G Protein Antagonists

A NOVEL HYDROPHOBIC PEPTIDE COMPETES WITH RECEPTOR FOR G PROTEIN BINDING*

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A substance P (SP) analog, [D-Pro4,D-Trp7,9,10]SP4-11, is known to inhibit the actions of various structurally unrelated messenger molecules as well as SP. Our studies on the effects of this peptide on the regulation of purified G proteins by receptor showed that at least some of the biological effects of the peptide can be explained by the ability of the peptide to block the activation of G proteins by receptors. Here we report that a novel truncated SP-related peptide, pGlu-Gln-D-Trp-Phe-D-Trp-Phe-Met-NH2, inhibited the activation of G, or G, by M2 muscarinic cholinergic receptor (M2 mAChR) or of G, by β-adrenergic receptor in the reconstituted phospholipid vesicles, assayed by receptor-promoted GTP hydrolysis. The inhibition by the peptide was apparently reversible and competitive with respect to receptor binding to G proteins; the inhibition could be overcome by increasing the concentration of receptor in the vesicles and was not altered by changes in the concentration of G protein. The competing effects of the peptide were used to analyze the effect of agonist on receptor-G protein interaction. The concentration change of muscarinic agonist did not alter the inhibitory effects of the peptide on M2 mAChR-promoted GTPase by G, which is consistent with the idea that the agonist increases the regulation efficiency of the receptor but does not alter its affinity for G proteins. This new group of compounds (G protein antagonists) is a promising tool to study receptor-G protein interaction quantitatively.

Antagonists for hormone action are typically receptor antagonists, molecules that bind to the same site on the receptor as the hormone and block its effects (1). These antagonists have been employed in the analysis of the functions of hormones (or receptors) to analyze ligand-receptor interaction and to treat various diseases. It is well established that many receptors are coupled to GTP-binding regulatory proteins (G proteins)1 that in turn regulate various effectors (see Refs. 2–4 for review). Compounds that interact with G proteins and block their activation by receptors would be very useful to delineate the functions of G proteins and G protein-coupled receptors. In addition, such a G protein antagonist would broadly inhibit the effects of various agonists whose receptors function via G proteins. In this article, we describe such compounds that seem to function as G protein antagonists in reconstituted systems and perhaps in cells.

There have been several reports of compounds that disturb receptor-G protein interactions. Anionic compounds like heparin (5–7), toxic peptides such as mastoparan (8), and peptide fragments of G protein-coupled receptors (9–13) or G proteins (14) are reported to disrupt the receptor-G protein interaction. This conclusion rests mainly on the observation that such compounds inhibit the ability of G proteins to increase the affinity of receptor for an agonist. The binding site(s) and the mechanism of inhibition by the anionic compounds are not yet clear. Although it seems reasonable to assume that peptides from receptors or G proteins can interact with G proteins or receptor, respectively, these reports have not shown the mechanism of inhibition for the receptor-G protein interactions. For any of these compounds to be useful as specific antagonists of receptor/G protein action, it must be shown that they interact only with G proteins or receptors and that they have no intrinsic ability to stimulate GDP-GTP exchange of G proteins.

We have reported earlier (15) that mastoparan, an amphiphilic tetradecapeptide toxin from wasp venom, can interact directly with G proteins. Mastoparan catalyzes nucleotide exchange of G proteins in a manner similar to that of receptors in vitro (8, 15). Mastoparan has a number of effects on cells, including histamine secretion from mast cells and proliferation of Swiss 3T3 cells. These effects can be explained by the activation of G proteins by mastoparan (16–22). Many of the activities of mastoparan both in vitro and in vivo are sensitive to pertussis toxin, which ADP-ribosylates G, and G, and renders them insensitive to hormonal regulation. Based on these studies, we have proposed that the target of mastoparan in mast cells is a G protein (15, 18). The same mechanism has been proposed to explain stimulation of secretion in mast cells by other amphiphilic compounds such as substance P (SP), compound 48/80, bradykinin, and MCD-peptide (23–26).

