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A Peptide Derived from a β_2-Adrenergic Receptor Transmembrane Domain Inhibits Both Receptor Dimerization and Activation*

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One of the assumptions of the mobile receptor hypothesis as it relates to G protein-coupled receptors is that the stoichiometry of receptor, G protein, and effector is 1:1:1 (Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) *Nature* 348, 125–132). Many studies on the cooperativity of agonist binding are incompatible with this notion and have suggested that both G proteins and their associated receptors can be oligomeric. However, a clear physical demonstration that G protein-coupled receptors can indeed interact as dimers and that such interactions may have functional consequences was lacking. Here, using differential epitope tagging we demonstrate that β_2-adrenergic receptors do form SDS-resistant homodimers and that transmembrane domain VI of the receptor may represent part of an interface for receptor dimerization. The functional importance of dimerization is supported by the observation that a peptide derived from this domain that inhibits dimerization also inhibits β-adrenergic agonist-promoted stimulation of adenylyl cyclase activity. Moreover, agonist stimulation was found to stabilize the dimeric state of the receptor, while inverse agonists favored the monomeric species, which suggests that interconversion between monomeric and dimeric forms may be important for biological activity.

The β_2-adrenergic receptor (β_2AR)[1] is one of the best characterized members of the G protein-coupled receptor family (1, 2). Activation of the receptor by agonist stimulates GDP/GTP exchange in the Gsα-subunit to which the receptor is coupled and results in activation of its effector, adenylyl cyclase. The stoichiometry of the interaction between the receptor, G-protein, and effector molecule is assumed to be 1:1:1 (3). However, several studies have demonstrated cooperativity in binding of agonists to G protein-coupled receptors and suggest that they may be part of an oligomeric array (4–12). Structural studies, including photoaffinity labeling of muscarinic receptors (11), radiation inactivation of α- and β-AR (13), cross-linking of glucagon receptors (14), and hydrodynamic properties of cardiac muscarinic receptors (15), also support the notion that G protein-coupled receptors may form dimers. An elegant study by Maggio *et al.* (16) using co-transfection of chimeric α2 adrenergic and M3 muscarinic receptors also demonstrates that intermolecular interactions can occur between receptors. Indeed, the two chimeras formed functional receptors when they were co-expressed, while no activity was seen when each chimeric receptor was expressed alone. Similarly, co-expression of two binding defective angiotensin II receptor mutants was recently demonstrated to rescue the binding for the peptide (17). The use of "split" receptors also suggests that GPCRs behave in some respects like two subunit proteins. Co-expression of truncated β_2AR containing transmembrane segments I–V and VI–VII resulted in formation of functional receptors, while expression of either truncation alone yielded no signaling (18).

The importance of oligomerization in normal G protein-coupled receptor signaling is poorly characterized and a direct demonstration that GPCR dimers exist is lacking. In other transmembrane signaling systems, however, the role of receptor dimer is better understood. Many growth factor receptors are known to act functionally and structurally as dimers (for review, see Ref. 19). These include the EGF-R (20, 21), the interferon γ receptor (22), the PDGF-R (23–25), and the FGF-R (26). It was suggested that the high affinity binding site on these receptors occurs only in their dimeric forms (20, 21). Indeed for the EGF-R, the high affinity state can be stabilized by the introduction of interreceptor disulfide bonds (27). The signal for the formation of these dimers seems to be activation by ligand. Dimers are also seen in many bacterial sensory receptors, including those for aspartate and serine (for review, see Refs. 28 and 29). However, it is less clear whether or not these receptors dimerize upon activation by ligand (30–32). The notion that GPCRs may also dimerize in response to activation by ligand has not been tested.

In this study we sought to determine whether monomeric GPCRs interact either functionally and/or physically as homodimers using a variety of approaches. These included ex-

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[1] The abbreviations used are: β_2AR, β_2-adrenergic receptor; BASED, bis[β-(4-azidosalicylamindo)ethyl]disulfide; M2-R, M2 muscarinic acetylcholine receptor; D1-R, D1 dopamine receptor; GPCR, G protein-coupled receptor; GpA, glycophorin A; HA, influenza hemagglutinin; TM VI, transmembrane domain 6; V2-R, V2 vasopressin receptor; CYP, cyanopindolol; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

pression of β_2ARs in Sf9 cells using the baculovirus system where differential epitope tagging was used, as well as stable expression of β_2AR in LTK$^-$ and CHW cells. We also transiently expressed wild-type and truncated forms of the V2 vasopressin receptor in COS-7 cells. In order to determine the functional correlate of receptor dimerization, we measured the effect of disrupting dimerization on the ability of the receptor to stimulate adenylyl cyclase activity. Finally, we were interested in the effects of different β_2AR ligands on the equilibrium between monomeric and dimeric forms of the receptor.

EXPERIMENTAL PROCEDURES

Recombinant Baculoviruses—The recombinant baculoviruses encoding the c-*myc* or hemagglutinin (HA)-tagged wild-type human β_2-adrenergic receptor, the c-*myc*-tagged human M2 muscarinic receptor, and c-*myc*-tagged D1 dopamine receptor (c-*myc* β_2AR and HA-β_2AR, c-*myc* M2-R, and c-*myc* D1-R, respectively) were constructed as described (33). Briefly, HA (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) and c-*myc* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) tags containing initiator methionine residues were introduced into the receptor cDNAs immediately before their initiator methionines by subcloning the corresponding double-stranded oligonucleotides. Cells were infected with recombinant baculoviruses at multiplicities of infection ranging from 3 to 5.

Sf9 Cell Culture—Sf9 cells are maintained at 27 °C in serum-supplemented (10% fetal bovine serum, v/v) Grace's insect medium (Life Technologies, Inc.) with gentamycin and fungizone. Cells were grown either as monolayers in T flasks or in suspension in spinner bottles supplemented with pluronic acid to prevent cell tearing due to agitation. Cells were infected at log phase at a density of 1×10^6 to 2×10^6 cells/ml for 48 h.

Mammalian Cell Culture—CHW and LTK$^-$ cell lines with and without stably transfected β_2AR were maintained as described (34). Cells were grown in Dulbecco's modified Eagle's medium supplemented with L-glutamate, 10% fetal bovine serum, gentamycin, and fungizone. Transfected CHW cells expressed ~5 pmol of receptor/mg of protein, while transfected LTK$^-$ cells expressed 200 fmol of receptor/mg of protein. Stably transfected cell lines were grown in the presence of 150 μg/ml G418.

