0026-895X/94/061191-07$03.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 45:1191-1197, 1994.

Multisite Interactions of Receptors and G Proteins: Enhanced Potency of Dimeric Receptor Peptides in Modifying G Protein Function

SUSAN M. WADE, HIROKO MORI DALMAN,[1] SHANG-ZHAO YANG, and RICHARD R. NEUBIG

Departments of Pharmacology (S.M.W., H.M.D., S.-Z.Y., R.R.N.) and Internal Medicine (Hypertension) (R.R.N.), The University of Michigan, Ann Arbor, Michigan 48109-0626

Received January 5, 1994; Accepted March 8, 1994

SUMMARY

Synthetic peptides that activate or inhibit G proteins reveal structural determinants of receptor-G protein interactions and show promise as potential therapeutic agents. A cysteine-containing peptide from the carboxyl-terminal part of the third cytoplasmic loop of the α_2-adrenergic receptor (peptide Q) uncouples α_2-adrenergic receptors from G_i. Peptide Q readily forms disulfide-linked dimers (Q_{dimer}), as detected by high performance liquid chromatography and mass spectrometry. Q_{dimer} is >100-fold more potent than monomeric Q peptide in inhibiting *p*-[^{125}I] iodoclonidine binding to the human α_{2a}-adrenergic receptor in platelet membranes and transfected Chinese hamster ovary cells. In addition, Q_{dimer} is 10–20 times more potent than monomeric Q peptide in inhibiting α_2 agonist-stimulated GTPase in cell membranes and in directly stimulating G_o/G_i GTPase in lipid vesicles. The effect of Q_{dimer} is reversible and not mimicked by cystine. Formylation of both tryptophans greatly reduces the potency of the dimer but a single formyl group is well tolerated, indicating an asymmetric interaction of the dimer with G_i in membranes. A mixed dimer of peptides from the amino- and carboxyl-terminal ends of the third cytoplasmic loop of the α_2-adrenergic receptor is most potent in all measures of G protein interactions, suggesting that the dimer of Q peptides mimics multiple intracellular portions of the α_2-adrenergic receptor with the G protein. These data confirm the importance of multiple receptor regions in G protein activation and suggest a strategy for examining the role of physically separated regions in protein-protein interactions.

G proteins transduce signals from classical hormones, neurotransmitters, and external stimuli (e.g., light, odor, and taste) to the interior of cells (1, 2). Our understanding of the structural basis of specificity in receptor-G protein coupling has been greatly enhanced by molecular cloning (3, 4) and mutagenesis (5, 6) approaches available in the past decade. In addition to mutagenesis and expression, synthetic peptides derived from the sequence of biologically active proteins have become very useful both as tools in studying protein-protein interactions and as potential therapeutic agents. Peptides from signal-transducing molecules such as receptors and heterotrimeric G proteins as well as low molecular weight G proteins have been used for these purposes (7). Although structure-activity studies of peptide agonists and antagonists at extracellular receptors have been extensively analyzed, there is less information available about the structural basis of peptides modifying signal transduction at intracellular sites.

The tetradecapeptide mastoparan (from wasp venom), which activates signals in mast cells, was one of the first small peptides found to have direct G protein-activating activity (8). Also, peptides from the carboxyl terminus of the visual sensory G protein transducin (9) and peptides from three intracellular regions of rhodopsin (10) were shown to uncouple rhodopsin-transducin interactions. Since these seminal discoveries, synthetic peptides have been shown to modulate receptor and G protein activity in numerous systems, including the β-adrenergic (11), α_2-adrenergic (12–14), muscarinic (15), and dopamine D_2 (16) receptor systems. The exact structural determinants of such effects have not been established, although both amphiphilicity (8) and the "consensus sequence" BBXB (17) have been proposed to be important (see, however, Ref. 18). There

This work was supported by National Institutes of Health Grants HL-GM46417 (to R.R.N.), P60-AR20557, and M01-RR00042 (for computer database access) and a Howard Hughes Fellowship (to H.M.D.).

[1] Present address: Department of Anesthesiology, Massachusetts General Hospital, Boston, MA 02115.

ABBREVIATIONS: i2 loop, second intracellular loop; i3 loop, third intracellular loop; P peptide, peptide with the sequence CRIYQIAKRRTRV (amino acids 218–229 from the porcine α_2-adrenergic receptor with an additional amino-terminal cysteine); Q peptide, peptide with the sequence RWRGRQNREKRFTC (amino acids 361–373 from the porcine α_2-adrenergic receptor with an additional carboxyl-terminal cysteine); Q_f, *N*-formyltryptophan derivative of Q peptide; PIC, *p*-iodoclonidine; Q_{dimer}, dimeric Q peptide; $Q_{monomer}$, monomeric Q peptide; HPLC, high performance liquid chromatography; CHO, Chinese hamster ovary; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; TFA, trifluoroacetic acid.

also appears to be a role for basic and hydrophobic residues in this interaction (8, 13, 19).

One aspect of these studies that has been difficult to understand is how peptides with such a small amount of the overall structural information of the native protein retain activity. There are several pieces of information that suggest a role for multiple peptide sequences in receptor-G protein interactions. First, mutagenesis data have implicated the i2 and i3 loops and the carboxyl-terminal tail of receptors in their interactions with G proteins (5, 20, 21). Second, rhodopsin and β-adrenergic receptor peptides show synergistic effects when peptides from more than one intracellular region are mixed (10, 11). Finally, the Hill coefficients of 2–4 suggest cooperative activation of G_o by mastoparan (8).

In this paper we report that covalently linked dimers of α_2-adrenergic receptor peptides show markedly enhanced potency compared with monomeric peptides. These results suggest that the dimers mimic the action of multiple intracellular portions of the α_2-adrenergic receptor on the G protein. This approach represents a new strategy for examining the role of physically separated regions in protein-protein interactions.

Materials and Methods

Radiochemicals. [^{125}I]PIC (2200 Ci/mmol) and [γ-^{32}P]GTP (30 Ci/mmol) were from DuPont-New England Nuclear.

