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Structural Requirements for G_o Activation by Receptor-Derived Peptides: Activation and Modulation Domains of the α_2-Adrenergic Receptor i3c Region

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SUMMARY

Synthetic peptides are important tools for understanding the sites and mechanisms of receptor/G protein interactions. We examined the structural determinants of receptor-fragment peptides for G protein binding and activation. A dimer of peptides from the carboxyl-terminal (i3c) and amino-terminal (i3n) regions of the α_{2A}-adrenergic receptor is most potent in stimulating guanine-nucleotide exchange of any peptides studied. Stimulation of GTPase by i3n is partially blocked by pertussis toxin treatment, whereas stimulation by i3c is not, which is consistent with action of i3c at the amino terminus of G_i. Both peptides inhibit adenylyl cyclase in Chinese hamster ovary cell membranes, but only the i3c effect is consistent with a pure G_i stimulation. We also examined the mechanism and defined a minimal structural subset of i3c required for G protein activation. Residues 361–365 from the receptor were essential for GTPase stimulation, whereas determinants in the region 368–373 modulated that activity. A specific role for arginines is defined beyond just their positive charge. Complex effects of modifications of Thr373 suggest a regulatory or conformational role of that residue in the previously defined constitutive activation of the α_{2a}-adrenergic receptor [*J. Biol. Chem.* 268:16483–16487 (1993)]. Thus, our data plus recent mutagenesis results support a role for hydrophobicity in the i3n region and a positively charged/arginine-rich region ~15–20 residues from the sixth transmembrane span in G protein activation. In contrast, the immediate perimembrane region of i3c seems to have largely conformational effects in producing constitutive activation of the receptors.

G proteins transduce many different signals in mammalian cells (1, 2). The structural basis of specificity in receptor/G protein coupling has been examined by molecular cloning (3, 4) and mutagenesis (5, 6). Synthetic peptides derived from the sequence of biologically active proteins are also useful as tools with which to study protein/protein interactions and as potential therapeutic agents. Peptides from signal transducing molecules, such as receptors and heterotrimeric G proteins (7–16) as well as low-molecular-weight G proteins, have been used for these purposes (17). 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 (18). Also, peptides from both the carboxyl terminus of the visual sensory G protein transducin (7) and three intracellular regions of rhodopsin (8) 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, 19), muscarinic (15), dopamine D_2 (16), and 5-hydroxytryptamine$_{1a}$ (20) receptors. The exact structural

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ABBREVIATIONS: i3c, carboxyl-terminal end of third intracellular loop; i3, third intracellular loop; i3n, amino-terminal end of third intracellular loop; P peptide, peptide with the sequence CRIYQIAKRRTRV (amino acids 218–229 from i3n region of porcine α_2-adrenergic receptor with an added cysteine); P-CM, carboxymethylated P peptide; P-P, disulfide-linked dimer of P peptide; P-Q, disulfide-linked dimer of P and Q peptides; Q peptide, peptide with the sequence RWRGRQNREKRFTC (amino acids 361–373 from i3c region of porcine α_2-adrenergic receptor with an added cysteine); Q-CM, carboxymethylated Q peptide; Q-Q, disulfide-linked dimer of Q peptide; AR, adrenergic receptor; mant-GTPγS, *N*-methyl,3′-*O*-anthranoyl-guanosine-5′-(3-*O*-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; ANOVA, analysis of variance, TM, transmembrane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

determinants of such effects have not been established, although both amphiphilicity (18) and a "consensus sequence" BBXB (21) have been proposed to be important (see, however, Ref. 22). There also seems to be a role for basic and hydrophobic residues in this interaction (13, 18, 23).

We recently showed that i3c peptide from the α_2AR binds to a highly specific site on the amino terminus of the G_o α subunit (24) and also interacts with a defined site on the β subunit (24, 25). Consistent with this site of action, the Q peptide effects are not blocked by pertussis toxin, whereas the GTPase stimulation by mastoparan and P peptide is blocked. To better understand the structure-activity relations at this potential site of drug action, we prepared a series of progressively shortened peptides and peptides with site-specific modifications from the i3c region of the α_2AR. We tested the ability of these peptides to activate G proteins in two assays: stimulation of nucleotide binding and GTPase stimulation. We show that residues in our peptide equivalent to 361–365 in the α_2AR are critical for activation of G protein. Specific amino acid substitutions further undermine the significance of the postulated "BBXB" motif and illustrate a key role of arginines (as opposed to just positive charges) in the function of this region. Also, replacement of the threonine in our peptide, which is equivalent to Thr373 in the native receptor, with amino acids found to result in a constitutively active receptor partially reproduced the phenotype of the intact receptor in our peptide fragments.

Experimental Procedures

Materials. [γ-^{32}P]GTP (30 Ci/mmol) and [^{32}P]NAD (30 Ci/mmol) were from DuPont-New England Nuclear (Boston, MA). [^{3}H]cAMP (34 Ci/mmol) and [α-^{32}P]ATP (30 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). GTP, NAD, activated charcoal, and iodoacetamide were obtained from Sigma Chemical Co. (St. Louis, MO); brimonidine tartrate (UK 14,304) was from Pfizer (Sandwich, UK); pertussis toxin was a gift from the Massachusetts Department of Public Health Center for Disease Control; trifluoroacetic acid was from Pierce Chemical (Rockford, IL); and forskolin and dithiothreitol were from Calbiochem (San Diego, CA).