For transient expression of V2 vasopressin receptors the following procedures were followed. COS-7 cells were maintained in supplemented Dulbecco's modified Eagle's medium as described above. Genomic DNA for the V2 vasopressin receptor was isolated from nephrogenic diabetes insipidus patients or unaffected individuals, subcloned into a construct containing a c-*myc* epitope tag, and ligated into a mammalian expression vector, pBC12BI (35). Using DEAE-dextran, COS-7 cells were transiently transfected with the expression vector encoding either wild-type V2 vasopressin receptor, a truncation mutant O-11, or with vector alone for 48 h.

Membrane Preparation—Sf9 or mammalian cells were washed twice with ice-cold phosphate-buffered saline. The cells were then disrupted by homogenization with a Polytron in 10 ml of ice-cold buffer containing 5 mM Tris-HCl, pH 7.4, 2 mM EDTA (plus a protease inhibitor mixture consisting of 5 mg/ml leupeptin, 10 mg/ml benzamidine, and 5 mg/ml soybean trypsin inhibitor). Lysates were centrifuged at $500 \times g$ for 5 min at 4 °C, the pellets homogenized as before, spun again, and the supernatants were pooled. The supernatant was then centrifuged at $45{,}000 \times g$ for 20 min and the pellets washed twice in the same buffer. In some cases receptors were then solubilized in 2% digitonin or 0.3% *N*-dodecyl-β-D-maltoside and purified by affinity chromatography on alprenolol-Sepharose as or by immunoprecipitation as described below.

Affinity Purification of β_2ARs—Solubilized receptors were affinity-purified by alprenolol-Sepharose chromatography as described (33, 36). The affinity-purified preparations were concentrated using Centriprep and Centricon cartridges (Amicon), and the amount of β_2AR in each sample was determined in soluble ^{125}I-CYP radioligand binding assays as described (33). Purified receptors were desalted on Sephadex G-50 columns prior to SDS-PAGE.

Immunoprecipitation of β_2ARs—Tagged β_2ARs were immunoprecipitated with either a mouse anti-c-*myc* monoclonal antibody (9E10; Ref. 37) or a mouse anti-hemagglutinin monoclonal antibody (12CA5; Ref. 38) as described previously (33). Removal of digitonin and concentration of the solubilized receptor was performed by dialysis using Centriprep cartridges (Amicon) against an ice-cold solution (Buffer A) containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA (plus protease inhibitors described above) until the digitonin concentration was reduced below 0.05%. Purified 9E10 or 12CA5 antibody (1:1000 dilution) was added to the concentrate and gently agitated for 2 h at 4 °C. Anti-mouse IgG agarose (Sigma; at an 11:1 secondary to primary antibody molar ratio) and protease inhibitor mixture were then added. The reaction was allowed to proceed overnight at 4 °C with gentle agitation. The immunoprecipitate was centrifuged at 12,000 rpm in a microcentrifuge for 10 min at 4 °C. The pellet was washed three times in buffer A and finally resuspended in 200 μl of nonreducing SDS-PAGE loading buffer for 30 min, sonicated, and centrifuged at 12,000 rpm. The supernatant was then subjected to SDS-PAGE and Western blotting as described below.

Cross-linking of β_2ARs—Ten ml of Sf9 cell suspension (2×10^6 cells/ml) were taken 48 h post-infection and either mock-treated with vehicle or treated with 1 mg of the membrane permeant photoactivatible cross-linking agent BASED (bis[β-(4 azidosalicylamindo)ethyl] disulfide; Pierce) for 60 min at room temperature with gentle agitation. Membranes were then prepared from cells as described above and resuspended in nonreducing SDS-PAGE sample buffer. Gels were subsequently immunoblotted as described below.

SDS-PAGE and Western Blotting—Membrane preparations from Sf9 or mammalian cells or in some cases affinity-purified or immunoprecipitated β_2AR were prepared for nonreducing SDS-PAGE on 10% slab gels as described previously (39). In the case of the V2 vasopressin receptors, reducing SDS-PAGE was performed. For Western blotting, gels were transferred to nitrocellulose and blotted with either the mouse anti-c-*myc* monoclonal antibody (9E10), the anti-hemagglutinin monoclonal antibody (12CA5) at dilutions of 1:1000, or in the case of mammalian cells expressing the β_2AR, a polyclonal rabbit anti- β_2AR antiserum raised against a peptide from the COOH-terminal region of the β_2AR at a dilution of 1:2000 (a generous gift of Dr. A. D. Strosberg, Institut Cochin de Génétique Moleculaire, Paris). Immunoblots against the anti-c-*myc* or anti-HA antibodies were revealed using a goat anti-mouse alkaline phosphatase-coupled second antibody (Life Technologies, Inc.) or a chemiluminescent substrate for a horseradish peroxidase-coupled second antibody (Renaissance, NEN DuPont). For the experiments performed using mammalian cells expressing the β_2AR Western blots were developed using a chemiluminescent substrate for goat anti-rabbit-coupled horseradish peroxidase antisera (Sigma). To assess total immunoreactivity of the various receptor species, blots were scanned by laser densitometry (Pharmacia Biotech Inc. Ultrascan).

Peptide Treatment of β_2ARs—Peptides were synthesized on solid-phase supports using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry (40) on a BioLynx 4175 manual peptide synthesizer (LKB). Peptides were solubilized in the following buffer: 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA (plus the protease inhibitor mixture described above), 0.05% digitonin, and 10% dimethyl sulfoxide. Peptide sequences were confirmed either by mass spectrometry or amino acid analysis. Peptides used were as follows: 1) β_2AR TM VI peptide consisting of residues 276–296; NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH, 2) a second peptide with Ala residues substituted at positions 276, 280, and 284 NH_2-AIIMATFTACWLPFFIVNIVH-COOH, 3) a peptide derived from residues 407–426 of the D2 dopamine receptor TM VII NH_2-YIIPNVASNVYGLWTFASYL-COOH, 4) a peptide derived from the COOH-terminal tail of the β_2AR consisting of residues 347–358 NH_2-LKAYGNGYSSNG-COOH, and 5) an additional peptide unrelated to the β_2AR but of similar size as the TM VI peptide NH_2-SIQHLSTGH-DHDDVDVGEQQ-COOH. To assess the effect of the different peptides or β_2AR ligands on the relative amount of different receptor species the following experiments were performed. Membrane preparations from mammalian or Sf9 cells or affinity-purified receptors derived from Sf9 cells expressing c-*myc*-tagged β_2AR were treated increasing concentrations of the different peptides at room temperature for various times as indicated under "Results and Discussion." Samples were then run on SDS-PAGE and then transferred to nitrocellulose. In some cases membrane preparations were also treated with either 10 μM timolol or 1 μM isoproterenol instead of, or in addition to, the different peptides. Membranes were also used to determine the effect of various peptides on the ability of the β_2AR to stimulate adenylyl cyclase activity described below.