Chemicals. Aprotinin, phenylmethylsulfonyl fluoride, L-cystine, GTP, ATP, phosphocreatine, creatine phosphokinase, 5′-adenylylimidodiphosphate, and activated charcoal were obtained from Sigma, benzamidine was from Aldrich, oxymetazoline was from Schering, PIC was from Research Biochemicals, brimonidine tartrate (UK 14,304) was from Pfizer, DTT was from Calbiochem, and TFA was from Pierce.

Peptide synthesis and modifications. Peptides with sequences from the α_2-adrenergic receptor were previously shown to disrupt receptor-G protein coupling (12). The P peptide is from the amino-terminal end of the i3 loop of the porcine α_2-adrenergic receptor (22) and the Q peptide is from the carboxyl-terminal end of the i3 loop (see Abbreviations). Both peptides contained an additional cysteine residue for preparation of antibodies. Several batches of the peptides were synthesized by the University of Michigan Protein and Carbohydrate Structure Core Facility or by Cambridge Research Biochemicals (Wilmington, DE) using fluorenylmethoxycarbonyl chemistry, as described (12). One batch of Q peptide was also prepared by *t*-butoxycarbonyl chemistry at the University of Nebraska (the source of Q_f peptide; see below). Peptides and modified peptides (see Results) were purified by reverse phase HPLC. Isolated peaks were dried using a Savant SS1 SpeedVac system and identity was confirmed by electrospray mass spectrometry. Peptides were dissolved in water and stored at −20° for use in all assays.

HPLC. Reverse phase HPLC was performed using a Rainin Rabbit HP system with either an analytical (0.46- × 25-cm) column or a semipreparative (1.0- × 25-cm) Vydac 218TP C_{18} column. The analytical column was eluted at 1 ml/min with 0.1% TFA in water (buffer A) and 0.1% TFA in acetonitrile (buffer B) using the following gradient profile: 0–10 min, 0–15% buffer B; 10–45 min, 15–30% buffer B; 45–50 min, 30–100% buffer B; 50–60 min, 100% buffer B. The semipreparative column was eluted at 2.5 ml/min with the same buffers using the following profile: 0–0.5 min, 5–15.5% buffer B; 0.5–21 min, 15.5% buffer B; 21–21.5 min, 15.5–18.5% buffer B; 21.5–36.5 min, 18.5–20% buffer B; 36.5–40 min, 20–90% buffer B; 40–50 min, 95% buffer B. All buffers were filtered and degassed. Absorbance was routinely monitored at 220 nm using a Spectroflow 75 detector, but for analysis of formyltryptophan content a Waters 990 photodiode array detector was used.

Purification of formylated Q_{dimer} derivatives. One batch of Q peptide exhibited multiple peaks on HPLC. Analysis of purified fractions by diode-array spectroscopy and mass spectrometry showed that this was due to incomplete removal of the formyl blocking group on the tryptophan. Three peaks were isolated, with masses of 3780, 3809, and 3836, consistent with Q_{dimer} containing 0, 1, and 2 formyl groups, respectively. As expected, the peaks with masses of 3809 and 3836 also showed the characteristic broad absorbance at 300 nm and a large peak at 242 nm, demonstrating the presence of *N*-formyltryptophan. Thus, the three peaks showed the expected UV and mass spectral characteristics of the homo- and heterodimers of Q peptide and Q_f. Also, reduction of the isolated peptides by DTT followed by HPLC resulted in the expected mixtures of Q and Q_f for Q-Q, Q-Q_f, and Q_f-Q_f (data not shown).

Mass spectrometry. Peptide masses and purity were determined by electrospray mass spectrometry, using a Vestec single-quadrupole mass spectrometer with electrospray interface, by the Protein and Carbohydrate Structure Core Facility at the University of Michigan.

MAG-2 membranes. The MAG-2 cell line was derived from CHO-K1 cells as described (23). For binding assays, crude membranes were prepared as described (24), resuspended in TME (50 mM Tris, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.6), quick frozen, and stored at −70°. For GTPase assays, confluent monolayers of cells were washed with phosphate-buffered saline, lysed at 4° for 15 min in 1 mM Tris, pH 7.4, with protease inhibitors (10 mM benzamidine, 10 units/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and homogenized by 10 strokes in a Teflon-glass homogenizer. Nuclei and undisrupted cells were pelleted for 10 min at 1000 × *g*. The pellet was homogenized in Tris buffer with protease inhibitors and pelleted for 5 min at 1000 × *g*. This step was repeated once more. Supernatants from all three centrifugations were combined and pelleted for 30 min at 40,000 × *g*. Membranes were resuspended in TME, quick frozen, and stored at −70°.

Human platelet plasma membranes. Membranes were prepared by discontinuous sucrose density gradient centrifugation as described (25), resuspended in TME, quick frozen, and stored at −70°.

[^{125}I]PIC binding assays. [^{125}I]PIC binding was measured in TME as described (12). Briefly, membranes (0.15 mg/ml MAG-2 membranes or 0.2 mg/ml platelet membranes) were preincubated with peptide for 10–15 min on ice in the presence or absence of 1.7 mM DTT (1 mM final concentration in the assay). [^{125}I]PIC (1.0 nM, isotopically diluted 2–6-fold) was then added and incubated for 45 min at 25°. Rapid filtration was performed on a Brandel filtration apparatus. Filters were dried for 15 min under a heat lamp and counted in 4 ml of ScintiVerse liquid scintillation cocktail. Nonspecific binding was defined using 10 μM oxymetazoline.

