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Effects of Partial Agonists and Mg^{2+} Ions on the Interaction of M_2 Muscarinic Acetylcholine Receptor and G Protein $G\alpha_{i1}$ Subunit in the M_2-$G\alpha_{i1}$ Fusion Protein

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We have expressed a M_2-$G\alpha_{i1}$ fusion protein in insect cells, in which the G protein α_{i1} subunit was fused with a mutant of the muscarinic receptor M_2 subtype without glycosylation sites and the central part of the third intracellular loop. The M_2-$G\alpha_{i1}$ fusion protein showed GTP-sensitive, high-affinity agonist binding. Displacement curves by GDP of [^{35}S]GTPγS binding shifted to the right in the presence of muscarinic agonists. The extent of the shift was greater for full agonists (120–150 fold) than for partial agonists (25–35 fold), and virtually no shift was observed for antagonists. The affinity for GDP decreased with increasing $MgCl_2$ concentration in the presence of an agonist but was not affected by $MgCl_2$ in the presence of an antagonist. These results indicate that the apparent affinity for GDP of the M_2-$G\alpha_{i1}$ fusion protein bound to a ligand represents the efficacy of the given ligand, and that Mg^{2+} is required for the agonist-bound M_2 to interact with $G\alpha_{i1}$, reducing its affinity for GDP. We propose that the agonist-M_2-$G\alpha_{i1}$ complex represents the transition state for the GDP-GTP exchange reaction catalyzed by agonist-bound receptors, and that the complex has different affinities for GDP depending on the species of the ligand bound to M_2 receptors.

Key words: fusion protein, G protein–coupled receptor, Mg^{2+} ion, muscarinic acetylcholine receptor, partial agonist.

Abbreviations: $G\alpha_{i1}$, α subunit of inhibitory GTP binding regulatory protein 1 (G_{i1}); M_2, muscarinic acetylcholine receptor M_2 subtype; M_2 mutant, a mutant of M_2 with replacement of aspargine in putative glycosylation sites by aspartic acid and with a deletion in the central part of the third intracellular loop; M_2-$G\alpha_{i1}$ fusion protein, a fusion protein of the M_2 mutant and $G\alpha_{i1}$; NMS, *N* methylscopolamine; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; QNB, 3-quinuclidinyl benzilate.

G protein-coupled receptors constitute one of the largest superfamilies of proteins and recognize extremely diverse ligands, including neurotransmitters, hormones, cytokines, and odorants [see recent reviews (*1–3*)]. When G protein-coupled receptors are bound to specific ligands, they activate G proteins (heterotrimeric GTP-binding proteins) [see recent reviews (*3–5*)]. The interaction of receptors and G proteins has been examined in various specimens including membrane preparations derived from mammalian tissues, reconstituted vesicles of purified proteins, and cultured cells expressing wild-type or mutant proteins. The interaction is monitored as guanine nucleotide-sensitive high affinity agonist binding, agonist-stimulated [^{35}S]GTPγS binding or GTPase activity, or agonist-dependent activation of effector systems. It is generally believed that an agonist-bound receptor facilitates the conversion of GDP bound to G protein α subunits into GTP, and thereby causes the dissociation of the $\alpha_{GDP}\beta\gamma$ trimer into α_{GTP} and βγ subunits. The details of the mechanism of the interaction, however, remain to be clarified. For example, the mode of interaction appears to differ from one receptor to another and/or from one G protein to another. Guanine nucleotide-sensitive, high-affinity agonist binding has clearly been shown for the interaction of M_2 muscarinic receptors and G protein G_i or G_o (*6–8*), but is not so apparent for the interaction of M_1 muscarinic receptors and G_q or G_{11}, whereas agonist-stimulated [^{35}S]GTPγS binding has clearly been observed for the M_1-G_q/G_{11} interaction (*9–10*), but not so apparently for the M_2-G_i/G_o interaction unless GDP is present (*6–8*, *11*). These differences have not been explained in mechanistic terms. Detailed studies of the receptor-G protein interaction have been hampered by the difficulty in reconstituting substantial amounts of purified receptors and G proteins into lipid vesicles in a quantitative and reproducible manner. In addition, the catalytic action of receptors on G proteins is a further complica-

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tion. In these respects, receptor-G protein fusion proteins may be useful for detailed studies of receptor-G protein interactions.

A fusion protein of the β_2 adrenergic receptor and the α subunit of G protein G_s ($G\alpha_s$) was originally produced by Bertin *et al*. (*12*). They showed that $G\alpha_s$ fused to β_2 adrenergic receptors can be activated by the addition of a β adrenergic agonist and can then activate adenylyl cyclase. Recently, various kinds of fusion proteins including those of the α subunit of G_i ($G\alpha_i$) with the α_2 adrenergic, adenosine A_1, and other receptors, as well as β_2 adrenergic receptor-$G\alpha_s$ fusion proteins, have been reported to be functional [see reviews (*13–15*)]. In these fusion proteins, the receptors have been reported to show guanine nucleotide-sensitive, high affinity agonist binding, and to stimulate GTPase or [^{35}S]GTPγS binding of fused G proteins in an agonist-dependent manner, as do receptors reconstituted with G proteins in phospholipid vesicles. Furthermore, fusion proteins have been shown to be useful for studying the interaction between agonist-bound receptors and G proteins (*16*, *17*), the efficacy of ligands (*18*, *19*), the effects of lipid modification (*20*), the specificity of receptor-G protein interactions (*21*, *22*), and the effects of alternative splicing or mutations (*23*, *24*).

In the present paper, we report that a fusion protein of an M_2 muscarinic receptor mutant and $G\alpha_{i1}$ provides a good model system for studies on the interaction of M_2 and $G\alpha_{i1}$: the effects of full or partial agonists are characterized by changes in the apparent affinity of the fusion protein for GDP, and Mg^{2+} ions are indispensable for the effects of agonists.

