Synthetic Peptides as Probes for G Protein Function

CARBOXYL-TERMINAL Go Peptides Mimic G, and Evoke High Affinity Agonist Binding to β-Adrenergic Receptors*

Mark M. Rasenick†, Masayuki Watanabe§, Milenko B. Lazarevic, Shinichi Hattaš, and Heidi E. Hamm††

From the Department of Physiology and Biophysics and the Committee on Neuroscience, University of Illinois College of Medicine at Chicago, Chicago, Illinois 60612-7342

The molecular interfaces between G, and the β-adrenergic receptor were investigated using synthetic peptides corresponding to various regions of its α subunit, αP. These experiments were carried out on saponin-permeable C6 glioma cells in which the β-adrenergic receptor appears tightly coupled to G, (Conklin and Hamm, 1993). Artificial site-specific peptidomimetics from αP (corresponding to amino acids 15-28, 354-372, and 284-294) and α (8-22, 315-324, and 345-355) were tested for their ability to interfere with coupling between the β-adrenergic receptor and G, (Conklin et al., 1992). The two carboxyl-terminal peptides from αP blocked β-adrenergic stimulation of adenylyl cyclase in permeable cells. However, only αP-354-372 had this effect in C6 membranes. It is suggested that the partial uncoupling of G, which occurs subsequent to cell disruption, may be related to a change in the interaction of the αP carboxyl terminus with the β-adrenergic receptor. Two carboxyl-terminal peptides, 354-372 and 364-394, could also mimic the effect of G, to increase agonist affinity for the β-adrenergic receptor. In combination, αP-345-372 and αP-384-394 increased the ability of isoproterenol to compete with [3H]pindolol binding in a partially additive manner. Synthetic peptides from αP and amino-terminal peptides from αP had no effect on β-agonist binding, suggesting a high specificity of peptide effects. Two findings suggest that these peptides bind directly to the β-adrenergic receptor and stabilize its high agonist affinity conformation. First, GTP and hydrolysis-resistant GTP analogs did not alter the high affinity binding in the presence of high concentrations of the peptides. Second, in S49 lymphoma cells, which lack G, these peptides evoked the high affinity agonist binding state of the β-receptor. Neither peptide had an effect on antagonist binding affinity, as measured by propranolol displacement of [3H]pindolol. These data suggest that at least two regions on the α subunit of G, participate in high affinity G, binding to the β-adrenergic receptor. The fact that these small peptides could mimic the holo-G, effect on the receptor is rather surprising, and the specificity of the effect suggests that the primary and secondary structure of small regions of αP contain much of the information for specific interaction with β-adrenergic receptors.

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† † Recipient of a Research Scientist Development Award from NIMH, National Institutes of Health (MH 00699).
§ Current address: Depts. of Neuropsychiatry and Pharmacology, Sapporo Medical College, Sapporo, 060 Japan.
‡ ‡ Recipient of a Gisao Cardiovascular Discovery Award.

The adenylyl cyclase system is a membrane-associated complex of receptors, G proteins, and enzymes that is activated or inhibited in response to hormones and neurotransmitters (Dohlman et al., 1961). The G proteins G, and G, are able, respectively, to stimulate or inhibit adenylyl cyclase. These signal-transducing G proteins are heterotrimeric in structure, consisting of α, β, and γ subunits. The activation of G proteins by receptors requires the presence of both α and βγ subunits and involves a tight interaction of the heterotrimer with an agonist-activated receptor followed by receptor-catalyzed GDP dissociation, GTP binding, and altered interaction between α and βγ subunits (Gilman, 1987). It is clear that the α subunit plays a major role in both receptor and effector interaction and also contains GTP binding and GTPase activities (Conklin and Bourne, 1993; Noel et al., 1993).

The functional domains on the α subunit involved in receptor interaction are a subject of current interest. Evidence from a variety of studies implicates the carboxyl terminus in receptor interaction (West et al., 1985; Hamm et al., 1988; Simonds et al., 1989; Conklin et al., 1993). It is probable, however, that multiple interaction sites exist on G protein α subunits for receptors, since synthetic peptides from the amino terminus of the α subunit of transducin block its interaction with rhodopsin (Hamm et al., 1988). Furthermore, an antibody that binds to the amino and carboxyl termini of α is capable of interfering with rhodopsin-transducin interaction (Hamm et al., 1987; Hamm et al., 1988; Navon and Fung, 1988; Hingerani and Ho, 1990; Mazzoni et al., 1981) and β-adrenergic receptor activation of adenylyl cyclase (Hamm et al., 1988).