GTPase assays. GTPase activity in MAG-2 membranes was measured as described (12), except that the final reaction mixture contained [γ-^{32}P]GTP (0.1 μCi/tube), 0.1 μM GTP, 0.2 mM ATP, 0.2 mM 5′-adenylylimidodiphosphate, 20 mM NaCl, 5 mM phosphocreatine, 50 units/ml creatine phosphokinase, 10 mM Tris, 2 mM $MgCl_2$, and 0.2 mM EGTA, in a total volume of 100 μl. Peptides were preincubated with MAG-2 membranes (10 μg/tube) for 15 min on ice in the presence or absence of 2 mM DTT (1 mM final concentration in the assay). The reaction was initiated by the addition of [γ-^{32}P]GTP and was incubated for 16 min at 30°. The reaction was terminated by the addition of 25% (w/v) activated charcoal, pH 2.3, and released $^{32}P_i$ was determined by counting of an aliquot of the supernatant in 4 ml of Scintiverse liquid scintillation cocktail. Low affinity GTPase activity, determined by the addition of 50 μM GTP, accounted for 9–25% of the total activity and was subtracted from the total activity to give the amount of high affinity GTPase activity. For G_o/G_i vesicle GTPase, purified G_o/G_i from bovine brain was reconstituted into lipid vesicles as described (26). The final reaction mixture contained [γ-^{32}P]GTP (0.1 μCi/tube), 0.5 μM GTP, 1.1 mM $MgSO_4$, 14 mM HEPES, 0.7 mM EDTA, and 35 mM NaCl, in a total volume of 50 μl. Peptides were preincubated with vesicles for 15 min on ice, and the reaction was initiated by the addition of [γ-^{32}P]GTP. The reaction was incubated for 15 min at 30°, terminated, and counted as described above.

Data analysis. Data were analyzed using the nonlinear least squares

method of the computer program InPlot (GraphPAD Software, San Diego, CA).

Results

We recently showed that two peptides from the α_2-adrenergic receptor were able to uncouple that receptor and others from the inhibitory G protein G_i (12, 15). The peptide from the carboxyl-terminal portion of the i3 loop (termed Q peptide) was active both in disrupting high affinity α_2-adrenergic agonist binding and in blocking G protein activation as measured by agonist-stimulated GTPase. In examining batch-to-batch variability of the activity of the Q peptide, we discovered that this peptide was quite prone to formation of disulfide-linked dimers, which resulted in the appearance of a new peak on reverse phase HPLC (Fig. 1). Even freshly prepared stock solutions of Q peptide contained variable amounts of the contaminating peak. This extra peak could be eliminated by pretreating peptide with 10 mM DTT in aqueous buffers. Isolation of the two peaks followed by electrospray mass spectrometry revealed that we had a monomeric peptide (R_T, 18.5 min; mass, 1892) and a dimeric peptide (R_T, 21.9 min; mass, 3780).

Consequently, we prepared milligram amounts of $Q_{monomer}$ and Q_{dimer} for direct functional analyses. Q peptide was reduced by incubation with a 10-fold molar excess of DTT for 30 min at 25° in 50 mM HEPES, pH 8. Oxidation to the disulfide-linked Q_{dimer} was accomplished by dissolving 1 mg of Q peptide in 0.4 ml of 50 mM Tris, pH 8, and allowing the mixture to remain at room temperature for approximately 2 weeks. The $Q_{monomer}$ and Q_{dimer} thus prepared were isolated by semipreparative reverse phase HPLC and were analyzed by mass spectrometry. Q_{dimer} was stable when stored in solution at −20° for up to 4 months.

We assessed the biological activity of $Q_{monomer}$ and Q_{dimer} in three different measurements of G protein activity, 1) high affinity α_2-adrenergic receptor agonist binding to membranes from MAG-2 cells, a CHO cell line stably transfected with the human α_{2A}-adrenergic receptor (23), 2) α_2-adrenergic receptor agonist-stimulated GTPase activity in membranes from the same cells, and 3) direct activation by peptide of the GTPase activity of purified bovine brain G_o/G_i.

The ability of Q peptide to inhibit high affinity α_2 agonist binding could be largely accounted for by the activity of the small amount of Q_{dimer} present in our stock solutions of Q peptide (Fig. 2A). Q_{dimer} was approximately 10 times more potent in inhibiting [^{125}I]PIC binding than was unpurified Q

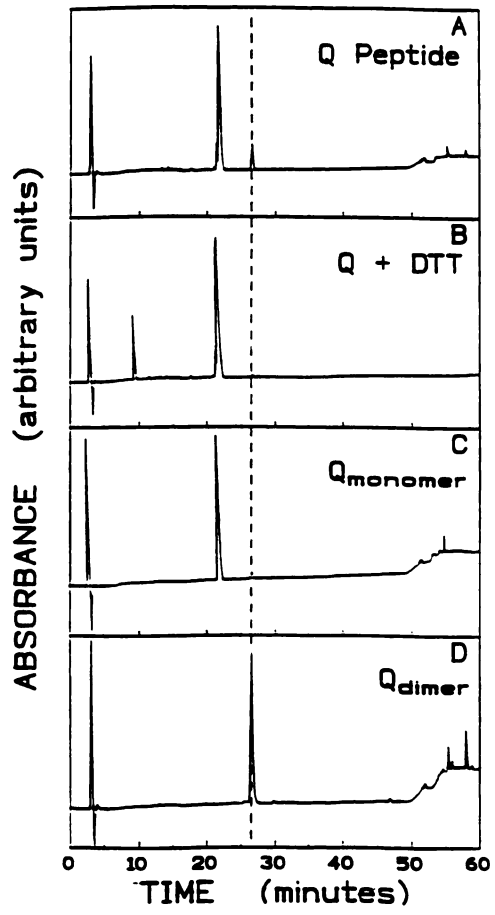

Fig. 1. HPLC analysis of Q peptides. Twenty-microgram samples of peptides were analyzed by reverse phase HPLC on a Vydac C_{18} column (0.46 × 25 cm), as described in Materials and Methods. A and B, Q peptide that had been stored in aqueous solution (A) displayed two peaks, which were reduced to a single peak (B) after treatment with a 10-fold molar excess of DTT, as described in Materials and Methods. C and D, Isolated and purified peaks from A. Electrospray mass spectrometry of isolated $Q_{monomer}$ and Q_{dimer} yielded molecular masses of 1893 and 3783, respectively. The retention time of Q_{dimer} is indicated by the *dotted line* at 26.5 min. The peak eluting at 9.7 min in B is DTT. *Ordinate*, absorbance monitored at 220 nm.