It has been reported that benzalkonium chloride (BAC) and a SP-related peptide, designated GPAn-1 (see Table I for sequence), can inhibit the secretion of histamine from mast cells stimulated by amphiphilic compounds (compound 48/80-GTPyS, givosine 5'-O-(thiodiphosphate); BAC, benzalkonium chloride; QNB, quinuclidinyl benzilate; Heps, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid; G, G protein α subunit; βγ, G protein βγ subunits; DTT, dithiothreitol; SP, substance P; D2, subtype 2 dopamine receptor; P, inorganic phosphate.

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80, SP) (27–29). We have shown that BAC inhibits the effects of mastoparan on G protein whereas BAC alone activates G protein (8). In addition, GAPt-1 inhibited the stimulation of G, and G, by mastoparan. Although it has been proposed that BAC and GAPt-1 work as classical receptor antagonists on mast cells, we thought that activities of BAC and SP-related compounds could be explained more reasonably by assuming that they modulate the receptor-G protein interaction. In fact, GAPt-1 is known to inhibit amylase secretion by various structurally unrelated peptides such as SP, bombesin, and cholecystokinin (30). To address this, we studied the effects of BAC and GAPt-1 on receptor-promoted activation of G proteins in vitro.

Here we report that a novel SP-related compound (GAPt-2, see Table I for the sequence) as well as GAPt-1 and BAC inhibit the ability of M2 muscarinic cholinergic receptor (M2 mAChR) to activate G proteins by interacting with G proteins (but not with receptor) in vitro. One application of this type of compound is more detailed analysis of receptor-G protein interactions in vitro. We show that an agonist does not increase the affinity of the receptor for G protein (G,) in the presence of guanine nucleotides. Another potential application is the analysis of signal transduction in cells. The functions of GAPt-1 in vivo may be explained by the peptide acting as a direct inhibitor of G proteins.

EXPERIMENTAL PROCEDURES

Materials—BAC, carbachol (CCh), and isoproterenol were obtained from Sigma. [γ-32P]GTP, [35S]GTPyS, [3H]quinuclidinyl benzilate ([3H][QNB]), and [125I]iodocyanopindolol were purchased from Du Pont-New England Nuclear. All lipids were obtained from Avanti Polar Lipids.

SP-related Peptides—All SP-related peptides shown in Table I were synthesized and purified as described previously (31). GAPt-2 was prepared from GAPt-3 by treating with acetic acid at room temperature for 1 week (32) and was then repurified by reverse phase high performance liquid chromatography. Peptides GAPt-1 and -3 were dissolved in dimethyl sulfoxide. GAPt-2 and -4 showed essentially no solubility in H2O even at 0.1 mM.

Purification of Proteins—The human M2 mAChR was expressed in SF9 cells (33) and purified by affinity chromatography as described by Higashijima and Haga (34). Purified turkey β-adrenergic receptor mutant (C116L) was a gift from Parker and Ross (33). G, and G, were purified as the resolved G,αβ and Gββ subunits from bovine brain (35) and as the Gαβγ trimer from rabbit liver (36), respectively, and were stored in 50 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 200 mM NaCl, 10 μM GDP, 0.1% Lubrol, and 800 pg deoxycholate in 50 μl. M2 mAChR, G, or G, alone was also mixed with the lipid dispersion as described above. These mixtures were then gel-filtered on a column (1 mM) of Ultrogel AcA 34 with 20 mM Hepes (pH 8.0), 2 mM MgCl2, 1 mM EDTA, and 100 mM NaCl to separate vesicles from detergent micelles, unassociated proteins, and small molecules (36). About 250 μl of eluate was collected as receptor-containing vesicles in the void volume (32); unassociated receptor and G proteins were eluted later with the detergent. The amounts of active receptor and G protein in the reconstituted vesicles were determined by [3H]QNB binding to the M2 mAChR, [125I]iodocyanopindolol binding to the avian β-adrenergic receptor, and [35S]GTPyS binding to G proteins as described previously (9, 33). The recovery of G proteins and receptor in the vesicles after reconstitution was affected largely by the initial concentrations of receptor and G proteins. Recovery of receptor (3–20%) tended to increase with higher concentrations of added receptor and to decrease with higher concentrations of added G proteins. Recovery of G proteins varied from 20 to 40%. To get a series of [3H]QNB binding G protein or receptor concentration held constant while varying the concentration of the other protein, we usually made eight vesicle preparations and picked four appropriate preparations. The actual concentration of receptors and G proteins in the utilized vesicles is indicated for each experiment. The vesicles were treated with 5 mM EDTA at 1 hr at 0°C before use to enhance the effects of receptor, unless otherwise noted (33).