Peptide synthesis and modifications. Peptides P and Q with sequences from the third intracellular loop of the porcine α_2AR (26) were synthesized by the Univers[redacted] of Michigan Protein and Carbohydrate Structure Core Facili[redacted]sing fluorenylmethoxycarbonyl chemistry as described previous[redacted]12). P peptide has the sequence CRIYQIAKRRTRV (amino acids [redacted]–229 with an additional amino-terminal cysteine) and is from t[redacted]i3n, and Q peptide has the sequence RWRGRQNREKRFTC (a[redacted]no acids 361–373 with an additional carboxyl-terminal cysteine[redacted]d is from the i3c of the porcine α_2AR. The Q-Thr373-substituted peptides and the tetradecapeptide mastoparan from wasp venom (INLKALAALAKKIL) were also synthesized by the University of Michigan Protein and Carbohydrate Structure Core Facility. All of these peptides were evaluated for purity by HPLC and electrospray mass spectrometry.

The Q truncation series peptides were synthesized by Cambridge Research Biochemicals (Wilmington, DE). They were further purified by reverse-phase HPLC 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 eluted with 0.1% trifluoroacetic acid and an acetonitrile gradient. Absorbance was monitored at 220 nm using a Spectroflow 75 detector. Isolated peaks were collected and dried using a Savant SS1 SpeedVac system.

The cysteines in the P and Q peptides were carboxymethylated by incubation with a 4-fold molar excess of iodoacetamide for 1 hr at room temperature in 50 mM Tris, pH 9. Carboxymethylated peptides were purified by reverse-phase HPLC using a BioCAD SPRINT Perfusion Chromatography System with a semipreparative (1.0 × 25 cm) Vydac 218TP C_{18} column. The carboxymethylated peaks were well separated from the parent peptides, and under these conditions the modification was essentially complete. The retention times were 25.0 and 22.8 for Q and Q-CM and 26.9 and 26.2 for P peptide and P-CM on a 37.5-min gradient. Isolated peaks from several runs were collected, pooled, and dried as described above. Carboxymethylation of the cysteine was confirmed by electrospray mass spectrometry and amino acid analysis. Dimer peptides were prepared as described previously (27). All peptides were dissolved in water and stored at −20° for use in all assays.

Mass spectrometry and amino acid analysis. Mass spectrometry and amino acid analysis were performed by the University of Michigan Protein and Carbohydrate Structure Core Facility. Peptide masses and purity were determined by electrospray mass spectrometry using a Vestec single-quadrupole mass spectrometer with electrospray interface. Amino acid analysis was performed on an ABI model 420H amino acid analyzer according to the phenylthiocarbamyl method after gas-phase hydrolysis. The phenylthiocarbamyl amino acids were resolved by HPLC and detected by UV absorbance at 254 nm.

Fluorescence measurements of mant-GTPγS association kinetics. Fluorescence measurements were made using a PTI ALPHASCAN fluorometer (Photon Technology International, Monmouth Junction, NJ) with a water-cooled 150-W xenon arc lamp. Mant-GTPγS was synthesized according to a modification of the method of Hiratsuka (28). G_o was purified from bovine brain as described previously (29).

The sample (0.2 ml in a 5-mm cylindrical quartz cell) was stirred continuously. Then, 5 μl of G_o (100 nM final) was added to 0.185 ml of 50 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 1.1 mM $MgCl_2$, 20 ppm Lubrol, pH 8, and the indicated concentrations of peptide. After equilibration for 50 sec, 400 nM mant-GTPγS was added and the fluorescence monitored at 440 nm (280 nm excitation). All fluorescence measurements were made at 20°. Peptides containing aromatic amino acids (Q-CM, Q-Q, and P-Q) contributed a significant but non-time-dependent blank signal that was subtracted from the time-dependent mant-GTPγS fluorescence before fitting for kinetic analysis.

GTPase assays. GTPase activity in G_o/G_i vesicles was measured using purified G_o/G_i from bovine brain reconstituted into asolectin vesicles as described previously (30). The final reaction mixture contained 0.1 μCi/tube [γ-^{32}P]GTP, 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. The reaction was initiated by the addition of [γ-^{32}P]GTP and incubated for 15 min at 30°. The reaction was terminated by the addition of 25% (w/v) activated charcoal, pH 2.3. Released $^{32}P_i$ was determined through counting an aliquot of the supernatant in 4 ml of Scintiverse liquid scintillation cocktail.

Pertussis toxin-catalyzed ADP ribosylation of bovine brain G_o/G_i. Purified bovine brain G_o/G_i was treated with 10 μg/ml pertussis toxin in the presence or absence of 1 mM NAD as described previously (31). Unreacted NAD was reduced ~1000-fold by repeated concentration and dilution in a Centricon-10. The completeness of ribosylation was determined by back-ribosylation with 10 μM [^{32}P]NAD followed by autoradiography as described previously (31).

MAG-2 membranes. The MAG-2 cell line was derived from Chinese hamster ovary-K1 cells by stable transfection with an SV40 promoter-based α_2AR expression vector as described previously (32). These cells express 1–2 pmol human α_2AR/mg membrane protein. For adenylyl cyclase assays, crude membranes were prepared by washing confluent monolayers with PBS and lysing the cells with 1 mM Tris, pH 7.4, for 15 min at 4°. Cells were scraped from dishes and pelleted at 100,000 × g for 45 min. Pellets were resuspended in buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.6) and either used immediately or quick frozen and stored at −70°. For pertussis

toxin treatment of cells, MAG-2 cells were grown overnight in the presence of 100 ng/ml pertussis toxin and harvested as described above.