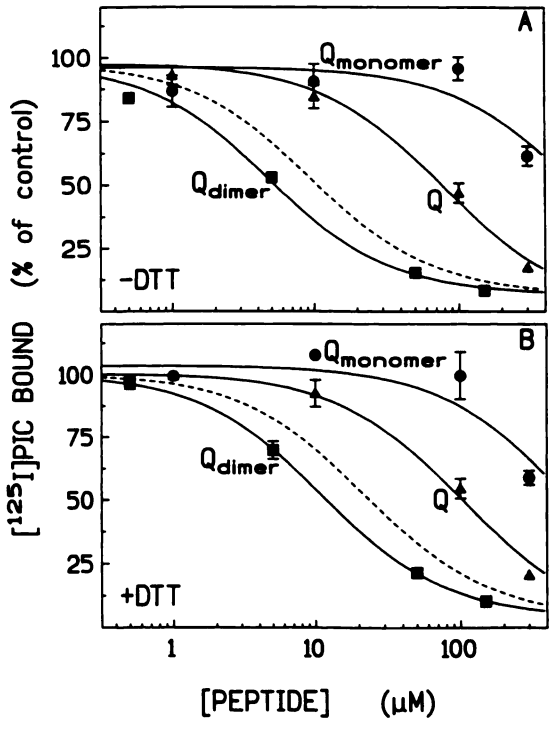

Fig. 2. Effect of peptides on [^{125}I]PIC binding to α_{2A}-adrenergic receptors in CHO cells. Peptide Q (▲), $Q_{monomer}$ (●), and Q_{dimer} (■) were preincubated with MAG-2 membranes in the absence (A) or presence (B) of 1.7 mM DTT for 15 min on ice, and specific binding of [^{125}I]PIC was determined as described in Materials and Methods. *Dashed line*, relative position of the Q_{dimer} data if Q_{dimer} concentrations were calculated by mass rather than by molar concentrations. Control binding was 720 and 640 fmol/mg of protein in the absence and presence of DTT, respectively. Data points represent the mean ± standard deviation of triplicates from one experiment but are similar to those from one (Q) or two ($Q_{monomer}$ and Q_{dimer}) additional experiments.

peptide, whereas $Q_{monomer}$ was an additional 1 order of magnitude less potent. One explanation for the greater potency of Q_{dimer} could be that at a given molar concentration there is a greater mass of peptide. This clearly cannot explain our results because this would result in only a 2-fold difference in potency. Fig. 2, *dashed lines*, shows the effect of Q_{dimer} if the concentrations are expressed in terms of mass of peptide rather than in molar concentrations. Surprisingly, inclusion of a >10-fold excess of DTT (1.7 mM) in the preincubation of peptide with membranes did not substantially reduce the potency of the Q_{dimer} (Fig. 2B). This unexpected insensitivity to DTT impeded the recognition that the disulfide-linked Q_{dimer} was the source of activity in the Q peptide preparations. Q_{dimer} was also substantially more potent in inhibiting α_2 agonist-stimulated GTPase activity in the MAG-2 cells (Fig. 3).

Because of the known sulfhydryl sensitivity of receptor coupling to G_i-like G proteins (27–29), we were concerned that the effect of Q_{dimer} might be due to modification of the receptor or G protein by reaction with the disulfide moiety of Q_{dimer}. We examined the effect of cystine on the binding of the α_2-adrenergic agonist [^{125}I]PIC and we found no effect at concentrations similar to those at which the Q_{dimer} potently inhibited high affinity agonist binding (data not shown). In addition, the reversibility of the effects of Q_{dimer} on agonist binding was examined. Preincubation of membranes with 15 μM Q_{dimer} followed by inclusion of 15 μM peptide in the binding assay resulted in approximately 60% inhibition of binding (Table 1). At a 10-fold lower concentration there was only a small decrease

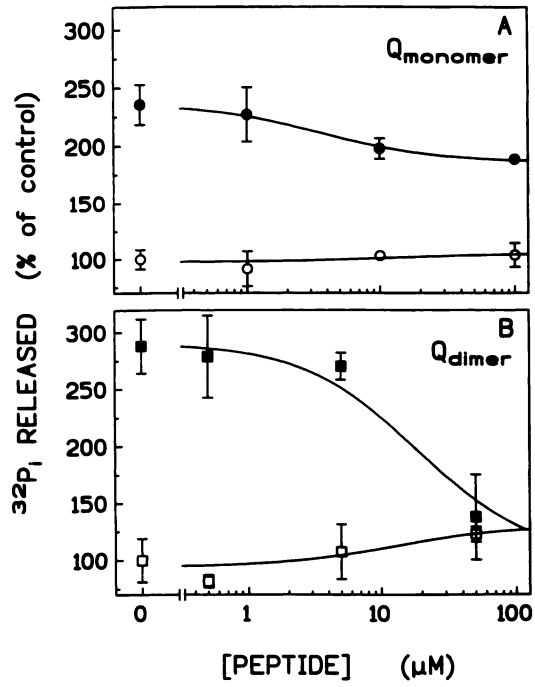

Fig. 3. Effect of $Q_{monomer}$ and Q_{dimer} on α_2 receptor-stimulated GTPase activity in CHO cell membranes in the presence of DTT. $Q_{monomer}$ (A) and Q_{dimer} (B) were preincubated with MAG-2 membranes in the presence of 2 mM DTT for 15 min on ice. GTPase assays were performed in the absence (*open symbols*) or presence (*closed symbols*) of 10 μM UK 14,304, as described in Materials and Methods. Low-affinity GTPase, determined by the addition of 50 μM GTP, accounted for 21–24% of the total activity and was subtracted from the total GTPase activity. Control activity was 1.0 ± 0.4 pmol/min/mg. Data shown represent the mean ± standard deviation of triplicate determinations from one experiment, which was repeated one ($Q_{monomer}$) or two (Q_{dimer}) additional times.