Receptor Quantification and Adenylyl Cyclase Assay—Receptor number was calculated from saturation binding experiments using ^{125}I-CYP as the radioligand (41). Briefly, 10 μl of a membrane preparation in a total volume of 0.5 ml was labeled with 250 pmol of ^{125}I-CYP, which is at a near saturating concentration. Nonspecific binding was defined using 10 μM alprenolol.

Adenylyl cyclase activity was assayed by the method of Salomon *et al.* (42). Membranes were prepared and washed as described above. Again 10 μl of membranes (3–5 μg of protein) were used in a total volume of

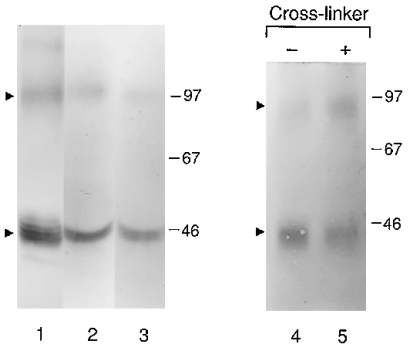

FIG. 1. **Immunoblotting of human β_2AR expressed in Sf9 cells.** Crude membrane preparations (*lane 1*), digitonin-solubilized membrane proteins (*lane 2*), and affinity-purified receptors (*lane 3*) derived from Sf9 cells expressing either c-*myc*-tagged (*lane 3*) or HA-tagged (*lanes 1* and *2*) β_2AR were immunoblotted following SDS-PAGE using the appropriate antibody (9E10 and 12CA5, respectively). The blots reveal immunoreactive bands corresponding to the expected monomeric form (43–50 kDa) as well as a higher molecular mass species (85–95 kDa). The *right panel* illustrates immunoblots of crude membrane preparations derived from Sf9 cells expressing HA-tagged β_2AR treated (*lane 5*) or not (*lane 4*) with the membrane-permeant photoactivatible cross-linker BASED. Position of receptor bands are denoted by *arrows* and molecular weight markers are as shown.

50 μl. In some experiments, the peptides or the buffer used to solubilize them were added to the enzyme assay mix. Enzyme activities were determined in the presence of 1 nM to 100 μM isoproterenol, 100 μM forskolin, or 10 mM NaF. Data were calculated as picomoles cAMP produced per min/mg of protein and were analyzed by least squares regression using SigmaPlot 4.17 (Jandel Scientific).

RESULTS AND DISCUSSION

Dimerization of β_2AR—Immunoblotting of c-*myc* epitope-tagged β_2AR expressed in Sf9 cells with the anti-c-*myc* antibody consistently revealed the presence of molecular species corresponding to the anticipated monomeric receptor (43–50 kDa) in Sf9 cells (33) as well as higher molecular weight forms. In particular, a prominent band was detected at an apparent molecular weight corresponding to twice that of the monomer (85–95 kDa), suggesting the existence of an SDS-resistant dimeric species of the receptor. In some membrane preparations, discrete bands, which could represent even higher order structures of the β_2AR, can also be detected (Fig. 1, *lane 1*). The dimer, which was readily observed in membrane preparations, was also detected in digitonin-solubilized receptors (*lane 2*) and following affinity purification of receptors on alprenolol-Sepharose (*lane 3*). As shown in *lanes 4* and *5*, when whole cells expressing the β_2AR were treated with the membrane-permeant cross-linking agent BASED, the dimer to monomer ratio as assessed by immunoblotting was increased by 2-fold. This suggests that the dimer is already present before cell fractionation and that cross-linking stabilizes this form of the receptor. Therefore, the dimeric species does not represent an artifact of membrane preparation or solubilization. Identical results were obtained when membranes were solubilized with 0.3% *N*-dodecyl-β-D-maltoside instead of digitonin (data not shown).

High molecular weight species have been previously observed for several GPCRs. Although some authors have suggested that these species may represent receptor dimers (14, 43–45), these have also been referred to as nonspecific aggregates (46–52). In order to directly determine if the higher molecular weight species observed in this study corresponded to a specific β_2AR homodimer, we devised a differential co-immunoprecipitation strategy using c-*myc* and HA epitope tagging. Human β_2ARs bearing either of these tags were co-expressed in Sf9 cells. The receptors were then immunoprecipitated with the anti-HA or anti-c-*myc* antibodies, subjected to SDS-PAGE, and blotted with one or the other antibody. In the results shown in Fig. 2 the anti-HA mAb was used to blot receptors immunoprecipitated with either the anti-HA or the anti-c-*myc* mAb. As seen in *lane 2*, blotting of the anti-HA immunoprecipitate revealed both the 45-kDa and the 90-kDa forms of the receptor. The β_2AR could also be detected by the anti-HA mAb in the c-*myc* immunoprecipitate of co-expressed receptors but the dimer then represented the predominant form (*lane 1*). This indicates that the two molecular species (HA-tagged and c-*myc*-tagged β_2ARs) were co-immunoprecipitated as part of a complex, which is stable in SDS, consistent with the higher molecular weight form being a β_2AR homodimer. Similar but complementary results are obtained when co-expressed receptors are immunoprecipitated with either anti-c-*myc* or anti-HA antibodies and then immunoblotted with the anti-c-*myc* antibody (data not shown). The specificity of the mAbs is illustrated by the absence of cross-reactivity in cells expressing one tagged receptor species only (Fig. 2, *lanes 3–6*). The occurrence of intermolecular interactions appears to be receptor-specific. Indeed, although dimers of c-*myc*-tagged M2 muscarinic receptor could be detected in Sf9-derived membranes expressing this receptor (data not shown and Ref. 51), no co-immunoprecipitation with the HA-tagged β_2AR was detected when the two receptors were co-expressed (Fig. 2, *lanes 7* and *8*).

V2 Vasopressin Receptors Are also Dimeric—The V2 vasopressin receptor is critical for regulation of water retention in the kidney (53). Recently, several mutations of this receptor have been linked to congenital nephrogenic diabetes insipidus, Ref. 54). In another approach to demonstrate GPCR dimer formation, transient expression of both wild-type and a truncated form of the V2 vasopressin receptor in COS-7 cells was studied. Both monomeric (~64–69 kDa) and dimeric (~120–135 kDa) forms of the wild-type human V2 vasopressin receptor were detected when expressed in COS-7 cells (Fig. 3, *lane 1*). A mutant form of the V2 receptor truncated in the COOH-terminal tail at residue 337 (O-11, isolated from a patient with congenital nephrogenic diabetes insipidus, Ref. 54) was also capable of forming dimers when expressed in COS-7 cells (Fig. 3, *lane 2*). Indeed, the O-11 V2 receptor was detected as ~55–58-kDa and ~89–100-kDa species, consistent with the idea that the higher molecular weight form represents a homodimer. These results confirm by a different approach that G protein-coupled receptors can form SDS-resistant dimers when expressed in mammalian cells.