The natural milieu of the adenylyl cyclase system is the plasma membrane, and it is possible that components that are present in intact cells, but lost following cell disruption, may be important for the normal regulation of the adenylyl cyclase system (Rasenick et al., 1981; Denker and Neer, 1991). Elements of the cytoskeleton are also likely to contribute to an ordered association among components of the adenylyl cyclase cascade (Rasenick and Wang, 1988; Wang et al., 1990; Roychowdhury et al.). A saponin-permeable C6 cell model has been developed for the study of receptor-effector coupling in a more native environment (Rasenick and Kaplan, 1986; Rasenick et al., 1993).

In this study, we have compared the ability of synthetic peptides corresponding to various regions of G, to mimic G, effects on agonist affinity of the β-adrenergic receptor and to disrupt the β-adrenergic activation of G, (Rasenick et al., 1993). It was observed that a peptide corresponding to the carboxyl terminus of αP blocked β-adrenergic activation of adenylyl cyclase only in permeable cells, while another peptide corresponding to an internal site blocked this activation in both permeable cells and cell membranes. Thus, it appears that within an intact membrane/cytoskeletal system, the molecular interfaces between the β-adrenergic receptor and G, are more elaborate than those seen following membrane isolation.
Radioligand binding studies on many receptors have shown multiple affinity states for the binding of agonist, and these are regulated by guanine nucleotides (Dohlman et al., 1991). For β-adrenergic receptors, antagonists display a single binding affinity. Several studies have suggested that when a β-receptor is coupled to Gs, the high affinity state for agonist binding results. The agonist-induced binding of GTP to the G protein causes uncoupling of that G protein from the receptor and a subsequent shift back to a lower affinity state for agonist binding. A functionally analogous stabilization of the active receptor conformation by G protein exists in the light receptor rhodopsin, in which the photoreceptor G protein transducin stabilizes metarhodopsin II (Hargrave et al., 1993). It has been observed that synthetic peptides corresponding to carboxyl-terminal regions of the α subunit of transducin can mimic holo-Gs and stabilize active metarhodopsin II (Hamm et al., 1988). This suggests that similar regions from other G proteins might mimic or antagonize the interaction of a hormone receptor with its cognate G protein. In this study, peptides from the carboxyl-terminal regions of the α subunit of Gs have been shown to increase the agonist binding affinity of the β-adrenergic receptor. Thus, occupation of a single site on the receptor by a G protein peptide is sufficient to mimic the ability of heterotrimeric Gs to stabilize the receptor in its active form.

**EXPERIMENTAL PROCEDURES**

**Preparation of Permeable Cells**—Saponin treatment of C6 glioma cells allows access for peptides or guanine nucleotides while preserving integrity of the cell. C6 glioma cells (passages 15-26) were grown to near confluency in Nuclon 175-cm² flasks in Dulbecco’s Eagle’s medium and gentle agitation. Cells were centrifuged at 500 g for 5 min, resuspended, and washed two times with Locke’s solution (154 mM NaCl, 2.6 mM KCl, 2.15 mM KH₂PO₄, 0.85 KH₂PO₄, 10 mM glucose, 2 mM CaCl₂, and 5 mM MgCl₂, pH 7.4) at 500 x g for 5 min. Resuspended and washed twice with potassium glutamate buffer. Permeable C6 cells were resuspended with Lockes’ solution (154 mM NaCl, 2.6 mM KCl, 2.15 mM KH₂PO₄, 0.85 KH₂PO₄, 10 mM glucose, 2 mM CaCl₂, and 5 mM MgCl₂, pH 7.4) at 500 x g for 5 min. Resuspended and washed twice with potassium glutamate buffer. Permeable C6 cells were resuspended with Lockes’ solution and assayed for receptor binding. All centrifugations were done at room temperature. Preparation of permeable S49 cyc- cells was similar to C6 cells except that horse serum was used instead of fetal bovine serum, and saponin exposure time was 20 s.

**Assay of Adenylyl Cyclase in Permeable C6 Cells**—150 μl of Locke’s solution containing [α-32P]ATP (2 x 10⁶ cpm), 0.5 mM ATP, 1 mM MgCl₂, and 0.5 mM isotobylymethylyxanthine were added to each well and incubated for 15 min at 27 °C. Reactions were stopped by adding 300 μl of ice-cold 15 mM Hepes buffer pH 7.4 and placing the plate on ice for 5 min. Plates were removed from ice and allowed to thaw, and cells were scraped into tubes. Tubes were boiled for 5 min in a heating block and centrifuged at 15,000 x g for 5 min at 4 °C. The supernatant was tested for cAMP content as described (Hatta et al., 1986). Protein content was determined by the Coomassie blue assay. C6 glioma membranes were prepared as described in Rasenick and Kaplan (1986) and stored under liquid N₂ until use. Membrane suspensions were assayed for adenylyl cyclase as described (Rasenick et al., 1984).