TABLE 1

Reversibility of Q_{dimer} inhibition of [^{125}I]PIC binding to α_{2A}-adrenergic receptors in CHO cells

MAG-2 membranes were preincubated with the indicated concentration of peptide for 45 min on ice. The mixtures were diluted 10-fold by addition to reaction tubes containing radioligand, peptide, and buffer, and binding assays were performed as described in Materials and Methods. Control binding was 730 ± 20 fmol/mg of protein. Values shown are the mean ± standard deviation from two experiments performed in triplicate.

Concentration of Q_{dimer}		Specific binding
Initial incubation	Binding assay	
μM		% of control
0	0	100 ± 7
0	1.5	80 ± 5
0	15	43 ± 1
1.5	0.15	100 ± 16
1.5	1.5	91 ± 1
1.5	15	43 ± 1
15	1.5	90 ± 12
15	15	44 ± 8

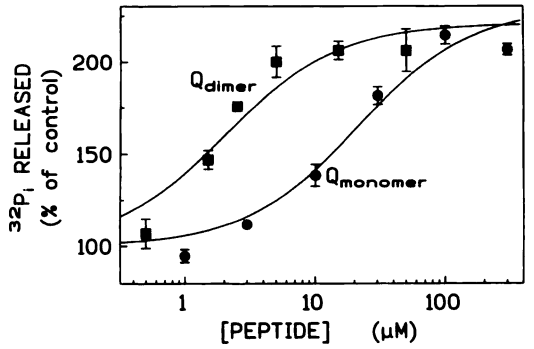

Fig. 4. Effect of $Q_{monomer}$ and Q_{dimer} on GTPase activity in purified bovine brain G_o/G_i. $Q_{monomer}$ (●) and Q_{dimer} (■) were preincubated for 15 min on ice with azolectin vesicles containing purified bovine brain G_o/G_i. GTPase activity was then determined as described in Materials and Methods. Data points represent the mean ± standard deviation of two to four experiments performed in triplicate.

(10–20%) in agonist binding. Dilution of the mixture preincubated with 15 μM Q_{dimer} to a final concentration of 1.5 μM in the binding assay reversed the effect of 15 μM Q_{dimer} to only 10% inhibition. Thus, the effect of Q_{dimer} on agonist binding was reversible.

We further examined the site of action of the Q peptide by measurements of the GTPase activity of purified bovine brain G_o/G_i in lipid vesicles (Fig. 4). As found for receptor peptides by others (11, 13, 17, 18, 30) and in our preliminary studies (14), this peptide stimulated the GTPase activity of the purified bovine brain G protein (Fig. 4). The maximum stimulation by Q peptide was about 2–2.5-fold over basal levels in buffer containing 2 mM $MgCl_2$. Under these conditions, 10 μM mastoparan produced approximately 3-fold stimulation (data not shown). $Q_{monomer}$ and Q_{dimer} produced similar maximal stimulations of GTPase activity. With the purified G proteins, the potency of the Q_{dimer} was only about 1 order of magnitude greater than that of $Q_{monomer}$, in contrast to the 2-order of magnitude difference observed in CHO cell membranes (EC_{50} values of 1.6 μM versus 25 μM for GTPase with G_o/G_i, compared with IC_{50} values of 7.8 μM versus >300 μM for agonist binding). As discussed below, this greater structural specificity for effects on high affinity binding to α_2-adrenergic receptors, compared with G protein GTPase stimulation, appears to be a common

observation and suggests that the determinants of receptor-G protein coupling and activation are different.

We considered various explanations for the enhanced potency of Q_{dimer}. An increased number of positively charged amino acid residues has typically been associated with greater effects of receptor peptides on G proteins (8). In addition, greater bulk could play a role. These do not appear to be the sole explanations, because formyltryptophan derivatives of Q_{dimer} show significant structural specificity in the action of the dimers. The Q_f-Q_f peptide was much less potent in inhibiting agonist binding to either human platelet membrane α_2-adrenergic receptors (Table 2) or the same receptors in the MAG-2 cell line (data not shown). The mixed dimer Q-Q_f showed nearly the same potency in blocking α_2-adrenergic receptor agonist binding as did Q_{dimer} (Table 2). This illustrates a significant structural specificity for the effects of Q_{dimer}. It also suggests that there is an asymmetry in the interaction of the two halves of Q_{dimer} with the G protein in the membrane. Specifically, one site of interaction with the G protein must be able to accommodate the formyltryptophan, whereas the other half of the dimer must interact with a site that requires a nonformylated tryptophan.

Because multiple intracellular portions of the G protein-coupled receptors have been implicated in the functional activation of the G protein, we wondered whether the Q_{dimer} might be mimicking the effect of two intracellular regions. Indeed, the structures of both the amino- and carboxyl-terminal ends of the i3 loop of the α_2-adrenergic receptor contain multiple positively charged amino acids. The P peptide described in our original work on α_2 receptor peptides was from the amino-terminal end of the i3 loop and contains a net charge of +5. Dimerization of mixtures of P and Q peptides yielded both homodimers Q-Q and P-P, as well as the heterodimer P-Q (Table 2). We were able to isolate sufficient quantities of the P-Q dimer for functional studies. In both the agonist binding measurements in membranes and the activation of purified G protein GTPase (Table 2), the P-Q heterodimer produced the most potent effects of any of the peptides that we studied. A summary of the concentrations of various peptides mediating efffects on agonist binding and GTPase activity is presented in Table 2.

TABLE 2

Effect of peptides on [^{125}I]PIC binding to α_{2A}-adrenergic receptors in membranes and on GTPase activity in G_o/G_i vesicles

IC_{50} values for [^{125}I]PIC binding to α_{2A}-adrenergic receptors in MAG-2 membranes (or human platelet membranes where indicated) were determined from peptide competition experiments, as described in Materials and Methods. See Materials and Methods and Abbreviations for structures of peptides. To determine GTPase EC_{50} values, azolectin vesicles containing purified bovine brain G_o/G_i were preincubated with peptide and GTPase activity was measured as described in Materials and Methods. Individual experiments were performed in triplicate; values in parentheses are the numbers of experiments. Values shown are mean ± standard deviation.

