1995), we demonstrated that this unique domain is critical for native ligand recognition for secretin and VIP receptors. This theme has also been extended for other members of this family (Couvineau et al., 1995; Couvineau et al., 1994; Lee et al., 1994). Although earlier physiological studies and binding to native tissues (Zhou et al., 1987; Christophe et al., 1981, 1984) recognized that the "secretin preferring receptor" was also capable of responding to high concentrations of VIP and binding VIP with low affinity, only the recent work with the recombinant rat secretin receptor-bearing cell line recognized that VIP was also capable of high affinity binding to this receptor that did not result in a cAMP response (Holtmann et al., 1995). This phenomenon is only apparent when performing competition-binding with a VIP radioligand (Holtmann et al., 1995; Ishihara et al., 1991). Such binding has previously been attributed to a co-existent "VIP-prefering receptor" when performed in native tissues (Christophe et al., 1981).

In the current work, we focus on elucidation of the molec-
ular basis of this high affinity binding of the nonnative peptide ligand to the rat secretin receptor. For this, we took advantage of species differences in secretin receptors. The recently cloned human secretin receptor is 84% identical to the rat receptor and displays similar biological responsiveness (Jiang and Ulrich, 1995), yet does not exhibit the high affinity binding of VIP observed in the rat receptor. Using chimeric constructs between secretin receptors from these two species, as well as truncated constructs and site-mutants, we have been able to focus on critical residues responsible for this unusual phenomenon.

**Materials and methods**

Rat and human secretin receptor cDNA were cloned as previously reported (Jiang and Ulrich, 1995; Ulrich et al., 1993). The rat VIP receptor cDNA was the kind gift of Professor Nagata (Osaka Bioscience Institute, Osaka, Japan) (Ishihara et al., 1992). Rat secretin and VIP peptide ligands were purchased from Peninsula Laboratories (Belmont, CA), and their radioligands were prepared as we reported (Holtmann et al., 1995; Ulrich et al., 1993). Restriction enzymes used for mutagenesis were from Boehringer-Mannheim (Indianapolis, IN), except for BsrGI, which was from New England Biolabs (Beverley, MA).

**Receptor constructs.** Receptor constructs (fig. 1) were cloned into the pBK-CMV eukaryotic expression vector from Stratagene (La Jolla, CA). The wild-type rat secretin receptor cDNA was inserted into BamHI and HindIII sites, although the wild-type human secretin receptor cDNA was inserted into BamHI and XhoI sites.

Chimeric receptor constructs in which the putative extracellular amino-terminus of each wild-type receptor was replaced with the corresponding sequence of the other receptor were constructed by

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**Fig. 1.** The top panel graphically illustrates the receptor constructs included in this study. The bottom panel shows the amino acid sequence for the N-terminal domain of the rat secretin receptor, with the conserved Cys residues highlighted and residues that are different in the human receptor noted. Only a single nonconserved acidic residue is present, D98, which is a N in the human receptor.
replacement of a BamHI-BsrGI fragment representing the first 123 amino-terminal residues of each receptor with the corresponding sequence of its counterpart (Rat-Hum SecR and Hum-Rat SecR).

A truncated segment of the N-terminal rat secretin receptor was produced by PCR (Polymerase Chain Reaction)-mutagenesis (Kunkel 1985). Additionally, the N-terminus of this construct was used to replace the analogous domain of the human receptor (RatD98N-Hum SecR). The sequences of all constructs were confirmed by the dideoxynucleotide chain termination method (Sanger et al., 1977).

Receptor expression. Constructs were expressed in COS-7 cells (American Type Cell Culture Collection, Rockville, MD), maintained in Dulbecco's modified Eagle's medium with 5% fetal calf serum (HyClone Laboratories, Logan, UT). Between 3 and 4 μg DNA were used for transfection of each 100-mm dish using a modification of the DEAE-dextran method we described (Holtmann et al., 1995; Lopata et al., 1984). Cells were harvested mechanically 48 to 72 hr after transfection for either biological activity studies or membrane preparation for subsequent ligand-binding studies.

