Effects of Magnesium Ion on the Interaction of Atrial Muscarinic Acetylcholine Receptors and GTP-Binding Regulatory Proteins†

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ABSTRACT: Muscarinic acetylcholine receptors (mAChR) purified from porcine atrium were reconstituted into lipid vesicles with GTP-binding regulatory proteins (G proteins, Gi, Go, or Gn) purified from porcine cerebrum. Apparent affinities of the reconstituted mAChR and G proteins for carbachol and GDP, respectively, were estimated from the effects of these ligands on the binding of [^{3}H]-L-quinuclidinyl benzilate ([^{3}H]QNB) to mAChR and [^{35}S]guanosine 5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) to G proteins in the presence of different concentrations of $MgCl_2$. A total of 30–35% of reconstituted mAChRs exhibited low affinity for carbamylcholine, irrespective of the presence or absence of guanine nucleotides, and the remainder of the mAChRs showed high affinities for carbamylcholine in the absence of GTP or GDP and a low affinity in their presence. The affinity for carbamylcholine in the absence of guanine nucleotides, but not in their presence, increased with increases in $MgCl_2$ concentration. Apparent K_d's for carbamylcholine were estimated to be approximately 100 μM in the presence of guanine nucleotides, 1.5 μM in the absence of guanine nucleotide and Mg^{2+} (<0.1 μM), and 0.1 μM in the absence of guanine nucleotide and the presence of $MgCl_2$ (10 mM). These results indicate that mAChRs may assume at least three different conformations that are characterized by different affinities for agonists. Furthermore, the data suggest that $MgCl_2$ is not necessary for the formation of the mAChR–G protein complex, but can induce a conformational change in the complex. On the other hand, the presence of $MgCl_2$ was necessary for carbamylcholine to influence the binding of guanine nucleotides. In the presence of 10 mM $MgCl_2$, the apparent affinity for GDP was decreased 20-fold by the addition of carbamylcholine: apparent K_d's were estimated to be 2.8 and 0.15 μM in the presence of carbamylcholine and atropine, respectively. No such effect of carbamylcholine was observed in the absence of $MgCl_2$. The apparent affinity for GTP was decreased to a much smaller extent compared to that observed for GDP: apparent K_d's of 0.21 and 0.12 μM were found in the presence of carbamylcholine and atropine, respectively. A specific decrease in the affinity for GDP in the presence of both $MgCl_2$ and carbamylcholine could also be demonstrated in experiments that measured the effects of GDP and GTP on the displacement of [^{3}H]QNB binding by carbamylcholine. These results suggest that magnesium is necessary for the action of mAChR on G proteins and that the agonist–mAChR–G protein complex formed in the presence of $MgCl_2$ is an intermediate for the action of mAChR on G proteins.

Muscarinic acetylcholine receptors (mAChRs)[1] are members of a superfamily of receptors that couple to GTP-binding regulatory proteins (G proteins). Five subtypes of mAChRs (m1–m5) have been identified by cloning of cDNAs or genes and by their expression in oocytes or cultured cells (Kubo et al., 1986a, 1986b; Peralta, et al., 1987a, 1987b; Bonner et al., 1987, 1988). The m1, m3, and m5 subtypes have been shown to be linked primarily to the activation of phosphatidylinositol-specific phospholipase C in a pertussis toxin-insensitive manner, whereas the m2 and m4 subtypes are primarily linked to the inhibition of adenylyl cyclase in a pertussis toxin-sensitive manner (for reviews, see: Nathanson, 1987; Bonner, 1989; Schimerlik, 1989; Hulme et al., 1990).

Interactions of mAChRs and G proteins have been demonstrated by reconstituting mAChRs purified from cerebrum (Florio & Sternweis, 1985, 1989; Haga et al., 1986, 1989; Kurose et al., 1986) or atrium (Tota et al., 1987; Ikegaya et al., 1990) with G proteins purified from cerebrum or atrium in lipid vesicles. Both cerebral and atrial mAChRs have been shown to interact with three different G proteins (Gi, Go, Gn) (Haga et al., 1989; Ikegaya et al., 1990). mAChRs purified from cerebrum may be composed of all five subtypes, but mAChRs purified from atrium are considered to be m2 because only m2 subtypes were detected by the Northern blot analysis of atrial mRNA (Kubo et al., 1986b; Peralta et al., 1987b; Maeda et al., 1988). Recently m1 and m2 subtypes expressed in Sf9 cells using baculovirus vectors were also purified and shown to interact with G proteins (Gi1, Gi3, Go, and Gz) (Parker et al., 1991). In these experiments the efficiency of the coupling was much greater for m2 than for m1.

The binding of agonists but not antagonists has been shown to be influenced by the interaction of mAChRs with G proteins. A total of 50–80% of mAChRs reconstituted with G proteins shows high affinities for agonists in the absence of guanine nucleotides and low affinities in the presence of GTP or GDP.

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[1] Abbreviations: mAChR, muscarinic acetylcholine receptors; G protein, GTP-binding regulatory protein; Gi, Go, and Gn, G proteins that are abundant in the brain and have the following subunit compositions, Gi($\alpha_{41}\beta\gamma$), Go($\alpha_{39}\beta\gamma$), Gn($\alpha_{40}\beta\gamma$) (the major α subunits of Gi, Go, and Gn are α_{i1}, α_{oA}, and α_{i2}, respectively); DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; QNB, L-quinuclidinyl benzilate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); EDTA, ethylenediaminetetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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By contrast, agonist binding to mAChRs reconstituted into lipid vesicles in the absence of G proteins is not affected by guanine nucleotides and most of the receptors exhibiting low affinities for agonists. These results indicate that mAChRs may assume two conformations characterized by high or low affinities for agonists, respectively. The conformation with high affinity for agonists represents the complex of mAChR with guanine nucleotide-free G protein. This complex dissociates into mAChR and G protein following binding of GTP or GDP to the G protein.

The binding of guanine nucleotides to G proteins is also affected by muscarinic agonists. Muscarinic agonists have been shown to facilitate the dissociation of [^{3}H]GDP from G protein in reconstituted vesicles and to stimulate the binding of [^{35}S]GTPγS to G proteins in reconstituted vesicles in the presence of GDP. The effect of agonist on the binding of [^{35}S]GTPγS was clearly observed only in the presence of GDP but not in its absence or the presence of GTP (Tota et al., 1987; Florio & Sternweis, 1989; Ikegaya et al., 1990). This is in contrast to clear effects of β adrenergic agonists on both the dissociation of [^{3}H]GDP from, and the binding of [^{35}S]-GTPγS to, stimulatory G proteins reconstituted with β adrenergic receptors (Brandt & Ross, 1986). The apparent difference between mAChR–Gi/Go and β adrenergic receptor–Gs interactions has not been explained in terms of molecular properties or molecular interactions.