Modulation of β_2AR Dimerization by TM VI Peptide—A number of chemical treatments failed to convert the dimeric species of the β_2AR to a monomeric form. These included reducing SDS-PAGE sample buffer with β-mercaptoethanol and dithiothreitol and the denaturants urea or guanidinium hydrochloride (data not shown). Other examples of SDS-resistant oligomers of membrane proteins have been noted in the literature. These include glycophorin A (GpA, Ref. 55), human erythrocyte band 3 (56), the tailspike protein from phage P22 (57), staphylococcal α-toxin (58), complement membrane attack complex (59–61), and a number of porins (62, 63).

In an elegant series of experiments it was demonstrated that residues located in the transmembrane domain of GpA are essential for the formation of dimers (64–67). The transmembrane regions are believed to form a right-handed coiled coil where noncovalent helix packing (hydrophobic) interactions dominate. Based on the relative importance of specific transmembrane residues, the existence of a dimerization motif (^{75}**L**I*XX***G**V*XX***G**^{83}V*XX*T) was proposed for GpA. In particular, Gly^{83} was found to be essential for dimerization as substitution with either hydrophobic or larger polar residues prevented dimer formation (67). Additional glycine and leucine residues

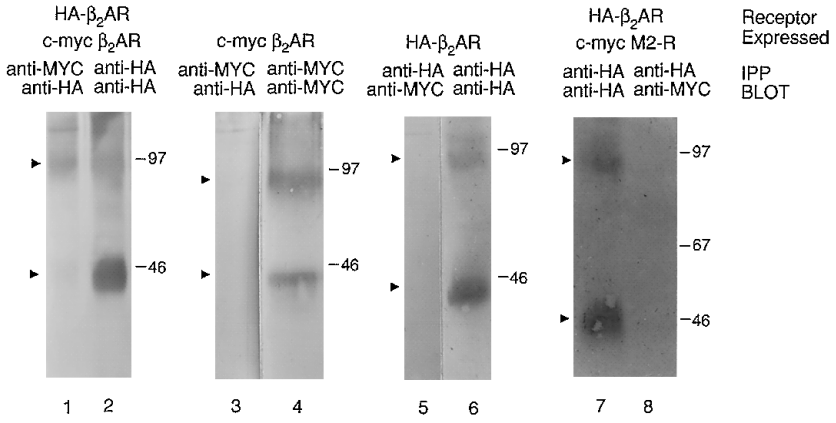

FIG. 2. **Co-immunoprecipitation of β_2ARs bearing two different immunological tags.** *Lanes 1* and *2*, c-*myc* β_2AR and HA-β_2AR were co-expressed in Sf9 cells and immunoprecipitated with either the anti-c-*myc* (*lane 1*) or anti-HA (*lane 2*) mAbs. The two immunoprecipitates were then immunoblotted with the anti-HA mAb. The occurrence of dimerization between the HA- and c-*myc*-tagged receptors is revealed by the fact that the HA-tagged β_2AR is co-immunoprecipitated with the c-*myc*-tagged receptor by the anti-c-*myc* mAb (*lane 1*). *Lanes 3* and *4*, c-*myc*-tagged β_2AR was expressed in Sf9 cells and immunoprecipitated with anti-c-*myc* mAb. The immunoprecipitates were then immunoblotted with either anti-HA (*lane 3*) or anti-c-*myc* (*lane 4*) mAbs. *Lanes 5* and *6*, HA-tagged β_2AR was expressed in Sf9 cells, immunoprecipitated with anti-HA mAb, and then immunoblotted with either anti-c-*myc* (*lane 5*) or anti-HA (*lane 6*) mAbs. These controls demonstrate the specificity of each antibody toward their respective targets. *Lanes 7* and *8*, HA-tagged β_2AR and c-*myc*-tagged M2 muscarinic receptors were co-expressed in Sf9 cells, immunoprecipitated with either anti-HA (*lane 7*) or anti-c-*myc* (*lane 8*) mAbs. Immunoblotting with the anti-c-*myc* mAb did not reveal the presence of a β_2AR/M2 muscarinic receptor heterodimer (*lane 8*). Results shown are representative of three separate experiments.

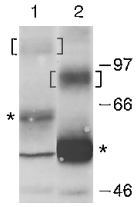

FIG. 3. **Immunoblotting of V2 vasopressin receptors (V2-R) expressed in COS-7 cells.** Crude membrane preparations from COS-7 cells transiently transfected with c-*myc*-tagged V2-R (*lane 1*) or c-*myc*-tagged V2-R truncation mutant O-11 (*lane 2*) were immunoblotted with the anti-c-*myc* mAb. The molecular weight markers are as shown. *Square brackets* highlight the dimeric species of both wild-type and O-11 V2 vasopressin receptors, while *asterisks* denote the monomeric species. Data are representative of three independent experiments.

(shown in bold) were also found to be important determinants of GpA dimerization. Analysis of β_2AR transmembrane sequences revealed that leucine and glycine residues positioned with a similar spacing exist in the cytoplasmic end of the sixth transmembrane domain (TM VI): ^{272}**L**KTL**G**IIM**G**TFT**L**. Interestingly, the placement of leucines and glycines is preserved in either direction. These studies suggested to us that perhaps this region of the β_2AR may be involved in receptor-receptor interactions. Consistent with this hypothesis, molecular modeling has suggested that TM VI is one of the most membrane exposed of all the transmembrane segments (68). Also, the leucine and glycine residues discussed above are predicted to be on the external face of the helical segment (68) where they could be available for intermolecular interactions. To specifically test the idea that these residues were important for β_2AR dimerization, we synthesized a peptide corresponding to most of TM VI (residues 276–296) and assessed its ability to interfere with β_2AR dimer formation and to affect receptor-stimulated adenylyl cyclase activity.

As shown in Fig. 4*A*, the addition of the TM VI peptide substantially reduced the amount of β_2AR dimer detected in Sf9 membranes in a time-dependent fashion (Fig. 4*A*, *lanes 1–4*). In this experiment the relative amount of receptor dimer was gradually reduced from 54% at time 0 to 17% after 30 min of treatment with TM VI peptide. When results of three such experiments were averaged, the TM VI peptide was found to reduce the relative amount of dimer by 69% after 30 min (Fig. 4*B*). A control hydrophobic peptide (from transmembrane domain VII from the D2 dopamine receptor) at maximal concentration had no effect on the relative amount of dimer detected. (Fig. 4*B*). This does not appear to result from a nonspecific hydrophobic interaction, since the unrelated dopamine receptor TM VII peptide was without effect. To address the importance of the glycine and leucine residues identified above, a second control peptide corresponding to TM VI of the β_2AR with Gly-276, Gly-280, and Leu-284 replaced by alanine residues (TM VI Ala) was synthesized. Although this peptide slightly decreased the amount of dimer, its effect was very modest compared with that of the TM VI peptide (Fig. 4*B*), thus suggesting that these three residues may be a part of the interface between two receptor monomers. One mechanism that could explain the effect of the TM VI peptide is that it may interact with monomeric β_2AR, thus preventing it from interacting with a second receptor monomer.