Adenylyl cyclase assays. Adenylyl cyclase assays were performed as described previously (31). MAG-2 membranes were preincubated with peptides for 10–15 min on ice before being added to the reaction mixture. Assays were conducted for 15 min at 30° in the presence of 10 μM forskolin.

Data analysis. Data were analyzed using the nonlinear least-squares and two-way ANOVA methods with the computer program Prism (version 1.03, GraphPAD Software, San Diego, CA).

Results

Activity of monomeric peptides. We recently showed that dimeric peptides were substantially more active than monomeric peptides in inhibiting high affinity α_2-adrenergic agonist binding and in stimulating GTPase activity of purified G proteins (27). We wanted to determine whether monomer peptides were really active or could a small amount of dimerization of monomer peptides have accounted for the activity of the monomers in GTPase activation (27) and whether the PQ dimer peptide was more active than the PP dimer, which we had not tested. In addition, we present a simple fluorescence assay for G protein activation based on binding of methylanthranoyl GTPγS. Fig. 1 illustrates the use of mant-GTPγS in examining the potency of monomeric and dimeric α_2AR peptides. Use of carboxymethylated peptides in this study completely eliminated the possibility of dimerization. The rate constant for mant-GTPγS binding is readily quantified (Fig. 1). The rate constant increases from a control value of 0.0031 ± 0.0004 ($T_{1/2}$ 203–257 sec, five experiments) to values of 0.0087 ± 0.0015 ($T_{1/2}$ 68–93 sec, four experiments) and 0.0058 ± 0.0009 ($T_{1/2}$ 103–141 sec, four experiments) with 100 μM of the carboxymethylated peptide monomers Q-CM and P-CM, respectively. This rate constant for the fluorescence increase can be considered a measure of GDP release (29, 33). The heterodimeric P-Q peptide was the most potent in stimulating the association rate of mant-GTPγS (Table 1). The Q-Q homodimer was next most potent, and P-P was the least potent of the dimeric peptides. The P-Q and Q-Q dimers also gave the greatest fold-stimulation of the rate of mant-GTPγS binding (4- and 5-fold, respectively), whereas the other peptides, including mastoparan and the P-P dimer, gave only ~2-fold stimulation. The fact that the "native" P-Q dimer is more potent than either of the homodimers lends additional support to activity being determined by the specificity of this effect rather than increased mass or charge.

Differential pertussis toxin sensitivity. Stimulation of G_o GTPase by mastoparan has been reported to be either partially (33) or completely (34) sensitive to pertussis toxin. Because a photoaffinity derivative of the Q peptide couples to the amino terminus of G_o (24), we wanted to determine whether pertussis toxin, which modifies the carboxyl terminus, would block activation of G protein by this peptide. Q peptide stimulation of the GTPase activity of pertussis-treated G_o/G_i was not significantly reduced compared with control ($p > 0.05$ by ANOVA; Fig. 2, *top*). In contrast, the effect of P peptide is partially but significantly blocked by pertussis toxin treatment ($p < 0.001$ by ANOVA; Fig. 2, *bottom*). GTPase activation by mastoparan was also partially inhibited by pertussis toxin ($p < 0.001$, data not shown) with the extent of inhibition similar to that of P peptide. To ensure pertussis toxin treatment, we tested for residual pertussis toxin substrate by back-ribosylation with [^{32}P]NAD.

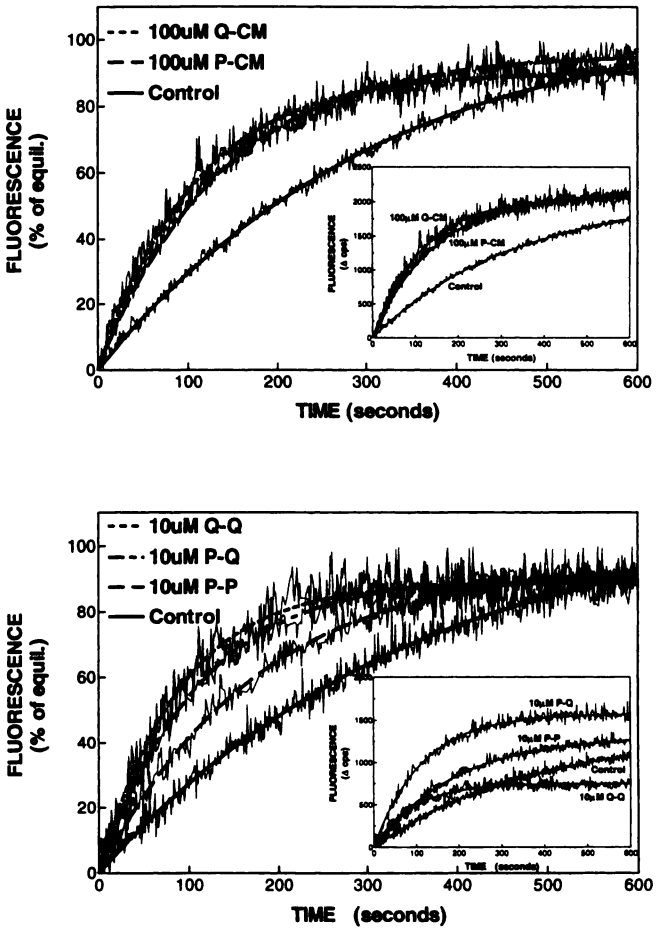

Fig. 1. Effect of α_2AR peptides on mant-GTPγS binding kinetics. Peptides derived from the α_2AR were prepared as described in Experimental Procedures. Carboxymethylated monomeric peptides (P-CM and Q-CM) or disulfide-linked dimer peptides (P-P, P-Q, and Q-Q) were incubated at the indicated concentrations with 100 nM G_o in buffer containing 50 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 1.1 mM $MgCl_2$, and 20 ppm Lubrol, pH 8. mant-GTPγS (400 nM) was added and the fluorescence monitored (excitation 280 nm, emission 440 nm) as described in Experimental Procedures. Background fluorescence due to mant-GTPγS, peptide, and G_o alone was subtracted. Fluorescence traces were smoothed with a 13-point Savitzky-Golay algorithm. Rate constants were determined by fitting the fluorescence curves to a one-phase exponential association equation using Prism (version 1.03, GraphPAD Software).