The interactions of receptors and G proteins are known to be affected by Mg^{2+} (for reviews, see: Gilman, 1987; Birnbaumer et al., 1990). The guanine nucleotide-sensitive high-affinity binding of agonists to mAChRs in membranes has been reported to increase with the increase of Mg^{2+} and other cations (Wei & Sulakhe, 1980; Hulme et al., 1983; Nukada et al., 1983). However, the effects of Mg^{2+} have not been examined in detail using reconstituted systems containing purified components, and hence it is not known whether Mg^{2+} affects the interaction of mAChRs and G proteins directly or indirectly through the third component. In addition, it is not clear whether Mg^{2+} is necessary for the interaction of G proteins with mAChRs and other receptors. Birnbaumer and his group reported that Mg^{2+} was necessary for the high-affinity binding of β adrenergic receptors in S49 lymphoma cell membranes (Birnbaumer et al., 1990) but not for the binding of glucagon receptors in liver membranes (Rojas et al., 1985). Brandt and Ross (1986) reported that a requirement for Mg^{2+} could not be detected for the agonist-stimulated, high-affinity binding of GTP to Gs (or, by inference, for the release of bound GDP), although the rate of binding was increased about 3-fold in the presence of 2 mM Mg^{2+}. The stimulation of the dissociation of GDP from Go by mastoparans was also shown to be independent of Mg^{2+}, although Mg^{2+} was required for activation by mastoparans of GTPase activity (Higashijima et al., 1990).

The purpose of this paper is to further characterize the interaction of atrial mAChRs and G proteins in a reconstituted system, particularly with regard for the effect of Mg^{2+} on the interaction. We present evidence that mAChR forms a complex with G protein in the virtual absence of Mg^{2+}, but Mg^{2+} is necessary for formation of the mAChR–G protein complex that has higher affinity for agonists and may function to facilitate the dissociation of GDP.

MATERIALS AND METHODS

[^{3}H]QNB (44.3 Ci/mmol) and [^{35}S]GTPγS (1114 Ci/mmol) were purchased from New England Nuclear, a brain extract (Folch fraction 1) from Sigma Chemical Co., McN-A-343 from Research Biochemicals Inc., and digitonin from Wako Pure Chemical Industries and Sigma. AF102B was kindly donated by Snow Brand Milk Products Co.

Purification of mAChRs and G Proteins. mAChRs were purified from porcine atrium by means of a single affinity chromatography step linked with a small column of hydroxyapatite, as described previously (Haga & Haga, 1985; Nishiyama et al., 1989). Specific [^{3}H]QNB binding of activity of the purified preparation was 2.4–3.7 nmol/mg protein. G proteins were prepared from porcine brain as described previously (Haga et al., 1989), except that Gi, Go, and Gn were separated from each other using a DEAE-Toyopearl column in 0.7% CHAPS solution instead of 0.6% Lubrol PX solution. Gi, Go, and Gn were eluted from the column in this order, and apparent molecular sizes of α subunits as determined by SDS–polyacrylamide gel electrophoresis were 41, 39, and 40 kDa, respectively. The Gi and Gn preparations contained less than 5% α_{39}. No contamination of the Go and Gn preparations by α_{41} or the Go and Gi preparations by α_{40} was detected. The α_{41} kDa protein is thought to be primarily α_{i1} but may also contain a small amount of α_{i3} (Kobayashi et al., 1989). The α_{39} (Goldsmith et al., 1988; Asano et al., 1992) and α_{40} (Katada et al., 1987; Kobayashi et al., 1989) preparations are considered to be α_{oA} and α_{i2}, respectively. Molecular species of $\beta\gamma$ subunits have not been identified and may be different among the Gi, Go, and Gn preparations (Haga et al., 1989; Asano et al., 1990).

Reconstitution of mAChRs and G Proteins. Reconstitution of mAChRs and G proteins was performed as described previously (Haga et al., 1989). Briefly, purified mAChRs (40 pmol) were mixed with crude lipid (0.3 mg) in a solution (HEN: 20 mM Hepes–KOH buffer (pH 8.0), 1 mM EDTA, and 160 mM NaCl; 0.2 mL) containing 0.09% deoxycholate and 0.02% sodium cholate, followed by passage through a small column of Sephadex G-50 preequilibrated with HEN. The void-volume fraction (400 μL) was mixed with G proteins (a mixture of Gi and Go in 0.4% sodium cholate solution or purified Gi, Go, or Gn in 0.7% CHAPS solution; 60–100 pmol; 20 μL) and HEN (80 μL) containing $MgCl_2$ and DTT (final concentrations 12 and 5 mM, respectively). The mixture of mAChRs and G proteins was kept for 60 min at 0 °C, diluted with five volumes of HEN, and used for the [^{3}H]QNB or [^{35}S]GTPγS binding assay.

[^{3}H]QNB and [^{35}S]GTPγS Binding Assay. Reconstituted vesicles (40 μL) were incubated with 1.5 nM [^{3}H]QNB and various concentrations of carbamylcholine in the presence or absence of 0.1 mM GTP for 60 min at 30 °C or with 40 nM [^{35}S]GTPγS and various concentrations of GDP in the presence of 1 mM carbamylcholine or 10 μM atropine for 20 min at 30 °C in a solution containing 20 mM Hepes–KOH buffer (pH 8.0), 0.16 M NaCl, 5 mM DTT, 5 mM EDTA, and different concentrations of $MgCl_2$ (total volume 200 μL; final concentrations of G proteins and mAChRs, 1–6 nM and 50–330 pM, respectively). Concentrations of free Mg^{2+} were calculated on the basis of the assumption that the apparent dissociation constant for binding of Mg^{2+} and EDTA at pH 8.0 is 0.37 μM. After incubation, the reaction mixture with [^{35}S]GTPγS was diluted with a Tris buffer solution (20 mM Tris-HCl buffer (pH 8.0), 0.1 M NaCl, and 25 mM $MgCl_2$; 1 mL) containing 1 mM GTP. The diluted solution with [^{35}S]GTPγS or the reaction mixture with [^{3}H]QNB was applied to glass fiber filter paper (Whatman GF/B) and washed with the Tris buffer solution (2 mL, three times). [^{35}S]GTPγS or [^{3}H]QNB trapped on the filter paper was counted with a liquid scintillation counter.