Peptide	[^{125}I]PIC binding, IC_{50}	G_o/G_i GTPase, EC_{50}
	μM	μM
Q	>100 (9)	7.4 ± 1.5 (8)
Q(cys$^-$)[a]	>100 (2)	46, 90 (2)
$Q_{monomer}$	>300 (3)	25.3 ± 5.8 (4)
Q-Q	7.8 ± 3.1 (3)	1.6 ± 0.3 (9)
Q-Q_f	7.7 ± 1.9 (2)[b]	3.7 ± 0.5 (5)
Q_f-Q_f	>50 (2)[b]	4.3 (1)
P	5.5 ± 2.1 (5)	4.7 ± 0.8 (6)
P-Q	3.5 ± 1.0 (4)	1.2 ± 0.2 (5)
Cystine	>150 (2)	ND[c]
Mastoparan	>100 (3)[b]	7.8 ± 1.5 (9)

[a] Q(cys$^-$), Q peptide without the carboxyl-terminal cysteine.
[b] Human platelet membranes were used in place of transfected CHO cells.
[c] ND, not determined.

Discussion

There has been significant interest recently in the structural determinants of receptor-G protein interactions and the requirements for direct activation or inhibition of G proteins by synthetic peptides (8, 13, 31) and other small molecules (24). The receptor regions that are important in G protein coupling are well known, as examined by site-directed mutagenesis and with chimeras of receptors specific for different G proteins. A complex pattern has emerged with effects of multiple domains of the receptors. The amino- and carboxyl-terminal regions of the i3 loop seem to play dominant roles, but the i2 loop and carboxyl-terminal tail of some receptors have also been implicated. In contrast, the structural determinants of small molecule interactions with G proteins remain more controversial. As discussed below, multiple structural features, such as amphiphilicity and proposed consensus sequences, have been considered. Our peptide data support the role, found in mutagenesis studies, of multiple noncontiguous stretches of peptide sequence in the interaction of receptors and G proteins. We also show that one must be cautious about the use of purified G proteins to predict effects in more complex membrane-bound signaling systems, and we raise an important technical caveat to researchers attempting to use synthetic peptides containing cysteine because the usual methods of reduction with excess DTT may not be sufficient to prevent effects of disulfide-linked dimers. Finally, these data suggest a strategy for examining the complex structure-activity relations involved in the interaction of one protein with multiple loops of a second protein.

The clear role for both the amino-terminal and carboxyl-terminal regions of the i3 loop of the G protein-coupled receptors (5, 21, 32, 33) in the interaction with G proteins made it difficult to understand how a peptide that encompasses only a single part of the receptor could adequately mimic the effects of receptors on G proteins. The elegant studies of rhodopsin peptides, which activate the G protein transducin, clearly showed potentiation of activity when mixtures of peptides from the i2 and i3 loops and the carboxyl-terminal tail were used (10). This has also been seen for the β-adrenergic receptor (11), with peptide effects occurring at 3 μM concentrations of peptide mixtures rather than 30 μM concentrations of single peptides. We (12) did not find any significant potentiation of activity in simple mixtures of receptor peptides. We now show that the marked potency of the Q peptide in our previous studies was due to Q_{dimer}, which seems to mimic the combination of two different intracellular regions (e.g., amino- and carboxyl-terminal parts of i3). Thus, the 14-amino acid $Q_{monomer}$ peptide does not appear to have sufficient structural information to impart high affinity for the G protein. Why is the Q_{dimer} so much more potent? Clearly, added mass and charge could account for the increase in potency, but the significantly lower potency of the diformylated Q_f-Q_f peptide indicates that there is structural specificity beyond just increased mass and charge. It also suggests that the site of interaction of the dimeric peptides with the G protein in the membrane is asymmetric, because a single formyl group (in the Q-Q_f peptide) is clearly accommodated, whereas the second formyl group (in Q_f-Q_f) is

not. Indeed, the P-Q dimer, which is a covalent complex of the amino- and carboxyl-terminal portions of the i3 loop of the α_2-adrenergic receptor attached at their membrane-proximal ends, is slightly more potent than Q_{dimer} but much more potent than $Q_{monomer}$.

Both amphiphilicity (8) and specific structural "motifs" (17) have been proposed as the dominant factors in G protein activation. The Q peptide is not amphiphilic and it does not contain either of the proposed activator motifs, BBXB or BBXXB, where B is a basic amino acid and X is a nonbasic amino acid (17). It does, however, contain five positively charged residues and a reversed BBXB motif. Indeed, in Q_{dimer} it is possible that the reversed BBXB of one of the Q peptides could mimic the charge distribution of the P peptide in the P-Q dimer. In a preliminary report, Higashijima and co-workers (34) showed that addition of a hydrophobic tail to the membrane-proximal end of a β-adrenergic receptor peptide makes it much more potent, and Nishimoto and co-workers (13) found that a hydrophobic amino acid such as phenylalanine or tyrosine on the membrane-proximal end of the α_2 receptor peptide (residues 356–372) dramatically enhances potency at purified G proteins. Thus, the P peptide probably contributes both additional charges and a hydrophobic region (residues 219–222) to enhance the interaction of Q peptide with the G protein. Mutagenesis studies have also emphasized the role of hydrophobic amino acids, rather than just positively charged amino acids, in G protein coupling (35).

The role of multiple molecules of peptide in binding to G proteins might also have been predicted from the positive cooperativity of mastoparan activation of G proteins. Typically, Hill coefficients of 2–4 are seen for activation of G proteins by mastoparan analogs (8). This suggests that, even for peptides such as mastoparan that do not contain a cysteine, the functional form of the peptide may be a noncovalently linked dimer. It is also possible, however, that monomeric mastoparan molecules could interact with two completely different sites on the G protein. It must also be noted that there are numerous examples where monomeric peptides from G protein-coupled receptors are just as potent as our dimeric peptides (13, 18, 34). In those cases, the size of the peptide is often bigger (20–30 amino acids) or there is significant hydrophobicity in the sequence.