In Vitro Assays—Receptor-stimulated GTPase activity was measured in 50 μl of buffer A (20 mM Tris (pH 8.0), 0.1 mM NaCl, 1 mM EDTA, 2 mM MgCl2, 1.5 mM GDP, 0.2 mg/ml BSA) containing 30 nm [γ-32P]GTP (~700 cpm/fmol), 0.5 mM residual DTT (from 5 μl of reconstituted vesicles) and agonist at 20°C (for G,) or 30°C (for G, and G). The high concentration of GDP (1.5 μM) was added to make the effects of agonist clear according to Florio and Sternweis (39). The concentration of agonist was 1 mM CCh for M2 mAChR (unless mentioned otherwise) or 10 μM isoproterenol for avian β-adrenergic receptor [3H]PITP; released after 15–20 min was assayed as reported (15), except that Tris instead of Hepes was used. Tris gave a lower background in the [3H]PITP assay. For the inhibitory effects of various compounds on receptor-stimulated GTP hydrolysis by G proteins, reconstituted receptor-G protein vesicles (5 μl) were preincubated for 1 hr, if not described otherwise, on ice with various concentrations of antagonist in 40 μl of buffer A containing agonist and residual DTT (without GTP). GTPase experiments were initiated by the addition of 10 μl of buffer A containing γ[32P]GTP (final 30 mM). Final concentrations of receptor, G protein, and compound are shown in each figure and table. GTPase data are expressed as molar turnover numbers (amounts of P, released for 1 min/amounts of G proteins in the vesicles in reaction). The inhibition of receptor-stimulated GTPase activity by various compounds was expressed relative to the activity in the absence of these compounds.

The effect of GAPt-2 on receptor-independent GTPase activity of reconstituted G, vesicles was measured in 50 μl of buffer B (50 mM Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 10 mM MgCl2, 100 μM GTP, and 0.1% Lubrol, and 500 nM peptide-bound fluorescent G protein; was assayed on nitrocellulose filters). Control experiments were performed with boiled vesicles (5 min), and the specific GTPyS-binding was obtained as the difference between intact and boiled vesicles.

The effect of GAPt-2 on ligand binding to reconstituted M2 mAChR vesicles (without G proteins) was measured at 30°C in 50 μl of buffer A without GDP, but with 2.5 μM [3H]QNB and various concentrations (0–10 mM) of CCh. After 60 min, reactions were stopped by filtration over Whatman GF/C filters, which were subsequently rinsed with 20 ml of wash buffer (20 mM Tris (pH 8.0), 10 mM MgCl2, and 100 mM NaCl). Specific [3H]QNB binding was defined by the difference between the values with and without the receptor antagonist atropine (2 μM).

Data Analysis—Data in Figs. 2A, 4A, 5, and inset of 6 were fit nonlinearly to the equation, y = A/(C/(x + K)), where y is observed turnover number, x is the concentration of inhibitor (peptide), A is the maximum turnover number without inhibitor, K is IC50 of the inhibitor, and p is the Hill coefficient. Data in Figs. 2B and 3B were fit to y = A/(x/(x + K)), where y is observed turnover number, x is the concentration of receptor, K is EC50 of the activation, and A is the maximum turnover number. Fig. 6 was fit to y = A/(C/(x + K)), where y is an observed turnover number, x is the concentration of agonist, K is IC50 of the concentration of agonist, and p is the Hill coefficient.