The effect of the TM VI peptide on dimer formation was also detected using purified β_2AR preparations and was shown to be dose-dependent. As seen in Fig. 5*A*, increasing concentrations of the TM VI peptide led to a gradual reduction in the amount of dimer. This was accompanied by a concomitant increase in the level of the monomer such that the proportion of the dimer decreased from a control level of 43.1 ± 4.3% to a final level of 12.6 ± 3.2% (Fig. 5*A*, *lanes 1–8*, Fig. 5*B*). The D2 receptor TM VII control peptide had no effect on receptor dimerization (Fig. 5*A*, compare *lanes 9* and *10*) similar to the results shown using membrane preparations (Fig. 4*B*). We also noted a modest but reproducible upward shift in the apparent molecular weight of the monomer resulting in a widening of the band as the concentration of peptide was increased (Fig. 5*B*, *inset*). This suggests that as proposed above the peptide forms a stable complex with the receptor monomer thus mimicking receptor-receptor interactions.

Functional Consequences of Receptor Dimerization—The functional significance for receptor dimerization is suggested by the inhibitory action of the TM VI peptide on receptor-stimulated adenylyl cyclase activity. As shown in Fig. 6a, the addition of TM VI peptide to membrane preparations at a concentration of 0.15 μg/μl significantly reduced isoproterenol-stimulated adenylyl cyclase activity ($p < 0.05$). In contrast, neither the peptide solubilization buffer (data not shown) nor

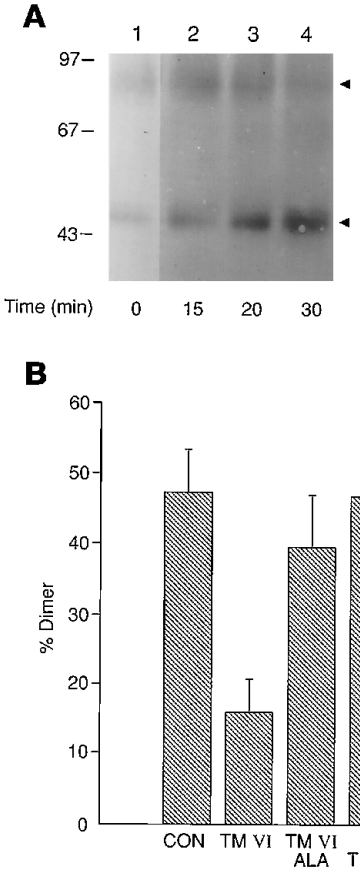

FIG. 4. **Effects of various peptides on receptor dimerization.** *A*, time course of the effect of the TM VI peptide on β_2AR dimerization. Membranes derived from Sf9 cells expressing the β_2AR were treated at room temperature with TM VI peptide (residues 276–296: NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH] at a concentration of 0.15 μg/μl for 0 (*lane 1*), 15 (*lane 2*), 20 (*lane 3*), or 30 min (*lane 4*). Membranes were then subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the anti-c-*myc* antibody. A representative immunoblot is shown. *B*, densitometric analyses of three experiments similar to that shown in the Fig. 4*A*, demonstrating the effects of treatment for 30 min with either vehicle (*CON, lane 1*), TM VI peptide (*TM VI, lane 2*), TM VI-Ala (NH_2-AIIMATFTACWLPFFIVNIVH-COOH) (*TM VI Ala, lane 3*), or D2 dopamine receptor TM VII peptide (residues 407–426: NH_2-YIIPNVASNVYGLWTFASYL-COOH) (*D2 TM VII, lane 4*). All peptides were used a concentration of 0.15 μg/μl. The relative intensity of the dimer is expressed as percent of total receptor (monomer + dimer) immunoreactivity. Data shown are mean ± S.E. (n = 3).

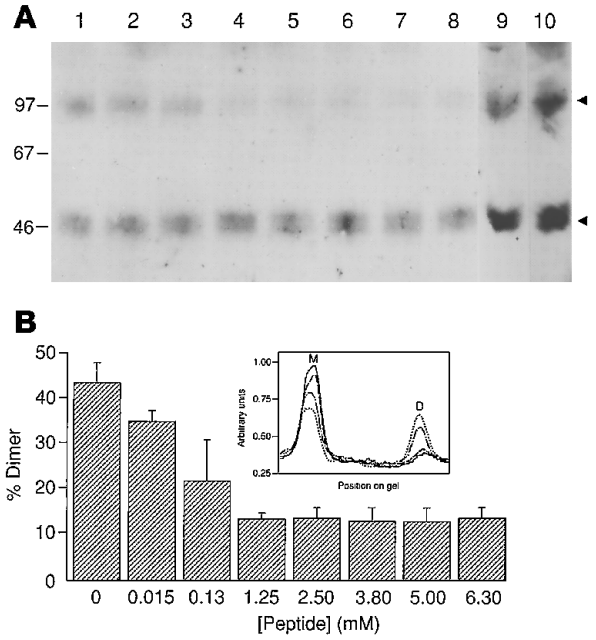

FIG. 5. *A*, effect of increasing concentrations of TM VI peptide on the amount of β_2AR dimer. Increasing concentrations (0–6.3 mM) of the peptide were added to purified c-*myc*-tagged β_2AR and the amount of dimer assessed by immunoblotting using the anti c-*myc* mAb (*lanes 1–8*). In *lanes 9* and *10* purified β_2AR was treated (*lane 10*) or not (*lane 9*) with the D2 TM VII peptide. The data shown are representative of three distinct experiments. Other control peptides used to determine the selectivity of the effect observed with the TM VI peptide included one derived the COOH-terminal tail of the β_2AR (residues 347–358: NH_2-LKAYGNGYSSNG-COOH) or an additional control peptide unrelated to the β_2AR, but of similar size as the TM VI peptide (NH_2-SIQHLSTGHDHDDVDVGEQQ-COOH) were also found to be without effect on the amount of dimer (data not shown). *B*, densitometric analyses of three experiments similar to that shown in *A*. The relative intensity of the dimer is expressed as percent of total receptor (monomer + dimer) immunoreactivity. *Inset* shows superimposed densitometric scans of immunoblotted receptors that were previously treated with increasing concentrations of the TM VI peptide. The monomer is denoted by *M*, while the dimeric species is marked by *D*. The concentration of peptide added for the curves shown was: none (····), 0.07 mM (—·—), 0.5 mM (– – –), and 1.25 mM (——).

control peptides (TM VI-Ala or TM VII of the D2 dopamine receptor) had significant effects on isoproterenol-stimulated adenylyl cyclase activity.