**Binding Assay for Permeable Cells**—[3H]-pindolol (IPIN) was used to assay antagonist binding to permeable cells. IPIN at concentrations of 10⁻⁶ M were used to determine the dissociation constant (Kd) and the density of β-adrenergic receptors (Bmax) in saturation experiments. Agonist binding to β-adrenergic receptors was assayed by measurement of competition of isoproterenol for IPIN binding sites. The concentration of IPIN used (150 pm for C6 cells and 200 pm for S49 cyc- cells) was close to the Kd value for the agonist. Seven concentrations of isoproterenol were used ranging from 1 nM to 1 mM. To investigate the dose dependence of peptide effects on agonist competition of IPIN binding, peptide concentrations of 1 μM to 1 mM were added in the presence of 100 nM isoproterenol. The reaction mixture consisted of a final volume of 250 μl containing Locke’s solution, IPIN, and appropriate agents. Assays were initiated by the addition of [3H]-isoproterenol (120-340 μg of total cellular protein) and were carried out for 50 min at 30 °C. Assays were terminated by the addition of 10 volumes of ice-cold 2 mM Hepes buffer pH 7.4, containing 5 mM MgCl₂ and 2 mM EGTA. Bound and free ligand were separated by rapid vacuum filtration (Brandel Cell Harvester model M24R) on Whatman GF/B filters. The filters were washed with 3 ml of the same buffer three times, and bound radioactivity was quantitated in a Beckman 6150 counter. Specific binding was defined as the difference between IPIN binding in the absence and presence of 10 μM 1-propranolol. Nonspecific binding was less than 20% for C6 cells and less than 15% for S49 cyc- cells in each assay. Binding parameters were analyzed to obtain the values of Cmax by using the LUNDON 1 program. Scatchard transformation of each saturation isotherm resulted in linear plots with correlation coefficients greater than 0.97 in C6 cells and 0.99 in S49 cyc- cells. IPIN binding data were best fit by a one-site model with Hill coefficients of approximately 1. The values of Cmax were 82 pm and 11.1 fmol/mg of total cellular protein in C6 cells, and 164 pm and 22.6 fmol in S49 cyc- cells. The Cmax of IPIN in C6 membranes was 145 fmol/mg of membrane protein. Since the membrane protein is likely to represent about 10% or less of the total cellular protein, the Cmax figures are actually similar. Protein content was determined by the Coomassie Brilliant Blue binding method with bovine serum albumin as the standard.

**Materials**—[3H]-pindolol (2200 Ci/mmol; 3200 KBq/ml) was purchased from DuPont NEN. GppNHz was purchased from Boehringer Mannheim, and (-)-isoproterenol was from Sigma. 1-Propranolol was a gift from Ayerst. [α-32P]ATP (800 Ci/mmol) was purchased from Dupont NEN. GTP and GppNHz were from Boehringer Mannheim. Forskolin, NaF, and isoproterenol were from Sigma. All other reagents were of analytical grade.

**RESULTS**

Synthetic peptides from various regions of the α subunits of Gs and Gβ were used as probes of contact regions between the β-adrenergic receptor and Gs, since peptides can serve as competitive inhibitors of receptor-G protein interaction (Hamm et al., 1988, 1989). Table I shows the amino acid sequences of the synthetic peptides used in this study.

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**Table I**

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<th>Amino acid sequences of synthetic peptides</th>
<th>QuMHLRLYVELL</th>
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<td>α–384–394</td>
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<td>EKDAEAKSMTD4IN</td>
</tr>
<tr>
<td>α–acetyl-354–372-amide</td>
<td>NTKRYTIEYHF</td>
<td>PFGCOKLNNKI</td>
</tr>
<tr>
<td>α–15–29</td>
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<td></td>
</tr>
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<td>α–8–22</td>
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<td>α–355–345 (nonaust)</td>
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</table>

