Different Single Receptor Domains Determine the Distinct G Protein Coupling Profiles of Members of the Vasopressin Receptor Family*

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The vasopressin receptor family is unique among all classes of peptide receptors in that its individual members couple to different subsets of G proteins. The V_{1a} vasopressin receptor, for example, is preferentially linked to G proteins of the G_{q/11} class (biochemical response: stimulation of phosphatidylinositol hydrolysis), whereas the V_{2} vasopressin receptor is selectively coupled to G_{i} (biochemical response: stimulation of adenyl cyclase). To elucidate the structural basis underlying this functional heterogeneity, we have systematically exchanged different intracellular domains between the V_{1a} and V_{2} receptors. Transient expression of the resulting hybrid receptors in COS-7 cells showed that all mutant receptors containing V_{1a} receptor sequence in the second intracellular loop were able to activate the phosphatidylinositol pathway with high efficiency. On the other hand, only those hybrid receptors containing V_{2} receptor sequence in the third intracellular loop were capable of efficiently stimulating cAMP production. These findings suggest that the differential G protein coupling profiles of individual members of a structurally closely related receptor subfamily can be determined by different single intracellular receptor domains.

An extraordinarily large number of neurotransmitters, peptide hormones, neuromodulators, and autocrine and paracrine factors exert their physiological actions via binding to specific plasma membrane receptors that are coupled to distinct classes of heterotrimeric G proteins (G protein-coupled receptors (GPCRs)). During the past decade, several hundred members of this receptor superfamily have been cloned and sequenced (Watson and Arkinstall, 1994). Characteristically, each GPCR contains a common sequence motif that is believed to be involved in G protein recognition by GPCRs that bind peptide ligands. However, such receptors form one of the largest subclasses of GPCRs, and more than sixty different peptide receptors have been cloned to date (Watson and Arkinstall, 1994). These receptors (including, for example, those for melanocortins, cholecystokinin, endothelins, neuropeptides, opioids, or somatostatin) are known to play key roles in the regulation of a multitude of fundamental physiological processes. Investigations into the structural basis of the G protein coupling selectivity displayed by these receptors have been hampered by the fact that the individual members of virtually all peptide receptor subfamilies couple to similar G proteins. All cholecystokinin, endothelin, neuropeptides, and bombesin receptors, for example, are preferentially coupled to G proteins of the G_{q/11} class, whereas the various opioid and somatostatin receptors are all selectively linked to G proteins of the G_{i/o} class (Watson and Arkinstall, 1994). This pattern has precluded the use of hybrid peptide receptors (in which distinct domains are exchanged between functionally different members of a receptor subfamily) to study the structural basis underlying the selectivity of protein recognition displayed by these receptors.

In contrast to all other peptide receptor subfamilies, the group of vasopressin receptors is exceptional in that its members clearly differ in their G protein coupling profiles. The vasopressin receptor family is formed by three distinct subtypes, V_{1a}, V_{1b}, and V_{2}, which share a high degree (40–50%) of sequence identity (Birnbaumer et al., 1992; Dohlman et al., 1992; Morel et al., 1992; Sugimoto et al., 1994; de Keyzer et al., 1994). However, the V_{1a} and V_{1b} receptors are selectively coupled to G proteins of the G_{q/11} family (Laszlo et al., 1991), which mediate the activation of distinct isomers of phosphoinositide C_{2}, resulting in the breakdown of phosphoinositide lipids (PI hydrolysis). The V_{2} receptor, on the other hand, preferentially activates the G protein G_{i} (Laszlo et al., 1991), resulting in the activation of adenyl cyclase(s).

The individual vasopressin receptors mediate numerous important physiological effects including hepatic glycogenolysis, contraction of vascular smooth muscle and mesangial cells, aggregation of platelets, and antidiuresis in the kidney (Laszlo et al., 1991). Moreover, recent studies have shown that mutations in the V_{2} receptor gene are responsible for the X-linked form of nephrogenic diabetes insipidus (for recent reviews see Birnbaumer (1995) and Spiegel (1996)).

To study the structural elements responsible for the functional diversity found within the vasopressin receptor family,
we have created a series of V$_{1a}$V$_2$ hybrid receptors in which distinct intracellular domains were exchanged between the two wild type receptors (Fig. 1). Functional characterization of the resulting hybrid receptors in transfected COS-7 cells led to the novel observation that different single receptor segments determine the differential G protein binding profiles of two structurally closely related peptide receptors.