The effect of the peptide was receptor-specific, as it had no effect on either NaF-mediated or forskolin-mediated adenylyl cyclase stimulation (Fig. 6*B*). Notably, the ligand-independent basal adenylyl cyclase activity was slightly inhibited by the TM VI peptide, suggesting that it may effect the spontaneous activity of the receptor as well. Indeed, spontaneous receptor activity is in large part responsible for the ligand-independent adenylyl cyclase activity observed in both Sf9 and mammalian cells expressing the β_2AR (71). A receptor-dependent effect is also supported by the fact that the TM VI peptide was without effect on basal cyclase activity in Sf9 cells that were infected with the wild-type baculovirus (data not shown). Also consistent with a receptor-specific action of the peptide is the observation that D1 dopamine receptor-stimulated adenylyl cyclase activity was not significantly affected by the TM VI peptide (Fig. 6*C*). As was the case for the inhibition of dimerization, the inhibitory action of the TM VI peptide on receptor-mediated adenylyl cyclase activity was dose-dependent (Fig. 6*C*). It should be noted that the peptide IC_{50} values for the inhibition of agonist-promoted adenylyl cyclase activity and for the inhibition of dimer formation are very similar (2.14 ± 0.05 and 3.2 ± 0.04 μM, respectively), thus suggesting that receptor dimerization may be an important step in β_2AR-mediated signaling. Although our data suggest a role for dimerization in receptor activity, one cannot exclude the possibility that the effect of the TM VI peptide is not directly due to an effect on the monomer: dimer equilibrium. Still, we clearly show that this domain of the receptor is important in modulating β_2AR signal transduction. Furthermore, the peptide represents a novel pharmacological tool for the study of receptor activity.

The effect of TM VI peptide on adenylyl cyclase stimulation does not result from a loss of receptor sites as neither the affinity or the maximum number of binding sites for ^{125}I-CYP were affected (K_D = 1.8 ± 0.5 × 10^{-10} M and B_{max} = 16.5 ± 2 pmol/mg of protein for untreated membranes compared with K_D = 4.2 ± 1.5 × 10^{-10} M and B_{max} = 21.3 ± 4.5 pmol/mg of protein for TM VI peptide-treated membranes, n = 3 for both determinations).

Effects of β_2AR Ligands on Receptor Dimerization—If there is a role for the dimeric species of the β_2AR in signaling, then it follows that receptor ligands should have effects on the monomer:dimer equilibrium. Fig. 7a shows the effect of the

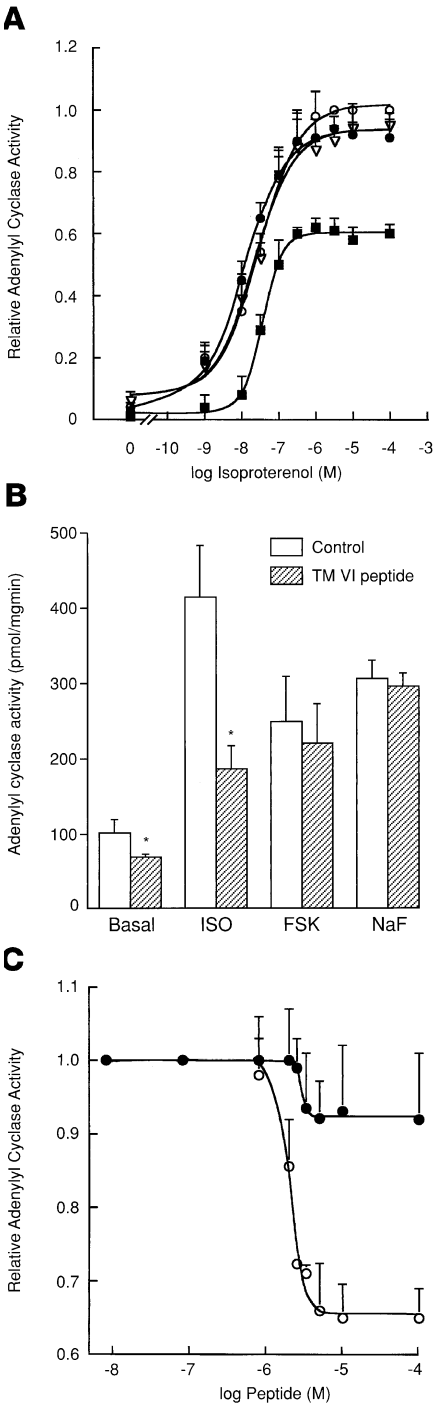

FIG. 6. **Effects of TM VI peptide on β_2AR-stimulated adenylyl cyclase activity in Sf9 cells.** *A*, membrane preparations derived from β_2AR expressing Sf9 cells were treated with vehicle (*open circles*), TM VI peptide (*closed squares*), control peptide TM VI Ala (*closed circles*), or a second control peptide from TM VII of the D2 dopamine receptor (*open triangles*). Isoproterenol-stimulated adenylyl cyclase activity was then assessed for these membranes. Data are expressed relative to the maximal stimulation obtained with the untreated membranes and represent mean ± S.E. of eight independent experiments. Peptides were used at a concentration of 0.15 μg/μl. *B*, effect of TM VI peptide (*hatched bars*) or vehicle alone (*open bars*) on basal (n = 13), maximal isoproterenol-stimulated (*ISO*, n = 13), forskolin-mediated (*FSK*, n = 13), and NaF-stimulated (NaF, n = 6) adenylyl cyclase activity. Data are expressed as picomoles of cAMP produced per mg of membrane protein per min ± S.E. Statistical significance of the difference are indicated by an *asterisk* and represent a $p < 0.05$ as assessed by a nonpaired Student's t test. None of the control peptides discussed in the legend to Fig. 4 had effects on adenylyl cyclase stimulation in β_2AR-expressing cells, nor did any of the peptides have effects on adenylyl cyclase stimulation in Sf9 cells that were infected with the wild-type baculovirus (data not shown). *C*, effects of increasing concentrations of peptide on isoproterenol and dopamine stimulated adenylyl cyclase activity. Membranes were prepared from Sf9 cells expressing either the human β_2AR (*open circles*) or the human D1 dopamine receptor (*closed circles*). Adenylyl cyclase activity was measured using maximally stimulating concentrations of either isoproterenol (10^{-4} M) or dopamine (10^{-4} M) in the presence of peptide concentrations ranging from 10^{-8} to 10^{-4} M. The inhibitory effects of the peptide was calculated relative to the maximal stimulation obtained for each receptor in the absence of peptide. The data are expressed as the mean ± S.E. (n = 3).