EXPERIMENTAL PROCEDURES

Materials—[^{35}S]GTPγS (specific activity, 1250 Ci/mmol) and [^{3}H]3-quinulcidinyl benzilate ([^{3}H]QNB) (specific activity, 43.5 Ci/mmol) were purchased from Du Pont/NEN Research; restriction enzymes were from Toyobo and Takara Shuzo. The cDNA for a human muscarinic M_2 receptor mutant, M_2 (N-D) (I3del), which lacks the central part of the third intracellular loop (233–380) and has mutations from Asn to Asp at putative N-glycosylation sites 2, 3, 6 and 9, was donated by Dr. Hayashi (*25*). The cDNA for the bovine $G\alpha_{i1}$ subunit was donated by Dr. Nukada (*26*).

M_2-$G\alpha_{i1}$ Fusion Protein—The M_2-$G\alpha_{i1}$ fusion protein was constructed by linking the carboxyl terminus of the muscarinic M_2 receptor mutant, M_2 (N-D) (I3del), with the amino terminus of the $G\alpha_{i1}$ subunit. The cDNA for M_2 (N-D) (I3del) was inserted into the baculovirus transfer vector pBacPAK8 with *Bgl*II and *Pst*I sites (pPAK-M_2 mutant). Then, a *Xho*I–*Xba*I fragment encoding the C-terminus of bovine $G\alpha_{i1}$ cDNA derived from pGa28 was cloned into the pPAK-M_2 mutant (pPAK-M_2-$G\alpha_{i1}$-part). The DNA region corresponding to the M_2-$G\alpha_{i1}$ fusion protein was generated by means of a three-step PCR protocol using KOD polymerase (Toyobo). In the first PCR, M_2 cDNA in the pPAK-M_2 mutant was amplified using the following oligonucleotides: sense, 5′-TCTTGGCTATTCT-GTTGGCTTTC-3′, corresponding to the sequence prior to the *Apa*I site in the M_2 mutant; antisense, 5′-CCTTG-TAGCGCCTATGTTCT-3′, corresponding to the C-terminus of the M_2 mutant. In the second PCR, bovine $G\alpha_{i1}$ cDNA in pGa28 was amplified using the following oligonucleotides: sense, 5′-AGAACATAGGCGCTACAAGGAT-GGGCTGTACGCTGAGCG-3′, containing 20 bp from the C terminus of the M_2 mutant and 19 bp from the N-terminus of $G\alpha_{i1}$; antisense, 5′-GGCGTCTAGATCAGAAGAG-ACCACAGTCTTTTAGG-3′, containing 25bp from the C-terminus of $G\alpha_{i1}$ and a recognition site for *Xba*I. In the third PCR, the products of the first and second PCRs were used as templates, and the sense primer corresponding to the sequence prior to the *Apa*I site in the M_2 mutant and antisense primer of the C-terminus of $G\alpha_{i1}$ were used. In this way, a fragment encoding the C-terminus of the M_2 mutant and the N-terminus of $G\alpha_{i1}$ was generated. This fragment was digested with *Apa*I and *Xho*I, and then inserted into pPAK-M_2-$G\alpha_{i1}$-part digested with *Apa*I and *Xho*I. As a result, we obtained an M_2-$G\alpha_{i1}$ fusion DNA inserted in the baculovirus transfer vector.

Expression in the Baculovirus-Sf9 System—Recombinant baculoviruses were generated in Sf9 cells using a BacPAK Baculovirus Expression System Kit (Clontech) as described previously (*25*). After the transfer vector and linearized baculovirus DNA were co-transfected, the recombinant baculoviruses were cloned by plaque isolation. Then, they were amplified and used for the production of the M_2-$G\alpha_{i1}$ fusion protein. Sf9 cells were grown at 28°C to a density of approximately 2–3 $\times$ 10^9 cells/liter culture, and then infected with the recombinant virus and cultured for 48 h. Cells, that had been harvested and stored at –80°C, were thawed and homogenized with a Potter-type homogenizer in a buffer comprising 20 mM Hepes-KOH (pH 8.0), 1 mM EDTA, 2 mM $MgCl_2$, 5 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 mM benzamidine (100 ml per cells derived from 1 liter culture). The homogenate was centrifuged at 150,000 $\times g$ for 1 h, and the pellet (membrane preparation) was resuspended in 20 mM Tris-HCl buffer (pH 7.4) and stored at –80°C before use.

[^{35}S]GTPγS and [^{3}H]QNB Binding Assay—In the standard assay for [^{35}S]GTPγS binding, a membrane preparation expressing the M_2-$G\alpha_{i1}$ fusion protein or the M_2 mutant was incubated with 50 nM [^{35}S]GTPγS (2–4 cpm/fmol), 1 μM GDP, 1 mM carbamylcholine or 10 μM atropine, and 10 mM $MgCl_2$ in a buffer solution [20 mM Hepes-KOH buffer (pH 8.0), 1 mM EDTA, 0.16 M NaCl, 1 mM DTT](total volume, 0.1 ml; final protein concentration, 0.2 mg/ml). In some experiments, various concentrations of [^{35}S]GTPγS, GDP, GTP, $MgCl_2$, or different muscarinic ligands were added to the reaction mixtures. In the standard assay for [^{3}H]QNB binding, the same components as for [^{35}S]GTPγS binding were used except that 50 nM [^{35}S]GTPγS, 1 μM GDP, and 1 mM carbamylcholine or 10 μM atropine were replaced by 2–10 nM [^{3}H]QNB with or without 0.1 mM GTP and 10 μM atropine. In some experiments, various concentrations of carbamylcholine, atropine or other muscarinic ligands were added. Incubation was performed at 30°C for 60 min in test tubes or 96-well plates, and the bound [^{35}S]GTPγS or [^{3}H]QNB was trapped on a GF/B glass fiber filter, which was washed three times with cold 20 mM potassium phosphate buffer (pH 7.0) and then counted in a liquid scintillation counter.