**EXPERIMENTAL PROCEDURES**

**Construction of Hybrid Receptors—Chimeric rat V$_{1a}$/human V$_2$ vasopressin receptor genes were constructed by using standard polymerase chain reaction mutagenesis techniques (Higuchi, 1989). The V$_2$ expression plasmid, V1pcD-SP6/T7, has been described previously (Mord et al., 1993). Expression plasmid for the wild type V$_2$PC-P-S, was similarly constructed as follows. A 1.7-kilobase pair EcoR1–XbaI fragment was cut out from hV2-pcDNA1/Amp (a plasmid containing the genomic human V$_2$ vasopressin receptor sequence; kindly provided by Dr. Allen Spiegel, NIH) and subcloned into the pcD-PS mammalian expression vector (Bonner et al., 1988) using the EcoRI and Spal sites present in the pcD-PS polylinker sequence. The two introns interrupting the V$_2$ receptor coding sequence were removed, and a stretch of nucleotides coding for a 9-amino acid epitope derived from the influenza virus hemagglutinin protein (YPFYDVDYA; Kolodziej and Young (1991)) was inserted after the initiating Met codon by employing standard polymerase chain reaction mutagenesis techniques (Higuchi, 1989). The ligand binding and G protein coupling properties of the epitope-tagged wild type V$_2$ receptor did not differ significantly from those found with the nontagged version. The composition of the individual V$_{1a}$/V$_2$ hybrid receptors is given in the legend to Fig. 1. To create a mutant m2 muscarinic receptor (human; m2p3) in which the 3 loop (amino acids 206–390) was replaced with the corresponding human β$_2$-adrenergic receptor sequence (amino acids 220–277), the Hm2p3D (Bannor et al., 1987) and β2pSVL (Fraser, 1989) expression plasmids were used for polymerase chain reaction-based mutagenesis. The correctness of all polymerase chain reaction-derived sequences was confirmed by dye Sequencing of the mutant plasmids (Sanger et al., 1977).

**Transient Expression of Wild Type and Mutant Receptors—** COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO$_2$ incubator. After 24 h, cells were transfected with the various vasopressin receptor constructs (4 μg of plasmid DNA/dish) by a DEAE-dextran method (Cullen, 1987). In G$_{i3}$m (human) coexpression experiments, COS-7 cells were cotransfected with 4 μg of individual receptor constructs and 1 μg of the pCD-PS expression plasmid (Amatruda et al., 1992).

**Radioligand Binding Assays—** For radioligand binding studies, COS-7 cells were harvested approximately 72 h after transfections, and membrane homogenates were prepared as described previously by Dörje et al. (1991). Binding buffer consisted of 50 mM Tris (pH 7.4), 3 mM MgCl$_2$, 1 mM EDTA, 0.1% bovine serum albumin, and 0.1 mg/ml bacitracin. Binding experiments were carried out at 1 h at 22°C in a 0.15 ml volume with increasing concentrations of the radioligand. [H]$^3$avigadorine vasopressin ([H]AVP, 81 Ci/mmol; DuPont NEN) was added to the growth medium. After a 40–48 h labeling period, cells were preincubated in Hanks' balanced salt solution containing 20 mM Hepes and 1 mM 3-isobutyl-1-methylxanthine for 15 min (37°C) and then stimulated with increasing concentrations of AP for 1 h at 37°C. The reaction was terminated by aspiration of the medium and addition of 1 ml of ice-cold 5% trichloroacetic acid containing 1 mM ATP and 1 mM CAMP. Increases in intracellular [H]$^3$avigadorine vasopressin (AVP) EC$_{50}$ levels were determined according to Bradford (1976). Binding data were analyzed by a nonlinear least squares curve-fitting procedure using the computer program Kaleidagraph (Synergy Software). The data are given as means ± S.E. of three or four independent experiments, each carried out in duplicate.