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1 The abbreviations used are: IPIN, [3H]-pindolol; Gβ, the α subunit of G protein; GppNHz, 5'-guanylylimidodiphosphate; Gβ, stimulatory GTP-binding regulatory protein of adenylyl cyclase; Gβi, inhibitory GTP-binding regulatory protein of adenylyl cyclase; Gα, a G protein abundant in brain with unknown functions; Gβ, a G protein of rod photoreceptors; α–8–22, a synthetic peptide corresponding to those residues of αs, between 20 and 640 pm was used to determine the dissociation constant (Kd) and the density of β-adrenergic receptors (Bmax) in saturation experiments. Agonist binding to β-adrenergic receptors was assayed by measurement of competition of isoproterenol for IPIN binding sites. The concentration of IPIN used (150 pm for C6 cells and 200 pm for S49 cyc- cells) was close to the Kd value for the agonist. Seven concentrations of isoproterenol were used ranging from 1 nM to 1 mM. To investigate the dose dependence of peptide effects on agonist competition of IPIN binding, peptide concentrations of 1 μM to 1 mM were added in the presence of 100 nM isoproterenol. The reaction mixture consisted of a final volume of 250 μl containing Locke’s solution, IPIN, and appropriate agents. Assays were initiated by the addition of [3H]-isoproterenol (120–340 μg of total cellular protein) and were carried out for 50 min at 30 °C. Assays were terminated by the addition of 10 volumes of ice-cold 2 mM Hepes buffer pH 7.4, containing 5 mM MgCl₂ and 2 mM EGTA. Bound and free ligand were separated by rapid vacuum filtration (Brandel Cell Harvester model M24R) on Whatman GF/B filters. The filters were washed with 3 ml of the same buffer three times, and bound radioactivity was quantitated in a Beckman 6150 counter. Specific binding was defined as the difference between IPIN binding in the absence and presence of 10 μM 1-propranolol. Nonspecific binding was less than 20% for C6 cells and less than 15% for S49 cyc- cells in each assay. Binding parameters were analyzed to obtain the values of Cmax by using the LUNDON 1 program. Scatchard transformation of each saturation isotherm resulted in linear plots with correlation coefficients greater than 0.97 in C6 cells and 0.99 in S49 cyc- cells. IPIN binding data were best fit by a one-site model with Hill coefficients of approximately 1. The values of Cmax were 82 pm and 11.1 fmol/mg of total cellular protein in C6 cells, and 164 pm and 22.6 fmol in S49 cyc- cells. The Cmax of IPIN in C6 membranes was 145 fmol/mg of membrane protein. Since the membrane protein is likely to represent about 10% or less of the total cellular protein, the Cmax figures are actually similar. Protein content was determined by the Coomassie Brilliant Blue binding method with bovine serum albumin as the standard (Bradford, 1976).
Adenylyl Cyclase in Membranes and Permeable Cells—When blocked isoproterenol-activated adenylyl cyclase by 50% (IC₅₀) renergic receptor binding was studied using a permeable cell membranes. Site-specific synthetic peptides in C6 glioma cells and membranes were as described in the text. Values are mean ± S.E. of triplicates from a representative experiment that was repeated three times.

Effects of Synthetic Peptides on β-adrenergic Stimulation of Adenylyl Cyclase in Membranes and Permeable Cells—When permeable C6 cells were incubated with the β-adrenergic agonist isoproterenol and GTP (10 μM each), two synthetic peptides from the carboxyl-terminal region of α₂, α₂-384-394 and α₂-acetyl-354-372-amide, were able to dose-dependently decrease isoproterenol stimulation of adenylyl cyclase (Fig. 1a). Peptides α₂-384-394 and α₂-acetyl-354-372-amide decreased stimulation maximally by 71 and 50%, with IC₅₀ concentrations of 50 and 20 μM, respectively. Amino-terminal α₂ peptides, as well as α₁ peptides, had no effect on adenylyl cyclase activity under any condition tested. All synthetic peptides from α₁ or α₂ were without effect on adenylyl cyclase in the presence of 10 mM forskolin or 10 mM NaF. Basal enzyme activity was also unaltered by these peptides.

In C6 membranes, the presence of β-agonists is not required for stimulation of adenylyl cyclase by hydrolysis-resistant GTP analogs (Rasenick and Kaplan, 1986). The effect of synthetic peptides on this less tightly coupled adenylyl cyclase was determined. The synthetic peptide α₂-acetyl-354-372-amide blocked isoproterenol-activated adenylyl cyclase by 56% (IC₅₀ 6 μM), while α₂-384-394 and other peptides had no effect on C6 membranes (Fig. 1b).

Use of Permeable Cells for Receptor Binding Studies—β-Adrenergic receptor binding was studied using a permeable cell suspension method. This method allows investigation of β-adrenergic receptor behavior in nearly intact cells under conditions where this receptor appears tightly coupled to G, to effect activation of adenylyl cyclase. It is noteworthy that some difference exists in receptor binding behavior between permeable cells and membrane preparations (Rasenick et al., 1993). For example (as can be seen in Fig. 2) in permeable cells, agonist competition curves are shallow and remain so in the presence of GppNHp or peptides. The reasons for this difference are unclear.

Effect of Peptides on IPIN Binding—Inhibition of the β-adrenergic radioligand antagonist IPIN by the full agonist isoproterenol-stimulated adenylyl cyclase in C6 membranes. Assay conditions for cells and membranes were as described in the text. Values are mean ± S.E. of triplicates from a representative experiment that was repeated three times.