Incorporation of radioactivity was reduced to ~2% of control, indicating that the initial ribosylation was nearly complete (Fig. 2, *inset*). The lack of effect of pertussis toxin on activation of GTPase by the Q peptide is consistent with its site of action on the amino terminus of the α subunit, whereas P peptide and mastoparan seem to act, at least in part, via another site. Thus, the actions of P peptide and mastoparan involve both the carboxyl terminus (because of the pertussis toxin block) and the amino terminus [because P peptide and mastoparan block binding of Q peptide to that site (24) and mastoparan couples there (35)].

Adenylyl cyclase inhibition. We have shown that the α_{2A} receptor peptides can activate G_o and G_i as measured by increases in GTPase activity, so a logical extension of that result would be to examine the downstream effect of these G proteins (i.e., adenylyl cyclase inhibition). It has recently been shown that 5-hydroxytryptamine$_{1a}$ receptor peptides

TABLE 1

Effect of peptides on mant-GTPγS binding and GTPase activity in G_o/G_i vesicles

Fluorescence and GTPase measurements were done in buffer containing 1.1 mM total and ~30 μM free Mg^{2+} as described in Experimental Procedures. EC_{50} values for stimulation of mant-GTPγS binding to G_o were determined with G_o in 20 ppm Lubrol. EC_{50} values for peptide stimulation of bovine brain G_o/G_i GTPase were determined with asolectin-reconstituted protein as described in Experimental Procedures. Single fluorescence traces were obtained in the indicated number of experiments. GTPase experiments were performed in triplicate with the number of experiments given in parentheses. Values are mean ± standard deviation.

Peptide	G_o mant-GTPγS binding rate EC_{50}	G_o/G_i GTPase EC_{50}
	μM	
P-Q	1.2 ± 0.5 (3)	1.2 ± 0.2 (5)[b]
Q-Q	2.2 ± 0.6 (3)	1.6 ± 0.3 (9)[b]
P-P	9.3 ± 4.7 (4)	3.4 ± 1.1 (4)
P	N.D.[a]	4.7 ± 0.8 (6)[b]
P-CM	34 ± 25 (2)	16.8 ± 3.4 (3)
Q-CM	53 ± 24 (3)	21.6 ± 3.0 (3)
$Q_{monomer}$	N.D.[a]	25.3 ± 5.8 (4)[b]
Mastoparan	33 ± 16 (3)	7.8 ± 1.5 (9)[b]

[a] Not determined.
[b] Data from Ref. 27.

can inhibit adenylyl cyclase in membranes (20). Both Q and P peptides significantly inhibit forskolin-stimulated adenylyl cyclase activity (Fig. 3) in membranes from a Chinese hamster ovary cell line (MAG-2 cells) stably transfected with 1–2 pmol/mg α_2AR (36, 37). The concentrations of peptides required for adenylyl cyclase inhibition in these membrane preparations were higher than those for GTPase activation of the pure G proteins. This may be due to proteases in the membranes or decreased access of peptides to the G protein. We were not able to determine IC_{50} values for these responses, but there was significant inhibition (30–40%) at 100 μM. As with the GTPase activation, the inhibition by Q peptide was not pertussis sensitive (Fig. 3). In addition, the effect of 100 μM Q peptide was not additive with effects of α_2AR-mediated inhibition of adenylyl cyclase (Fig. 4). These data are consistent with the Q peptide effect being mediated by the same mechanism as that of the α_2AR (i.e., activation of G_i; see Discussion).

Structure-activity relations of Q peptide-induced GTPase activation. We were also interested in defining the smallest structural unit necessary for peptide modulation of G protein activity. To examine this, we prepared truncated Q peptide analogs from which amino acids were removed from either the amino-terminal or carboxyl-terminal ends. None of the truncation series peptides, including Q peptide itself, showed any significant inhibition of [^{125}I]*p*-iodoclonidine binding in Chinese hamster ovary cell membranes expressing the human α_{2a}AR (data not shown). This lack of effect of peptides on [^{125}I]*p*-iodoclonidine binding is presumably due to the absence of peptide dimers, which we have shown are required for peptide effects on agonist binding but not GTPase activation (27). This explanation is supported by the observation that Q peptide dimer decreased control binding by >40% in this series of experiments.