RESULTS

Effect of BAC and SP Analogs on G Protein Activation by Receptors—SP did not show any effect on receptor- or mastoparan-promoted GTP hydrolysis by G proteins (data not

T. Higashijima, unpublished data.
shown). GPAnt-1, however, showed clear inhibition of receptor-promoted GTP hydrolysis, with an IC_{50} of 40-50 μM for both G, and G_{o} (Fig. 1, A and B, respectively). GPAnt-1 also inhibited mastoparan-promoted GTP hydrolysis with similar potency. The peptide alone, however, promoted GTP hydrolysis by G, and G_{o} by 5- and 8-fold at 100 μM when assayed without receptor (Table I).

The structure-activity relationships of GPAnt-1 analogs revealed that removal of the N-terminal d-Pro (GPAnt-3) or the further acetylation of the N-terminal Gln (GPAnt-4) did not increase potency, as shown in Fig. 1, A and B, for G, and G_{o}, respectively. We noticed, however, that GPAnt-3 showed greater inhibitory activity with longer storage in solution, suggesting that pyroglutamation could be responsible for the enhanced activity. In fact, as seen in Figs. 1, A and B, GPAnt-2 was 6- and 3-fold more potent than GPAnt-1 for the inhibition of M2 mAChR-promoted GTP-hydrolysis by G, and G_{o}, respectively. GPAnt-2 alone did not promote any GTP hydrolysis by G, and G_{o} (Table I). GPAnt-2 also inhibited mastoparan-promoted GTP hydrolysis by G, and G_{o} with 10- and 2-fold higher potency than GPAnt-1, respectively (data not shown).

BAC inhibited the M2 mAChR-promoted GTP hydrolysis by G, with an IC_{50} of approximately 4 μg/ml. No inhibitory effect on the receptor-promoted GTP hydrolysis by G_{o} was demonstrable at concentrations less than 100 μg/ml (data not shown). Accordingly, inhibitory effects by BAC seem to be very selective for G, over G_{o}, consistent with our previous report for mastoparan-promoted GTP-hydrolysis by G, and G_{o}. GPAnt-2, however, activated G, at ~30 μM/μl (8). Detailed analysis of the inhibitory mechanism was not pursued because of these apparent complexities.

**Mechanism of the Inhibition**—Because GPAnt-2 was the most potent inhibitor among peptides that we tested and showed no activity as an agonist, we studied its mechanism of inhibition of M2 mAChR-promoted GTP hydrolysis. We first examined the reversibility of the inhibition, as shown in Table II. The M2 mAChR-G, vesicles were preincubated for 1 h with 25 μM GPAnt-2, which inhibited more than 80% of GTP hydrolysis in the vesicles. Dilution of the mixture to reduce GPAnt-2 to 2 μM nearly abolished the effect of the peptide. The results support the idea that there was no irreversible damage to the vesicles or to the interaction between receptor and G proteins by GPAnt-2. In addition, this experiment demonstrates that preincubation of the peptide with vesicles is not required for the inhibitory effects of GPAnt-2.

We then examined whether the peptide blocked the interaction between receptor and G proteins. If GPAnt-2 competed with M2 mAChR for interaction with G proteins, changes in the concentration of receptor would be expected to alter the apparent potencies of the inhibitor. For this purpose we co-reconstituted M2 mAChR and G proteins into phospholipid vesicles and removed unreconstituted proteins by gel-filtration on an Aca 34 column. We could keep the concentration of G protein fairly constant (3-4 nM for G, and 3-5 nM for G_{o}) while changing the concentration of M2 mAChR over 30- and 60-fold for G, and G_{o}, respectively. As shown in Figs. 2A and 3A for G, and G_{o}, respectively, the IC_{50} of the peptide increased with increasing concentrations of M2 mAChR. The Hill coefficient of inhibition was approximately 1.6 (1.4 ± 0.4 for G, and 1.9 ± 0.7 for G_{o}). Figs. 2B and 3B are