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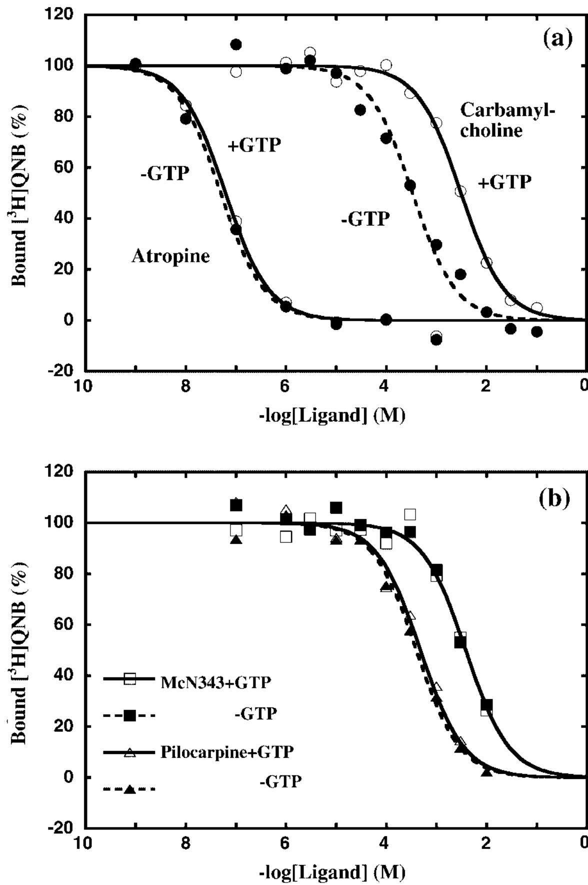

Fig. 1. **Displacement by muscarinic ligands of [^{3}H]QNB binding to the M_2-$G\alpha_{i1}$ fusion protein in the presence or absence of GTP.** An Sf9 membrane preparation expressing the M_2-$G\alpha_{i1}$ fusion protein (20 µg protein/tube) was incubated at 30°C for 60 min with 2–10 nM [^{3}H]QNB in the presence of different concentrations of muscarinic ligands with or without 0.1 mM GTP. [^{3}H]QNB bound to the membrane was trapped on a GF/B glass fiber filter and then counted. Displacement curves were fitted to an equation for a one-site model, $Y = 100/(1 + X/IC_{50})$. The IC_{50} values in the absence and presence of 0.1 mM GTP were estimated to be 0.33 and 3.1 mM for carbamylcholine, 50 and 62 nM for atropine, 3.85 and 3.83 mM for McN-343, and 0.40 and 0.48 mM for pilocarpine, respectively. When the displacement curve with carbamylcholine in the absence of GTP was fitted to an equation for a two-site model, $Y = A/[1 + X/IC_{50}(H)]+(100 - A)/[1 + X/IC_{50}(L)]$, the values for A, $IC_{50}(H)$, and $IC_{50}(L)$ were estimated to be 38.3%, 0.066 mM, and 0.87 mM, respectively.

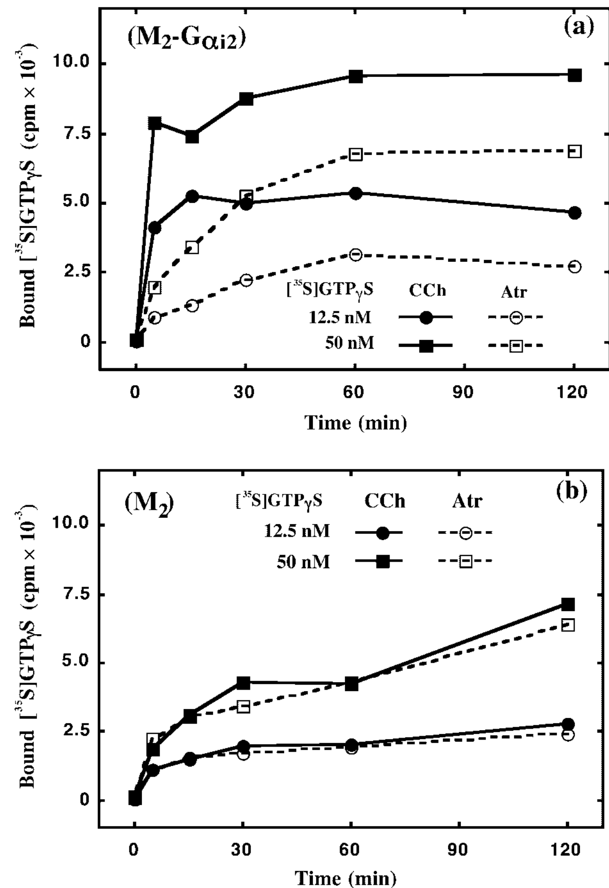

Fig. 2. **Time courses of [^{35}S]GTPγS binding to membranes expressing M_2-$G\alpha_{i1}$ fusion proteins or M_2 mutants.** Membrane preparations expressing M_2-$G\alpha_{i1}$ fusion proteins (a) or M_2 mutants (b) were incubated with 12.5 nM (circles) or 50 nM (squares) [^{35}S]GTPγS (6.5–7.7 cpm/fmol) in the presence of 1 mM carbamylcholine (solid lines) or 10 µM atropine (dotted lines) for the indicated times at 30°C. Bound [^{35}S]GTPγS was trapped and counted.