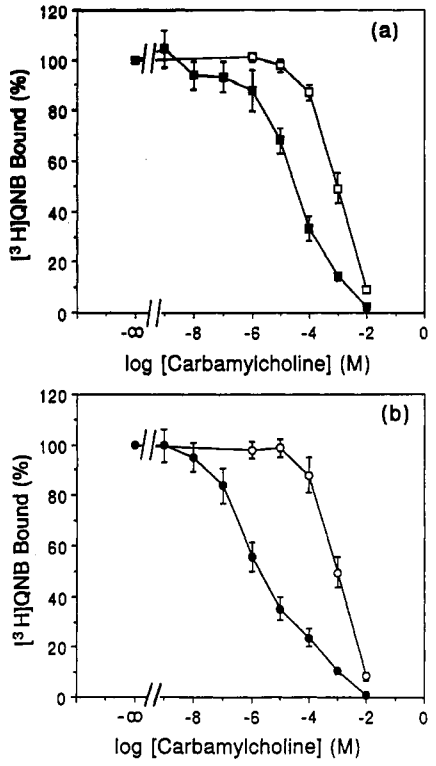

FIGURE 1: Effect of Mg^{2+} on the displacement by carbamylcholine of the [^{3}H]QNB binding to mAChRs reconstituted with Go. The [^{3}H]QNB binding was carried out in the presence of 1.5 nM [^{3}H]-QNB in the absence of (■, ●) or presence (□, ○) of 0.1 mM GTP as described in Materials and Methods. The concentrations of mAChRs and Go and the ratio of Go to mAChRs in the reaction mixture were 100–130 pM, 1.5–2.0 nM, and 11–20, respectively. Data points represent the average ± standard deviation for six separate experiments. The binding in the absence of carbamylcholine was taken as 100%, and the actual counts ranged from 700 to 1300 cpm. The displacement curves in the presence of GTP fit well with the equation, $Y = 100IC_{50}/(X + IC_{50})$, and no appreciable difference was found between the bindings in the presence of 40 nM (a) and 10 mM (b) free Mg^{2+}. The IC_{50} values were calculated to by 871 (a) and 955 (b) nM, respectively. The displacement curves in the absence of GTP fit well with the equation, $Y = AIC_{50}(L)/(X + IC_{50}(L)) + (100 - A)IC_{50}(H)/(X + IC_{50}(H))$, where A is the proportion of low-affinity sites and $IC_{50}(H)$ and $IC_{50}(L)$ are concentrations of carbamylcholine giving half-maximal effects on the binding of [^{3}H]QNB to the high- and low-affinity sites, respectively. The K_d values were calculated using the equation, $K_d = IC_{50}K_d^*/(K_d^* + [[^3H]QNB])$, where K_d^* is the dissocation constant for the binding of [^{3}H]QNB. Estimated values were as follows: A (%) 31.6 (a), 33.8 (b); $K_d(L)$ (μM) 113 (a), 49 (b); $K_d(H)$ (μM) 1.6 (a), 0.064 (b). Variations in K_d and A values were estimated by evaluating them from each experiment repeated six times and averaging the values obtained. Estimated values (the average ± sem) were as follows: A (%) 30.9 ± 3.96 (a), 33.9 ± 1.6 (b); $K_d(L)$ (μM) 125 ± 28 (a), 70.0 ± 8.1 (b); $K_d(H)$ (μM) 1.93 ± 0.14 (a), 0.081 ± 0.015 (b). The two $K_d(H)$ values were significantly different from each other ($p < 0.01$) and were also different from the values in the presence of GTP: 140.2 ± 16.9 μM (40 nM Mg^{2+}) and 152.9 ± 14.0 μM (10 mM Mg^{2+}).

Displacement curves of the [^{3}H]QNB binding by carbamylcholine and of the [^{35}S]GTPγS binding by GDP were analyzed by nonlinear least-squares methods using a fitting program of MULTIFIT (version 2.00). In these analyses, we assumed a single affinity site for [^{3}H]QNB and [^{35}S]GTPγS, a single or two different affinity sites for carbamylcholine and GDP, and a simple mass action with a Hill coefficient of 1 for each binding event.

RESULTS

Effect of GTP on the Displacement by Carbamylcholine of the [^{3}H]QNB Binding to mAChRs Reconstituted with G Proteins. The binding of [^{3}H]QNB to mAChRs reconstituted with G proteins (Go) and its displacement by carbamylcholine were examined in the presence of 40 nM free Mg^{2+} (5 mM EDTA and 0.5 mM $MgCl_2$) or 10 mM free Mg^{2+}. The [^{3}H]-QNB binding in the absence of carbamylcholine was not affected by Mg^{2+}, and virtually the same dissociation constants of 230 and 270 pM were obtained in the presence of 40 nM and 10 mM free Mg^{2+}, respectively (data not shown). Figure 1 shows displacement curves of the [^{3}H]QNB binding by carbamylcholine in the presence or absence of 0.1 mM GTP. The displacement curve shifted to the right in the presence of GTP whether the free Mg^{2+} concentration was 40 nM or 10 mM, although the degree of the shift was greater in the presence of 10 mM Mg^{2+}. Displacement curves in the presence of 0.1 mM GTP are consistent with the assumption that there is a single affinity site, and K_d values were estimated to be 136 and 149 μM in the presence of 40 nM Mg^{2+} and 10 mM Mg^{2+}, respectively. On the other hand, displacement curves in the absence of GTP were shallower than those expected for a single site and fitted the theoretical curves on the basis of the assumption that there are two sites with different affinities for carbamylcholine. The proportions of high-affinity sites were estimated to be 66 ± 7.1 ($n = 6$) and 68 ± 5.3 ($n = 6$) in the presence of 40 nM and 10 mM Mg^{2+}, respectively. The estimated K_d value for the high-affinity site was 25 times lower in the presence of 10 mM Mg^{2+} (0.064 μM) than in the presence of 40 nM Mg^{2+} (1.6 μM). K_d values for the low-affinity sites in the presence of 40 nM and 10 mM Mg^{2+}, 113 and 49 μM, respectively, were similar to the values obtained in the presence of GTP.

Table I: Apparent K_d's for the Binding of Carbamylcholine to mAChR Reconstituted with One of Three Kinds of G Proteins (Gi, Go, Gn)[a]

	Gi (μM)		Go (μM)		Gn (μM)	
	–GTP	+GTP	–GTP	+GTP	–GTP	+GTP
40 nM Mg^{2+}	15.2	201	5.2	136	7.4	59
10 mM Mg^{2+}	0.47	192	0.31	149	0.36	53

[a] Experimental conditions are the same as these described in the legend to Figure 1, except that one of three G proteins (1–5 nM) and receptors (50–130 pM) were used. Experiments were repeated twice (Gi, Gn) or four times (Go), and a single displacement curve by carbamylcholine was depicted for each set of conditions by using an average value at a given concentration of carbamylcholine, as shown in Figure 1. Apparent K_d's were calculated using the equation, $K_d = IC_{50}K_d^*/(K_d^* + [[^3H]QNB])$, where K_d^* is the dissociation constant for the binding of [^{3}H]QNB and IC_{50} is the concentration of carbamylcholine giving a half-maximal effect on the [^{3}H]QNB binding.

mAChRs reconstituted with Gi or Gn showed essentially the same ligand-binding characteristics as mAChRs reconstituted with Go. The effect of GTP on the displacement of the [^{3}H]QNB binding by carbamylcholine was observed in the presence of 40 nM Mg^{2+}, although the degree of the effect was smaller than that in the presence of 10 mM Mg^{2+} (Table I). Furthermore, similar binding characteristics were also observed for other muscarinic agonists, such as oxotremorine, pilocarpine, AF102B, and McN-A-343 (Table II). Each ligand showed the lowest affinity for mAChR in the presence of GTP irrespective of the presence or absence of Mg^{2+}, high affinity in the absence of GTP and Mg^{2+}, and the highest affinity in the absence of GTP and presence of Mg^{2+}.