To examine minimal structural requirements for this activation of G proteins, we measured stimulation of lipid-reconstituted bovine brain G_o/G_i GTPase by a 100 μM concentration of each peptide analog. Under these conditions, 100 μM gives a maximal stimulation with full-length Q peptide, and

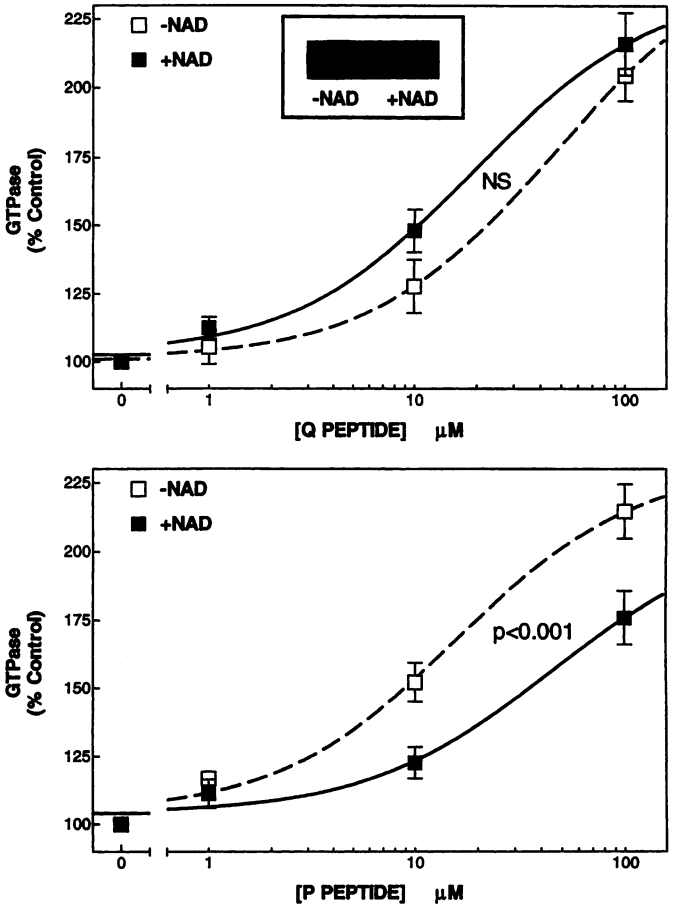

Fig. 2. Effect of pertussis toxin pretreatment on peptide-stimulated GTPase activity in purified bovine brain G_o/G_i. Bovine brain G_o/G_i was treated with 10 μg/ml pertussis toxin in the absence (□) or presence (■) of 1 mM NAD as described in Experimental Procedures. Q (*top*) and P (*bottom*) peptides were preincubated with pertussis-treated G_o/G_i and GTPase activity assessed as described. Data represent mean ± standard error of three separate experiments performed in triplicate. *Inset*, autoradiogram of the pertussis toxin-treated G_o/G_i after incubation with [^{32}P]NAD to assess the efficiency of ribosylation.

activity is linear up to 30 min for both control and Q peptide-stimulated GTPase (not shown).

Progressive decreases in GTPase stimulation were observed as amino acids were removed from the amino-terminal end of the Q peptide (Fig. 5A). Activity was markedly reduced on removal of three amino acids with stimulation decreasing from 218 ± 17% of control to 140 ± 2%. Stimulation was completely lost after deletion of five amino acids (residues 361–365 in the receptor). It is notable that three of the five arginines are in this portion of the peptide, confirming the key importance of positive charges in compounds that activate G proteins *in vitro* (18). However, charge was not the sole determinant of the interaction of peptides with G proteins. Substitution of an alanine for the lysine had no effect on GTPase activity, whereas replacement of the adjacent arginine slightly reduced activity (Fig. 5C). More compellingly, substitution of lysines for all of the arginines (Fig. 5C) substantially decreased GTPase stimulation. The arginines in this region of the receptor may play an important structural role (38) in stabilizing interactions with the backbone or side chains of G protein α and/or β subunits.

Removal of amino acids from the carboxyl-terminal end of

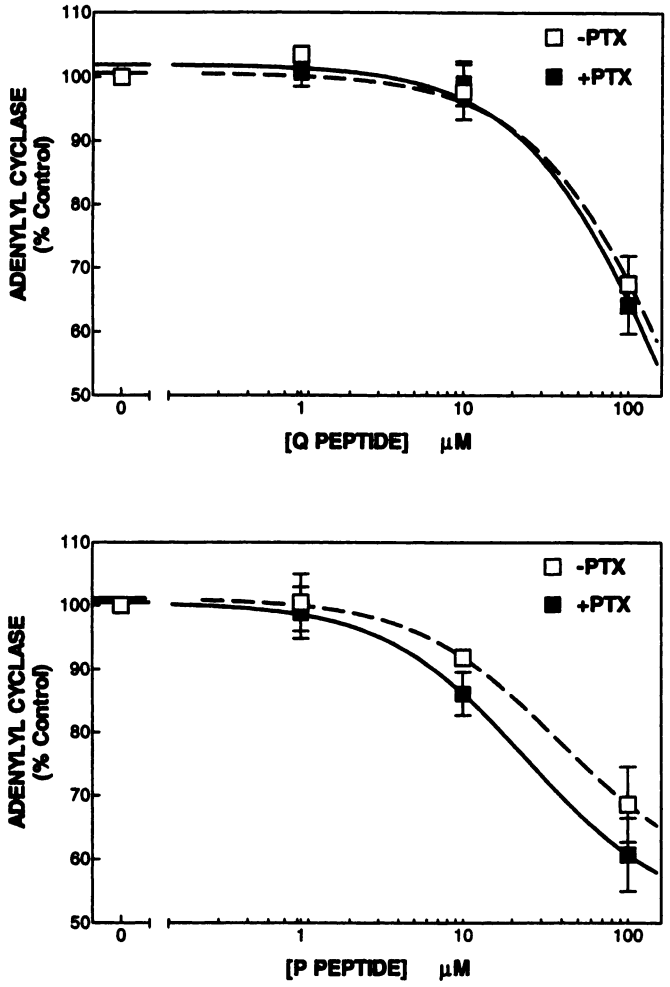

Fig. 3. Peptide effects on forskolin-stimulated adenylyl cyclase activity in pertussis toxin (*PTX*)-treated MAG-2 membranes. MAG-2 cells were grown overnight in the absence (□) or presence (■) of 100 ng/ml pertussis toxin and harvested as described. Q (*top*) and P (*bottom*) peptides were preincubated with membranes, and forskolin-stimulated adenylyl cyclase activity was measured. Data represent mean ± standard error of two to seven separate experiments performed in triplicate.