RESULTS

Interaction of M_2 and $G\alpha_{i1}$ in the M_2-$G\alpha_{i1}$ Fusion Protein—Membrane preparations derived from Sf9 cells expressing the M_2 mutant or M_2-$G\alpha_{i1}$ fusion proteins were subjected to binding with [^{3}H]QNB or [^{35}S]GTPγS. Figure 1 shows displacement curves with muscarinic ligands of [^{3}H]QNB binding in the absence or presence of 0.1 mM GTP for membrane preparations expressing M_2-$G\alpha_{i1}$ fusion proteins. The displacement curve with a full agonist, carbamylcholine, shifted to the right in the presence of GTP for M_2-$G\alpha_{i1}$ expressing membranes (Fig. 1a), whereas no such shift was detected for cell membranes expressing the M_2 mutant (data not shown). No shift was observed for displacement curves with an antagonist, atropine, for membranes expressing either the M_2 mutant or M_2-$G\alpha_{i1}$ fusion proteins. The displacement curves with partial agonists, McN-343 or pilocarpine, for membranes expressing M_2-$G\alpha_{i1}$ fusion proteins tended to shift to right in the presence of GTP, but the difference was not significant (Fig. 1b). It should be noted that the effect of GTP was not significant even in the presence of 10 mM Mg^{2+} ion, despite the fact that the amount of high affinity agonit-M_2-$G\alpha_{i1}$ complex increased with an increase in Mg^{2+} ion concentration (see Ref. *8* and the following section).

Figure 2 shows [^{35}S]GTPγS binding to membranes expressing M_2-$G\alpha_{i1}$ fusion proteins or the M_2 mutant in the presence of 1 mM carbamylcholine or 10 µM atropine. The extent of [^{35}S]GTPγS binding was greater in the presence of carbamylcholine than atropine for membranes expressing M_2-$G\alpha_{i1}$ fusion proteins (Fig. 2a). The difference was more apparent at lower concentrations of [^{35}S]GTPγS and shorter incubation times. On the other hand, no difference was observed in the presence of carbamylcholine and atropine for membranes expressing the M_2 mutant (Fig. 2b). These results indicate that the M_2 mutant and $G\alpha_{i1}$ subunit in the fusion protein inter-

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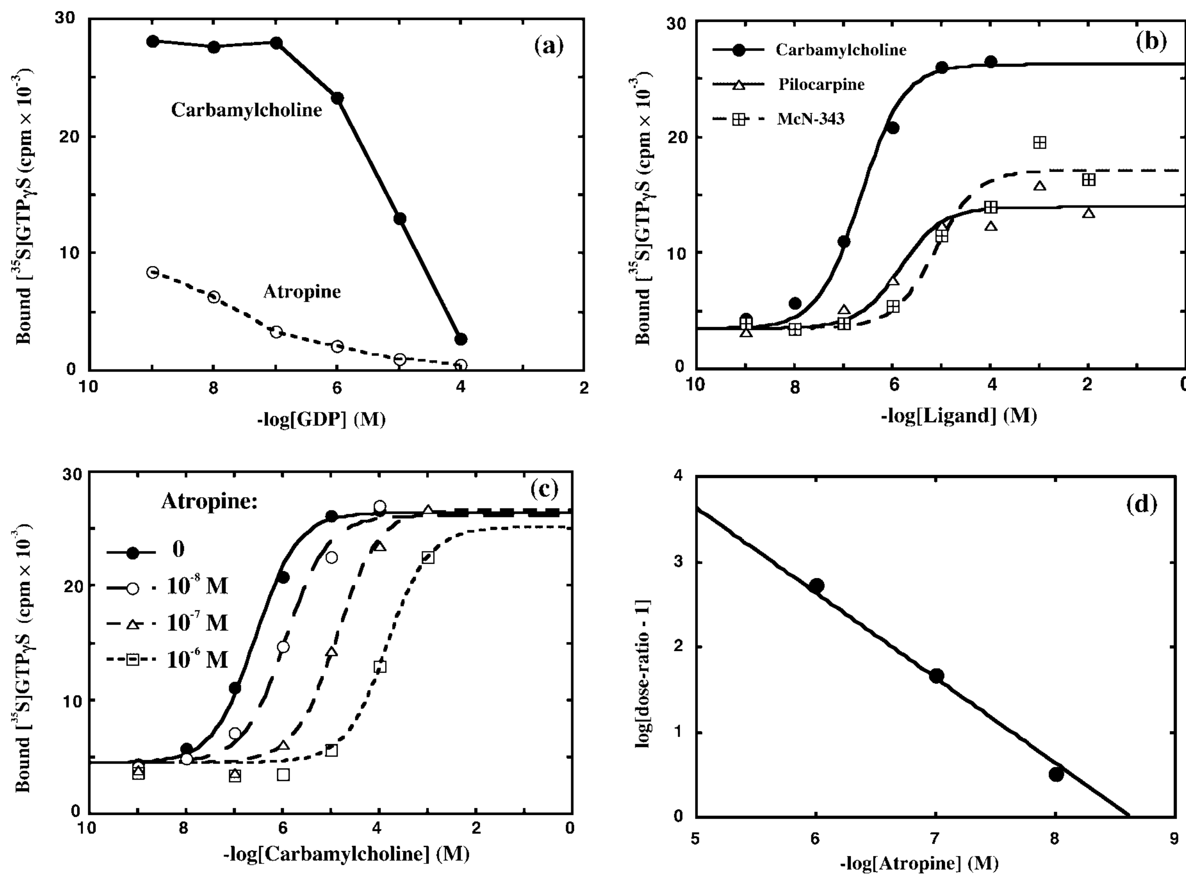

Fig. 3. **Effects of an agonist, partial agonists, and an antagonist on [^{35}S]GTPγS binding of M_2-Gα_{i1} fusion proteins.** Membrane preparations expressing M_2-Gα_{i1} fusion proteins were incubated with 1 nM [^{35}S]GTPγS for 20 min in the presence of (a) different concentrations of GDP and 1 mM carbamylcholine or 10 μM atropine, (b) different concentrations of muscarinic ligands and 1 μM GDP, or (c) different concentrations of carbamylcholine and atropine. In (b), the EC_{50} and B_{max} values were estimated to be 0.26 μM and 100% for carbamylcholine, 1.5 μM and 47% for pilocarpine, and 7.8 μM and 61% for McN-343, respectively. A Schild plot of the data shown in (c) gave estimates of pA_2 = 8.64 and A_2 = 2.3 nM (d).

act with each other, whereas the M_2 mutant does not interact with endogenous G proteins, or that the interaction could not be detected under the present experimental conditions. The failure of the M_2 mutant to interact with endogenous G proteins in Sf9 cells is not due to the mutation of the M_2 receptors, because the M_2 mutant purified from Sf9 cells can interact with purified G_i or G_o proteins in reconstituted vesicles (*25*). Other receptors coupled to G_i/G_o have also been reported not to interact with endogenous G proteins in Sf9 cells (*22*).