**RESULTS**

**Functional Profile of Wild Type Vasopressin Receptors**

The V$_2$ receptor in COS-7 cells (Fig. 1) was approximately linear over the 1-h assay interval. Increases in intracellular inositol monophosphate (IP$_1$) levels were determined according to Salomon et al. (1994). Studies with cells expressing the wild type V$_2$ receptor showed that AVP-induced IP$_1$ accumulation was approximately linear over the 1-h assay interval.

Drugs—PTX was purchased from List. Unless otherwise noted, all other drugs were obtained from Sigma.

**Table I**

| Receptor | Maximum increase in cAMP levels | AVP EC$_{50}$ | Maximum increase in cAMP levels | AVP EC$_{50}$ |
|----------|---------------------------------|-------------|---------------------------------|-------------|
|          | -fold above basal AVP EC$_{50}$ |                          | -fold above basal AVP EC$_{50}$ |              |
| V2 wild type | 12.2 ± 2.3 0.18 ± 0.03           | 0.02 ± 0.1  nd$^a$ | V2 wild type | 12.3 ± 2.0 0.46 ± 0.05           | 1.7 ± 0.3  nd$^a$ |
| V1a wild type | 14.6 ± 1.6 0.35 ± 0.04           | 7.4 ± 0.6 1.82 ± 0.21 | V2 wild type | 2.6 ± 0.4  nd               | 2.5 ± 0.2  nd$^a$ |
| V13 wild type | 11.1 ± 3.1 0.38 ± 0.05           | 1.6 ± 0.1  nd$^a$ | V1 wild type | 7.6 ± 0.5 0.97 ± 0.12           | 0.6 0.72 ± 0.15 0.19 |
| V13 wild type | 9.5 ± 1.4 0.88 ± 0.12           | 7.2 ± 0.7 1.75 ± 0.19 | V2 wild type | 7.0 ± 0.7 2.25 ± 0.44           | 0.7 0.75 ± 0.10 0.19 |
| V14 wild type | 2.3 ± 0.18 0.44 ± 0.06           | 0.2 nd     | V2 wild type | 7.8 ± 0.7 0.29 ± 0.05           | 0.7 0.75 ± 0.10 0.19 |

$^a$ nd, not determinable with sufficient accuracy.
$^b$ no significant increase in cAMP above basal levels.

**Functional Profile of Wild Type Vasopressin Receptors and Ligand Binding Properties of Wild Type and Mutant Receptors**—All wild type and mutant vasopressin receptors analyzed in this study were transiently expressed in COS-7 cells and assayed for their ability to mediate AVP-dependent stimulation of adenyl cyclase (mediated by G$_s$) and PTX-insensitive stimulation of PI hydrolysis (mediated by G proteins of the Gi class; Smrcka et al. (1991); Berstein et al. (1992)). Consistent with its reported functional profile, the wild type V$_2$ receptor (rat) mediated a pronounced increase in inositol phosphate production (7.6 ± 0.5-fold above basal) but left intracellular CAMP levels unaffected (Table I and Figs. 2 and 3). On the other hand, AVP stimulation of the wild type V$_2$ receptor (human) led to a marked increase in cAMP production (13.2 ± 2.3-fold above basal) but resulted in only a rather weak stimulation of the PI pathway (Table I and Figs. 2 and 3).

To explore the structural basis underlying this selectivity, a series of hybrid V$_{1a}$/V$_2$ receptors (Fig. 1) were created in which distinct intracellular domains were systematically exchanged between the two wild type receptors (note, however, that V2i1 receptor showed that AVP-induced cAMP accumulation was approximately linear over the 1-h assay interval.}

Whereas the wild type V$_2$ receptor bound [H]$^3$avigadorine vasopressin with 2-3-fold higher affinity than the wild type V$_{1a}$ receptor (p <

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Coupling Specificity of Vasopressin Receptors

Stimulation of Adenylyl Cyclase—Initially, we examined the ability of the different mutant receptors to mediate AVP-induced increases in intracellular cAMP levels. Mutant V2 receptors in which the i1 loop together with the N-terminal domain (the first transmembrane domain), the second intracellular loop (i2), or the C-terminal tail (i4) were replaced with the corresponding V1a receptor sequence were able to stimulate cAMP production in a fashion similar to that of the wild type V2 receptor (Table I and Fig. 2). In contrast, a mutant V2 receptor (V2i3) containing V1a receptor sequence in the third intracellular loop (i3) almost completely lost the ability to mediate agonist-dependent stimulation of adenylyl cyclase (Table I and Fig. 2).