Biological activity studies. Biological activity was determined by assaying intracellular cAMP levels after hormone stimulation. Harvested cells were washed with phosphate-buffered saline and resuspended in Krebs-Ringer-Hepes medium containing 25 mM Hepes, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 2 mM CaCl2, 1 mM KH2PO4, 0.2% bovine serum albumin, 0.01% soybean trypsin inhibitor and 1 μM 3-isobutyl-1-methylxanthine. Stimulation was performed with appropriate concentrations of secretin and VIP for 10 min at 37°C, and stopped with ice-cold 6% perchloric acid. After pH adjustment to 6 with KHCO3 and centrifugation at 2000 rpm for 10 min, supernatants of cell lysates were used to determine cAMP levels with a [3H]-cAMP competition-binding assay kit from Skatron cell harvester with glass fiber filter mats presoaked in 0.3% polybren. Bound radioactivity was quantified in a γ-counter. Nonspecific binding was assessed in the presence of excess unlabeled analogous peptide (1 μM secretin and 0.1 μM VIP).

Statistical analysis. All observations were repeated at least three times in independent experiments and are expressed as means ± S.E.M. Binding curves were analyzed and plotted using the nonlinear regression analysis routine for radioligand binding in the Prism software package (GraphPad Software, San Diego, CA). Differences were determined by using the Mann-Whitney or t test for unpaired values, with P < .05 considered to be significant.

Results

Biological activity. Changes in intracellular cAMP concentrations in response to agonist stimulation are shown in table 1 and figure 2. Sham-transfected cells had no cAMP responses to either secretin or VIP in any of the concentrations studied (up to 1 μM) (basal levels of 1 pmol cAMP per tube remained constant). The wild-type rat secretin receptor (WT Rat SecR) was activated by secretin with an EC50 of 0.5 ± 0.1 nM and by VIP with an EC50 of 83 ± 12 nM. The wild-type human secretin receptor (WT Hum SecR) responded to secretin with an EC50 of 0.4 ± 0.2 nM and to VIP with an EC50 of 29 ± 11 nM. Both wild-type receptors thus displayed similar responsiveness to secretin (P = .7) and VIP (P = .1), with potency approximately 100-fold more for secretin than VIP. Maximal responses were similar for both agonists, reaching 2.5 ± 0.2 pmol cAMP per tube.

The chimeric Hum-Rat SecR construct, with the extracellular N-terminus of the human secretin receptor and the remainder of the rat secretin receptor, responded to stimulation with secretin (EC50 = 0.3 ± 0.1 nM) and VIP (EC50 = 106 ± 58 nM) similarly to the wild-type receptors. The converse construct, Rat-Hum SecR, also responded similarly (EC50 = 1 ± 0.5 nM for secretin and 97 ± 20 nM for VIP).

Replacement of the aspartate residue at position 98 with asparagine in the construct RatD98N SecR also did not affect the ability of the wild-type receptor to generate intracellular cAMP responses to either ligand. The EC50 was 1.1 ± 0.4 nM for secretin and 157 ± 72 nM for VIP. The same was true for the RatD98N-Hum SecR construct, which responded to secretin with an EC50 of 0.6 ± 0.3 nM and to VIP with an EC50 of 110 ± 44 nM.

As expected, the truncated rat secretin and VIP receptor constructs, however, were not capable of eliciting any cAMP response to secretin or VIP (table 1).

### Table 1

<table>
<thead>
<tr>
<th>Construct</th>
<th>Intracell. cAMP Responses, EC50 [nM]</th>
<th>Homologous Binding, K [nM]</th>
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<tr>
<td></td>
<td>Sec</td>
<td>VIP</td>
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<tr>
<td>WTRat SecR</td>
<td>0.5 ± 0.1</td>
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<tr>
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<td>Hum-Rat SecR</td>
<td>0.3 ± 0.1</td>
<td>106 ± 58</td>
</tr>
<tr>
<td>RatD98N SecR</td>
<td>1.1 ± 0.4</td>
<td>157 ± 72</td>
</tr>
<tr>
<td>RatD98N-Hum SecR</td>
<td>0.6 ± 0.3</td>
<td>110 ± 44</td>
</tr>
<tr>
<td>Rat SecR 1-147</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rat VIPR 1-140</td>
<td>ND</td>
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Thus all full-length receptor constructs used in this work displayed similar responsiveness to stimulation with secretin and VIP, maintaining a potency of secretin greater than VIP of approximately 2 orders of magnitude (fig. 2).

**Ligand binding.** The ligand binding properties of our constructs were determined by direct assessment of homologous competition-binding of secretin and VIP radioligands. These data are summarized in table 1 and illustrated in figures 3 and 4.

