A Cyclic Peptide Mimicking the Third Intracellular Loop of the V₂ Vasopressin Receptor Inhibits Signaling through Its Interaction with Receptor Dimer and G Protein*§

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In this study, we investigated the mechanism by which a peptide mimicking the third cytoplasmic loop of the vasopressin V₂ receptor inhibits signaling. This loop was synthesized as a cyclic peptide (i3 cyc) that adopted defined secondary structure in solution. We found that i3 cyc inhibited the adenylyl cyclase activity induced by vasopressin or a nonhydrolyzable analog of GTP, guanosine 5′-O-(3-thio)triphosphate. This peptide also affected the specific binding of [³H]AVP by converting vasopressin binding sites from a high to a low affinity state without any effect on the global maximal binding capacity. The inhibitory actions of i3 cyc could also be observed in the presence of maximally uncoupling concentration of guanosine 5′-O-(3-thio)triphosphate, indicating a direct effect on the receptor itself and not exclusively on the interaction between the Gs protein and the V₂ receptor (V₂-R). Bio luminescence resonance energy-transfer experiments confirmed this assumption, because i3 cyc induced a significant inhibition of the bioluminescence resonance energy-transfer signal between the Renilla reniformis luciferase and the enhanced yellow fluorescent protein fused V₂-R. This suggests that the proper arrangement of the dimer could be an important prerequisite for triggering Gs protein activation. In addition to its effect on the receptor itself, the peptide exerted some of its actions at the G protein level, because it could also inhibit guanosine 5′-O-(3-thio)triphosphate-stimulated AC activity. Taken together, the data demonstrate that a peptide mimicking V₂-R third intracellular loop affects both the dimeric structural organization of the receptor and has direct inhibitory action on Gs.

G protein-coupled receptors (GPCRs),¹ a superfamily of seven transmembrane domain proteins, act as molecular switches that are able to transmit signals from extracellular stimuli to G proteins present on the cytoplasmic side of the plasma membrane. These receptors promote ligand-dependent GDP/GTP exchange in the Ga subunit of the heterotrimeric G protein leading to the activation of effectors. It is well established that intracellular loops of these receptors are important structural elements for their coupling to the heterotrimeric G proteins (1). Yet the precise understanding of the mechanisms by which GPCRs regulate G protein activity remains limited because of the general difficulties in characterizing the structure of integral membrane proteins. One approach considered to circumvent these problems is the use of synthetic peptides mimicking receptor cytosolic domains to probe the events responsible for G protein activation. For the rat vasopressin V₁a receptor, a peptide mimicking its second intracellular loop was found to specifically inhibit vasopressin-specific binding (2). In other cases, short peptides mimicking GPCR third intracellular loops were found to act on the coupling between the receptor and the G protein (3–8). Expressed as a minigene, the α₂-adrenergic i3 loop was shown to inhibit Gq signaling (9). In contrast to the inhibitory actions observed in most studies, Okamoto and colleagues showed that a soluble peptide corresponding to the C-terminal end of the human β₂-adrenergic receptor third cytoplasmic loop activates the Gs protein in cell-free conditions (10). Palmitoylated peptides (called pepducins) derived from the third cytoplasmic loop of the protease-activated receptors were recently found to target the receptor-G protein interface to either stimulate or inhibit G proteins (11). Although it is generally assumed that these peptides function by competitively inhibiting or mimicking the interactions between the receptor and their cognate G proteins, their precise mechanism of action remains unknown.

Expressed in the kidney and belonging to the GPCR family, the V₂ vasopressin receptors (V₂-R) are involved in the antidiuretic response by promoting water reabsorption. These recep-

¹ The abbreviations used are: GPCR, G protein-coupled receptor; V₂-R, V₂ vasopressin receptor; AC, adenylyl cyclase; BRET, bioluminescence resonance energy-transfer; AR, adrennergic receptor; i3 cyc, cyclic peptide mimicking the V₂-R third intracellular loop; Fmoc, N-(9-fluorenylethoxycarbonyl); AVP, [Arg²]-vasopressin; CYP, (−)-iodocyanopindolol; i3 lin, open chain form of the i3 cyc peptide; i2 cyc, cyclic peptide mimicking the V₂-R second intracellular loop; i3 endo, linear peptide mimicking the third intracellular loop of the rat ETA endothelin receptor; HEK, human embryonic kidney; GTP-γS, guanosine 5′-O-(3-thio)triphosphate; YFP, yellow fluorescent protein; h−, human; r−, rat; TM, transmembrane domain.
tors are positively coupled to adenylyl cyclase (AC) via Gαs and thus promote cAMP production (12). Structure-function studies involving mutational and/or chimeric approaches provided considerable insight into the structural elements involved in receptor/G protein interactions. For instance, Erlenbach and Wess (13) provided evidence for the importance of the V2-R third intracellular loop for coupling to Gs and consequently for AC activation.

In recent years, increasing evidence has suggested that GPCRs exist and function as dimeric entities (14, 15). Infrared-laser atomic-force microscopy studies have revealed that the native arrangement of rhodopsin in mouse disc membranes is formed of paracrystalline arrays of dimers (16). Moreover, Bañeres et al. (17) have established that one heterotrimetric Golf protein interacts with one leukotriene B4 receptor (BLT1) dimer to form a pentameric complex and suggested that receptor dimerization could be crucial for signal transduction. The functional importance of GPCR dimerization is also supported by studies carried out with chimera receptors able to complement each other, suggesting the existence of both intramolecular and intermolecular interactions in GPCRs (18). When considering the specific case of V2-R, Zhu and Wess pointed out that N-terminal V2-R fragments were able to display dominant-negative effects on the wild-type V2-R activity as a result of their heterodimerization with the full-length V2-R (19). More recently, Terrillon et al. (20) confirmed by bioluminescence resonance energy-transfer (BRET) experiments that these intermolecular interactions truly reflected V2-R homo-dimerization occurring in living cells.