β-adrenergic agonist isoproterenol on the monomer:dimer ratio. Incubation of membrane preparations with 1 μM isoproterenol induced a modest but reproducible increase in the amount of dimer in a time-dependent manner. In the experiment shown the relative amount of receptor dimer increased from 50% at time 0 to 66% after 30 min of treatment with 1 μM isoproterenol. When results from three similar experiments were averaged, isoproterenol increased the relative amount of dimer by 45% (Fig. 7*B*). Isoproterenol treatment also protected the dimer from the disruptive effect of the TM VI peptide (Fig. 7*B*). Indeed, the peptide reduced the dimer:monomer ratio by 65 ± 7%, while pretreatment of the membranes with isoproterenol limited the effect of the peptide to 15 ± 8%. These results then suggest that isoproterenol stabilizes the β_2AR dimer. We then addressed the possibility that β_2AR inverse agonists might favor the monomeric form of the receptor. The classical definition of an antagonist has changed in recent years as many so-called competitive antagonists interact with their cognate receptors by reducing spontaneous activity rather than by simply preventing the binding of agonists, a property known as inverse agonism (69, 70). A number of β_2AR antagonists have been shown previously to behave as inverse agonists (71, 72). Consistent with the idea that inverse agonists may stabilize the monomeric form of the receptor is the observation that incubation of membranes with 10 μM timolol (one of the most efficacious β_2AR inverse agonists, see Ref. 71) decreased the proportion of dimer by 23 ± 5% (Fig. 7*B*). Taken together, these results suggest that agonist stabilizes the dimeric form of the receptor, while inverse agonists favor the monomer. Similar effects of ligands on monomer/dimer equilibrium have been postulated for D2 and D4 dopamine receptors (73, 74). These effects of ligands on the relative amount of receptor dimer may be taken as an indication that agonist-induced dimerization may be the mechanism by which the receptor activates Gs. Alternatively, dimerization may be the consequence of the formation of the agonist-receptor-Gs complex or of the interaction with other accessory membrane-associated proteins. However, this second hypothesis appears less likely, since we have also observed an agonist-induced increase in the amount of receptor dimer using purified β_2AR (data not shown). Wregget and Wells (12) showed in their study that M2 muscarinic receptor oligomerization could be observed even in the absence of G proteins. G protein-independent effects of ligands on GPCR conformation was also demonstrated in an elegant study by Gether *et al.* (75) which showed, using purified fluorescently labeled β_2AR, that agonists and inverse agonists have opposite effects on the fluorescent spectra of the receptor.

β_2AR Dimerization Is Detectable in Mammalian Cells—Although receptor dimerization may be more easily detectable in high-level expression systems such as the baculovirus/Sf9 cell system, the occurrence of higher molecular weight species has also been reported for G protein-coupled receptors expressed in mammalian systems (14, 46–49). In the present study β_2AR dimers were observed in CHW cells stably transfected with the receptor (Fig. 8*A*, *inset*) by immunoblotting with a polyclonal anti-β_2AR antisera. Similar to our observations in Sf9 cells, the TM VI peptide also reduced the amount of β_2AR dimer detected

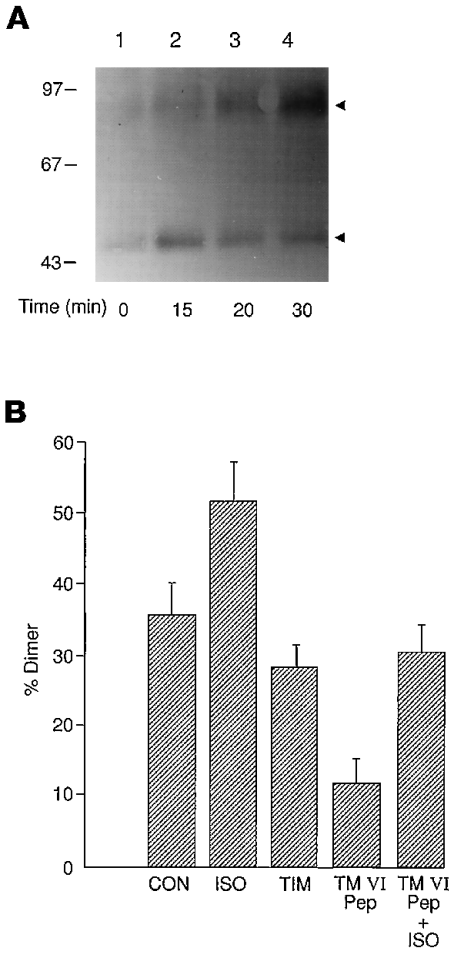

FIG. 7. **Effects of β_2AR ligands on receptor dimerization.** *A*, time course of the effect of 1 μM isoproterenol on β_2AR dimerization. Membranes derived from Sf9 cells expressing the c-*myc* β_2AR were treated at room temperature with 1 μM isoproterenol for 0 (*lane 1*), 15 (*lane 2*), 20 (*lane 3*), or 30 min (*lane 4*). Membranes were then subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the anti-c-*myc* antibody. A representative immunoblot is shown. *B*, densitometric analyses of three experiments where membranes from Sf9 cells expressing the β_2AR were treated for 30 min at room temperature with either vehicle (*CON*), 1 μM isoproterenol (*ISO*), 10 μM timolol (*TIM*), TM VI peptide at a concentration of 0.15 μg/μl (*TM VI*), or isoproterenol followed by 30 min with TM VI peptide (*ISO/PEP*). The TM VI data (*lane 4*) is reproduced from Fig. 4*B* for comparison. The relative intensity of the dimer is expressed as percent of total receptor (monomer + dimer) immunoreactivity. Data shown are mean ± S.E. (n = 3).