Effects of Muscarinic Ligands on [^{35}S]GTPγS Binding—A merit of receptor-Gα fusion proteins is that they can be used to discriminate agonists and antagonists by means of a simple binding assay. Figure 3(a) demonstrates a marked difference between carbamylcholine and atropine in their effects on [^{35}S]GTPγS binding of the M_2-Gα_{i1} fusion protein. The difference was evident at a short incubation time (20 min) and with the use of a low concentration of [^{35}S]GTPγS (1 nM). A full agonist (carbamylcholine) and partial agonists (McN-343 and pilocarpine) can be discriminated on the basis of the extent of [^{35}S]GTPγS binding in the presence of GDP (Fig. 3b). The stimulation by carbamylcholine of [^{35}S]GTPγS binding is competitively inhibited by atropine, as expected (Fig. 3c). The pA_2 and A_2 values for atropine were estimated to be 8.64 and 2.3 nM, respectively, from a Schild plot (Fig. 3d). The A_2 value is comparable to those reported for M_2 receptors (*27*).

Effects of Muscarinic Ligands on the Affinities of M_2-Gα_{i1} Fusion Protein for Guanine Nucleotides—Figure 4 shows the displacement curves with GDP, GTP, or GTPγS of [^{35}S]GTPγS binding in the presence of different kinds of muscarinic ligands. The displacement curves with GDP shifted to the right by 120–150 fold in the presence of carbamylcholine or acetylcholine and by 25–35 fold in the presence of McN-343 or pilocarpine, while virtually no shift was observed in the presence of atropine (Fig. 4a). Shifts in the presence of full or partial agonists were also observed for displacement by GTP (Fig. 4b) and GppNHp (data not shown), although the extent of the shift was smaller compared with in the case of the displacement by GDP. On the other hand, no shift was observed for displacement by GTPγS (Fig. 4c). The simplest interpretation of these results is that the M_2-Gα_{i1} fusion proteins exhibit the lowest affinity for GDP when bound to a full agonist, medium affinity when bound to a partial agonist, and the highest affinity when bound to an antagonist or not bound to a ligand, whereas they exhibit the same affinity for GTPγS independent of the species of ligand bound to them.

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Effects of $MgCl_2$ on the Affinity for GDP—Figure 5 shows the displacement curves with GDP of [^{35}S]GTPγS binding in the presence of different concentrations of $MgCl_2$ with 1 mM carbamylcholine or 10 μM atropine. The displacement curves in the presence of atropine were hardly affected by the concentration of $MgCl_2$, and the IC_{50} values for GDP were estimated to be 0.42 and 0.66 μM in the absence and presence of 10 mM $MgCl_2$ (Fig. 5b). On the other hand, the displacement curves in the presence of carbamylcholine shifted to the right with increasing $MgCl_2$ concentrations from 0 to 10 mM, and the IC_{50} value increased from 0.76 to 39 μM (Fig. 5a). A Schild plot gave a straight line with an A_2 value of 135 μM, which is consistent with, although does not prove, the idea that Mg^{2+} competes with GDP for binding to agonist-bound M_2-$G\alpha_{i1}$ fusion proteins (Fig. 5c).