A previous study showed that a synthetic peptide corresponding to the TM6 of the β2-adrenergic receptor (β2-AR) could inhibit the receptor function by preventing its dimerization (21). Maggio et al. (22) also proposed that the muscarinic receptor dimerization might depend on the presence of a long third intracellular loop. Taken together, these observations suggest that in addition to interfering directly with receptor-G protein coupling, small peptides derived from GPCR transmembrane domains or the third cytoplasmic loop could also affect the receptor dimerization process or proper dimer arrangement.

To better understand the mechanism of action of such peptides, we synthesized a cyclic peptide mimicking the V2-R third intracellular loop (i3 cyc) and tested its functional properties. We found that the i3 cyc peptide could inhibit receptor signaling at least in part by interfering with V2-R dimer structural organization.

**Experimental Procedures**

**Chemicals**

Fmoc-Rink amide and pseudo-proline dipeptide building blocks were obtained from NovaBiochem (Lauferlingen, Switzerland). Trinitrated [Arg5]-vasopressin (60 Ci/mmol) ([3H]AVP) was obtained from PerkinElmer Life Sciences. [Arg5]-vasopressin was obtained from Bachem. [125I]CGP64213 was synthesized from ethyl-(1,1,2,2-tetrafluoroethyl)-2,2,2-trifluoroethanol (50%; v/v) or dodecylphosphocholine (10%; v/v).

**Peptide Synthesis**

To mimic the constraints imposed by transmembrane helices, we synthesized the third intracellular loop of the rat ET2 receptor third intracellular loop, respectively. These peptides were prepared using solid phase procedures (the detailed procedure for the synthesis of i3 cyc peptide is available in the Supplementary Data). The loading of the Tentagel S-NH₂ resin (initially 0.28 mmol/g) was reduced to 0.12 mmol/g by coupling the corresponding amount of Fmoc-Rink amide linker (benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate and N-ethylisodisopropylamine). Acetic anhydride was then used to cap unreacted amino groups. To suppress aggregation, pseudo-proline building blocks (24, 25) were used as structure-disrupting agents at different stages of the solid phase synthesis of such long peptides. Thus, residues 11–12, 39–40, and 45–46 were introduced as Fmoc-Ala-Ser(ψ2,6-Me2)-OH, Fmoc-Val-Ser(ψ2,6-Me2)-OH, and Fmoc-Lys-Thr(ψ2,6-Me2)-OH, respectively, and coupled to the resin using a coupling reagent consisting of benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate, N-ethylisodisopropylamine, and 1-hydroxy-7-azabenzotriazole.

**CD**

The i3 cyc CD spectra were obtained on a Jobin Yvon CD6 spectropolarimeter. The spectra were scanned at room temperature in a quartz optical cell with a 0.03-cm path length. Baseline spectra for each solvent were obtained before the peptide spectra. The i3 cyc peptide was diluted at a concentration of 75 μM in a 50 mM phosphate buffer, pH 6.4, containing either 0.05% TritonX-100 or dodecylphosphocholine (10%; v/v).

**Receptor Expression Plasmids**

V2-R-Rluc, V2-R-YFP—Ssense and antisense primers were made to both introduce an AgeI site at the end of the human V2-R coding sequence expressed in pRK5 vector and remove the stop codon. Likewise, an AgeI site was created at the beginning of the Rluc coding sequence expressed in pHL-CMV vector (Promega). The pEYFP (BD Biosciences Clontech) and pHL-CMV vectors were cut with AgeI and XbaI, respectively, to excise the YFP and Rluc coding region and insert them in frame into the pPK5-V2-R vector to yield the pPK5-V2-R-YFP and pPK5-V2-R-Rluc constructs, respectively (20).

**Cell Culture and Transfection**

HEK 293T cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 500 units/ml penicillin/streptomycin in an atmosphere of 95% air and 5% CO2 at 37 °C. Calcium phosphate precipitation and G418 selection pressure (450 μg/ml) were used to produce stable HEK 293T cell lines expressing β2-AR-Rluc (20). For all transient expression, 2 × 10⁶ cells were plated in each 100-mm Petri dish, and transfections were performed using the calcium phosphate precipitation method (28).

**Cell Membrane Preparations**

HEK 293 cell membranes were prepared for radioligand binding, adenyl cyclase assays, and BRET measurements as described previously (20). In brief, cells were resuspended in lysis buffer (15 mM Tris-HCl, pH 7.4, 0.3 mM EDTA, and 2 mM MgCl₂), homogenized in a Polytomor homogenizer, and centrifuged at 100,000 × g for 5 min at 4 °C. Supernatants were recovered and centrifuged at 44,000 × g for 20 min at 4 °C. Membranes were resuspended in an appropriate volume of membrane buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂) and protein concentration was determined using the method of Bradford. Membranes were used immediately or stored at −80 °C.

**Rat Brain and Kidney Membranes**

Rat brain and kidney membranes were prepared as described previously (20). In brief, the different tissues were minced and homogenized in three volumes of an isotonic buffer containing 5 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 3 mM MgCl₂, with 15 strokes of a tight glass Dounce homogenizer. The homogenate was centrifuged at 1500 ×
g for 15 min at 4 °C and the pellet was resuspended in a hypotonic buffer containing 5 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 3 mM MgCl₂. Cells were lysed for 15 min at 4 °C in the hypotonic buffer, and the homogenate was centrifuged at 1500 × g for 15 min at 4 °C. The pellet was then homogenized with 10 strokes of a tight glass Dounce homogenizer and centrifuged at 1500 × g for 15 min at 4 °C. The final pellet was resuspended in an appropriate volume of membrane buffer (50 mM Tris-HCl, pH 7.4, and 0.3 mM GTP S⁻), and the protein concentration was determined by the method of Bradford. Membranes were used immediately or stored at −18 °C in presence of 40% glycerol. Just before the use of frozen membranes, glycerol was removed by centrifugation at 1500 × g for 15 min at 4 °C.