FIG. 1. Inhibition of isoproterenol-stimulated adenylyl cyclase by site-specific synthetic peptides in C6 glioma cells and membranes. α, permeable C6 cells. Peptides α₂-354-372 (closed squares), α₂-384-394 (closed circles), and nonsense peptide α₂-355-345 (open triangles) were incubated with permeable C6 cells at the indicated concentrations in the presence of isoproterenol and GTP (10 μM), b, C6 membranes. Effects of the same peptides on isoproterenol-stimulated adenylyl cyclase in C6 membranes. Assay conditions for cells and membranes were as described in the text. Values are mean ± S.E. of triplicates from a representative experiment that was repeated three times.

FIG. 2. Effect of site-specific synthetic peptides on agonist or antagonist competition of 150 μM IPIN binding sites to permeable C6 cells. Each assay was performed at 30 °C for 30 min as described under "Experimental Procedures." Results are expressed as specific binding of IPIN in the presence of indicated ligand concentrations. Data points are the means of triplicate determinations in one of three representative experiments. α₁ tissues were prepared and isoproterenol inhibition of IPIN binding was assayed in the absence (open circles) or presence (open squares) of 100 μM GppNHp, 150 μM α₂-315-324 (open triangles); or 150 μM α₂-384-394 (closed circles); b, isoproterenol inhibition of IPIN was assayed in the absence (open circles) or presence (closed squares) of 100 μM α₂-354-372; c, antagonist competition of IPIN was determined in the absence (open circles) or presence (closed circles) of 100 μM α₂-354-372 or 100 μM α₂-384-394 (open triangles). The IC₅₀ values of propranolol were 1.3 μM for control, 1.6 μM for α₂-315-324, and 1.7 μM for α₂-384-394, respectively. dpm, disintegrations/minute.
terenol is shown in Fig. 2a. The well established effect of GppNHp in lowering agonist affinity is observed by a shift in the agonist displacement curve to the right. Two synthetic peptides from the carboxyl-terminal region of \( \alpha_{i,384-394} \) and \( \alpha_{i,354-372} \) (Fig. 2a) and \( \alpha_{i,384-372} \) (Fig. 2b), by contrast, increased the affinity of the \( \beta \)-receptor for isoproterenol and shifted the curve to the left. A peptide corresponding to a similar sequence of \( \alpha_{i,315-324} \), had no effect on agonist affinity (Fig. 2a). To determine whether \( \alpha_{i,384-394} \) would also change the affinity of \( \beta \)-adrenergic antagonists, the ability of the antagonist propranolol to displace IPIN was tested. The dose-response curve for propranolol was not altered in the presence of either \( \alpha_{i,384-394} \), or the control peptide, \( \alpha_{i,315-324} \) (Fig. 2c).

The competition binding isotherms in Fig. 2a were analyzed by LIGAND (Table II). In the presence of GppNHp (without peptide \( \alpha_{i,384-394} \) or in the presence of peptide \( \alpha_{i,384-394} \) (without GppNHp) a single-site model was significant (\( p = 0.003 \) and 0.032, respectively). This suggests that, in the presence of 100 \( \mu \)M GppNHp (without \( \alpha_{i,384-394} \)), 100% of the receptors are in the low affinity state, while in the presence of 100 \( \mu \)M GppNHp, 100% high affinity binding is displayed. Data for control conditions and for conditions in which \( \alpha_{i,384-394} \) and GppNHp were added (both at 100 \( \mu \)M) best fit a two-site model by LIGAND analysis. In control conditions, 72.4% of the receptors display high affinity binding, while 27.6% are low affinity. For \( \alpha_{i,384-394} \) plus GppNHp, the values are 75.2 and 24.8%, respectively.

Table III shows IC\(_{50} \) values of isoproterenol inhibition of IPIN binding in the absence or presence of various peptides from \( \alpha_{i} \) or \( \alpha_{g} \) in C6 cells. Peptides from the amino-terminal region of either \( \alpha_{i} \) or \( \alpha_{g} \), as well as peptides from the carboxyl-terminal region of \( \alpha_{i} \), had no significant effect on IC\(_{50} \) values, while the IC\(_{50} \) in the presence of \( \alpha_{i,384-394} \) was 3-fold lower than control values. In the presence of \( \alpha_{i,354-372} \), the IC\(_{50} \) value was similar to that of \( \alpha_{i,384-394} \) (data not shown). Thus, either of two peptides from the carboxyl-terminal region of \( \alpha_{i} \) could mimic the holo-G protein to promote an increased agonist affinity for \( \beta \)-receptors.

The above studies were conducted in the presence of a fixed amount of each synthetic peptide (100 \( \mu \)M). To examine the peptide dose dependence of the increase in \( \beta \)-agonist affinity, one concentration of isoproterenol (100 \( \mu \)M) was chosen, which half-maximally inhibited IPIN binding under control conditions. The dose-response curve for the effect of peptide \( \alpha_{i,384-394} \) on agonist binding is shown in Fig. 3. Under these conditions, the peptide \( \alpha_{i,384-394} \) increased agonist-displaced binding from approximately 15 to 65%. Thus, the peptide increased the potency of isoproterenol to maximally displace IPIN binding from \( 10^{-4} \) to \( 10^{-7} \) M.