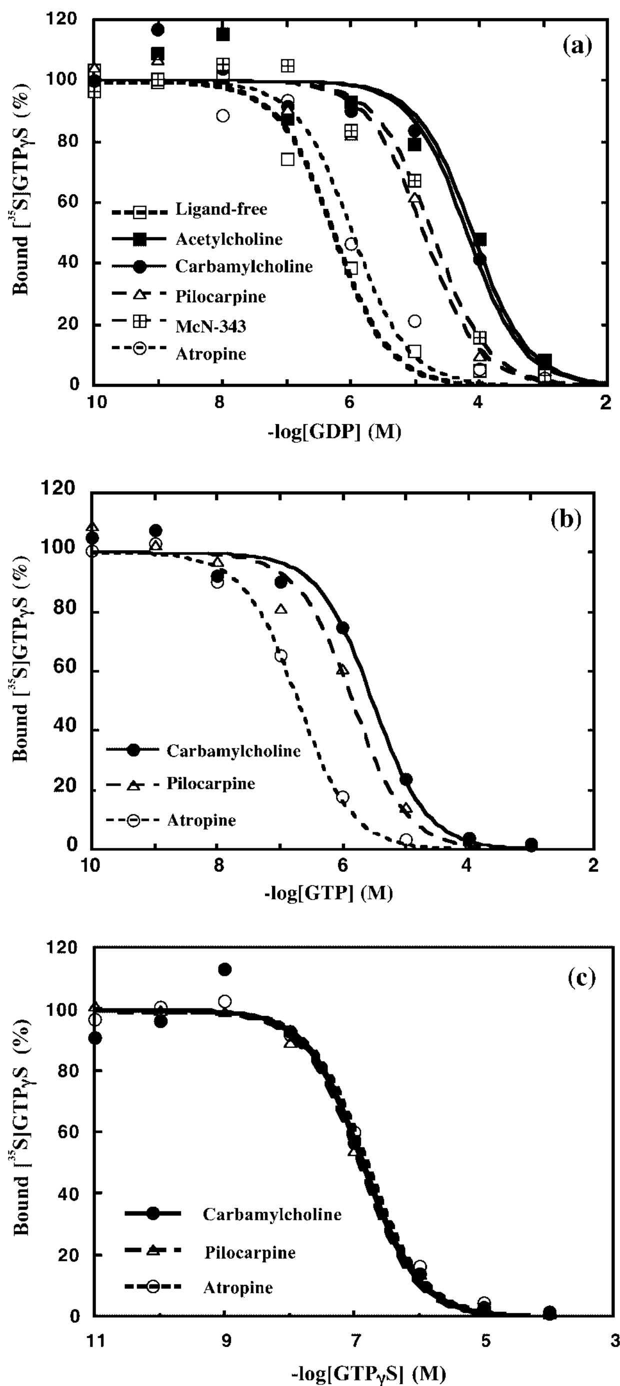

Fig. 4. **Displacement of [^{35}S]GTPγS binding by GDP, GTP, or GTPγS in the presence of various muscarinic ligands.** Membrane preparations expressing M_2-$G\alpha_{i1}$ fusion proteins were incubated with 50 nM [^{35}S]GTPγS for 60 min in the presence of the indicated concentrations of GDP (a), GTP (b), or GTPγS (c) with or without 1 mM acetylcholine, carbamylcholine, pilocarpine, McN343 or 10 μM atropine. The displacement curves were fitted to the equation, $Y = 100/(1 + X/IC_{50})$. The actual values for 100% were 4,900–5,700 cpm. The same experiments were repeated 2–5 times and the data from a representative experiment are shown here. The IC_{50} values (μM) for GDP [average ± standard deviation (experimental number)] were estimated to be 1.03 ± 0.69 (2) in the absence of ligand, and 1.08 ± 0.25 (5), 11.9 ± 6.2 (3), 11.7 ± 3.0 (2), 38.8 ± 16.0 (5), 62.8 ± 24.6 (2) in the presence of atropine, pilocarpine, McN-343, carbamylcholine, and acetylcholine, respectively. The values in the presence of pilocarpine or McN343 are significantly different from the value in the presence of atropine with p values of 0.0001, and also from the value in the presence of carbamylcholine with p values of 0.008 or 0.01, respectively. The IC_{50} values for GTP (μM) were estimated to be 0.29 ± 0.13 (2), 1.57 ± 0.21 (2), and 3.1 ± 0.30 (2) in the presence of atropine, pilocarpine, and carbamylcholine, respectively, with the value for pilocarpine differing from the values in the presence of atropine and carbamylcholine with p values of 0.04 and 0.03, respectively.

DISCUSSION

In the present study, we have shown that M_2 muscarinic receptors and $G\alpha_{i1}$ subunits in M_2-$G\alpha_{i1}$ fusion proteins interact with each other. The interaction of M_2 and $G\alpha_{i1}$ is apparent from the findings that the affinity for GDP or GTP is affected by agonist binding, and that the affinity for an agonist is affected by the binding of GDP or GTP. A fusion protein exhibits low affinity for GDP or GTP in the presence of an agonist and low affinity for an agonist in the presence of guanine nucleotides, that is, a negative tropic interaction. These results are consistent with previous reports on fusion proteins of β_2 adrenergic (*12*, *24*), α_2 adrenergic (*18*), adenosine A_1 (*16*), and formyl peptide receptors (*22*) with $G\alpha_s$ or $G\alpha_i$. Recently, a fusion protein of M_2 receptors with $G\alpha_z$ was also reported to be activated by muscarinic agonists and to activate G protein-activated K^+ channels (*28*). The present results are qualitatively consistent with those for M_2 muscarinic receptors reconstituted with G_i or G_o proteins in lipid vesicles (*6–8*, *11*), although there is the apparent difference in that βγ subunits are required for the interaction of M_2 receptors and $G\alpha_{i1}$ in reconstituted vesicles (*11*), but not for their interaction in the fusion protein, as shown here. It is not likely that endogenous βγ subunits contribute to the interaction of M_2 and $G\alpha_{i1}$ in the fusion protein, because the interaction of M_2 receptors and endogenous G proteins was not observed under the present experimental conditions. In preliminary experiments, we have observed that the coexpression of βγ subunits increases the extent of guanine nucleotide-sensitive high affinity agonist binding, but the effect on the agonist-dependent

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decrease in the affinity for GDP is much lower (Tanabe *et al.*, a paper in preparation).

The M_2-$G\alpha_{i1}$ fusion protein exhibits low affinity for GDP in the presence of both carbamylcholine and $MgCl_2$, and high affinity in the absence of either an agonist or $MgCl_2$. Mg^{2+} appears to compete with GDP, causing an apparent decrease in the affinity of GDP for the fusion protein. As $G\alpha_{i1}$ is thought to interact with agonist-bound, but not antagonist-bound M_2 in the fusion protein, we may speculate that the agonist-bound M_2 causes a conformational change in $G\alpha_{i1}$ so that $G\alpha_{i1}$ cannot retain both GDP and Mg^{2+} together (Fig. 6). Alternatively, Mg^{2+} may be necessary for the interaction of $G\alpha_{i1}$ and agonist-bound M_2, which should be a prerequisite for a decrease in the apparent affinity for the GDP of the fusion protein. Whichever the mechanism, the present results indicate that Mg^{2+} is involved in the formation of the agonist-bound state with low affinity for GDP of the M_2-$G\alpha_{i1}$ fusion protein. These results are consistent with previous results that showed the role of Mg^{2+} in the interaction of M_2 and G_i or G_o in reconstituted vesicles of purified proteins (*8*). It is tempting to speculate that a complex of an agonist, M_2, $G\alpha_{i1}$, and Mg^{2+} exhibits low affinity for GDP, and that the complex represents the transition state for the GDP-GTP exchange reaction catalyzed by agonist-bound receptors.