**Adenyl Cyclase Assays**

Adenyl cyclase activity was assessed by measuring the conversion of [α-32P]ATP to [α-32P]cAMP as described previously (30). In brief, membranes (20 µg of protein per assay) from rat kidney or HEK 293T cells transiently co-expressing V₂-R-Rluc + V₂-R-YFP were pre-incubated for 15 min at 37 °C in a 180-µl incubation buffer containing 50 mM Tris-HCl, pH 7.4, 0.3 mM MgCl₂, 1 µM cAMP, 0.3 mM CaCl₂, 0.66 mM MgCl₂, 2.5 mM ATP, 1 mM creatine kinase, 1 mM creatine phosphate, 0.01 mg/ml leupeptin, 10 mM sodium azide, 0.1 µM ouabain, and 0.2 µCi/ml [3H]cAMP with or without (basal) the compounds to be tested. 1 µCi of [α-32P]ATP per assay was then added for a further 6 min to a final volume of 200 µl. cAMP levels were determined by measuring the conversion of [α-32P]ATP to [α-32P]cAMP. Labeled cAMP and ATP were separated by sequential chromatography on Dowex and alumina columns. 32P and 3H radioactivities present in the cAMP fractions were immediately or stored at −18 °C in presence of 40% glycerol. Just before the use of frozen membranes, glycerol was removed by centrifugation at 1500 × g for 15 min at 4 °C.

**Radioligand Binding Assays**

[3H]AVP binding to plasma membrane preparations was performed as described previously (29). In brief, membranes (10 µg of protein per assay) from rat kidney or HEK 293T cells transiently co-expressing V₂-R-Rluc + V₂-R-YFP were pre-incubated for 15 min at 30 °C in 125 µl of a buffer containing 50 mM Tris-HCl, pH 7.4, 0.3 mM MgCl₂, 1 mM bovine serum albumin, and 0.1 mM phenylmethylsulfonyl fluoride. Increasing amounts of synthetic peptides, 100 µM GTP γ S, or a combination of these effectors were added in the medium when necessary. Then, either a fixed concentration (competition experiments) or increasing amounts of [3H]AVP (saturation binding experiments) were added in the incubation buffer.

[35S]GTP γ S Assay

Guanosine 5'-O-(3-[35S]thiotriphosphate) (GTPγS) assays were conducted as described previously (31). In brief, rat kidney membrane preparations (10 µg of protein per assay) were pre-incubated for 15 min at 30 °C with or without 10 µm i3 cyc in an incubation mixture containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5 µM GDP, 0.01 mg/ml leupeptin, 1 mM EDTA, 100 mM NaCl, and 0.5% bovine serum albumin in the presence (total GTPγS-stimulated condition) or absence (total basal condition) of 100 nM AVP (final volume, 150 µl). [35S]GTPγS was then added in the medium and incubated for an additional 15 min. Nonspecific binding, determined in the presence of 100 µM unlabelled GTPγS, did not represent more than 6% of the total [35S]GTPγS binding under both basal and GTPγS-stimulated conditions. The reaction was stopped by adding 4 ml of cold washing solution (50 mM Tris-HCl, pH 7.4, and 5 mM MgCl₂) and immediately filtered onto Whatman glass fiber filter (0.8–1.2 µm) previously preoashed for 2 h in a 50 mM Tris-HCl buffer, pH 7.4. The filters were then rinsed three times with 4 ml of washing solution, and the remaining radioactivity was measured by liquid scintillation spectrometry. The specific [35S]GTPγS binding related to hormone effect was calculated as the difference between the total [35S]GTPγS bound in the GTPγS-stimulated condition and the total [35S]GTPγS bound in the basal condition. The specific basal [35S]GTPγS binding was calculated as the difference between the total basal binding and the corresponding nonspecific binding values.

[35S]GTPγS binding experiments with membranes from HEK293T cells transiently expressing Gβ1, Gβ2, and Gαo were performed in 96-well filtration plates previously equilibrated with 50 mM Tris-HCl, pH 7.4. Membranes (5 µg/well) were prepared as described above and were pre-incubated 15 min at 30 °C with or without 10 µM i3 cyc (final volume, 20 µl). The plate was incubated for 1 h at 30 °C after the addition of 60 µl of an incubation buffer containing 50 mM Tris-HCl, 1 mM EDTA, 10 µM GDP, 5 mM MgCl₂, 0.01 mg/ml leupeptin, 100 mM NaCl, and 1 nM [35S]GTPγS in presence (total stimulated condition) or absence (total basal condition) of 100 µM breflcon. Nonspecific binding was determined in the presence of 100 µM unlabeled GTPγS. After vacuum filtration, plate-filter washing (three times with 250 µl of 50 mM Tris) and drying, the radioactivity was measured using a 1450 MicroBeta liquid scintillation counter (PerkinElmer Wal- lace). The specific [35S]GTPγS binding related to hormone effect was calculated as described above.

**Radioligand Binding Assays**

[3H]AVP binding to plasma membrane preparations was performed as described previously (29). In brief, membranes (10 µg of protein per assay) from rat kidney or HEK 293T cells transiently co-expressing V₂-R-Rluc + V₂-R-YFP were pre-incubated for 15 min at 30 °C in 125 µl of a buffer containing 50 mM Tris-HCl, pH 7.4, 0.3 mM MgCl₂, 1 mM bovine serum albumin, and 0.1 mM phenylmethylsulfonyl fluoride. Increasing amounts of synthetic peptides, 100 µM GTPγS, or a combination of these effectors were added in the medium when necessary. Then, either a fixed concentration (competition experiments) or increasing amounts of [3H]AVP (saturation binding experiments) were added in the medium. In both cases, the reaction was allowed to proceed for an additional period of 45 min at 30 °C. Nonspecific binding was determined in the presence of an excess of unlabeled AVP (1 µM).

Brain membranes (10 µg of protein per assay) were pre-incubated 15 min at 30 °C in 100 µl of a buffer containing 50 mM Tris-HCl, pH 7.4, 0.3 mM MgCl₂, 1 mM bovine serum albumin, 1.8 mM CaCl₂, 0.1 mM phenylmethylsulfonyl fluoride with or without 10 µM i3 cyc. [3H]CYP (0.1 µM) was added in the incubation medium, and the reaction was allowed to proceed for an additional 60 min. Nonspecific binding was determined using 1 µM GABA (32).