Effect of Mg^{2+} Concentrations on the Affinity for Carbamylcholine of mAChRs Reconstituted with G Proteins. Figure 2 shows the effect of concentrations of free Mg^{2+} on the displacement by carbamylcholine of the [^{3}H]QNB binding. Results of the analysis of displacement curves are summarized

Table II: Apparent K_d's for the Binding of Several Muscarinic Agonists to mAChR Reconstituted with G Protein (Gi)[a]

	oxotremorine (μM)		pilocarpine (μM)		AF102B (μM)		McN-A-343 (μM)	
	-GTP	+GTP	-GTP	+GTP	-GTP	+GTP	-GTP	+GTP
40 nM Mg^{2+}	1.6	7.3	15	36	7.5	13	26	84
10 mM Mg^{2+}	0.12	8.4	0.73	22	3.5	15	3.9	64

[a] Experimental conditions are the same as those described in the legend to Figure 1, except that carbamylcholine was replaced by one of the above muscarinic agonists. Data obtained in one of two experiments with similar results are presented.

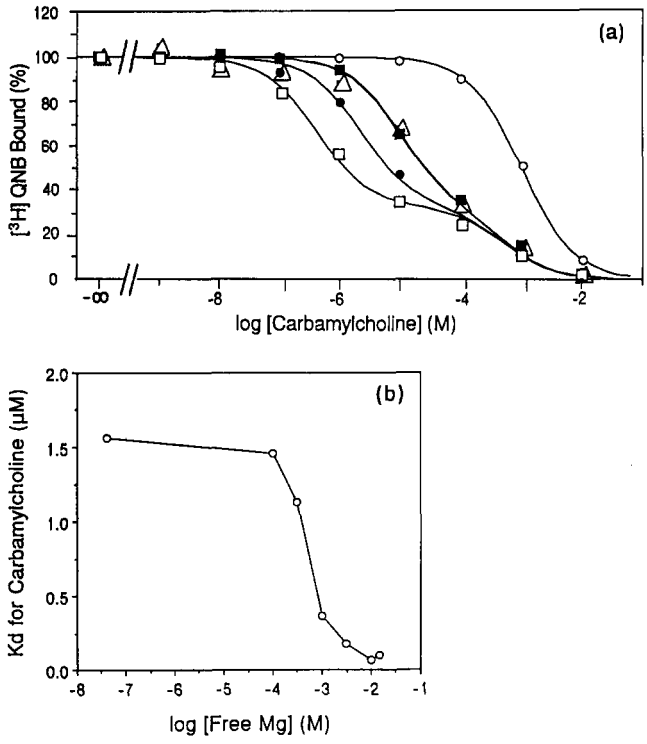

FIGURE 2: (a) Effect of Mg^{2+} concentration on the displacement by carbamylcholine of the [^{3}H]QNB binding to mAChRs reconstituted with Go. Experimental procedures are the same as described in the legend to Figure 1, except that concentrations of added $MgCl_2$ were 0.5, 5.1, 6.0, and 15 mM. Concentrations of free Mg^{2+} in the presence of 5 mM EDTA were calculated as described in Materials and Methods and are indicated as 40 nM (△), 0.1 mM (■), 1 mM (●), and 10 mM (□, ○). Data points represent the average for 2–9 separate experiments. Displacement curves in the presence of GTP (○) fit well with the equation for the one-site model. Displacement curves in the absence of GTP (△, ■, ●, □) fit well with the equation for the two-site model. Estimated parameters are shown in Table III. (b) Dissociation constants for carbamylcholine binding to the high-affinity sites in the absence of GTP and presence of different concentrations of Mg^{2+}.

Table III: Affinity for Carbamylcholine of mAChRs Reconstituted with Go in the presence of Different Concentrations of Mg^{2+} [a]

free Mg^{2+}	K_d(L) (μM)	K_d(H) (μM)	proportion of low-affinity sites (%)	number of experiments
40 nM	113	1.56	31.6	4
0.1 mM	102	1.46	33.8	9
0.3 mM	106	1.13	33.6	2
1.0 mM	71.3	0.366	32.4	8
3.0 mM	43.0	0.175	34.5	6
10.0 mM	49.3	0.0636	33.8	9
15.0 mM	69.7	0.0984	34.7	3

[a] Experimental procedures are described in the legend to Figure 2. Displacement curves by carbamylcholine of the [^{3}H]QNB binding, some of which are shown in Figure 2, were analyzed according two-site models, as described in the legend to Figure 1. Better fittings were obtained with the two-site model than with the one-site model. Variations in K_d values in the presence of 0.1, 1, and 10 mM Mg^{2+} were estimated by evaluating them from each experiment repeated 8–9 times and averaging the obtained values. Estimated values (the average ● sem) were as follows: A (%) 35.0 ± 2.1 (0.1 mM Mg^{2+}), 36.1 ± 2.7 (1 mM Mg^{2+}), 33.9 ± 1.6 (10 mM Mg^{2+}); K_d(L) (μM) 153 ± 19.3 (0.1 mM Mg^{2+}), 71.0 ± 17.0 (1.0 mM Mg^{2+}), 70.0 ± 8.1 (10.0 mM Mg^{2+}); K_d(H) (μM) 1.45 ± 0.19 (0.1 mM Mg^{2+}), 0.35 ± 0.06 (1.0 mM Mg^{2+}), 0.081 ± 0.015 (10.0 mM Mg^{2+}). The K_d(H) value in the presence of 1 mM Mg^{2+} was significantly different from those in the presence of 0.1 mM Mg^{2+} ($p < 0.01$) or 10 mM Mg^{2+} ($p < 0.01$).

in Table III. Displacement curves in the presence of GTP were essentially the same, irrespective of the concentration of Mg^{2+} (data not shown). Proportions of high-affinity sites and K_d values for the low-affinity site did not change significantly with a change in Mg^{2+} concentration from 10^{-7} to 10^{-2} M. On the other hand, K_d values for the high-affinity site decreased from 1.5 to 0.06–0.1 μM with the increase of Mg^{2+} concentration from 0.1 to 15 mM (Table III, Figure 2b).

These results indicate that 30–35% of mAChRs show only a low affinity for carbamylcholine, irrespective of the presence or absence of GTP. This proportion may represent a fraction of mAChRs that have been modified either in vivo or during the procedures of solubilization, purification, and reconstitution and are unable to interact with G proteins. On the other hand, 65–70% of mAChRs have a higher affinity in the absence of GTP than in its presence, indicating that they interact with G proteins. The low-affinity sites in the presence of GTP and the high-affinity sites in the absence of GTP are considered to represent free mAChR and the mAChR–G protein complex, respectively.