**Effect of GppNHp on Peptide-induced Increases in \( \beta \)-agonist Affinity**—The peptide-induced increase in agonist affinity is not expected to be altered by guanine nucleotides, since peptides would not be influenced by these compounds. Data in Fig. 4e indicate that even in the presence of 100 \( \mu \)M \( \alpha_{i,384-394} \), GppNHp can still decrease agonist affinity. This suggests that, at this peptide concentration, the \( G \) protein \( G_{i} \) and the peptide compete for binding to the \( \beta \)-receptor. This possibility was further studied as a function of peptide concentration in the presence of 100 \( \mu \)M isoproterenol (Fig. 4b). At peptide concentrations higher than 200 \( \mu \)M, there was a negligible difference in agonist-displaceable binding in the presence and absence of GppNHp. This suggests that the peptide has two effects, a direct binding to \( \beta \)-adrenergic receptors and a competition with endogenous \( G_{i} \) for binding to receptors. Thus, in the presence of peptide concentrations that uncouple all endogenous \( G_{i} \) from \( \beta \)-receptors, there is no longer any effect of GppNHp.

**Multiple Sites of Interaction with Receptor**—Previous studies with the photoreceptor system indicated that synthetic peptides corresponding to homologous regions of \( \alpha_{i} \) were synergistic in their effect (Hamm et al., 1988). At low concentrations, \( \alpha_{i,384-394} \) and \( \alpha_{i,315-324} \) have additive, but not synergistic, effects (Fig. 5). At higher concentrations, however, only partial additivity is seen.

**Peptide Effects on Agonist Affinity in S49 cyc- Cells**—S49 cyc- lymphoma cells lack \( G_{i} \) but retain \( \beta \)-adrenergic receptors. If \( G_{ir} \) peptides exert their effect by binding to \( \beta \)-adrenergic receptors rather than affecting \( G_{i} \) directly, a similar result would be predicted for C6 and cyc- cells. The potency of isoproterenol in displacing IPIN was enhanced by 100 \( \mu \)M \( \alpha_{i,384-394} \) in permeable S49 cyc- cells. Calculated IC\(_{50} \) values for isoproterenol were 546 nM for control and 123 nM in the presence of peptide 384-394.

**DISCUSSION**

This report demonstrates that site-specific synthetic peptides can disrupt the coupling between the \( \beta \)-adrenergic receptor and \( G_{i} \), and mimic \( G_{i} \) in eliciting high affinity agonist binding by the receptor. Evidence from many laboratories supports the notion that the high affinity component of agonist binding occurs within a ternary complex of agonist, receptor, and \( G_{i} \), while the low affinity component represents agonist binding to free receptor (Dohlman et al., 1991). Guanine nucleotides perturb the ternary complex and cause a decrease in agonist affinity. In this work, we have found that two small synthetic peptides from the carboxyl-terminal \( \alpha_{i} \) sequence increase agonist affinity compared with control membranes with a normal complement of \( \beta \)-receptors and \( G_{i} \).

Several lines of evidence suggest that these peptides bind directly to the \( \beta \)-adrenergic receptor and stabilize the agonist-bound conformation. First, GppNHp did decrease agonist affinity in the presence of high concentrations of peptide \( \alpha_{i,384-394} \), suggesting that the peptide competes with \( G_{i} \) for a common binding site on the receptor. Second, in S49 cyc- lymphoma cells, which lack \( G_{i} \), but retain \( \beta \)-adrenergic receptors, peptide \( \alpha_{i,384-394} \) increased the affinity for agonist. Third, there was no effect of these peptides on antagonist affinity. Thus, it appears likely that these peptides bind to sites on the \( \beta \)-receptor that normally interact with \( \alpha_{i} \). Analysis of the data shows that the effect of the peptides is to convert biphasic competition binding isotherms into high affinity monophasic isotherms (Table II). This would suggest that the peptides can mimic \( G_{i} \) effects; when receptors are not occupied by \( G_{i} \), the peptides can convert free receptors with low agonist affinity into the high affinity state.

This peptide-induced high affinity agonist binding is analogous to the ability of \( G_{i} \)-derived carboxyl-terminal peptides to stabilize the active metarhodopsin II conformation of rhodopsin (Hamm et al., 1988). This suggests an important role of the carboxyl terminus of \( \alpha \) subunits in mediating \( G \) protein-in-
TABLE III

<table>
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*Not significantly different from b, c, and d.
†Not significantly different from a, c, and d.
‡Not significantly different from a, b, and d.
§Not significantly different from a, b, and d.
¶Significantly different from a (p < 0.001), b (p < 0.003), c (p < 0.001), and d (p < 0.02).