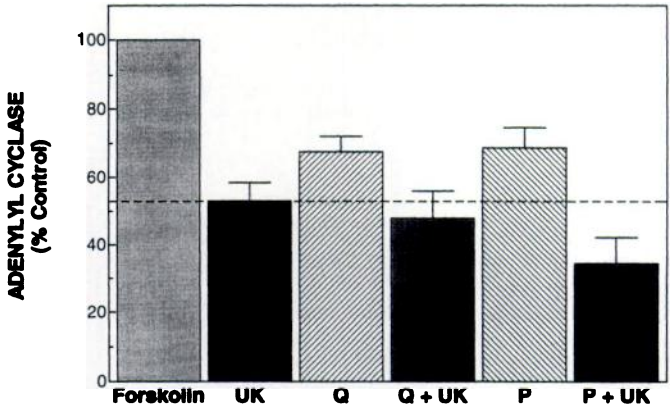

Fig. 4. Effects of Q and P peptides on forskolin-stimulated adenylyl cyclase activity in MAG-2 membranes. The effect of 100 μM Q or P peptides on forskolin stimulated adenylyl cyclase in MAG-2 membranes was measured in the absence or presence of 10 μM UK 14,304 (*UK*) as described in Experimental Procedures. Values represent the mean ± standard error of two to seven individual experiments performed in triplicate.

the Q peptide resulted in a more complex and interesting pattern of activity (Fig. 5B). Elimination of the cysteine residue caused a substantial decrease in GTPase activity, but further removal of the threonine (which corresponds to Thr373 in the α_{2A}AR) restored the activity. Further truncations again produced a decrease and then an increase in GTPase-stimulation activity. Of note, the activity was greatest when arginine or phenylalanine was the carboxyl-terminal residue. We and others have shown that the addition of hydrophobic amino acids to (13) or hydrophobic chemical modifications of (24, 39) the membrane-proximal end of receptor peptides increases their potency in G protein interactions. The decrease in activity on removal of the cysteine was not due to loss of dimer formation because modification to a serine (Fig. 5C) does not decrease activity. Also, monomeric Q peptide (40) and Q-CM (Table 1) show full GTPase stimulation at a concentration of 100 μM.

Because the new carboxyl-terminal residue in this singly truncated peptide was Thr373, we were intrigued by the possibility that the low activity of the threonine-terminated peptide was related to the observation by Ren *et al.* (41) that replacement of Thr373 with other amino acids resulted in a "constitutively" activated α_2AR. To examine the relation of our results to results with intact receptors, we prepared peptides with several amino acids substituted for Thr373. Fig. 6 and Table 2 show the ability of five such peptides to activate the G_o/G_i GTPase. All produced a similar maximum stimulation of 2.5–3.0-fold, but there were significant differences in potency. When phenylalanine, which caused a small increase in basal adenylyl cyclase inhibition in the intact receptor, was at the carboxyl terminus of the peptide, the EC_{50} was ~50% lower than for the wild-type peptide. Lysine produced a 3.3-fold increase in potency consistent with its substantial increase in basal adenylyl cyclase. In contrast, alanine had no effect on GTPase stimulation, whereas glutamate, which was second best in its ability to induce constitutive activation of receptor, caused a substantial decrease in potency of the peptide. Thus, substitutions at this position show divergent effects between the native receptor structure and the synthetic peptide, which is not consistent with this region being a simple contact site for the G protein (see Discussion).

Discussion

The results provide evidence for specific roles for regions of the i3 loop of the α_2AR. We showed differential pertussis toxin sensitivity for the P and Q peptides, indicating distinct but overlapping sites at which these peptides from the α_2AR i3n and i3c regions activate G_o. Adenylyl cyclase inhibition by Q but not P peptide seems to involve G_i activation. Finally, our structure-activity data dissect the i3c region and indicate that the the membrane-proximal and membrane-distal parts of the α_2AR i3c peptide play different roles in the interaction with G protein. The membrane distal (arginine-rich) region of the i3c peptide is absolutely required for G protein activation and may be the actual domain of the receptor involved in activation, whereas the residues in the membrane-proximal region seem to play a modulatory role.