Membranes (10 µg of protein per assay) from HEK 293T cells stably expressing β₃-AR-Rluc were pre-incubated for 15 min at 30 °C in 180 µl of a buffer containing 75 mM Tris-HCl, pH 7.4, 12.5 mM MgCl₂, 2 mM EDTA, 1 mg/ml leupeptin, and 0.02 mg/ml ascorbic acid with or without 10 µM i3 cyc. [3H]CYP (0.2 µM) was added in the incubation medium, and the reaction was allowed to proceed for an additional period of 75
min. Nonspecific binding was determined using 6 μM isoproterenol. The reactions were stopped by adding 4 ml of cold washing solution (10 mM Tris-HCl, pH 7.4, 3 mM MgCl₂), and immediately filtered onto a Whatman glass fiber filter (0.8–1.2 µm) previously presoaked for 2 h with 10 mg/ml BSA. The filters were then rinsed three times with 4 ml of washing solution, and the remaining radioactivity was measured by liquid scintillation spectrometry. The specific binding was calculated as the difference between the total and nonspecific values.

Bioluminescence Resonance Energy Transfer
Forty-eight hours after transfection, cells were washed twice with PBS and detached with PBS containing 5 mM EDTA. Total fluorescence and luminescence signals were determined using a Fluorocount and Luminocount (PerkinElmer Life and Analytical Sciences) to assess the expression level of the YFP and Rluc fusion constructs. Membranes were then prepared as described above before being incubated for 15 min at 37 °C in an incubation buffer containing 50 mM Tris-HCl, pH 7.4, 0.3 mM MgCl₂, 1 mg/ml bovine serum albumin, 0.01 mg/ml benzamidine, 5 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor, with or without the compound to be tested. Incubated membranes were distributed in a 96-well microplate (12 μg of protein per well) and Coelenterazine h (Molecular Probes Inc., Eugene, OR) added to a final concentration of 5 μM. Readings were collected using a multi-detector plate reader FUSION (PerkinElmer Life and Analytical Sciences), allowing the sequential integration of the signals detected in the 440–500 nm and 510–590 nm windows. The BRET ratio is defined as \[(emission at 510–590) - (emission at 440–500)\]/(emission at 440–500), where \(C_f\) corresponds to \[(emission at 510–590)/emission at 440–500)\) for the Rluc construct expressed alone in the same experiment.

RESULTS
Synthesis of i3 cyc—To synthesize i3 cyc, we used a r-V2-R model derived from the rhodopsin coordinates (Protein Data Bank accession number 1F88) to select the appropriate N- and C-terminal residues at the cytoplasmic ends of TMs 5 and 6,
respectively. A chemoselective ligation strategy was devised to synthesize a cyclic form of the peptide (described in Supplementary Data). Therefore, the N- and C-terminal residues (Gln225 and Leu274) of the i3 loop were replaced with Gly and S-carboxymethyl cysteine, respectively, to reproduce a distance similar to that evaluated for the r-V2-R model (Fig. 1). The Met266 and Met272 of the r-V2-R were also replaced with Nle43 and Nle49 in the peptide to avoid any possibility of methionine oxidation. Because the cyclization strategy was predicted to alter the orientation of the Gln2 side chain in the peptide, this residue was introduced as d-Gln. Finally, i3 cyc was purified by high performance liquid chromatography and characterized by mass spectrometry (matrix-assisted laser desorption ionization/time of flight) (see Supplementary Data).

i3 cyc Structure—In phosphate buffer alone, i3 cyc adopted a random conformation as indicated by a minimum of ellipticity observed at 198 nm. The addition of dodecylphosphocholine, a molecule known to induce the formation of membrane-mimicking micelles at a concentration higher than its critical micellar concentration led to a CD spectra right-shift with two marked minima at 208 and 224 nm. These minima are typical features of the presence of an appreciable amount of \( \alpha \)-helices as secondary structures (22% as calculated by the K2D software: http://www.embl-heidelberg.de/~andrade/k2d.html). This effect was even more pronounced for the CD spectrum performed in the presence of 50% 2,2,2-trifluoroethanol, an organic solvent known to preferentially induce the formation of \( \alpha \)-helices (36% of \( \alpha \)-helices as calculated by the K2D software) (see Supplementary Data). The consensus method offered by the network protein sequence analysis (33) predicted a 24.5% \( \alpha \)-helix proportion, thus indicating that the long i3 cyc peptide presented structural features, such as \( \alpha \)-helices, in the expected proportions.

Effects of i3 cyc on AVP and GTP\( \gamma \)S-stimulated Adenylyl Cyclase Activity in Rat Kidney Membranes—To determine whether i3 cyc would affect \( V_2 \)-R signaling, AC assays were performed on rat kidney membranes in the presence or absence of i3 cyc. As shown in Fig. 2A, increasing amounts of i3 cyc almost completely inhibited the cAMP accumulation induced by AVP (30 nm) or GTP\( \gamma \)S (10 nm) with an apparent IC\(_{50}\) of 17 ± 7 \( \mu \)M and 19 ± 5 \( \mu \)M, respectively. In these experiments, the basal cAMP accumulation was only weakly inhibited. As illustrated in Fig. 2B, these effects were specific for i3 cyc because a peptide derived from the third intracellular loop of a non-related GPCR (i3 endo from r-ET\(_A\)-R) and presenting similar physicochemical properties than i3 cyc, did not inhibit the cAMP production in the same condition. The fact that i3 endo is not a cyclic peptide could not account for its lack of effect on cAMP production, because a linear peptide corresponding to the third intracellular loop of V2-R (i3 lin) was able to inhibit the cAMP production induced by AVP (30 nm) or GTP\( \gamma \)S (10 nm) albeit less efficiently (Fig. 2B). These results also support our prediction that i3 peptide should be more active under cyclized form, most likely by mimicking the constraints imposed by transmembrane helices in the receptor. Therefore, only i3 cyc was used for the following experiments (Fig. 2B).