The simplest equilibrium model for the binding of a muscarinic agonist (a) and GDP (g) to a M_2-$G\alpha_{i1}$ fusion protein (RG) is to assume four states of the fusion protein at minimum, RG (ligand-free form), aRG (agonist-bound form), RGg (GDP-bound form), and aRGg (both agonist- and GDP-bound form). In this model, the ratio of the equilibrium constants for agonist binding to RG and RGg should be equal to the ratio of the equilibrium constants for GDP binding to RG and aRG. The ratio of the equilibrium dissociation constants for agonist binding to RG and RGg can be approximated by the ratio of the IC_{50} values for carbamylcholine, which are estimated from the displacement of [^{3}H]QNB binding in the presence and absence of GDP, because [^{3}H]QNB binding is not affected by the presence or absence of GDP. Similarly, the ratio of the equilibrium dissociation constants for GDP binding to RG and aRG can be approximated by the ratio of the IC_{50} values for GDP, which are estimated from the displacement of [^{35}S]GTPγS binding in the presence and absence of carbamylcholine, because [^{35}S]GTPγS binding is not affected by carbamylcholine. The ratio of the IC_{50} values for carbamylcholine is 9, much lower than the value of 120 for the ratio of the IC_{50} values for GDP. For the interaction of purified M_2 and G_i reconstituted in lipid vesicles, the ratio of the IC_{50} values for carbamylcholine is approximately 1000 in contrast with 19 for the ratio of the IC_{50} values for GDP (*8*). It is true that agonist binding negatively affects GDP binding and *vice versa* for both the fusion protein and reconstituted vesicles, but the extents of the effects are not equal to each other in either case. Furthermore, the apparent affinity for McN-343 and pilocarpine in the M_2-$G\alpha_{i1}$ fusion protein is not affected by the presence or absence of guanine nucleotides, despite the fact that the apparent affinity for GDP and GTP is clearly affected by the presence of McN-343 or pilocarpine. These results are also not consistent with

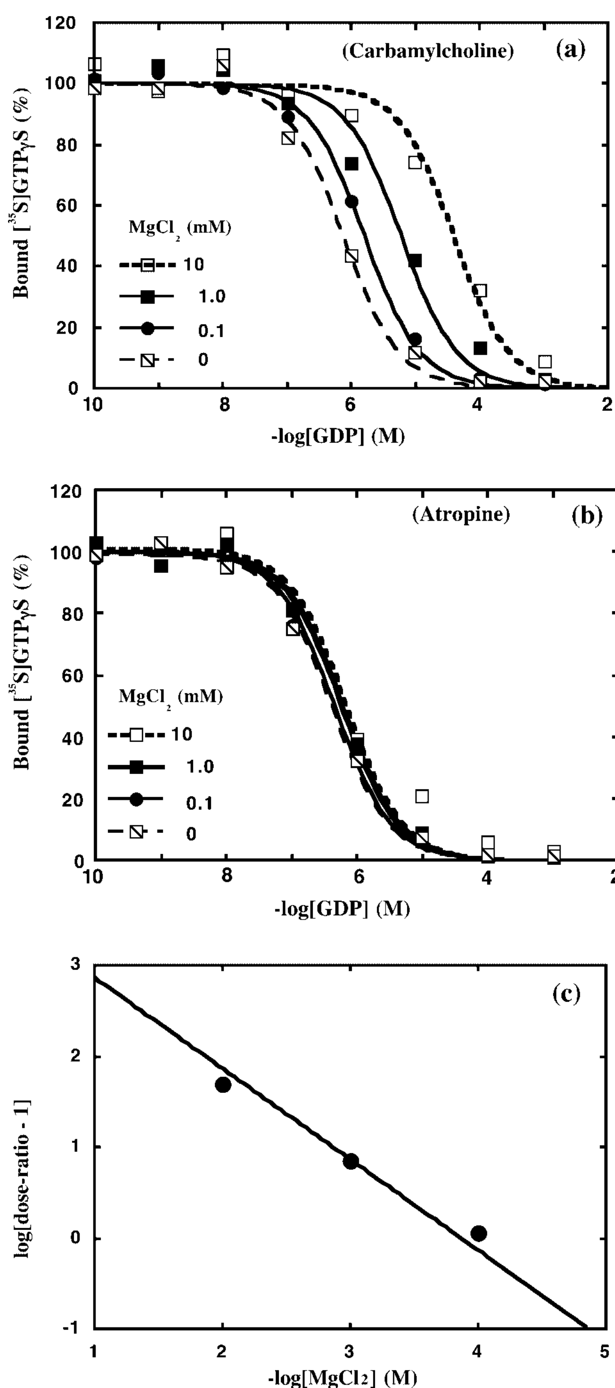

Fig. 5. **Effect of the $MgCl_2$ concentration on the displacement of [^{35}S]GTPγS binding by GDP in the presence of carbamylcholine (a) or atropine (b).** The experimental conditions were the same as described in the legend to Fig. 4 except that different concentrations of $MgCl_2$ were used instead of 10 mM $MgCl_2$. The IC_{50} values for GDP (μM) in the presence of 1 mM carbamylcholine were estimated to be 0.78 ± 0.03 (2) in the absence of $MgCl_2$, and 2.1 ± 0.7 (2), 5.3 ± 1.1(2), and 36.4 ± 3.7 (2) in the presence of 0.1, 1.0, and 10 mM $MgCl_2$, respectively (a). The value in the absence of $MgCl_2$ differs from the values in the presence of 0.1, 1.0, and 10 mM $MgCl_2$ with p values of 0.06, 0.01, and 0.0004, respectively. A Schild plot of the data shown in (a) gave estimates of $pA_2 = 3.87$ and $A_2 = 135$ μM (d).