Consistent with these results, substitution of the i1, i2, or i4 domain of the V2 receptor into the V1a receptor resulted in mutant receptors that, similar to the wild type V1a receptor, lacked the ability to mediate stimulation of adenylyl cyclase (Table I and Fig. 2). However, a mutant V1a receptor in which the i3 domain was replaced with the homologous V2 receptor sequence yielded a hybrid receptor (V2i3) that gained the ability to stimulate inositol phosphate production with high efficacy (7.4 ± 0.6-fold increase in IP1 above basal) and high AVP potency (EC50 = 1.82 ± 0.21 nM), in a fashion very similar to that of the wild type V1a receptor (Table I and Fig. 3).

“Bifunctionality” of Hybrid Receptors Is Not Due to Promiscuous G Protein Coupling—As outlined above, all mutant receptors containing V1a receptor sequence in the i2 loop were able to efficiently activate the PI pathway (mediated by Gq/11), whereas all mutant receptors containing V2 receptor sequence in the i3 loop were capable of productively coupling to stimulation of adenylyl cyclase (via Gi). Consequently, two mutant receptors were identified, V2i2 and V1i3 (Fig. 1), which could efficiently couple to both second messenger responses.

To exclude the possibility that the ability of V2i2 and V1i3 to couple to both stimulation of PI hydrolysis and adenylyl cyclase was due to a complete loss of G protein coupling selectivity (coupling promiscuity; Wong and Ross (1994)), we examined the ability of these two mutant receptors to mediate coupling to Gi, a G protein (family) recognized by neither of the two wild type receptors. It is known that wild type or mutant GPCRs that can couple to both Gi and Gq can stimulate adenylyl cyclase activity with markedly increased efficacy after inacti-
Coupling Specificity of Vasopressin Receptors

Fig. 3. AVP-induced stimulation of PI hydrolysis mediated by wild type V1α and hybrid V1α/V2 vasopressin receptors. Transfected COS-7 cells transiently expressing the various receptors were incubated in 6-well plates for 1 h at 37°C with the indicated AVP concentrations, and the resulting increases in intracellular IP$_1$ levels were determined as described (Berridge et al., 1987; Blin et al., 1995). The data are presented as fold increase in IP$_1$ above basal levels in the absence of AVP. Basal IP$_1$ levels for the wild type V2 receptor amounted to 1500 ± 310 cpm/well. The basal IP$_1$ levels observed with the various mutant receptors were not significantly different from this value. Each curve is representative of three independent experiments, each carried out in duplicate.

Fig. 4. Effect of PTX on receptor-mediated stimulation of adenyl cyclase. COS-7 cells transiently expressing the indicated receptors were studied for their ability to mediate AVP-induced increases in intracellular cAMP levels, either in the absence or in the presence of PTX (500 ng/ml). Assays were carried out as described under “Experimental Procedures.” The structures of V2i2 and V1i3 are given in Fig. 1. m2β3i3 represents a human m2 muscarinic receptor (Bonner et al., 1987) in which the i3 loop was replaced with the corresponding human β$_2$-adrenergic receptor sequence (Chung et al., 1987). Basal cAMP levels for the wild type V2 receptor amounted to 984 ± 243 cpm/well and remained virtually unaffected by PTX pretreatment. The basal cAMP levels observed with the different mutant receptors were not significantly different from this value. The data are given as means ± S.E. and are representative of three independent experiments, each carried out in duplicate.

DISCUSSION

The vasopressin receptor family represents an ideal model system to study the molecular basis of G protein recognition by peptide receptors, because its individual members (V1α, V1β, and V2) clearly differ in their G protein coupling properties. In this study, we have created and functionally analyzed a series of V1α/V2 hybrid receptors in which distinct intracellular domains (I–IV; Fig. 1) were systematically exchanged between the two wild type receptors. CAMP assays showed that all mutant receptors that contained V2 receptor sequence in the i3 loop were able to stimulate adenyl cyclase activity with high efficacy and AVP potency, whereas all mutant receptors in which the i3 loop was derived from the V1α receptor had little or no effect on intracellular cAMP levels (Fig. 1). These data strongly suggest that the i3 loop of the V2 receptor plays a key role in proper recognition and activation of G$_s$.