To further characterize the inhibitory action of i3 cyc on AVP-stimulated cAMP production, the effect of the peptide was assessed on full AVP dose-responses. 10 \( \mu \)M i3 cyc inhibited the maximal response by 65 ± 3% and significantly right-shifted the \( K_{act} \) for AVP (6.0 ± 2.0 nm in the presence of i3 cyc versus 0.8 ± 0.2 nm in the absence of the peptide) (Fig. 2C). The i3 cyc peptide (10 \( \mu \)M) was also found to inhibit the maximal GTP\( \gamma \)S-stimulated cAMP production by 70 ± 4% (Fig. 2D). In this case, however, the \( K_{act} \) of GTP\( \gamma \)S was not significantly affected by the peptide (16 ± 3 nm in the presence of i3 cyc versus 12 ± 4 nm in the absence of the peptide). In contrast, i3 cyc had no significant effect on the AC activity stimulated by Mn\(^{2+}\), a cation known to directly activate AC (data not shown), indicating that the peptide did not act as an inhibitor of the AC catalytic activity. The specificity of these inhibitory effects was further confirmed by the observation that an unrelated synthetic peptide mimicking the second intracellular loop of the rat V1a receptor (i2 cyc) had no effect on either AVP or GTP\( \gamma \)S-
stimulated cAMP production (Fig. 2, C and D). As for i3 endo, i2 cyc was chosen as a negative control because it harbors physicochemical properties similar to those of i3 cyc. Moreover, it corresponds to an intracellular region of GPCR important for V1a/Gq coupling but not Gs coupling (13, 34) and has previously been shown to selectively inhibit [3H]AVP binding to membrane expressing the human vasopressin V1a receptor (2). Hence, i3 cyc selectively inhibits AVP- and GTPγS-stimulated cAMP production in rat kidney membranes, most likely by acting at the receptor and/or G protein level.

Effects of i3 cyc on AVP-stimulated [35S]GTPγS Binding in Rat Kidney Membranes—To determine whether i3 cyc would affect the receptor-mediated GDP/GTP exchange on Gs, the influence of i3 cyc on the specific vasopressin-stimulated [35S]GTPγS binding was next assessed in rat kidney membranes.

AVP dose-dependently increased the specific basal [35S]GTPγS binding with a Kd of 0.29 ± 0.11 nM. Maximal effects were reached for 100 nM AVP and represented a 84 ± 5% increase compared with the basal [35S]GTPγS-specific binding measured in the absence of AVP (data not shown).

Saturation experiments performed with increasing amounts of [35S]GTPγS in the presence of 100 nM AVP revealed that i3 cyc reduced the maximal binding capacity of [35S]GTPγS by 40 ± 9% without affecting its affinity (Kd = 2.0 ± 0.3 and 2.2 ± 0.3 nM with or without i3 cyc, respectively) (Fig. 3A), indicating that the peptide inhibits the receptor-mediated GDP/GTP exchange on Gs. To demonstrate that the peptide does not act as a general inhibitor of G protein activity, the potential effect of i3 cyc was assessed on a Gi/Go-coupled receptor: the GABA-b receptor. For this purpose, membranes from HEK293T cells expressing the GABA-b receptor were pre-incubated or not with i3 cyc before measuring [35S]GTPγS binding in response to stimulation with the GABA-b receptor agonist baclofen. As shown in Fig. 3B, i3 cyc did not modify the specific baclofen-stimulated [35S]GTPγS binding, thus supporting the specificity of i3 cyc effects on Gs-mediated signaling.

Effects of i3 cyc on [3H]AVP-specific Binding in Rat Kidney Membranes—To determine whether the i3 cyc-mediated inhibition of AVP-stimulated GTPγS binding and cAMP accumulation could result in part from an alteration in hormone binding, [3H]AVP binding experiments were performed. For this purpose, a subsaturating concentration (0.4 nM) of [3H]AVP was used. As illustrated in Fig. 4A, i3 cyc had a biphasic effect on the hormone binding. Indeed, it marginally increased the specific binding of [3H]AVP at the lowest concentrations used but almost completely abolished specific binding (90 ± 3% inhibition) at higher concentrations. This inhibition occurred with an apparent IC50 value of 12 ± 2 μM and was specific for the i3 cyc peptide sequence, because two other peptides derived from other GPCR intracellular loops (i2 cyc from rat V1a receptor and i3 endo from r-ETA-R) had only marginal effects on the specific vasopressin-stimulated [3H]AVP binding (Fig. 4A).

Specific binding was calculated in each experimental condition and expressed as percentage of control values (100% = 73 ± 5 fmol of [3H]AVP specifically bound per mg of protein). Results are the mean ± S.E. of triplicate determinations from three independent experiments.
enously expressed in rat brain membranes (Fig. 4B).

Further analysis of i3 cyc binding inhibitory action was carried out by performing \[^{3}H\]AVP saturation binding experiments. In the absence of i3 cyc, Scatchard analyses revealed the presence of two binding sites that differed from each other by their affinities for \[^{3}H\]AVP (see Table I for individual values). In contrast, in the presence of 10 \(\mu M\) i3 cyc, only the low affinity binding sites were detected (Fig. 4C and Table 1). It is interesting that treatment with i3 cyc had no significant effect on the global \(B_{\text{max}}\) indicating that the high affinity binding sites observed in the control were converted into low affinity ones by the peptide.

Because uncoupling from G protein is known to shift \(V_2\)-R binding sites from a high to a low affinity state (35, 36), we then sought to determine whether the effect of i3 cyc on \[^{3}H\]AVP binding could result solely from such uncoupling. For this purpose, the effects of i3 cyc on \[^{3}H\]AVP binding were assessed in the presence and absence of 100 \(\mu M\) GTP\(\gamma\)S, the concentration of GTP\(\gamma\)S leading to its maximal inhibitory effect on \[^{3}H\]AVP specific binding (data not shown). As illustrated in Fig. 5, in the presence of 100 \(\mu M\) GTP\(\gamma\)S, 10 \(\mu M\) i3 cyc still inhibited the specific binding of a subsaturating concentration of \[^{3}H\]AVP. In fact, the inhibitory actions of GTP\(\gamma\)S and i3 cyc were found to be almost additive. Taken together, these results indicate that the effect of the peptide on AVP binding could not be explained only by a dissociation of the \(V_2\)-RGs complex, thus suggesting a direct action of the peptide on the receptor itself.