The insensitivity of Q_{dimer} to DTT in the binding assay is rather surprising. Both the inactivity of cystine and the reversibility of the effect of Q_{dimer} indicate that a covalent effect on receptor and/or G protein disulfides is not responsible for the effects of Q_{dimer}. Perhaps the Q_{dimer} is protected from the effects of DTT when it binds to the G protein or sites in the membrane preparations. Reduction by a large excess of DTT in buffer alone clearly does result in the breakdown of the dimer into monomers. It is interesting that a cysteine was required for the activity of a transducin peptide to disrupt coupling of transducin and rhodopsin (9).

As shown in our previous studies, where the A peptide from the i2 loop of the α_2-adrenergic receptor altered agonist binding but not G protein activation, it appears that there are differential determinants of receptor-G protein coupling and direct G protein activation. In the data reported in this paper, several peptides including $Q_{monomer}$ and Q_f-Q_f show relatively potent direct effects on G protein activation but do not uncouple the receptor for high affinity agonist binding. Thus, we have reagents that uncouple binding but not activation (A peptide) and those that affect activation but do not uncouple binding ($Q_{monomer}$ and Q_f-Q_f). These conclusions from peptide methods are consistent with recent mutagenesis data, which similarly show differences in the regions responsible for G protein activation and G protein binding to receptor to induce the high affinity state for agonists (36–38).

As would be expected from the known structure of G protein-coupled receptors, there appear to be multiple sites on the G protein with which receptor peptides interact. The development of both functional and structural maps of these sites will be important to the understanding of the mechanisms of G protein activation and the design of drugs to modulate this key process in signal transduction.

Acknowledgments

The authors thank Drs. Ann Remmers and Guim Kwon for providing the purified bovine brain G_o/G_i. We thank Chuck Mountjoy (University of Nebraska) and Drs. Phil Andrews and Hank Mosberg for many helpful discussions about peptide synthesis and analysis. We also thank Dr. Jonathan Maybaum for use of the Waters diode array detector.

References

1. Hepler, J. R., and A. G. Gilman. G proteins. *Trends Biochem. Sci.* **17**:383–387 (1992).
2. Brown, A. M., and L. Birnbaumer. Ionic channels and their regulation by G protein subunits. *Annu. Rev. Physiol.* **52**:197–213 (1990).
3. Lefkowitz, R. J., and M. G. Caron. Adrenergic receptors: models for the study of receptors coupled to guanine nucleotide regulatory proteins. *J. Biol. Chem.* **263**:4993–4996 (1988).
4. Schimerlik, M. I. Structure and regulation of muscarinic receptors. *Annu. Rev. Physiol.* **51**:217–227 (1989).
5. Strader, C. D., I. S. Sigal, and R. A. F. Dixon. Structural basis of β-adrenergic receptor function. *FASEB J.* **3**:1825–1832 (1989).
6. Kobilka, B. K., T. S. Kobilka, K. Daniel, J. W. Regan, M. G. Caron, and R. J. Lefkowitz. Chimeric α_2-,β_2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science (Washington D. C.)* **240**:1310–1316 (1988).
7. Plutner, H., R. Schwaninger, S. Pind, and W. E. Balch. Synthetic peptides of the Rab effector domain inhibit vesicular transport through the secretory pathway. *EMBO J.* **9**:2375–2383 (1990).
8. Higashijima, T., J. Burnier, and E. M. Ross. Regulation of G_i and G_o by mastoparan, related amphiphilic peptides, and hydrophobic amines: mechanism and structural determinants of activity. *J. Biol. Chem.* **265**:14176–14186 (1990).
9. Hamm, H. E., D. Deretic, A. Arendt, P. A. Hargrave, B. Koenig, and K. P. Hofmann. Site of G protein binding to rhodopsin mapped with synthetic peptides from the *alpha* subunit. *Science (Washington D. C.)* **241**:832–835 (1988).
10. Konig, B., A. Arendt, J. H. McDowel, M. Kahlert, P. A. Hargrave, and K. P. Hofmann. Three cytoplasmic loops of rhodopsin interact with transducin. *Proc. Natl. Acad. Sci. USA* **86**:6878–6882 (1989).
11. Munch, G., C. Dees, M. Hekman, and D. Palm. Multisite contacts involved in coupling of the β-adrenergic receptor with the stimulatory guanine-nucleotide binding regulatory protein: structural and functional studies by β-receptor-site-specific peptides. *Eur. J. Biochem.* **198**:357–364 (1991).
12. Dalman, H. M., and R. R. Neubig. Two peptides from the α_{2A}-adrenergic receptor alter receptor-G protein coupling by distinct mechanisms. *J. Biol. Chem.* **266**:11025–11029 (1991).
13. Ikezu, T., T. Okamoto, E. Ogata, and I. Nishimoto. Amino acids 356–372 constitute a G_i-activator sequence of the α_2-adrenergic receptor and have a Phe substitute in the G protein-activator sequence motif. *FEBS Lett.* **311**:29–32 (1992).
14. Dalman, H. M., M. A. Gerhardt, and R. R. Neubig. Differential effects of α_{2A}-adrenergic receptor peptides on G proteins. *FASEB J.* **5**:A1594 (1991).
15. Neubig, R. R., and H. M. Dalman. Effect of α_{2a}-adrenergic receptor peptides on agonist binding to α_{2b}-adrenergic, muscarinic (M_4) and opiate (*delta*) receptors in NG108–15 membranes. *FASEB J.* **5**:A1594 (1991).
16. Malek, D., G. Münch, and D. Palm. Two sites in the third inner loop of the dopamine D_2 receptor are involved in functional G protein-mediated coupling to adenylate cyclase. *FEBS Lett.* **325**:215–219 (1993).
17. Okamoto, T., and I. Nishimoto. Detection of G protein-activator regions in M_4 subtype muscarinic, cholinergic, and α_2-adrenergic receptors based upon characteristics in primary structure. *J. Biol. Chem.* **267**:8342–8346 (1992).
18. Voss, T., E. Wallner, A. P. Czernilofsky, and M. Freissmuth. Amphipathic α-helical structure does not predict the ability of receptor-derived synthetic peptides to interact with guanine nucleotide-binding regulatory proteins. *J. Biol. Chem.* **268**:4637–4642 (1993).
19. Mukai, H., E. Munekata, and T. Higashijima. G protein antagonists: a novel