**FIG. 3. Dose dependence of synthetic peptide effects.** The effect of varying concentrations of synthetic peptides \(\alpha_{315-324}\) (open triangles) or \(\alpha_{384-394}\) (closed circles) was examined on the competition of 0.1 \(\mu\)M isoproterenol for 150 \(\mu\)M IPIN binding to permeable C6 cells. Data points are means of triplicate determinations in one of three representative experiments. dpm, disintegrations/minute.

**FIG. 4. a, competition between \(\alpha_{384-394}\) and GppNHp on agonist affinity was studied in the absence (open circles) or presence (open squares) of 100 \(\mu\)M GppNHp, in the presence of 100 \(\mu\)M \(\alpha_{384-394}\) (closed circles), or in the presence of 100 \(\mu\)M GppNHp plus 100 \(\mu\)M \(\alpha_{384-394}\) (closed squares). While GppNHp decreased the potency of isoproterenol, the peptide elicited an increased agonist affinity. In the presence of peptide, the GppNHp-induced decrease in agonist affinity was diminished. Data represent the mean of triplicate determinations in a typical experiment which was repeated four times. b, effect of increasing concentrations of \(\alpha_{384-394}\) on 100 \(\mu\)M isoproterenol competition of IPIN binding in the absence (open circles) or presence (closed circles) of 100 \(\mu\)M GppNHp in permeable C6 cells. At the higher concentrations of \(\alpha_{384-394}\) used here, GppNHp had only a negligible effect. dpm, disintegrations/minute.
this region (Thr-Cys-Ala-Thr) is found also in a subclass of small G proteins related to the ADP-ribosylation factor (Sewell and Kahn, 1988). This region of $\alpha_i$, Thr354-Thr363, has recently been shown to be involved in binding to the guanine ring of the guanine nucleotide (Noel et al., 1993). The possibility that this region also interacts with receptors suggests a putative mechanism for receptor-mediated GDP release, since reorientation of the guanine binding side chains would cause a diminution of GDP affinity. Mutagenesis of $\alpha_i$, Cys354 has confirmed this finding (Thomas et al., 1993). Furthermore, comparative studies of interaction of various classes of GTP binding proteins with their cognate guanine nucleotide exchange factor proteins suggest that the exchange factor interacts with the TCAT region, which is directly involved in binding the guanine ring (Hwang et al., 1993).

It is clear that other regions of the G protein may be involved in receptor interaction. The amino-terminal region of $\alpha_i$ was shown to be involved in rhodopsin interaction (Hamm et al., 1988), and this may be the case with other G proteins as well, since Higashijima et al. (1990) have shown that the receptor mimetic tetradecapeptide mastoparan can be cross-linked to the amino terminus of $\alpha_i$. The crystal structure of the $\alpha$ subunit of G shows that the amino and carboxyl termini are in spatial proximity (Noel et al., 1993). In this study, amino-terminal peptides from $\alpha_i$ had no effect on $\beta$-adrenergic receptor-G$\alpha$ coupling or agonist affinity. Thus, there are key differences in the experimental findings regarding regions on different G protein $\alpha$ subunits of interaction with their cognate receptors. These findings provide a rationale for the study of multiple receptor systems to elucidate a general mechanism and to discern variations on the common theme.

The fact that homologous peptides from the sequence of $\alpha_i$ had no effect on agonist affinity (Table III) suggests that short peptide sequences can carry a surprising degree of specificity. An average of 40% of the amino acids of the carboxyl-terminal region are identical between $\alpha_i$ and $\alpha_i$; thus, the specificity determinants must reside in the unique residues of the sequence. Peptide proton NMR and peptide substitution studies have shown that a $\beta$-turn at the carboxyl terminus of $\alpha_i$ involving Cys347, Gly348, Leu349, and Phe350 is critical for the peptide-induced stabilization of active metarhodopsin II (Dartz et al., 1988). It may be possible to extrapolate from these structural studies on rhodopsin-$G\alpha$ interaction to the $\beta$-adrenergic receptor-$G\alpha$ interface, since stabilization of the active conformation of both receptors is similarly achieved by carboxyl-terminal peptides. It is noteworthy that a conformational change around Lys354 occurs upon binding of the carboxyl-terminal peptide from $\alpha_i$ (340–350) to metarhodopsin II (Dartz et al., 1993). The unc mutation of S49 lymphoma cells, in which receptor and $G\alpha$ are uncoupled, changes the homologous Arg356 to Pro, which would not have the same conformational flexibility. Thus, conformational change of the G protein carboxyl terminus may be important for productive G protein-receptor interaction and subsequent receptor-mediated GDP release and G protein activation.