The finding that the affinity for carbamylcholine is converted from high to low by addition of GTP in the presence of as little as 40 nM Mg^{2+} suggests that Mg^{2+} is not required for the formation of the mAChR–G protein complex. Mg^{2+} did not influence the proportion of mAChRs with a high affinity for carbamylcholine but did increase the affinity for carbamylcholine in the absence of GTP but not in the presence of GTP, indicating that Mg^{2+} increases the affinity for carbamylcholine of the mAChR–G protein complex but not of the free mAChR.

Effect of Carbamylcholine on the [^{35}S]GTPγS Binding to G Proteins Reconstituted with mAChRs in the Presence of GDP. The rate of [^{35}S]GTPγS binding to G proteins reconstituted with mAChRs has been reported to increase with addition of muscarinic agonists (Kurose et al., 1986). However, the effect was small and difficult to observe reproducibly. On the other hand, the effect of carbamylcholine on [^{35}S]GTPγS binding was clearly observed in the presence of GDP and 10 mM Mg^{2+} (Figure 3b), as was reported previously (Tota et al., 1987; Florio & Sternweis, 1989; Ikegaya et al., 1990). The effect of carbamylcholine, however, was not observed in the presence of 40 nM Mg^{2+}, in spite of the presence of GDP (Figure 3a).

The binding isotherms of [^{35}S]GTPγS in the absence of GDP showed the presence of a single homogeneous site, and the K_d values did not change appreciably with or without carbamylcholine, or with or without Mg^{2+}, and ranged from 11 to 26 nM. The binding capacity, however, was 3.5-fold greater in the presence of 10 mM Mg^{2+} than in the presence of 40 nM Mg^{2+}.

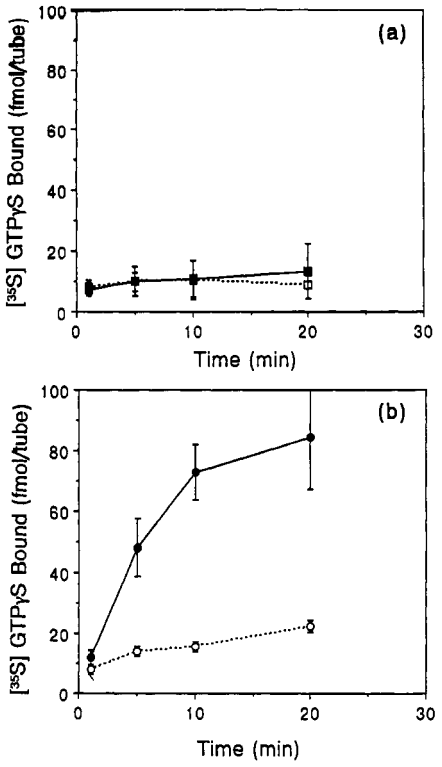

FIGURE 3: Effect of carbamylcholine on the [^{35}S]GTPγS binding to G proteins (a mixture of Gi and Go) reconstituted with mAChRs. The [^{35}S]GTPγS binding was carried out in the presence of 40 nM [^{35}S]GTPγS, 1 μM GDP, either 1 mM carbamylcholine (■, ●) or 10 μM atropine (□, ○), and either 40 nM (a) or 10 mM (b) Mg^{2+}. Concentrations of mAChRs and G proteins (a mixture of Gi and Go) and the ratio of G protein to mAChRs were 0.05–0.21 nM, 2.5–2.9 nM, and 23–26, respectively. Other experimental procedures are described in Materials and Methods. Data points are the mean for two experiments.

Table IV: Apparent Affinities for GDP and GTP of G Proteins Reconstituted with mAChRs

(a) Apparent K_d's for GDP and GTP[a,b]

	GDP (nM)		GTP (nM)	
	+atr	+carb	+atr	+carb
40 nM Mg^{2+}	29.3	24.1	24.2	42.4
10 mM Mg^{2+}	151	2770	124	209

(b) Concentrations of GDP and GTP for Half-Maximal Effects on [^{3}H]QNB Binding[c,d]

	GDP (nM)	GTP (nM)
40 nM Mg^{2+}	56.9	36.2
10 mM Mg^{2+}	1060	80.8

[a] K_d's were assessed from displacement by GDP and GTP of [^{35}S]GTPγS binding. [b] The data presented in Figure 4 were analyzed according to a one-site model, and apparent K_d values were calculated using the equation, $K_d = IC_{50}K_d^*/(K_d^* + [[^{35}S]GTP\gamma S])$, where K_d^* is the dissociation constant for the binding of [^{35}S]GTPγS. The data represent the mean of two experiments. [c] In the presence of 0.1 mM carbamylcholine. [d] These values were estimated from the data presented in Figure 6. Data obtained in one of two experiments with similar results are presented.

Figure 4 shows displacement curves by GDP or GTP of the [^{35}S]GTPγS binding in the presence of carbamylcholine or atropine at free Mg^{2+} concentrations of 10 mM or 40 nM. Apparent K_d values are summarized in Table IVa. The displacement curve by GDP was shifted to the right in the presence of carbamylcholine compared with the displacement curve in the presence of atropine, when 10 mM Mg^{2+} was present. Apparent K_d values were 151 and 2770 nM in the presence of atropine and carbamylcholine, respectively. The shift was not observed in the presence of 40 nM Mg^{2+}, and the apparent K_d values were 29 and 24 nM in the presence of atropine and carbamylcholine, respectively. A similar shift was also observed for the displacement by GTP in the presence of 10 mM Mg^{2+}, but the degree of the shift was much smaller and the apparent K_d values were 124 and 209 nM in the presence of atropine and carbamylcholine, respectively.

Table V: Apparent K_d's for the Binding of GDP to One of Three G Proteins (Gi, Go, Gn) Reconstituted with mAChRs[a]

	Gi (nM)		Go (nM)		Gn (nM)	
	+atr	+carb	+atr	+carb	+atr	+carb
40 nM Mg^{2+}	10	21	26	42	18	17
10 mM Mg^{2+}	25	570	220	1010	35	608

[a] Experimental conditions are the same as those described in the legend to Figure 4, except that one of three G proteins (1–5 nM) was used. Data of Gi and Go are the mean of two and three experiments. Data for Gn are the mean of the duplication of a single experiment. Experiments were repeated twice (Gi), three times (Go), or once (Gn), and a simple displacement curve by GDP was depicted for each set of conditions by using the average value obtained at a given concentration of GDP, as shown in Figure 4.

Table VI: Apparent K_d's for the Binding of GDP to G Protein (Gi) Reconstituted with mAChR in the Presence of Atropine (10 μM) or Muscarinic Agonists (1 mM) and in the Presence of 10 mM Mg^{2+} [a]

atropine (nM)	oxotremorine (nM)	pilocarpine (nM)	AF102B (nM)	McN-A-343 (nM)
54	494	715	67	622

[a] Experimental conditions are the same as those described in the legend to Figure 4, except that Gi (2–3 nM) was used and carbamylcholine was replaced by one of the above ligands. Data obtained in one of two experiments that yielded similar results are presented.