Secretin competed for secretin radioligand binding to the wild-type rat receptor with a $K_i$ of $2.1 \pm 0.5$ nM and to the wild-type human receptor with a $K_i$ of $1.4 \pm 0.1$ nM ($P = 1.0$). The binding properties for the VIP radioligand, however, differed considerably. The wild-type rat receptor bound VIP...
secretin and VIP receptor constructs and the VIP radioligand. Values

1996 Secretin ± was 10.2 that of the secretin radioligand (P

pressed any saturable binding of the secretin radioligand (data not

experiments. Binding data represent saturable binding relative to the

are expressed as the means ± S.E.M. of at least three independent

Fig. 4. Binding characteristics of truncated receptor constructs. Shown are data from competition-binding experiments using enriched plasma membranes from COS cells transfected with N-terminal rat secretin and VIP receptor constructs and the VIP radioligand. Values are expressed as the means ± S.E.M. of at least three independent experiments. Binding data represent saturable binding relative to the control condition in the absence of competitor. Neither construct expressed any saturable binding of the secretin radioligand (data not shown).

radioligand with high affinity ($K_i = 0.7 ± 0.2 \text{nM}$), similar to that of the secretin radioligand ($P = .1$). The binding affinity of VIP radioligand to the wild-type human receptor, however, was $10.2 ± 2.1 \text{nM}$, which was almost 15-fold lower than the VIP binding affinity to the wild-type rat receptor ($P < .001$).

Of note, replacement of the extracellular N-terminus of the wild-type human receptor with that of the wild-type rat receptor (Rat-Hum SecR) was capable of reestablishing the high affinity binding of VIP seen in the wild-type rat receptor ($K_i = 1.9 ± 0.6 \text{nM}$) ($P = .4$). The binding affinity of this construct for secretin was similar to that of the wild-type rat secretin receptor ($K_i = 1.5 ± 0.9 \text{nM}$) ($P = 1.0$).

For the converse chimeric construct incorporating the extracellular N-terminus of the human secretin receptor into the rat receptor (Hum-Rat SecR), the binding affinity for secretin was also high ($K_i = 0.3 ± 0.1 \text{nM}$). In contrast, the binding affinity for VIP was one order of magnitude lower ($K_i = 5.2 ± 1.5 \text{nM}$) ($P < .005$). This chimeric construct thus shares the lower binding affinity for the VIP radioligand observed for the wild-type human receptor ($P = .2$).

Substitution of an asparagine for aspartate 98 in the rat secretin receptor had no impact on either secretin- or VIP-stimulated biological responsiveness of the rat receptor (see cAMP data) or its high affinity binding of secretin ($K_i = 1.4 ± 0.1 \text{nM}$) ($P = 1.0$ compared to WT Rat SecR). However, VIP bound to this construct with a $K_i$ of $7.7 ± 1.2 \text{nM}$, representing a reduction in binding affinity relative to that of the wild-type rat receptor ($K_i = 0.7 ± 0.2 \text{nM}$) of one order of magnitude ($P = .03$).

The same impact of this mutation could be demonstrated in the RatD98N-Hum SecR construct. This receptor bound the secretin radioligand with high affinity ($K_i = .6 ± 0.1 \text{nM}$); the binding of VIP radioligand, however, yielded a $K_i$ of $4.4 ± 0.5 \text{nM}$, which represented a clear shift to lower binding affinity for VIP ($P = .01$).

For the truncated rat secretin receptor construct, Rat SecR, 1–140, no saturable binding of the secretin radioligand was detectable, although the VIP radioligand bound with a $K_i$ of $3.2 ± 0.9 \text{nM}$ (fig. 4). This was not significantly different from the VIP binding affinity to the wild-type rat secretin receptor, demonstrating the critical role of the N-terminal domain of this receptor for high affinity binding of VIP. Similarly, the truncated rat VIP receptor mutant, Rat VIPR, 1–140, bound VIP with a $K_i$ of $7.9 ± 2.6 \text{nM}$, but did not exhibit saturable binding of secretin (fig. 4).

Discussion

Mutagenesis techniques and receptor expression strategies provide powerful tools to gain insights into the molecular basis of ligand-receptor interactions. We have used this approach to identify key determinants for high affinity binding of the nonnative VIP ligand to the rat secretin receptor in a manner distinct from that of its native ligand. Despite the structural homology between secretin and VIP and between their receptors, this high affinity binding interaction does not induce the conformational change leading to initiation of signaling by this receptor. The biological response to VIP observed with the wild-type secretin receptor likely reflects its low affinity binding to the structurally distinct high affinity secretin-binding site on that receptor (Holtmann et al., 1995).