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**Table I**

<table>
<thead>
<tr>
<th>Primary structures of substance P (SP) and its N-truncated analogs</th>
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<tbody>
<tr>
<td><strong>SP</strong></td>
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<tr>
<td><strong>GPAnt-1</strong></td>
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<tr>
<td><strong>GPAnt-2</strong></td>
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<td><strong>GPAnt-3</strong></td>
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<td><strong>GPAnt-4</strong></td>
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*10 and 100 μM of GPAnt-1 increased the turnover number of GTP hydrolysis from 0.05 to 0.05 and 0.24 min⁻¹, respectively, for G, and from 0.07 to 0.12 and 0.36 min⁻¹, respectively, for G_{o}. On the other hand, 10 and 100 μM of GPAnt-2 showed almost no effects (0.04 and 0.04 min⁻¹, respectively, for G, and 0.06 and 0.05 min⁻¹, respectively, for G_{o}). These assays were performed as reported (4, 5) for reconstituted G protein vesicles in the absence of GDP.*
TABLE II
Reversibility of the inhibition of GPAn-2 of the GTPase activity of G, induced by M2 mAChR

<table>
<thead>
<tr>
<th>Concentration of GPAn-2</th>
<th>Initial incubation</th>
<th>GTPase assay</th>
<th>Turnover number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td></td>
<td>10⁻²/min</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9.71</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>2.6</td>
<td></td>
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<tr>
<td>2.5</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>20</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>3.5</td>
<td></td>
</tr>
</tbody>
</table>

Replots of the data in Figs. 2A and 3A, respectively, showing Schild plots as insets. Both Schild plots gave a slope of about 1.5 (1.4 for G and 1.6 for G, which corresponds to the Hill coefficient of ~1.6. Three independent experiments gave a value of pA₂ of 2.2 ± 0.5 for G, and 3.0 ± 1.1 µM for G. These results support the hypothesis that GPAn-2 competes with M2 mAChR for interaction with G and G.

The effect of GPAn-2 was further examined as a function of the concentration of G, to confirm that the effective site of the peptide is only on the G protein and not the M2 mAChR. If the peptide binds only to the G protein to block receptor-G protein coupling, then the inhibitory effects of the peptide should not be affected by changes in concentration of G proteins. For this purpose, we reconstituted M2 mAChR and G, and kept the concentration of M2 mAChR fairly constant (0.24–0.42 nM) while changing the concentration of G, 10-fold (5–52 nM). As shown in Fig. 4, the inhibitory effects of the peptide were not altered by changes in concentration of G, as long as the concentration of M2 mAChR was kept constant. The small shift of IC₅₀ at 52 nM G protein (from 9.9 to 12.3 µM, see Fig. 4) can be explained very well by the small increase of receptor concentration (from 0.24 to 0.42 nM); calculated IC₅₀ values based on Fig. 3 are 10.4 and 12.3 µM at 0.24 and 0.42 µM M2 mAChR, respectively. The data support the idea that there is no functional interaction between GPAn-2 and M2 mAChR at least at the concentration range examined in this study.

In additional experiments not shown, we could exclude other possible causes of the inhibitory effects by GPAn-2 as follows. (i) GPAn-2 does not interfere with the binding of ligands to the receptor. Effects of the peptide on the ligand binding to M2 mAChR were studied by measuring the binding of [³H]QNB, an antagonist, with M2 mAChR vesicles (reconstituted without G proteins) at different concentrations of CCh. The IC₅₀ of CCh for [³H]QNB binding was 750 µM under our experimental conditions, and the addition of 20 µM GPAn-2 affected neither the IC₅₀ (750 µM) nor [³H]QNB binding itself. (ii) GPAn-2 does not interfere with the interactions of G protein and guanine nucleotide. The guanine nucleotide exchange of G proteins was studied by GTP hydrolysis and GTPγS binding to G, (reconstituted without M2 mAChR) in the presence or absence of 20 µM GPAn-2. The peptide did not alter the turnover number of GTP hydrolysis by G, 0.065 and 0.061 min⁻¹ at 20 °C, with and without GPAn-2, respectively, the rate of GTPγS binding (0.051 and 0.045 min⁻¹ at 20 °C with and without GPAn-2, respectively), or the total GTPγS binding. Hence the peptide has no effect, in the absence of receptor, on the interaction of the G protein with guanine nucleotide. These results also support the notion that GPAn-2 does not disturb the interaction between G protein α and β subunits, since the dissociation of subunits will increase the rate of GDP-GTP exchange of G, under these conditions (40).