On the other hand, all hybrid constructs in which the i2 loop consisted of V1α receptor sequence were able to activate the PI cascade in a fashion very similar to the wild type V1α receptor, whereas all mutant receptors that contained V2 sequence in this receptor region displayed only residual PI activity, similar
to the wild type V2 receptor (Fig. 1), indicating that the i2 loop of the V2 receptor is critically involved in selective activation of Gq11.

Consistent with this pattern, substitution of the i2 loop of the V2 receptor into the wild type V2 receptor resulted in a mutant receptor (V2i2) that gained the ability to efficiently couple to Gq/11, but still retained the ability to productively couple to Gs. Analogously, replacement of the i3 loop in the V1a receptor with the homologous V2 receptor sequence yielded a hybrid construct (V1i3) that gained efficient coupling to Gs, but was still able to activate Gq11 in a fashion similar to the wild type V1a receptor. The ability of V2i2 and V1i3 to couple to both Gq/11 and Gs was shown.

Interestingly, Wong and Ross (1994) recently described chimeric m2 muscarinic/β2-adrenergic receptors, which are completely nonelective among the known mammalian G proteins. These mutant receptors could couple Gs and Gq as well as Gq11, which is not a target of either of the two parent receptors. Moreover, Wong and Ross (1994) found that a mutant m2 muscarinic receptor containing β2-adrenergic receptor sequence in the i3 loop does not only activate Gs but also Gq11, which is not a target of either the m2 muscarinic or the β2-adrenergic receptor. Like this mutant receptor, the bifunctional receptors described in the present manuscript (V2i2 and V1i3) were composed of sequences derived from Gq11- and Gs-coupled receptors. Therefore, to rule out the possibility that the ability of V2i2 and V1i3 to couple to both Gs and Gq11 is fully consistent with the notion that different single receptor domains determine the differential G protein coupling profiles of the V1a and V2 vasopressin receptors.

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increase in agonist-induced adenyl cyclase activity. Thus, the functional properties of the V2i2 and V1i3 mutant receptors clearly differ from those of the generally "promiscuous" hybrid receptors described by Wong and Ross (1994), indicating that the ability of V2i2 and V1i3 to couple to both Gq and G11 is not due to a general loss of G protein coupling selectivity.

It might also be argued that the i2 loop of the V2 receptor plays a specific role in preventing access to G11. Analogously, the i3 loop of the V2 receptor may be involved in preventing interactions with G11. To exclude such a mechanism as a possible cause of receptor-G protein coupling selectivity, an additional hybrid receptor (V2i2ss) was created in which the i2 loop of the V2 receptor was replaced with the corresponding segment of the Gq11-coupled ss4 somatostatin receptor (O'Carroll et al., 1992). The i2 loop of the ss4 somatostatin receptor shares considerably more sequence homology with the corresponding region of the V1a receptor (35–40% sequence identity) than with that of the V2 receptor (20–25%; Fig. 5A). In contrast to the mutant V2 receptor containing V2i2 receptor sequence in the i2 loop (V2i2), the V2i2ss hybrid receptor did not gain the ability to efficiently couple to Gq11, indicating that the bi-functionality of V2i2 is not due to the ability of the i2 loop of the V2 receptor to prevent access to Gq11 proteins or to a loss of specific interactions between the i2 and i3 loop (in the wild type V2 receptor) that constrain G protein coupling selectivity. Taken together, these data suggest that the i2 loop of the V1a receptor is in fact directly involved in Gq11 recognition and activation.

Consistent with the proposed roles of the i2 loop of the V1a receptor and the i3 loop of the V2 receptor in selective recognition of Gq11 and G11, respectively, two mutant receptors, V2i2 and V1i3, were identified that showed only residual or no functional activity at all (Fig. 1). However, we could demonstrate that both mutant receptors retained the ability to productively couple to G16 upon coexpression with Gq11, a G protein known to be activated by most GPCRs (Offermanns and Simon, 1995). This observation strongly suggests that the inability of the V2i2 and V1i3 mutant receptors to interact with G11 and Gq11 is not caused by a generalized misfolding of the intracellular receptor surface.