hydrophobic peptide competes with receptor for G protein binding. *J. Biol. Chem.* **267**:16237–16243 (1992).
20. O'Dowd, B. F., M. Hnatowich, J. W. Regan, W. M. Leader, M. G. Caron, and R. J. Lefkowitz. Site-directed mutagenesis of the cytoplasmic domains of the human β_2-adrenergic receptor: localization of regions involved in G protein-receptor coupling. *J. Biol. Chem.* **263**:15985–15992 (1988).
21. Wong, S. K.-F., E. M. Parker, and E. M. Ross. Chimeric muscarinic cholinergic:β-adrenergic receptors that activate G_s in response to muscarinic agonists. *J. Biol. Chem.* **265**:6219–6224 (1990).
22. Guyer, C. A., D. A. Horstman, A. L. Wilson, J. D. Clark, E. J. Cragoe, Jr., and L. E. Limbird. Cloning, sequencing and expression of the gene encoding the porcine α_2-adrenergic receptor. *J. Biol. Chem.* **265**:17307–17317 (1990).
23. Gerhardt, M. A., and R. R. Neubig. Multiple G_i subtypes couple to a single effector mechanism. *Mol. Pharmacol.* **40**:707–711 (1991).
24. Huang, R.-R. C., R. N. DeHaven, A. H. Cheung, R. E. Diehl, R. A. F. Dixon, and C. D. Strader. Identification of allosteric antagonists of receptor-guanine nucleotide-binding protein interactions. *Mol. Pharmacol.* **37**:304–310 (1990).
25. Neubig, R. R., and O. Szamraj. Large-scale purification of α_2-adrenergic receptor-enriched membranes from human platelets: persistent association of guanine nucleotides with non-purified membranes. *Biochim. Biophys. Acta* **854**:67–76 (1986).
26. Kim, M. H., and R. R. Neubig. Membrane reconstitution of high-affinity α_2-adrenergic agonist binding with guanine nucleotide regulatory proteins. *Biochemistry* **26**:3664–3672 (1987).
27. Limbird, L. E., and J. L. Speck. *N*-Ethylmaleimide, elevated temperature, and digitonin solubilization eliminate guanine nucleotide but not sodium effects on human platelet α_2-adrenergic receptor-agonist interactions. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **9**:191–201 (1983).
28. Smith, M. M., and T. K. Harden. Modification of receptor-mediated inhibition of adenylate cyclase in NG108–15 neuroblastoma × glioma cells by *N*-ethylmaleimide. *J. Pharmacol. Exp. Ther.* **228**:425–433 (1984).
29. Asano, T., and N. Ogasawara. Uncoupling of *gamma*-aminobutyric acid B receptors from GTP-binding proteins by *N*-ethylmaleimide: effect of *N*-ethylmaleimide on purified GTP-binding proteins. *Mol. Pharmacol.* **29**:244–249 (1986).
30. Okamoto, T., T. Katada, Y. Murayama, M. Ui, E. Ogata, and I. Nishimoto. A simple structure encodes G protein-activating function of the IGF-II/mannose 6-phosphate receptor. *Cell* **62**:709–717 (1990).
31. Nishimoto, I., E. Ogata, and T. Okamoto. Guanine nucleotide-binding protein interacting but unstimulating sequence located in insulin-like growth factor II receptor: its autoinhibitory characteristics and structural determinants. *J. Biol. Chem.* **266**:12747–12751 (1991).
32. Hargrave, P. A., H. E. Hamm, and K. P. Hofmann. Interaction of rhodopsin with the G-protein, transducin. *Bioessays* **15**:43–50 (1993).
33. Hurley, J. B., H. K. W. Fong, D. B. Teplow, W. J. Dreyer, and M. I. Simon. Isolation and characterization of a cDNA clone for the *gamma* subunit of bovine retinal transducin. *Proc. Natl. Acad. Sci. USA* **81**:6948–6952 (1984).
34. Wakamatsu, K., K. Shinagawa, T. Tanaka, M. Oya, M. Sukumar, and T. Higashijima. Interaction of cytoplasmic loop peptides of G protein-coupled receptors with G proteins and with phospholipid membranes. *J. Cell. Biochem.* **17C**:286 (1993).
35. Cheung, A. H., R. R. Huang, and C. D. Strader. Involvement of specific hydrophobic, but not hydrophilic, amino acids in the third intracellular loop of the β-adrenergic receptor in the activation of G_s. *Mol. Pharmacol.* **41**:1061–1065 (1992).
36. Franke, R. R., B. Konig, T. P Sakmar, H. G. Khorana, and K. P. Hofmann. Rhodopsin mutants that bind but fail to activate transducin. *Science (Washington D. C.)* **250**:123–125 (1990).
37. Hausdorff, W. P., M. Hnatowich, B. F. O'Dowd, M. G. Caron, and R. J. Lefkowitz. A mutation of the β_2-adrenergic receptor impairs agonist activation of adenylyl cyclase without affecting high affinity agonist binding: distinct molecular determinants of the receptor are involved in physical coupling to and functional activation of G_s. *J. Biol. Chem.* **265**:1388–1393 (1990).
38. Fraser, C. M. Site-directed mutagenesis of β-adrenergic receptors: identification of conserved cysteine residues that independently affect ligand binding and receptor activation. *J. Biol. Chem.* **264**:9266–9270 (1989).

Send reprint requests to: Richard Neubig, Department of Pharmacology, University of Michigan, M6322 Medical Science Building 1, Ann Arbor, MI 48109–0626.