Effect of GPAn-2 on β-Adrenergic Receptor-G, Vesicles—GPAn-2 was also effective in inhibiting β-adrenergic receptor-promoted GTP hydrolysis by G, (Fig. 5). Although the concentration of β-adrenergic receptor could not be changed over a wide range, due to the limited availability of receptor, the data are consistent with the idea that GPAn-2 competes with receptor for binding to G proteins.

Concentration Dependence of CCh for the Inhibitory Effects of GPAn-2 on M2 mAChR-G—Because GPAn-2 apparently competes with receptor for binding to G proteins, the peptide may be a powerful tool to study the receptor-G protein interaction. It is widely accepted that the affinity of receptors for G proteins is increased by the binding of agonist in the
hydrolysis by concentration of M2 mAChR constant while varying that of Go. GTP hydrolysis by Go was assayed. Data were analyzed as in Fig. 2A. The concentrations of reconstituted M2 mAChR and Go in the reaction mixture were: O, 240 pm and 3 nm; ●, 91 pm and 3 nm; V, 364 pm and 4 nm; ▼, 1514 pm and 6 nm, respectively. 100% turnover number (after fitting) and Hill coefficient for each vesicle preparations were: 0.034 min⁻¹ and 1.7 ± 0.1 (●), 0.051 min⁻¹ and 2.3 ± 0.3 (V), 0.061 min⁻¹ and 2.3 ± 0.7 (▼), respectively. B is the replot of data from A, as in Fig. 2. O, without peptide; ●, 1 μM; ▼, 2 μM; ▼, 3 μM; □, 5 μM; ■, 7 μM; △, 10 μM; ▲, 20 μM; ○, 30 μM of peptide. The inset is the Schild plot of data from B (at turnover number of 0.03 min⁻¹). A pA2 value of 3.1 and a slope of 1.56 were obtained (r = 0.98).

The concentrations of CCh (agonist) were: O, 0; ●, 1 μM; ▼, 3 μM; ▼, 10 μM; and □, 30 μM. The turnover number of GTP-hydrolysis of Go is plotted versus the concentration of CCh. The EC50 of CCh and maximum turnover number are 0.97 μM, 0.031 min⁻¹ (O), 0.90 μM and 0.028 min⁻¹ (●), 0.79 μM and 0.027 min⁻¹ (V), 1.9 μM and 0.014 min⁻¹ (▼), respectively. The inset is a replot of the data. The concentrations of CCh were: O, 0; ●, 1 μM; ▼, 10 μM; ▼, 100 μM; and □, 1 mM. The IC50 and Hill coefficient from curve fitting are 9.1 μM and 1.7 (O), 8.3 μM and 2.7 (●), 8.1 μM and 2.6 (V), 9.0 μM and 2.2 (▼), and 9.6 μM and 2.4 (□), respectively.

absence of guanine nucleotides, and this interaction is weakened by the addition of guanine nucleotides (2). Is the affinity of receptor for G proteins increased by an agonist in the presence of guanine nucleotides? This question was examined by using GPAnt-2 as a competitive inhibitor of receptor. GTP hydrolysis by Go, promoted by M2 mAChR was measured in the presence of increasing concentrations of CCh (agonist) and GPAnt-2 (Fig. 6). If the agonist increases the affinity of receptor for G protein, the apparent inhibitory effect of GPAnt-2 would be expected to decrease with increasing agonist. The inset of Fig. 6 shows, however, that the IC50 was independent of the concentration of CCh. As long as the total concentration of receptor ([agonist-bound receptor] + [ligand-free receptor]) is constant, the change in the fraction of agonist-receptor does not alter the potency of the antagonist (GPAnt-2). This observation supports the idea that affinity
of M2 mAChR for G<sub>4</sub> is not affected by CCh in the presence of guanine nucleotides.