The decrease in the apparent affinity for GDP in the presence of carbamylcholine and Mg^{2+} was observed when mAChRs were reconstituted with one of the three G proteins, Gi, Go, and Gn (Table V). Furthermore, the effect of carbamylcholine could be replaced by other muscarinic agonists, such as oxotremorine, pilocarpine, AF102B, or McN-A-343 (Table VI).

Effect of Mg^{2+} Concentrations on the Binding of [^{35}S]-GTPγS and GDP in the Presence of Carbamylcholine. Effects of Mg^{2+} concentrations on the displacement by GDP of the [^{35}S]GTPγS binding in the presence of carbamylcholine and atropine are shown in Figure 5. The displacement curves by GDP of the [^{35}S]GTPγS binding shifted to the right with an increase in Mg^{2+} (Figure 5a). The apparent K_d for GDP, which was estimated from the concentration of GDP giving a half-maximal effect on the [^{35}S]GTPγS binding, increased from 26 to 270 nM with an increase in the free Mg^{2+} concentration from 40 nM to 3.3 μM, irrespective of the presence of atropine or carbamylcholine. The K_d value in the presence of atropine did not increase any more with the further increases in free Mg^{2+} concentration. On the other hand, the K_d value in the presence of carbamylcholine increased from 270 to 1010 nM with the increase of Mg^{2+} concentration from 3.3 μM to 10 mM (Figure 5a). The [^{35}S]GTPγS binding capacity increased with the increase in free Mg^{2+} concentration from 40 nM to 3.3 μM, but did not increase any more with the further increase in free Mg^{2+}.

Effects of Concentration of GDP or GTP on the Displacement by Carbamylcholine of [^{3}H]QNB Binding. The carbamylcholine displacement curve is shifted to the right by the addition of either GTP or GDP. In order to estimate effective concentrations of GTP and GDP, the binding of [^{3}H]QNB

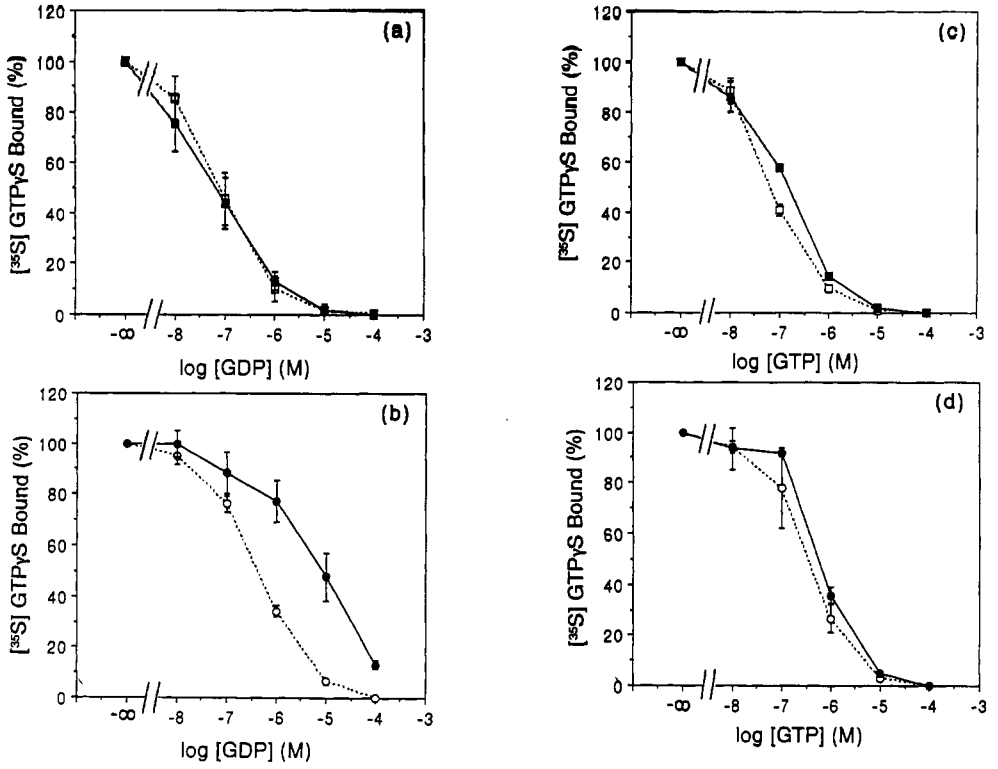

FIGURE 4: Effect of carbamylcholine on the displacement by GDP (a, b) or GTP (c, d) of the [^{35}S]GTPγS binding in the presence of either 1 mM carbamylcholine (■, ●) or 10 μM atropine (□, ○) and in the presence of either 40 nM (a, c) or 10 mM Mg^{2+} (b, d). Experimental procedures are the same as described in the legend to Figure 3, except that different concentrations of GDP or GTP were used and the incubation time was 20 min. Data points represent the average ± standard deviation for four (GDP) or two (GTP) independent experiments. Variations in IC_{50} values in the presence of GDP were estimated by evaluating them in each experiment (repeated four times) and averaging the values obtained. Estimated values (μM; the average ± sem) were as follows: (a) 70.3 ● 32.3 (carbamylcholine), 87 ± 25.3 (atropine); (b) 9640 ± 1203 (carbamylcholine), 560 ± 76.7 (atropine). The K_d value in the presence of 10 mM Mg^{2+} and carbamylcholine was significantly different from that in the presence of 10 mM Mg^{2+} and atropine ($p < 0.01$) and was also different from that in the presence of 40 nM Mg^{2+} ($p < 0.01$). Actual values of 100% were 6000 (atropine) and 5800 (carbamylcholine) cpm in 40 nM Mg^{2+} and 9700 (atropine) and 10 500 (carbamylcholine) cpm in 10 mM Mg^{2+}.

in the presence of 0.1 mM carbamylcholine was examined in the presence of various concentrations of GDP or GTP (Figure 6). An approximately 20-fold higher concentration of GDP was necessary to influence carbamylcholine binding in the presence of 10 mM Mg^{2+} as compared to the case when the Mg^{2+} concentration was 40 nM (Table IVb). Concentrations of GDP giving a half-maximal effect were 57 and 1060 nM in the presence of 40 nM and 10 mM Mg^{2+}, respectively. On the other hand, concentrations of GTP giving a half-maximal effect were 36 and 81 nM in the presence of 40 nM and 10 mM Mg^{2+}, respectively. These values are comparable to the K_d values estimated from displacements by GDP or GTP of [^{35}S]GTPγS binding in the presence of carbamylcholine (Table IVa).

DISCUSSION

The present results show that Mg^{2+} modifies the interaction of mAChR and G protein, which was monitored by measuring the effects of GTP and GDP on the [^{3}H]QNB binding in the presence of carbamylcholine and the effects of carbamylcholine on the [^{35}S]GTPγS binding in the presence of GDP. The relevant findings of this study are the following: (1) Mg^{2+} is not required for formation of the guanine nucleotide-sensitive high-affinity agonist-binding sites; (2) Mg^{2+} increases the affinity of the GTP-sensitive high-affinity sites for the agonist; and (3) Mg^{2+} is required for the carbamylcholine-induced decrease in the affinity for GDP.