**Effects of i3 cyc on the Binding and Activation Properties of the Human \(V_2\) Receptor Expressed in HEK 293 Cells—**We next wanted to assess the potential effects of i3 cyc on the \(V_2\)-R dimerization state. For this purpose, we used another biological model more adapted for the study of both \(V_2\)-R signaling efficacy and oligomerization process. HEK 293T cells were thus transiently transfected with the Rluc- and YFP-fused \(V_2\)-R constructs, so as to monitor \(V_2\)-R homodimerization in presence or absence of i3 cyc by BRET experiments. Given that the \(V_2\)-R loop amino acid sequences of r-\(V_2\)-R and h-\(V_2\)-R are highly homologous (92% of identity), we predicted that i3 cyc could also be active on the human \(V_2\) receptor. To validate this prediction, the effects of i3 cyc on AVP or GTP\(\gamma\)S-stimulated adenyl cyclase and hormone binding were first assessed in membranes derived from HER 293T cells expressing h-\(V_2\)-R-YFP and h-\(V_2\)-Rluc (Fig. 6B). This inhibition was accompanied by a significant \(K_{\text{act}}\) rightward-shift (1.58 \(\pm\) 0.37 nm in the presence versus 0.32 \(\pm\) 0.05 nm in the absence of i3 cyc). When adenyl cyclase was stimulated by GTP\(\gamma\)S, 10 \(\mu M\) i3 cyc inhibited cAMP production by 41 \(\pm\) 5%. In this case, the \(K_{\text{act}}\) was only modestly affected (43 \(\pm\) 4 to 24 \(\pm\) 3 \(\mu M\) for \(K_{\text{act}}\) measured with or without i3 cyc, respectively) (Fig. 6C). We also evaluated the effect of 10 \(\mu M\) i3 cyc on the cAMP production induced by isoproterenol or GTP\(\gamma\)S on membranes derived from an HEK 293 cell line stably expressing the Rluc-tagged \(\beta_2\)-adrenergic receptor and observed in both cases an inhibition of the maximal cAMP production (27 \(\%\) and 26 \(\%\), respectively). The \(K_{\text{act}}\) for GTP\(\gamma\)S was not significantly modified but, more interestingly, the inhibition of the isoproterenol-induced cAMP production was not accompanied by a significant modification of the \(K_{\text{act}}\) for isoproterenol (data not shown). Hence, these results confirm the existence of a non-receptor-mediated inhibition of i3 cyc, which probably reflects a direct action of the peptide at the G protein level.

Taken together, these results indicate that i3 cyc has identical effects on the r-\(V_2\)-R and the h-\(V_2\)-R tagged receptors. Therefore, the Rluc and YFP fused h-\(V_2\)-R heterologously expressed in HEK 293T cells constitute a fair biological model to study the effects of i3 cyc on the \(V_2\)-R homo-dimerization process using BRET experiments.

### Table I

**Influence of 10 \(\mu M\) i3 cyc on \[^{3}H\]AVP binding parameters in rat kidney membranes**

| Assay conditions | \(K_{d1}\) (nM) | \(K_{d2}\) (nM) | \(B_{\text{max}}\) (fmol/mg) | \(B_{\text{max}+i3}\) (fmol/mg) |
|------------------|----------------|---------------|-----------------------------|-----------------------------|
| Control          | 0.5 \(\pm\) 0.1 | 6.6 \(\pm\) 1.5 | 297 \(\pm\) 18              | 335 \(\pm\) 50              |
| + i3 cyc (10 \(\mu M\)) | \(K_{d1+i3} = 6.0 \pm 0.6\) | | | |

**FIG. 5. Combined effects of i3 cyc and GTP\(\gamma\)S on AVP-specific binding to rat kidney membranes.** Rat kidney membranes (10 \(\mu g\) of protein per assay) were preincubated for 15 min at 30 °C with either i3 cyc, i3 cyc + GTP\(\gamma\)S, or vehicle (control). 0.4 nm \[^{3}H\]AVP in the presence (nonspecific binding) or absence (total binding) of 1 \(\mu M\) unlabeled AVP was then added to the incubation medium, and the reaction was allowed to proceed for another 45 min. Specific binding was calculated in each experimental condition and expressed as percentage of control values (100% \(\pm\) 107 \(\pm\) 19 fmol of \[^{3}H\]AVP specifically bound per mg of protein). Results are the mean \(\pm\) S.E. of triplicate determinations from four independent experiments.
i3 cyc Specifically Inhibited the BRET between the Rluc and YFP Constructs within V₂-R Homodimers—To further investigate whether the effects of i3 cyc could in part result from a direct action on the receptor, BRET experiments were carried out with HEK 293T cells transiently co-expressing V₂-R-Rluc + V₂-R-YFP, pretreated or not with i3 cyc. Given that V₂-R, like several GPCRs, was demonstrated to form homodimers (20, 37, 38), one could indeed assume that a direct action of i3 cyc on the receptor would affect either the dimer conformation or oligomerization state, both leading to a change in BRET measured between the Rluc and YFP-fused V₂-R.

As shown in Fig. 7A, the BRET signals were dose-dependently inhibited by i3 cyc, whereas the two other control peptides (i2 cyc and i3 endo) had no effect. This reduction in BRET did not result from the uncoupling of the receptor from the G protein because the addition of 100 μM GTPγS had no effect on the BRET signal (Fig. 7A). Moreover, upon addition of 100 μM GTPγS or 1 μM AVP, i3 cyc was still able to inhibit the BRET signals (data not shown) confirming that this peptide could directly act on the receptor. The i3 cyc effects were specific for the V₂-R because the BRET signal resulting from the heterodimerization of GABA-B Gb-R1/Gb-R2 receptors and the homodimerization of β₂-AR were not affected by this peptide (Fig. 7B).