**DISCUSSION**

It has been reported that GPAnt-1 (10 μM or more) inhibits the secretion of histamine from mast cells stimulated by basic compounds (Refs. 28 and 29; confirmed by us for mastoparan SP). However, GPAnt-1 caused weak secretion at high concentrations (30 μM or more) (Ref. 28; confirmed by us). These effects of GPAnt-1 in <i>vivo</i> are consistent with effects on G proteins observed in <i>vitro</i>. The present investigation reveals that GPAnt-1 inhibited the ability of M2 mAChR (or mastoparan<sup>4</sup>) to activate G<sub>4</sub> and G<sub>2</sub> (Fig. 1, A and B), whereas at high concentrations the GPAnt-1 activated these G proteins in the absence of receptor in <i>vivo</i> (Table I). A very good correlation was also observed between Bac-induced inhibition of histamine secretion in <i>vivo</i> (Ref. 27; confirmed by us) and its inhibition of mastoparan- or M2 mAChR-promoted GTP hydrolysis by G<sub>4</sub> (8).

Among the peptides we investigated, GPAnt-2 was found to be the most potent inhibitor of the M2 mAChR-promoted GTP hydrolysis by G<sub>4</sub> and G<sub>2</sub> (Fig. 1) and did not promote GTP hydrolysis by itself (Table I). Detailed analyses of the inhibitory mechanism revealed that GPAnt-2 competes with M2 mAChR for the interaction with G proteins. We conclude that the peptide interacts with at least a part of the receptor-binding domain on G proteins to block the effects of receptor. From Child plots, the peptide interacts with G<sub>4</sub> and G<sub>2</sub> with similar potency (Figs. 2 and 3, insets). The Hill coefficient calculated for the inhibition is approximately 1.5, suggesting that one or two molecules need to bind per G protein to inhibit the effects of receptor. The Hill coefficient, however, has a tendency to increase with a high concentration of GPAnt-2 required to inhibit, which might suggest that the peptide interferes with the receptor-G protein coupling by other mechanism(s) than described above, especially at the high concentration of GPAnt-2. This peptide appears to interact with G<sub>4</sub> as well (Fig. 5). This observation raises the possibility that the binding site of the peptide might include βγ subunits of G proteins, which seem to be common to all G proteins. However, three different batches of βγ subunits from bovine brain or rabbit liver (anticipated to be composed of different subunits forms) did not influence the effects of the peptide (data not shown). Studies utilizing preparations of pure β and pure γ subunit isoforms will be necessary to determine the role of βγ subunits in the interaction of the peptide with G proteins.

The competition between receptor and GPAnt-2 for binding to G proteins allowed the effects of GPAnt-2 to be used to analyze receptor-G protein interactions in the same way that receptor antagonists have been used for quantitative analyses of hormone-receptor interaction. Many G protein-coupled receptors interact with specific G proteins in <i>vivo</i> but appear to have less selectivity when studied in <i>vitro</i> (3). These apparent discrepancies could be explained by quantitative analyses of the coupling. Thus specificity of receptor-G protein coupling needs to be analyzed in terms of efficacy and affinity of the coupling. GPAnt may provide a way to analyze the affinity and efficacy of receptor-G protein coupling. Here we used the GPAnt-2 to analyze the effects of a receptor agonist on receptor-G protein coupling. These analyses lead to the conclusion, as discussed below, that an agonist enhances the productive interaction between receptor and G protein by increasing efficacy of coupling, not by increasing affinity between receptor and G protein.

The IC<sub>50</sub> of GPAnt-2 increased with increasing concentrations of GPAnt-2 with respect to M2 mAChR. On the other hand, the IC<sub>50</sub> of GPAnt-2 stayed constant with increasing concentrations of CCh. With increasing concentrations of CCh, the fraction of CCh-bound M2 mAChR increases while that of agonist-free M2 mAChR decreases. If the affinity of CCh-bound M2 mAChR for G<sub>4</sub> is higher than that of agonist-free M2 mAChR, the IC<sub>50</sub> of GPAnt-2 should be higher with increasing concentrations of CCh-bound M2 mAChR. In such cases, the IC<sub>50</sub> should increase with CCh concentration. In practice, the IC<sub>50</sub> did not alter with changing CCh concentration (Fig. 6). This suggests that the affinity of M2 mAChR for G<sub>4</sub> is not affected by an agonist in the presence of guanine nucleotides. In contrast, Fig. 3 demonstrates that a small 4-fold increase of the concentration of M2 mAChR gave a clear increase in IC<sub>50</sub> of GPAnt-2.