Taken together, these data provide compelling evidence that different single receptor domains are responsible for the functional diversity found within the vasopressin receptor family. The possibility therefore exists that the G protein coupling selectivity of other classes of peptide receptors is also determined by a clearly delineated intracellular receptor region. In agreement with this view, it has been demonstrated that the G protein coupling properties of a series of splice variants of the pituitary adenylyl cyclase-activating polypeptide receptor critically depend on the sequence present at the C terminus of the i3 loop (Spengler et al., 1993).

It should be of interest to investigate the functional effects of substituting the i2 loop of the V1a receptor or the i3 loop of the V2 receptor into other GPCRs (nonvasopressin receptors). Such experiments could provide information as to whether these specific loop sequences are sufficient for proper recognition and activation of Gq11 and G11, respectively. Alternatively, this question could be addressed by randomizing intracellular vasopressin receptor sequences while leaving the i2 loop of the V1a receptor or the i3 loop of the V2 receptor intact.

A sequence comparison (Fig. 5) shows that the i2 and i3 loops of the V1a and V2b vasopressin receptors and the oxytocin receptor (which is structurally closely related to the vasopressin receptors and, like the V1a and V2b receptors, is selectively coupled to Gq11; Kimura et al. (1992)) are quite similar to each other but substantially differ from the corresponding V2 receptor sequences. Each of the two loops contains a number of residues that are conserved only within the two functional receptor subclasses. It is therefore likely that these amino acids play key roles in determining the distinct G protein coupling profiles of the different vasopressin/oxytocin receptors.

In contrast to the findings reported here for different members of a peptide receptor family, multiple intracellular domains are known to be involved in determining the G protein coupling properties of receptors activated by biogenic amine ligands such as the adrenergic or muscarinic acetylcholine receptors (Wong et al., 1990; Liggett et al., 1991; Wong and Ross, 1994; Blin et al., 1995). Such regions have been shown to include the i2 loop, the N- and C-terminal segments of the i3 loop, and the membrane-proximal portion of the C-terminal tail (i4). It could be demonstrated that these regions act in a cooperative fashion to select and activate the proper set of G proteins (Wong et al., 1990; Liggett et al., 1991; Wong and Ross, 1994; Blin et al., 1995).

Interestingly, several peptide receptors (including, for example, the receptors for calitonin, glucagon, vasoactive intestinal polypeptide, or secretin) have recently been identified that, similar to two of the hybrid receptors examined in this study (V2i2 and V1i3), can couple to both Gq11 and G11 (Chabre et al., 1992; Abou-Samra et al., 1992). This property is also shared by the receptors that are activated by the glycoprotein hormones follicle-stimulating hormone, luteinizing hormone, and thyrotropin (Kosugi et al., 1993a; Allgeier et al., 1994). Loss-of-function mutagenesis studies showed, for example, that mutational modification of the N- and C-terminal segments of the i3 loop of the thyrotropin receptor virtually abolished coupling to Gq11, but had little effect on efficient activation of G11 (Kosugi et al., 1993a, 1993b). In agreement with the results of the present study, these data suggest that the region(s) in the thyrotropin receptor critical for activation of Gq11 differ(s) from that (those) required for productive coupling to G11.

Despite the existence of distinct vasopressin receptor sequences dictating specificity of G protein recognition, it is likely that most (if not all) intracellular receptor regions are generally required for the proper formation of the receptor-G protein complex. This notion is based on a large number of biochemical and molecular genetic studies with other GPCRs (Strosberg, 1991; Dohlman et al., 1991; Savarese and Fraser, 1992; Strader et al., 1994) demonstrating that there are multiple receptor-G protein contact sites involving at least three domains on the G protein subunits (Rens-Domiano and Hamm, 1995). Because all GPCRs and all G proteins are predicted to share a similar three-dimensional structure, the molecular architecture of the receptor-G protein interface may be generally conserved.

In conclusion, this study introduces the novel concept that the differential G protein coupling profiles of individual members of a peptide receptor subfamily are determined by different single intracellular receptor domains. All our experimental data are consistent with the notion that these domains are directly involved in G protein binding and/or activation. The identification of the site(s) on the G protein(s) involved in these interactions should eventually lead to the delineation of three-dimensional models of the receptor-G protein complex and provide novel insights into the molecular basis of peptide receptor-mediated G protein activation.

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