The lack of pertussis toxin sensitivity for Q peptide combined with the sensitivity for P peptide is consistent with the idea that different intracellular loops of the receptors interact with adjacent but distinct regions on the G protein (8, 10). It supports our recent data on the localization of Q peptide

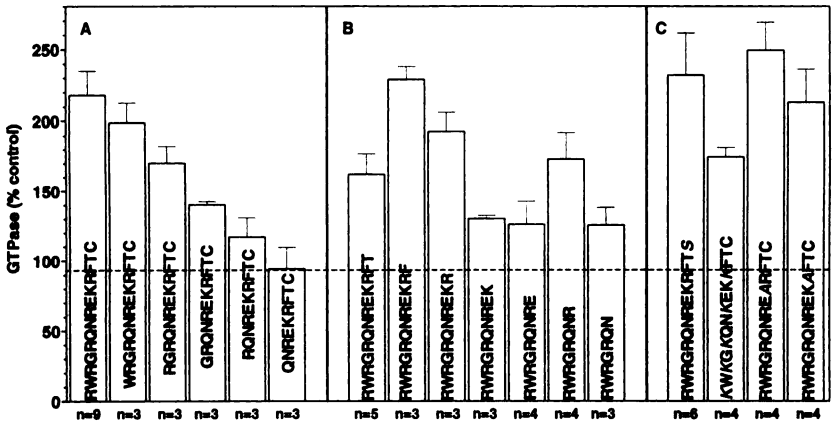

Fig. 5. Activation of GTPase by α_2AR peptides. A series of truncated or modified versions of the Q peptide were prepared and tested at 100 μM for G_o/G_i GTPase stimulation in asolectin vesicles as described in Experimental Procedures. Values are mean ± standard error of the indicated number of replicates. A, Amino-terminal truncations. B, Carboxyl-terminal truncations. C, Site-specific modifications.

TABLE 2

Effects of Thr373 mutations on activity of α_2AR receptor peptides

GTPase activity in G_o/G_i vesicles was measured as described in Experimental Procedures. Data represent the average of three or four separate experiments performed in triplicate.

Amino acid	V_{max}	K_m
	% of control	*μM (95% CI)*
K	293	26 (18–37)
F	236	38 (18–80)
T (wt)	273	88 (54–142)
A	257	90 (33–249)
E	269	147 (65–334)

binding to the amino terminus of $G_{o\alpha}$ (24), whereas pertussis toxin modifies the carboxyl terminus (42). Mastoparan interacts with both the amino- and the carboxyl-termini because it is able to form a disulfide cross-link to cysteine 4 in G_o (35), yet its actions are blocked by both pertussis toxin and a carboxyl-terminal antibody to G_i (34). Also, both mastoparan and P peptide inhibited binding of Q-DAP to the amino terminus of α (24). Thus, the sites for P peptide and mastoparan seem to span the carboxyl and amino termini, whereas Q peptide interacts more specifically at the amino terminus.

Adenylyl cyclase inhibition by these α_{2A}AR peptides was expected, but only Q peptide showed properties expected from previous biochemical studies; i.e., inhibition was not additive with that of α_{2A} receptor. Similar conclusions were drawn regarding the mechanism of adenylyl cyclase inhibition by an i3c peptide from the 5-hydroxytryptamine$_{1a}$ receptor (20). In contrast, the adenylyl cyclase inhibition by P peptide is at least partially additive with that of the α_2AR, indicating that the P peptide has nonspecific effects on the adenylyl cyclase in the membranes in addition to its ability to activate G_i. Similar nonspecific effects have been reported for some other receptor-derived peptides (22), indicating that the specificity of peptide effects must be evaluated on a case-by-case basis.

Specific structural conclusions that we can derive from our peptide analog studies include the following. (a) Further evidence is provided that the proposed BBXB motif (21) is not required for efficient G protein activation (see also Ref. 27). Retention of GTPase-stimulation activity on elimination of the lysine residue in the dibasic pair KR in the Q peptide provides further evidence against this requirement. The retention of activity without a BB structure will be helpful in trying to design more stable peptides for interfering with receptor/G protein interactions. Elimination of the BB removes a potentially labile site of proteolytic digestion. (b) There is a specific function of arginines beyond just the positive charge. The multivalent potential of arginines has been recognized as being important in maintaining protein structure (38). Our results suggest that it may be important for intermolecular as well as intramolecular interactions. (c) The membrane-proximal region of the α_2-adrenergic i3c peptide does not seem to be absolutely required for GTPase-stimulation activity yet seems to play an important role in determining the affinity and/or conformation of the intracellular loops. It will be interesting to test these results with mutagenesis in the context of the intact receptor structure.

Although many cationic compounds can activate G proteins, the specificity of effects of the Q (i3c) peptide from the α_2AR is supported by the highly restricted site of labeling of the G_o α subunit (24) and β subunit (25) by a photoaffinity derivative of Q peptide. In addition, we have shown that labeling of both subunits can be completely blocked by low micromolar concentrations of unlabeled peptide. The site of interaction of Q peptide on the amino terminus of the α subunit probably overlaps with that for mastoparan (34, 35) but does not seem to be identical (24).[3] Very interestingly, the Q-peptide site on the carboxyl terminus of the β subunit is in close proximity to the amino terminus of the α subunit in the recently described crystal structure of heterotrimeric G proteins (43, 44).

The importance of the i3n and i3c regions in the coupling of heptahelical receptors to G proteins has been established for several different receptor types (5, 6, 45–47). However, the second cytoplasmic loop (8, 12, 45–49) and carboxyl-terminal tail (8, 47) of some receptors also play a role. The i3c region of the m1 muscarinic receptor has been studied in detail in the activation of phospholipase C (50). Two arginines at positions 16 and 18 from the predicted membrane-spanning domain seemed to be important for signalling (TM6).[4] Two of the crucial

[3] The site of diazopyruvoyl-Q peptide labeling of the G_o α subunit was proposed to be on Cys3, but our mass calculations in that work (24) neglected to account for the removal of methionine on myristoylation of the glycine. Thus, the labeled amino-terminal fragment of $G_{o\alpha}$ did include a pyridoethylated cysteine, indicating that the diazopyruvoyl-Q peptide was incorporated at a site distinct from the Cys3 that was labeled by mastoparan (35).