Matesic et al. (41) have reported that the high affinity complex of M2 mAChR and G proteins (G<sub>4</sub> or G<sub>2</sub>), in solubilized form, was promoted by an agonist in the absence of guanine nucleotide. This demonstrates that an agonist increases the affinity of M2 mAChR for G proteins. Our results, however, cannot be compared directly with their results. Matesic et al. showed the formation of high affinity complex in the absence of guanine nucleotides, whereas our conclusions were derived from the observations in the presence of guanine nucleotides. In fact, Matesic et al. reported that the tight complex was not observed when guanine nucleotides were added (41). Together these studies provide evidence that the effects of CCh to enhance the affinity of receptor for G protein can be observed only without guanine nucleotides. In fact, guanine nucleotides are known to abolish the high affinity agonist binding to M2 mAChR promoted by G proteins (42, 43).

Receptor even without agonist (or with receptor antagonist) stimulates the activation of G proteins weakly in <i>vivo</i>, an agonist increases the productive coupling of its receptor with G protein. Since agonists (CCh) did not increase the affinity of receptor with G proteins in the presence of guanine nucleotides, our results support a notion that CCh increases the efficacy of the coupling. Consistent with our results, Rubenstein et al. (38) observed higher efficacy for liganded β-adrenergic receptor to activate G<sub>4</sub> than for antagonist-bound β-adrenergic receptor at all receptor concentrations they observed. On the other hand, studies by Senogles et al. (44) in dopamine-2 receptor (D<sub>2</sub>R)-G<sub>2a</sub> interaction suggest that an agonist increases the affinity of a receptor for G protein but does not alter efficacy. They observed the saturable dependence of the coupling (assayed by GTP hydrolysis) on the concentration of G protein, and an agonist shifted the curve to the left (lower concentration of G<sub>2a</sub>). These discrepant interpretations may suggest that the effects of an agonist on the affinity between receptor and G protein depends on the system. Further studies with different purified receptors and G proteins are necessary to examine the generality of the effects of an agonist on receptor-G protein coupling in the presence of guanine nucleotides.

The present study supports the specific interaction of GPAnt-2 with G proteins, but no functional interactions with receptor were observed. The specificity of GPAnt-2 and related compounds was further confirmed in reconstitutions of M1 mAChR and G<sub>4</sub><sub>11</sub>. GPAnt-2 was weak in inhibiting M1 mAChR-promoted GTP hydrolysis by G<sub>4</sub><sub>11</sub> (25% inhibition at 10 μM). On the other hand, an GPAnt-2 analog (Arg-Pro-Lys-Pro-Gln-Gln-d-Trp-Phe-d-Trp-d-Trp-Met-NH<sub>2</sub>, one of undeca-SP antagonists (46)) potently inhibited (81% inhibition at 10 μM) M1 mAChR-promoted GTP hydrolysis.
by G_{b1} (data not shown), whereas it was not effective in inhibiting M2 mACHr-promoted GTP hydrolysis by G_{i} (7% inhibition at 10 μM). Interestingly unde-CA-SP antagonists are known to inhibit not only the functions of SP but also those of other peptides such as bombesin, vasopressin, and endothelin, whose cognate receptors are coupled to pertussis toxin-insensitive G_{i} (maybe G_{b1}) (47).

In conclusion, our data show that GPAn-2 can bind to G proteins and competes with receptor to block the effects of thelin, whose cognate receptors are coupled to pertussis toxin-inhibition at 10 μM of other peptides such as bombesin, vasopressin, and endothelin. GPAnt-1 works by this mechanism. If so, these G protein antagonists would be effective for the study of detailed mechanisms of G protein pathway in cells.

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