Effects of Mg^{2+} on the agonist binding of mAChRs in atrial membranes have been studied by several groups, and they report that Mg^{2+} increases the high-affinity binding which is sensitive to guanine nucleotides (Wei & Sulakhe, 1980; Hulme et al., 1983; McMahon & Hosey, 1985). However, it has not been examined in detail whether these effects are due to the increase in the binding capacity or the increase in the affinity for agonists. In addition, there is some discrepancy among authors regarding the effect of guanine nucleotides in the absence of Mg^{2+}. Hulme et al. (1983) and Wei and Sulakhe (1980) reported only a slight effect of guanine nucleotides on the agonist binding in the absence of Mg^{2+}, but Harden et al. (1982) reported a clear effect of GTP in the absence of Mg^{2+}. Effects of Mg^{2+} have also been studied on the high-affinity binding of other G protein-like receptors. Bird and Maguire (1978) reported that the affinity of β adrenergic receptors in S49 lymphoma cell membranes for isoproterenol increased with Mg^{2+} concentration with a half-maximal effect at 2–3 mM, and the effect of Mg^{2+} was not observed for β adrenergic receptors in the cell membranes derived from cyc$^-$ mutants that lack Gs-α. The presence of guanine nucleotide-sensitive, high-affinity binding in the absence of Mg^{2+} has been reported for β adrenergic receptors in the heart (Harden et al., 1982), glucagon receptors in liver membranes (Rojas & Birnbaumer, 1985), and fMLP receptors in HL-60 cell membranes (Gierschik et al., 1989). On the other hand, the necessity for Mg^{2+} has been reported for β adrenergic receptors of S49 lymphoma cells (Birnbaumer, 1990). These experiments were carried out using membrane preparations, and hence there is a possibility that the effect of Mg^{2+} is not on receptors and/or G proteins but is indirect through the third component.

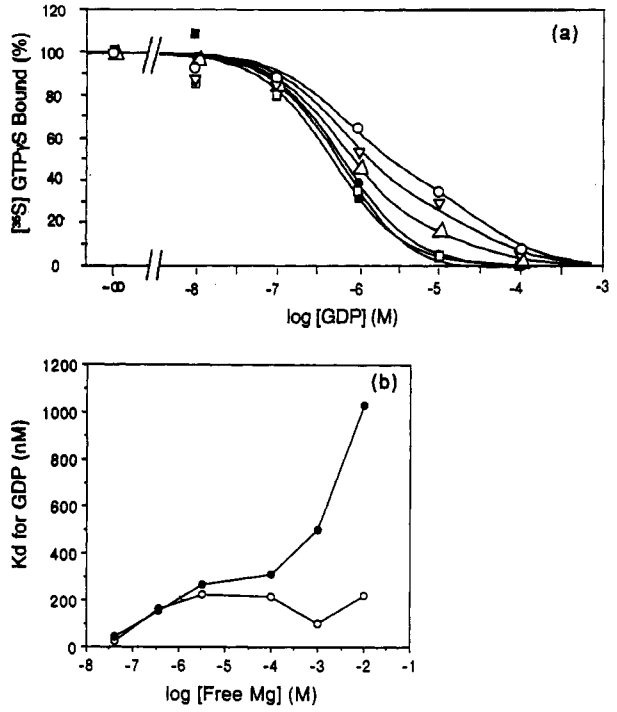

FIGURE 5: (a) Effect of Mg^{2+} on the displacement by GDP of the [^{35}S]GTPγS binding to Go reconstituted with mAChRs. Experimental procedures are the same as described in the legend to Figure 4, except that different concentrations of Mg^{2+} (370 nM (■, □), 0.1 mM (△), 1 mM (▽), and 10 mM (●, ○)) and 1.2–1.9 nM Go were used. Experiments were performed with atropine (■, ●) or carbamylcholine (□, △, ▽, ○). Data points represent the mean for three experiments. The concentration of Go and the ratio of Go to mAChRs in the reaction mixture were 1.2–1.9 nM and 10–17, respectively. Actual values of 100% were 1200–1700 cpm (40 nM Mg^{2+}) and 3300–4900 cpm (10 mM Mg^{2+}). (b) Apparent dissociation constants for GDP were calculated from IC_{50} values for displacement by GDP of the [^{35}S]GTPγS binding in the presence of different concentrations of Mg^{2+} with atropine (○) or carbamylcholine (●).

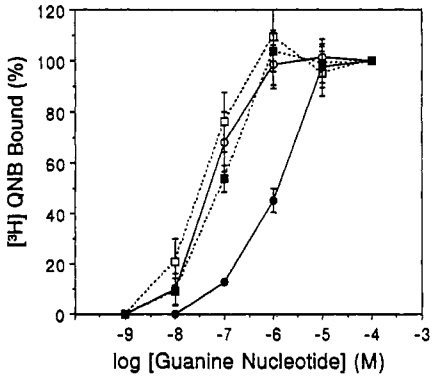

FIGURE 6: Effects of concentration of GDP (○, ●) or GTP (□, ■) on the [^{3}H]QNB binding in the presence of 0.1 mM carbamylcholine to mAChRs reconstituted with a mixture of Gi and Go at 40 nM (○, □) or 10 mM (●, ■) of Mg^{2+}. Concentrations of mAChRs and G proteins and the ratio of G proteins to mAChRs were 100–200 pM, 2.5–2.9 nM, and 14–16, respectively. The [^{3}H]QNB binding in the presence of 0.1 mM carbamylcholine and 0.1 mM GTP was taken as 100%, and that in the presence of 0.1 mM carbamylcholine and the absence of guanine nucleotide was taken as 0%. Concentrations of mAChRs and G proteins and the ratio of G proteins to mAChRs were 100–200 pM, 2.5–2.9 nM, and 14–16, respectively. Data points represent the average ± standard deviation for triplicate determinations in one of 2–3 experiments in which similar results were obtained. The actual counts at 100% were 1200–1600 cpm and those at 0% were 400–600 cpm, respectively.

The present findings that (1) the effect of GTP on agonist binding was apparent in the presence of 40 nM Mg^{2+} and (2) the proportion of guanine nucleotide-sensitive high-affinity sites did not change appreciably with the increase in Mg^{2+} concentration from 10^{-7} to 10^{-2} indicate that the formation of guanine nucleotide-sensitive high-affinity sites does not require the presence of Mg^{2+} or, more strictly, the presence of more than 40 nM Mg^{2+}. We could not exclude the possibility that the very high-affinity site for Mg^{2+} of Gi or Go with an apparent K_d of approximately 10 nM, which was reported by Higashijima et al. (1987), is necessary to be occupied by Mg^{2+} for the formation of the high-affinity sites. This, however, does not seem to be the case because the GTP-sensitive high-affinity agonist binding was observed in the presence of 5 mM EDTA without addition of Mg^{2+}, where free Mg^{2+} concentration is estimated to be less than 1 nM if the concentration of contaminating Mg^{2+} is less than 13 μM.