[4] The limits of the six-transmembrane-spanning domains in the human m1 muscarinic and porcine α_{2A}-adrenergic receptors were predicted using the Goldman Engleman Steitz algorithm in Protean (version 1.23, Lasergene Software).

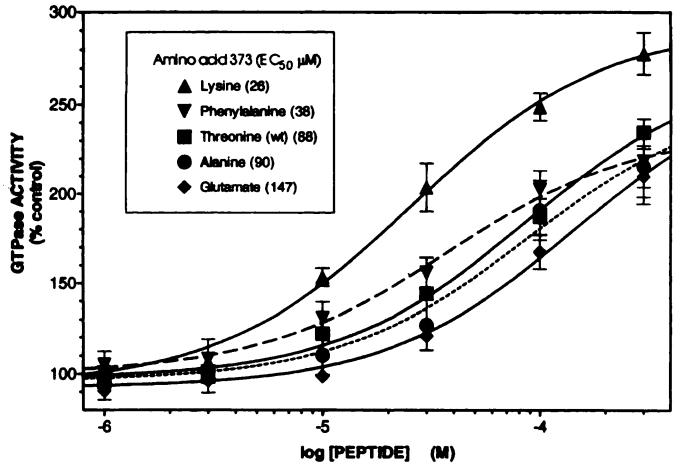

Fig. 6. GTPase activation by α_2AR peptides with mutations in Thr373. The concentration dependence of GTPase stimulation by five tridecapeptides with different carboxyl-terminal residues is shown. Threonine is indicated as *wt* because that is the residue present in the native α_{2A}AR. Values are mean ± standard error of three or four separate determinations performed in triplicate, and fitted curves were determined by nonlinear least-squares fitting using Prism. *Numbers in parentheses after amino acid names*, EC_{50} value.

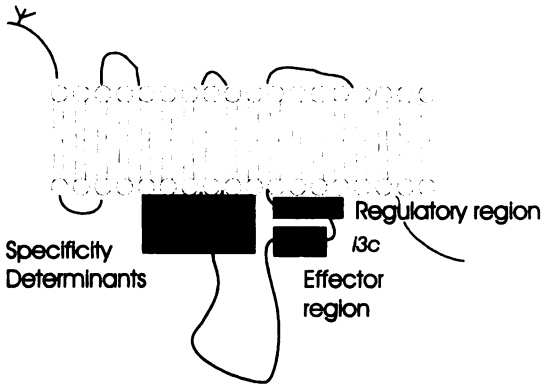

Fig. 7. Proposed functions of intracellular loops of the α_{2A}AR. Based on mutagenesis and peptide structure-activity studies, the i3c seems to include a possible activator domain (or effector region) that directly activates the G protein. Closer to the membrane, the regulatory domain containing the Thr373 would regulate exposure of the activator to the G protein. The i2 and i3n are involved in specificity for G proteins because numerous mutations in these regions lead to loss of specificity.

arginines (RWR) in our Q peptide are at positions 15 and 17 from the predicted TM6 domain. Thus, in both the m1 receptor mutagenesis and in our study, the BBXB structure 7 or 8 residue from TM6 seems to be less critical than the cluster of basic residues 10 amino acids farther from the membrane. This important basic region thus seems to represent an effector region that is required for activation but perhaps is not as crucial for regulation or specificity. Further support for this model derives from a deletion mutation in the m4 muscarinic receptor (51) that retains affinity for G_i but loses its ability to activate the G protein. That deletion removes four basic residues 13–16 positions from the membrane as well as the first residue in the KERK sequence near TM6.

In contrast, the amino terminus of i3 (i.e., i3n) of the α_{2A}AR has recently been implicated in specificity of G protein coupling (52). Interestingly, in the i3n region of the βAR, it is the hydrophobic residues and not the basic residues that are essential for G_s activation (53). These data are interesting in light of our observation that the potency of the α_2AR i3c peptide can be enhanced by adding either the i3n peptide (27) or a hydrophobic photoaffinity group (24). Similarly, hydrophobic residues in the i2 loop of human m1 muscarinic and β_2ARs are required for efficient G protein coupling (45). Thus, the hydrophobic residues in the i3n region of the α_{2a}AR and i2 regions of m1 muscarinic and β_2ARs may be responsible for G protein binding and specificity rather than activation.

The complex effects of truncation of the membrane-proximal region of the i3c peptide are suggestive of a modulatory rather than direct role in G protein activation. Perhaps more convincingly, mutations in this region have been implicated in the constitutive activation of α_{1b}ARs, α_{2a}ARs, and β_2ARs (54–56) and the lutropin (57) and thyrotropin (58) receptors. A model based on the data presented here as well as numerous mutagenesis studies from the literature (cited above) is shown in Fig. 7. We hypothesize that the i2 and i3n regions are involved in receptor specificity for G proteins, whereas the i3c region has two functions. The membrane-distal region of i3c carries the positive charge required for activation of many G proteins, whereas the membrane-proximal region regulates the exposure of this effector domain. It will be important to test this model with additional studies with synthetic peptides and targeted mutagenesis.

In summary, we propose distinct roles for the membrane-proximal and membrane-distal portions of the α_{2a}AR i3c region. Although our data show some of the limitations of the use of synthetic peptides as probes of receptor/G protein function, they have permitted us to develop hypotheses that may be further tested with mutagenesis studies.

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