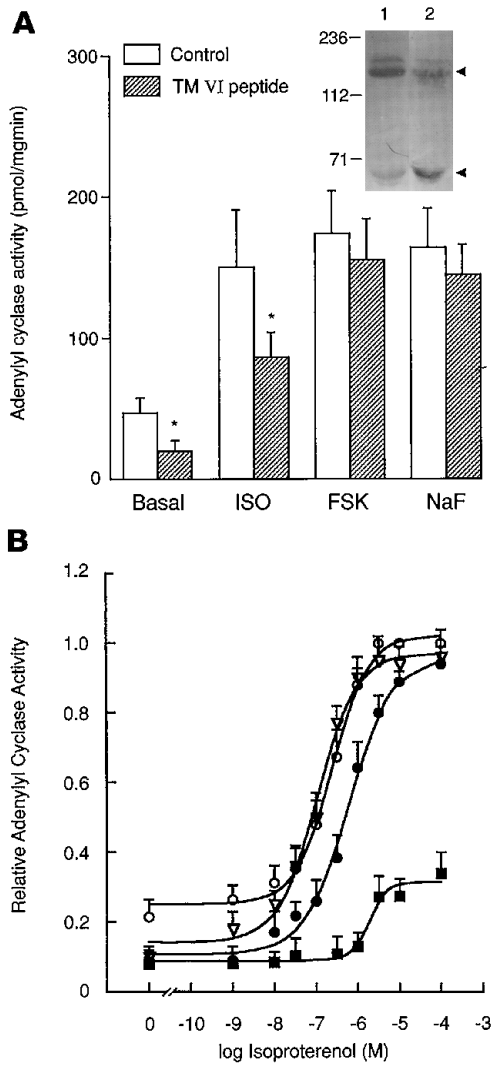

FIG. 8. **Effects of TM VI peptide on β_2AR expressed in mammalian cells.** *A*, effect of 0.15 μg/μl TM VI peptide (*hatched bars*) or vehicle (*open bars*) on basal (n = 2), maximal isoproterenol-stimulated (*ISO*, n = 2), forskolin-mediated (*FSK*, n = 2), and NaF-stimulated (*NaF*, n = 2) adenylyl cyclase activity in CHW cells expressing 5 pmol of β_2AR/mg of protein. Data are expressed as picomoles of cAMP produced per mg membrane protein per min ± S.E. Statistical significance of the difference are indicated by an *asterisk* and represent a $p < 0.05$ as assessed by a nonpaired Student's t test. *Inset*, immunoblotting of human β_2AR expressed in CHW cells. Membranes from CHW cells expressing the β_2AR were prepared and immunoblotted with an anti-β_2AR polyclonal antiserum. Membranes were treated with either vehicle (*lane 1*) or the TM VI peptide at a concentration of 0.15 μg/μl (*lane 2*) for 30 min at room temperature. Membranes from untransfected CHW cells had no detectable receptors (data not shown). *B*, effects of TM VI peptide on β_2AR-stimulated adenylyl cyclase activity in mouse Ltk$^-$ cells. Membranes were prepared from Ltk$^-$ cells stably expressing 200 fmol of human β_2AR/mg of membrane protein. Isoproterenol-stimulated adenylyl cyclase activity was then assessed in membranes treated with vehicle (*open circles*), TM VI peptide (*closed squares*), control peptide TM VI Ala (*closed circles*), or the D2 TM VII control peptide (*open triangles*). Data are expressed relative to the maximal stimulation obtained with vehicle treated membranes and represent mean ± S.E. for three independent experiments. Peptides were used at a concentration of 0.15 μg/μl.

in membranes derived from CHW cells (Fig. 8*A*, *inset: lane 2*). This peptide also reduced basal and isoproterenol-stimulated adenylyl cyclase activity in these cells while leaving forskolin- and NaF-mediated stimulation unaffected (Fig. 8*A*). Similar findings were also obtained with LTK$^-$ cells expressing as little as 200 fmol of β_2AR/mg of protein (Fig. 8*B*). Dimers could also be observed in these cells (data not shown), suggesting a similar functional significance for β_2AR dimerization in mammalian cells as in Sf9 cells.

The results presented here demonstrate that both human β_2AR and V2 vasopressin receptors can form SDS-resistant homodimers. For the β_2AR, the relative amount of dimer can be altered by a peptide derived from TM VI and by receptor ligands, suggesting that under basal conditions there appears to be a dynamic equilibrium between monomeric and dimeric species of receptors. The data also suggest that shifting the equilibrium away from the dimeric form of the receptor interferes with the ability of the β_2AR to productively interact with its signaling pathway. In a recent study functionally important multimeric species (including species larger than a dimer that were suggested to represent trimers and tetramers) of the M2 muscarinic receptor were seen in purified preparations (12). Whether the largest species of the β_2AR (*i.e.* greater than 85–90 kDa), which we occasionally see in membrane preparations (see Fig. 1), are analogous to the higher order structural

forms of the M2 receptor is not clear. However, this raises the possibility that oligomers larger than dimeric species might indeed represent active forms that may be inherently less stable in SDS than the dimers.

Our results also suggest that key residues located near the cytoplasmic side of TM VI appear to play an important role in the dimerization of β_2AR. Interestingly, higher molecular weight species have been detected in both mammalian and Sf9 expression systems for many GPCRs. These include the V2 vasopressin receptor (this study, Fig. 3), platelet-activating factor receptor (46), metabotropic glutamate receptor (47), substance P receptor (48), neurokinin-2 receptor (49), the C5a anaphylaxotoxin receptor (50), glucagon receptor (14), the dopamine D1 receptor (43), D2 receptor (44), the $5HT_{1B}$ receptor (45), the M2 muscarinic receptor (12), and the M3 muscarinic receptor (52). Therefore, it is clear that receptor-specific determinants for dimerization must exist. This receptor specificity is illustrated in the present study by the observation that the M2 muscarinic receptor forms homodimers (data not shown) yet does not form heterodimers with the β_2AR (Fig. 2). Similarly, the β_2AR TM VI peptide had little effect on D1 dopamine receptor-stimulated adenylyl cyclase activity (Fig. 6*C*) or on D2 dopamine receptor dimer formation.[2] It should also be noted that the GpA sequence is not strictly conserved in other proteins that form SDS-resistant oligomers (64). The precise sequence requirements for individual receptors remain to be determined and may confer the required selectivity for proper homodimerization.

The recognition that the dimerization of GPCRs may have functional significance may aid to reconcile several observations that are difficult to explain in terms of the mobile receptor hypothesis. These include the isolation of oligomeric G proteins (76, 77) and GPCR complexes (12), the recent crystallographic evidence that Giα1 is an oligomer (78) and the involvement of the cytoskeleton in organizing G protein-coupled signaling complexes (77, 79). Also, receptor dimerization can explain the functional complementation recently observed in mutant angiotensin II (A II) receptors (17). The co-expression of two binding-defective point mutants of the A II receptors restored binding activity. Indeed, the simplest explanation for these results is that intermolecular interactions between the two defective receptors restores a functional binding site.

In addition to providing insights into the mechanisms underlying receptor activation, the observation that a peptide derived from the TM VI domain of the β_2AR inhibits receptor-stimulated adenylyl cyclase activity offers a new pharmacological approach to the study of receptor function.

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