We have shown that two small peptides from the carboxyl-terminal region of $\alpha_i$ can each mimic G$\alpha$ effects on $\beta$-adrenergic receptors. Although either peptide can elicit a nearly maximal effect, combinations of the two peptides are partially additive (Fig. 5), leading to a multistate model of receptor-G$\alpha$ interaction. These data are consistent with several point-to-point interactions between receptors and G proteins at regions that might be some distance from each other on the primary sequence of the proteins. Thus, it is likely that three-dimensional folding of these proteins is critical for high affinity molecular recognition. This may help to explain why "consensus sequences" of receptors and G proteins have not been found, despite the wealth of information on primary amino acid sequences of both protein families. The elegant studies of the mechanism of action of mastoparan (Higashijima et al., 1988, 1991) demonstrate that the two- and three-dimensional conformation of interacting sites is critical for the interaction to take place. Our studies demonstrate that some small peptides have the ability to fold into the appropriate conformation in the context of a recognition site on the receptor.

It is noteworthy that either $\alpha_i$,354-372 or $\alpha_i$,384-394 was able to achieve a maximal effect on increasing $\beta$-receptor affinity (Fig. 5). This suggests that, although multiple sites on G proteins may normally contact the $\beta$-receptor, occupation of one of those sites is sufficient to mimic the binding of a G protein. Increasing concentrations of peptide eliminate the ability of GTP to shift receptor into the low affinity state for agonist binding. This result is consistent with the notion of a continuous competition between $\alpha_i$ and peptide for a binding site on the receptor, which can be overcome by great excess of peptide relative to $\alpha_i$ (Fig. 4b). Such a mechanism might also be involved in the ability of the $\alpha_i$ peptides to shift the $\beta$-receptor into a higher affinity state for agonist. Peptides bind to the appropriate sites to mimic high agonist affinity without displaying the comprehensive characteristics of the receptor-G$\alpha$ protein complex. Even in the absence of agonist, this complex may have some inherent function. The observation that reconstituted receptor-G$\alpha$ protein complexes (without agonist) have GTPase activity that is not seen with the G protein alone (Kurose et al., 1991) is consistent with this idea. Thus, receptor plus peptide represents the "pristine" high affinity agonist binding state.

It is of interest that the ability of a given peptide to block the coupling between the $\beta$-adrenergic receptor and G$\alpha$ (Fig. 1) is dependent upon the environment surrounding these proteins. Thus, while the $\alpha_i$ peptide 354–372 blocks $\beta$-adrenergic stimulation of adenylyl cyclase in both permeable cells and membranes, the carboxyl-terminal peptide, 384–394, is capable of blocking $\alpha_i$ activation by the activated $\beta$-receptor only when in a near native environment. Furthermore, the carboxyl-terminal peptide appears to be disrupting only the "tightly coupled state" of the $\beta$ receptor-G$\alpha$-adenyl cyclase complex.

Two possible reasons for this discrepancy exist. First, it is possible that disruption of the membrane and cytoskeletal en-
vimentin alter the receptor such that the site available for binding is no longer accessible to the aqueous environment. Alternatively, disruption of the membrane may increase the strength of the receptor-Gα interface so that the carboxyl-terminal peptide no longer competes with the site available for binding. This is evidenced by the ability of GTP or GTP analogs to activate adenylyl cyclase in the absence of P-agonist. A mechanistic explanation for this might be that disruption of the cell frees Gα from the constraint normally imposed by the β-receptor. This is evidenced by the ability of Gα to interact with the receptor-Gα interface so that the carboxyl-terminal region of the α subunit and the receptor. This would constitute a primary recognition event, which could prime a secondary interaction between the receptor and the guanine nucleotide binding region within α, -354 to -372, thus promoting guanine nucleotide release.

According to this model, in the undisrupted cellular milieu, G proteins in their GDP-bound state have a low affinity for receptors. G protein activation occurs as a two-stage process. First, receptor recognition and primary activation due to contact between the activated receptor and the carboxyl-terminal of the α subunit, followed by a secondary interaction of the receptor with the TCAT region leading to guanine nucleotide release. Upon cellular disruption, especially in certain cell and tissue types, a changed environment (including, perhaps, low GDP concentration) promotes the high affinity mode of receptor-G protein interaction preventing access of the ligand to its binding pocket.

Thus, this study may suggest a molecular rationale for the well-documented "uncoupling" between receptor and G protein seen following cell disruption. Through the use of site-specific synthetic peptides, the coupling between receptor, G protein, and intracellular effector molecules can be explored and some of the intricacies of in situ G protein-mediated signal transduction might be revealed.

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REFERENCES


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