The key for our ability to focus on critical binding determinants was the species difference identified in this work. Because the high affinity binding of VIP by the rat secretin receptor was not present for the human secretin receptor, construction of rat-human secretin receptor chimeras provided the first level of identification of the importance of the N-terminal domain. High affinity binding of VIP was conserved in the human construct incorporating the N-terminus of the rat receptor, whereas the reverse construct bound VIP with low affinity. For this divergent characteristic, VIP binding to these chimeric receptors was determined by the N-terminus.

Because rat and human secretin receptors are 84% identical in primary structure, the chimeric approach provided an opportunity to rapidly focus on distinct residues as being critical. Within the extracellular N-terminal 123 residues of these receptors, there are only 38 amino acids that are different. Of these, most are chemically similar. Six basic residues in the rat receptor are not conserved in the human receptor, although only a single acidic residue in the rat receptor (aspartate 98) is not conserved (fig. 1). In the human receptor, the latter is replaced by a polar, but uncharged, asparagine. This acidic residue in the rat receptor might provide a countercharge for a basic residue (lysine 15) that has been shown to be a critical binding determinant in the VIP peptide (Bodanszky et al., 1978; Bodanszky et al., 1977). Indeed, substitution of this single residue in the rat receptor...
with the residue in this position in the human receptor (RatD98N SecR) eliminated the high affinity VIP binding characteristic of the rat receptor. The postulated charge interaction may well lead to the aberrant alignment of VIP in a nonproductive orientation in the rat secretin receptor.

The data with the truncated secretin receptor construct were also particularly interesting and potentially quite important. Until now, the N-terminus of this family of receptors has been recognized as being a critical determinant for ligand binding, but only one of several such determinants (Couvineau et al., 1995; Lee et al., 1994). As with most receptors for large peptide ligands, the assumption was that other loop regions contributed other determinants. For secretin binding by the secretin receptor, we know this to be true (Holtmann et al., 1995). Similar data exist for other receptors in this family (Lee et al., 1994). This is also analogous to the apparent binding determinants for other moderate-sized peptide ligands that exist on multiple loop domains of other heptahelical receptors in this superfamily (Coughlin, 1994).

The high affinity binding interaction between VIP and the isolated N-terminus of the rat secretin receptor, in the absence of the extensive architecture provided by the remainder of this heptahelical receptor, may support the interpretation that this domain can independently achieve its native conformation. The six highly conserved cysteine residues in this domain of this new receptor family likely contribute to the stability of such a conformation (Segre and Goldring, 1993). Although the disulfide bonding pattern among such residues is not yet established, several of the receptors in this family are quite sensitive to chemical reduction and treatment with cysteine-reactive reagents (Robberecht et al., 1984).

Of interest, mutagenesis experiments have also demonstrated the critical nature of the N-terminal domain of the VIP receptor in binding its natural ligand (Couvineau et al., 1995; Holtmann et al., 1995). We have, therefore, constructed the analogous N-terminal truncation mutant of that receptor and found that it is also capable of high affinity binding of VIP. The high affinity binding of VIP exhibited by the isolated N-terminal domains of these two receptors provides a potential mechanism to modulate the amount of VIP that might be available to bind productively to its natural, intact receptor. In a manner analogous to some cytokine receptors in which soluble truncated forms can be produced and modify the response to bioavailable ligand (Maliszewski and Fanslow, 1990), a similar phenomenon is at least theoretically possible here, even though no direct data for such a phenomenon yet exist.

The observations provided by our report provide important clarifications and insights relevant to the secretin receptor and to this new receptor family. For the secretin receptor, it will be critical to reexamine data derived from native rat tissues where secretin and VIP receptors have been postulated to coexist (Zhou et al., 1987). It is possible that some of the high affinity VIP binding attributed to a distinct VIP receptor may, in fact, represent binding to a secretin receptor. This work also adds to our interest in and focus on the complex N-terminal domain of this new family of receptors, and could provide a basis for novel mechanisms for regulation of G protein-coupled receptors.

Acknowledgments
The authors acknowledge the excellent technical assistance of E. Holicky, D. Pinon and I. Ferber, and the excellent secretarial assistance of S. Erickson.

References

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