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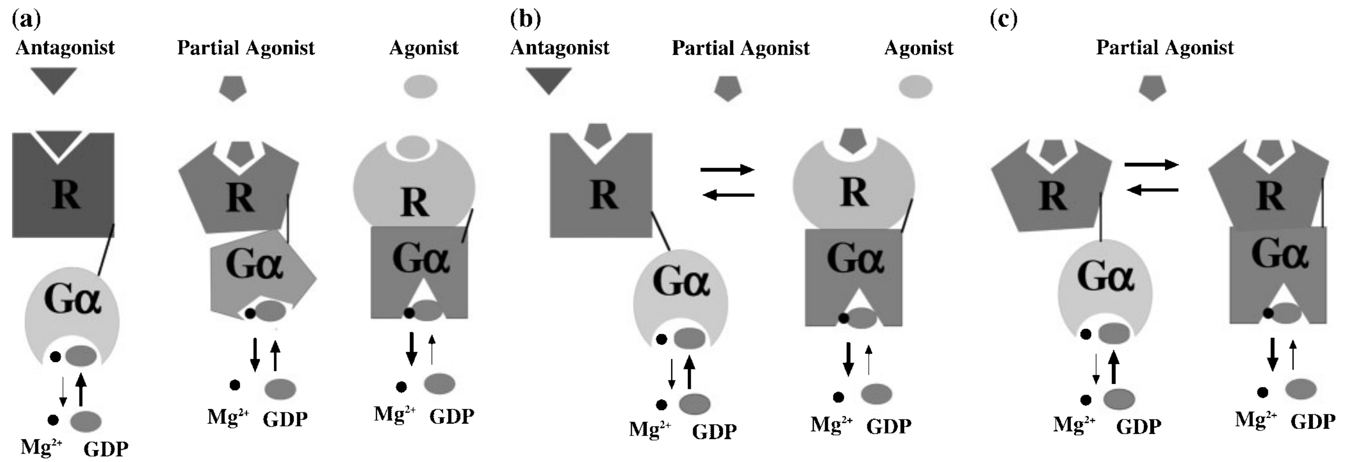

Fig. 6. **A model for the interaction of the M_2-$G\alpha_{i1}$ fusion protein with muscarinic ligands, GDP and Mg^{2+}.** We assume in all three models (a)–(c) that antagonist-bound M_2 is not subject to a conformational change and does not interact with $G\alpha_{i1}$, which exhibits high affinity for GDP, whereas agonist-bound M_2 undergoes a conformational change and interacts with M_2-$G\alpha_{i1}$, thereby causing a conformational change in $G\alpha_{i1}$, that results in low affinity for GDP in the presence of Mg^{2+}. When a partial agonist is bound to M_2, we assume in model (a) that both M_2 and $G\alpha_{i1}$ are subject to partial conformational changes and that $G\alpha_{i1}$ exhibits intermediate affinity for GDP. In model (b) we assume that the partial agonist-bound M_2-$G\alpha_{i1}$ fusion protein takes on two conformations that are in equilibrium, and that each is the same as in the presence of agonist or antagonist. In model (c), we assume that the partial agonist-bound M_2 takes on a single conformation but that $G\alpha_{i1}$ takes on two conformations, each of which is the same as in the presence of agonist or antagonist.

the simplest assumption described above and require a more complicated model, such as a two-state model, in which the equilibrium between the two states is shifted by the binding of an agonist or guanine nucleotide. It is interesting to note that the difference in the affinity for GDP is greater than the difference in the affinity for agonist in the fusion protein while the reverse is the case for reconstituted lipid vesicles.

The present results indicate that the apparent affinity for GDP of the M_2-$G\alpha_{i1}$ fusion protein depends on the species of muscarinic ligand bound to M_2 receptors. The EC_{50} values for GDP in the presence of pilocarpine and McN-343 are intermediate between those in the presence of carbamylcholine and atropine. One interpretation of this result is that the conformation of $G\alpha_{i1}$ in the pilocarpine-bound M_2-$G\alpha_{i1}$ fusion protein is distinct from and intermediate between the two conformations of $G\alpha_{i1}$ in the carbamylcholine- and atropine-bound M_2-$G\alpha_{i1}$ fusion protein (Fig. 6a). Alternatively, we may assume that $G\alpha_{i1}$ in the pilocarpine-bound M_2-$G\alpha_{i1}$ fusion protein takes on two conformations that are in equilibrium and each of which is the same as in the presence of carbamylcholine or atropine, where R may also take two conformations (Fig. 6b) or one conformation intermediate between the two conformations of carbamylcholine- and atropine-bound R (Fig. 6c). Whichever is the case, the decrease in the apparent affinity for GDP in the presence of pilocarpine indicates an increase in the dissociation rate or a decrease in the association rate, or both. It is tempting to speculate that $G\alpha_{i1}$ in the pilocarpine-bound M_2-$G\alpha_{i1}$ fusion protein exhibits an intermediate GDP dissociation rate between those of $G\alpha_{i1}$ in the carbamylcholine- and atropine-bound M_2-$G\alpha_{i1}$ fusion proteins. The GDP dissociation rate is thought to be a rate-limiting step for the activation of G proteins by receptors. Thus, this speculation may explain why pilocarpine functions as a partial agonist. Tota and Schimerlik (*29*) reported that pilocarpine-bound M_2 receptors show a lower affinity for G protein G_i than carbamylcholine-bound M_2 receptors by measuring agonist-dependent GTPase activity in reconstituted vesicles of purified M_2 and G_i. Their results are consistent with either model (b) or (c) in Fig. 6.

The present results indicate that the interaction between M_2 and $G\alpha_{i1}$ in the M_2-$G\alpha_{i1}$ fusion protein is much more robust as compared with that in the reconstituted vesicles of M_2 and G_i. In addition, the one to one interaction in the fusion protein is much easier to analyze than the catalytic interaction of a single receptor molecule with multiple G protein molecules. We have examined the interaction of the fusion protein with agonists or GDP indirectly by examining the binding of a radioactive antagonist and GTPγS. The use of radioactive agonists and GDP will make detailed and quantitative analyses more feasible. The receptor-Gα fusion proteins will be useful for ligand screening systems, because agonists, partial agonists and antagonists can be discriminated by means of a simple binding assay, as shown here and in other studies (*18*, *22*, *30*; see also accompanying papers *31*, *32*).

In summary, we have shown that the M_2-$G\alpha_{i1}$ fusion protein is a good model system for studies on the interaction of receptors and G proteins. We suggest that the agonist- M_2-$G\alpha_{i1}$-Mg^{2+} complex with low affinity for GDP represents the transition state in the interaction of M_2 and $G\alpha_{i1}$, and that the efficacy of a given ligand is defined by the affinity for GDP of the agonist-M_2-$G\alpha_{i1}$ complex.

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