Apparent K_d's of the guanine nucleotide-sensitive high-affinity sites for carbamylcholine decreased from 1.6 μM in the presence of 40 nM Mg^{2+} to 0.064 μM in the presence of 10 mM Mg^{2+}. The simplest interpretation of this result is that there are two forms of complexes, mAChR–G protein (RG) and mAChR–(G protein)–Mg^{2+} (RGMg), with a low and a high affinity for carbamylcholine, respectively. If the two complexes were in rapid equilibrium during the [^{3}H]-QNB binding experiment, an intermediate affinity would be observed at a given concentration of Mg^{2+}. On this assumption, an apparent dissociation constant for the binding of Mg^{2+} to RG is equivalent to the concentration of Mg^{2+}, giving a half-maximal effect on the change in apparent association constants ($1/K_d$) for carbamylcholine, and is estimated to be approximately 2 mM.

Approximate K_d values of free mAChR (R), RG, and RGMg for carbamylcholine were 100, 1.5, and 0.1 μM, respectively. A 1000-fold difference in affinities between R and RGMg is consistent with previous results on reconstituted vesicles of purified atrial mAChRs and G proteins (Ikegaya et al., 1990), atrial membranes reconstituted with G proteins (Haga et al., 1990), or chicken heart membranes (McMahon & Hosey, 1985). The three affinity states for agonists (SH, H, and L) were originally proposed for mAChRs of rat cerebral membranes by Birdsall et al. (1980) and were also found for heart membranes (McMahon & Hosey, 1985; Mattera et al., 1985). SH and L sites are considered to correspond to RGMg and R, respectively, but the molecular nature of H sites remains obscure. It is interesting to note that the ratios of affinities for carbamylcholine or acetylcholine of SH, H, and L sites are similar to those of the affinities of RGMg, RG, and R. This may be accidental, but may reflect that mAChRs tend to take three stable conformations.

Binding of [^{35}S]GTPγS in the presence of 40 nM Mg^{2+} was not affected by carbamylcholine in the presence or absence of GDP, in spite of the fact that the mAChR–G protein complex is formed as assessed by the presence of guanine nucleotide-sensitive high-affinity agonist binding. The present result provides an example of a case where the formation of receptor–G protein complex does not necessarily result in their functional coupling. A similar disparity between complex formation and functional coupling has been reported to be caused by mutations of β adrenergic receptors or Gs-α subunits instead of the absence of Mg^{2+}. Miller et al. (1988) reported that a mutant of the Gs-α subunit may form a complex with β adrenergic receptors, as assessed by the high-affinity agonist binding, but may not be activated by β adrenergic receptors. Hausdorff et al. (1990) reported that a mutant of β adrenergic receptors exhibited high-affinity agonist binding identical to that of the wild-type receptor, but the maximal adenylyl cyclase

response mediated by this mutant receptor was less than one-half of that seen with the wild-type receptor.

Effects of carbamylcholine on the binding of [^{35}S]GTPγS were observed in the presence of Mg^{2+} and GDP. The requirement for Mg^{2+} and GDP has been observed using cardiac membranes by Hilf et al. (1989). The increase in the [^{35}S]GTPγS binding reflects the decrease in the affinity for GDP in the presence of carbamylcholine. The apparent affinity for GDP as estimated from the displacement of [^{35}S]-GTPγS binding decreased 18-fold in the presence of carbamylcholine, but the decrease in the affinity for GTP was less than 2-fold. Similar differential decreases in the affinities for GDP and GTP in the presence of carbamylcholine and Mg^{2+} were also observed for the effects of GDP and GTP on the displacement by carbamylcholine of the [^{3}H]QNB binding. The apparent affinities for GDP estimated by the two methods were comparable. These results indicate that the effect of carbamylcholine on the displacement by GDP of the [^{35}S]-GTPγS binding and the effect of GDP on the displacement by carbamylcholine of the [^{3}H]QNB binding are based on the same molecular event.

The affinity for GDP of G proteins reconstituted with mAChRs decreased in two phases with a change of Mg^{2+} concentration: muscarinic agonist-independent decrease occurred from 40 nM to 3.3 μM Mg^{2+}, and carbamylcholine-dependent decreased occurred from 3.3 μM to 10 mM Mg^{2+}. The latter decreased was observed in roughly the same region of Mg^{2+} concentration as the increase in the affinity for carbamylcholine. The simplest explanation is that there is a single Mg^{2+} binding site that is involved in both the formation of the high-affinity agonist binding and the apparent decrease in the affinity for GDP.

Displacement curves by GDP of the [^{35}S]GTPγS binding in the presence of atropine fit the assumption of a single affinity site. However, the displacement curves in the presence of carbamylcholine and free Mg^{2+} of higher than 3.3 μM did not fit to an assumption of a single site. The displacement curves are consistent with the existence of two sites: a higher affinity site with the same K_d as one in the presence of atropine (210 nM) and a lower affinity site. The K_d values of the lower affinity site were estimated to be 6–12 μM, and the proportion of the lower affinity sites increased from 10 to 44% with the increase of free Mg^{2+} concentration from 3.3 μM to 10 mM. The proportion of G proteins with apparently low affinity for GDP in the presence of carbamylcholine is greater than the total number of mAChR, which is one-tenth of total G proteins.

Dissociation rates of [^{3}H]GDP from G proteins reconstituted with mAChRs have been reported to increase in the presence of agonist (Haga & Haga, 1987, 1989; Tota et al., 1987). In these cases also, the amount of G proteins with the higher dissociation rate was greater than the total amount of mAChRs. Thus, G proteins of amounts several times greater than mAChRs are considered to show a higher dissociation rate and a higher K_d value in the presence of carbamylcholine.

In summary, the present experiments suggest that mAChR may form a complex with G proteins even in the absence of Mg^{2+}, but the formation of the complex of mAChR, G protein, and Mg^{2+} with the higher affinities for agonists is necessary for the action of mAChRs on G proteins.

ACKNOWLEDGMENT

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CORRECTIONS

The Antitumor Agent CC-1065 Inhibits Helicase-Catalyzed Unwinding of Duplex DNA, by Ira P. Maine, Daekyu Sun, Laurence H. Hurley, and Thomas Kodadek*, Volume 31, Number 16, April 28, 1992, pages 3968–3975.

Page 3968. In column 2, line 19, Reynolds et al., 1986, should read Reynolds et al., 1985.

Page 3970. In column 1, line 14, Reynold et al., 1985, should read Reynolds et al., 1985.

Page 3974. In column 1, last line, Lee et al., 1990, should read Lee et al., 1991.

Pages 3974 and 3975. Under References, the following were either worded incorrectly or missing entirely.

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