As already evoked, the i3 cyc induced-reduction in BRET between h-V₂-R-Rluc and h-V₂-R-YFP could result either from a dissociation of the protomers leading to monomerization or from a conformational change within the dimer that increases the distance or negatively affects the dipole orientations of the energy donor and acceptor without disrupting the dimer. In an effort to distinguish between these two possibilities, BRET titration experiments were carried out to determine the relative affinity between each of the protomers, as described previously (27). Indeed, if the peptide promotes a dissociation of the protomers, one would predict that i3 cyc should decrease the apparent affinity of the receptors for one another. Because the maximal BRET level reached (BRETₘₐₓ) in BRET titration experiments depends not only on the total number of dimers formed but also on the distance and orientation between V₂-R-Rluc and V₂-R-YFP, this value cannot be used to quantify the relative numbers of dimers present in each condition. In contrast, the BRETₙ₀, which corresponds to the concentration of h-V₂-R-YFP giving 50% of the maximal energy transfer, should be a reflection of the relative affinity between the two protomers. As shown in Fig. 7C, the addition of i3 cyc led to a significant reduction in BRETₘₐₓ without significantly affecting the BRETₙ₀ (see Table II for values). These results thus strongly indicate that the peptide acts by affecting the conformational organization of the dimer and not by promoting its dissociation into monomers. Once again the selectivity of the effect was confirmed by the lack of influence of i2 cyc on the BRET signal.

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**Fig. 6.** Influence of i3 cyc on the human V₂-R binding properties and activation. A, membranes from HEK 293T cells co-expressing h-V₂-R-Rluc and h-V₂-R-YFP were pre-incubated for 15 min at 30 °C with or without (control) 10 μM i3 cyc as described under “Experimental Procedures.” 1 nM [³H]AVP in presence (nonspecific binding) or absence (total binding) of 1 μM unlabeled AVP was then added to the incubation medium, and the reaction allowed to proceed for another 45 min. Specific binding was calculated in each experimental condition and expressed as a percentage of control values (100% = 1.62 ± 0.28 pmol of [³H]AVP specifically bound per mg of protein). Results are the mean ± S.E. of triplicate determinations from three independent experiments. B and C, membranes from HEK 293T cells co-expressing h-V₂-R-Rluc and h-V₂-R-YFP (20 μg of protein per assay) were pre-incubated for 15 min at 37 °C with or without (control) 10 μM i3 cyc in presence or absence of increasing amounts of either AVP (B) or GTPγS (C). 1 μCi of [α-³²P]ATP per assay was then added to the incubation medium, and the reaction allowed to proceed for a further 6 min. cAMP produced was determined as described under “Experimental Procedures.” Results are expressed as percentage of the maximal responses and correspond to the mean ± S.E. of triplicate determinations from three distinct experiments (100% = 44 ± 7 and 368 ± 89 pmol of cAMP produced per mg of protein for AVP and GTPγS-stimulated AC activity, respectively).
**FIG. 7.** Influence of receptor mimetic peptides and GTP\(\gamma\)S on the BRET signals. A, membranes from HEK 293T cells co-expressing h-V2-R-Rluc and h-V2-R-YFP were incubated for 15 min at 30 °C with or without (control) increasing amounts of i3 cyc, 10 μM i2 cyc, 10 μM i3 endo, or 100 μM GTP\(\gamma\)S and then subjected to BRET measurements as described under “Experimental Procedures.” Data are expressed as the percentage of the control BRET signal and correspond to the mean ± S.E. of at least three independent experiments, each performed in triplicate. B, membranes from HEK 293T cells co-expressing either GbR1-RLuc+GbR2-YFP or β2AR-Rluc+β2AR-YFP were incubated for 15 min at 30 °C with or without (control) 10 μM i3 cyc or i2 cyc before being subjected to BRET measurements. Data are expressed as the percentage of the control BRET signal and correspond to the mean ± S.E. of four independent experiments, each performed in triplicate. C, HEK 293T cells were co-transfected with increasing amounts of plasmid DNA for the h-V2-R-YFP construct (0.1–12 μg), whereas the h-V2-R-Rluc construct was kept constant (0.05 μg). All samples were subjected to fluorescence and luminescence analysis to control for receptor expression. Then, membrane preparations for each condition were incubated for 15 min at 30 °C with or without (control) 10 μM i3 cyc or i2 cyc before being subjected to BRET measurements. All BRET values expressed in relative fluorescence units and represent the mean ± S.E. of four independent determinations. Statistical analyses were done using unpaired Student’s t test with the Prism 3.0 Graph Pad Software.

**TABLE II**

| Assay conditions | BRET\(\text{max}\) (arbitrary units) | BRET\(\text{50}\) (arbitrary units) |
|------------------|--------------------------------------|-----------------------------------|
| Control          | 0.23 ± 0.02                          | 0.69 ± 0.03                       |
| + i3 cyc (10 μM) | 0.15 ± 0.08a                        | 0.44 ± 0.03a                      |
| + i2 cyc (10 μM) | 0.24 ± 0.02                          | 0.69 ± 0.03                       |

\[a p = 0.35 (\text{BRET}_{\text{max}}, \text{non-significant}).
\]

\[b p < 0.0002 (\text{BRET}_{\text{max}}, \text{***})], \text{i3 cyc versus control values.}\n
**DISCUSSION**

The i3 loop of the V2 vasopressin receptor was shown to be an important determinant for Gs coupling (13). BRET technology recently allowed the establishment of the presence of V2-R dimeric entities in living cells (20). To test the hypothesis that a peptide corresponding to V2-R third intracellular loop could affect the receptor dimer structural arrangement and function, we synthesized a cyclic peptide of 51 residues mimicking the i3 loop structure (i3 cyc). This peptide was shown to specifically inhibit AVP- or GTP\(\gamma\)S-stimulated AC activity in membranes from both rat kidney, where V2-R is endogenously expressed, and HEK 293T cells heterologously co-expressing h-V2-R-YFP and h-V2-R-Rluc. To better understand i3 cyc mechanism of action, we studied its effects at distinct steps of the V2-R signaling: hormone binding, GDP/GTP exchange at the G protein level and receptor dimer structural organization.

The i3 cyc peptide induced principally a dose-dependent decrease of [\(^3\)H]AVP-specific binding by modifying the relative proportion of high and low affinity states of the receptor, leading to a loss of high affinity binding sites. Given that GTP\(\gamma\)S was previously shown to shift the high affinity binding site into low affinity by disrupting the V2-R/Gs complex (36), one possible explanation for i3 cyc effects would be that the peptide induced V2-R uncoupling from Gs. However, the observation that the effects of i3 cyc on AVP binding were additive to those of GTP\(\gamma\)S discredits such a hypothesis and suggests rather that the shift from high to low affinity promoted by the peptide results from a direct action of i3 cyc on the receptor. This conclusion is supported by the fact that i3 cyc induced a significant inhibition of the BRET signal measured between V2R-Rluc and V2R-YFP, strongly indicating that i3 cyc modified the distance and/or orientation between V2R-Rluc and V2R-YFP, leading to a rightward shift in the AVP-stimulated AC activity.

Moreover, the receptor-induced [\(^3\text{S}\)GTP\(\gamma\)S] binding experiments revealed that i3 cyc altered the maximal binding
capacity of the Gs protein and had no effect on the affinity of 

\[ ^{35}S \text{GTP}_{\gamma}S \] for Gs (Fig. 3A), suggesting that the peptide did not act allosterically by reducing the affinity of Gs for 

\[ ^{35}S \text{GTP}_{\gamma}S \]. Rather, the data indicate that i3 cyc inhibits the receptor-mediated GTP/GTP exchange by competing with the receptor, thus reducing the proportion of Gs molecules available for receptor activation. Thus, the decrease in Gs molecule availability could account for the reduction in \( E_{\text{max}} \) observed in AVP-stimulated AC activity. Whether the direct effect of the peptide on the receptor could account for the entire loss of efficacy in AC response is difficult to determine. Indeed, in addition to its effect on the receptor itself, i3 cyc was also found to inhibit the GTP\( ^{35}S \)-stimulated AC activity in membrane preparations from rat kidney and HEK 293 cells co-expressing h-V2-R-YFP and h-V2-R-Rluc. Moreover, i3 cyc inhibited the cAMP production induced by isoproteorenol or GTP\( ^{35}S \) in membranes derived from a HEK 293 cell line stably expressing the Rluc-tagged \( \beta_{2} \)-adrenergic by decreasing the \( E_{\text{max}} \) (27 ± 8 and 26 ± 6 %, respectively) without significant modification of the \( K_{\text{act}} \) for isoproteorenol or GTP\( ^{35}S \). This indicates that the peptide can also directly act on the G protein leading to an alteration of its coupling to AC. Thus, such a dual effect of i3 cyc made it difficult to evaluate the relative portion of AVP signaling inhibition because of its effects on the receptor itself or on the Gs proteins.

In an attempt to better understand the effects of i3 cyc on the structural dimer organization, BRET titration experiments were then performed with cells co-expressing V2-R-Rluc and V2-R-YFP. No significant differences between the BRET\( _{\text{iso}} \) were observed after i3 cyc treatment, although it significantly inhibited the BRET\( _{\text{max}} \), strongly suggesting that i3 cyc would modify the distance and/or orientation between V2-R-Rluc and V2-R-YFP engaged in dimer formation without affecting the oligomerization state itself. These data therefore support the notion that the peptide changes the conformation of a pre-existing dimer without promoting disassembly of protomers. These changes cannot be attributed to an effect on the receptor/G protein coupling because GTP\( ^{35}S \) on its own had no effect on the BRET signal between V2-R-Rluc and V2-R-YFP or on the BRET inhibitory effect of i3 cyc. It could thus be hypothesized that i3 cyc acts by directly binding to V2-R, most likely by substituting for the interactions in which the V2-R third intracellular loop is normally engaged (Fig. 8). Whether the interaction of i3 cyc occurs between the two protomers of the dimer or within the same protomer but transmitted across the dimer remains to be determined.

Other studies have also identified peptides that can affect both GPCR dimers and function. For instance, a peptide derived from the TM6 domain of the \( \beta_{2} \)-AR was found not only to decrease the amount of receptor dimers detected in co-immunoprecipitation studies but also to inhibit the receptor-stimulated AC activity, leading the authors to conclude that dimerization may be important in receptor function. Likewise, Banéres et al. (17) recently found that a peptide mimicking the TM6 domain inhibited the dimerization of the BLT1 receptor and affected the ability of the receptor to interact with the G protein. Therefore, instead of inducing a conformational rearrangement of the dimers, as is the case with i3 cyc, these peptides corresponding to the TM6 of the \( \beta_{2} \)-AR and the BLT1 receptor inhibit by directly disrupting the preformed dimers. In all cases, however, the results are consistent with the notion that dimers represent the active form of the receptor. By contrast, a TM6 mimetic peptide of the D1 dopamine receptor has been shown to affect the receptor function without disrupting the dimers that could be detected by immunoblot analyses (39), but one cannot exclude that this peptide acts in affecting the dimer structural organization.

In conclusion, in this study, we have demonstrated that a peptide mimicking the V2-R third intracellular loop inhibits the receptor function through a modification of its dimeric structural organization and a direct action on Gs protein.

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i3 Peptide Acts on Vasopressin V2 Receptor Dimer

FIG. 8. Speculative model of i3 cyc effects. In a basal state, the dimer might contain a high and a low affinity binding site for vasopressin. A 10 \( \mu \text{M} \) i3 cyc concentration could induce a structural reorganization within the receptor (R) dimer leading to the presence of two low affinity sites within the dimer and to an inhibition of G protein signaling. The peptide could also act through a direct interaction with the G proteins thus reducing their availabilities for receptor activation (GDP/GTP exchange) and consequently to an inhibition of adenylyl cyclase (A.C.) activation.

| A.C. | cAMP | ATP | GDP | GTP |
|------|------|-----|-----|-----|
| R    | R    |     |     |     |
| αβγ  |      |     |     |     |

these changes cannot be attributed to an effect on the receptor/G protein coupling because GTP\( ^{35}S \) on its own had no effect on the BRET signal between V2-R-Rluc and V2-R-YFP or on the BRET inhibitory effect of i3 cyc. It could thus be hypothesized that i3 cyc acts by directly binding to V2-R, most likely by substituting for the interactions in which the V2-R third intracellular loop is normally engaged (Fig. 8). Whether the interaction of i3 cyc occurs between the two protomers of the dimer or within the same protomer but transmitted across the dimer remains to be determined.

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