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Effects of an Agonist, Allosteric Modulator, and Antagonist on Guanosine-γ-[^{35}S]thiotriphosphate Binding to Liposomes with Varying Muscarinic Receptor/G_o Protein Stoichiometry

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ABSTRACT

We investigated whether alcuronium, an allosteric modulator of muscarinic acetylcholine receptors, can induce receptor-mediated activation of G_o proteins in liposomal membranes incorporating purified M_2 receptors and G_o proteins and whether its action is affected by the receptor/G_o protein (R/G_o) ratio. The binding of guanosine-γ-[^{35}S]thiotriphosphate ([^{35}S]GTPγS) served as the indicator of G protein activation. It was stimulated by empty receptors at high receptor densities, and the dose-response curve was shifted to the left by the agonist carbachol and to the right by the antagonist atropine. At an R/G_o ratio of 300:100, the rate of [^{35}S]GTPγS binding was the same in the presence or absence of 0.1 mM carbachol. Alcuronium increased the binding of [^{35}S]GTPγS at R/G_o ratios of $<$3:100 and diminished it at R/G_o ratios of $>$10:100, similar to previous observations on intact cells expressing muscarinic receptors at different densities. The apparent biphasicity of alcuronium action indicates that the allosteric modulator has at least two effects on muscarinic receptor/G protein interaction but its mechanistic basis is unclear. The "active state" of muscarinic receptors induced by alcuronium probably is different from that induced by carbachol. Changes in the densities of receptors and G_o proteins had little effect on the kinetics of [^{35}S]GTPγS binding and on receptor affinity for carbachol, provided the R/G_o ratio was kept constant. This suggests that the receptors and G proteins are located in microdomains in which their concentrations remain constant, despite variations in the amounts of lipidic membranes in the system.

It has been known for some time that the binding properties of muscarinic acetylcholine receptors may be modulated by agents acting allosterically (for a review, see Tuček and Proška, 1995) and that the allosteric modulators not only diminish (Clark and Mitchelson, 1976; Stockton *et al.*, 1983), but also enhance the affinity of the receptors for their orthosteric ligands (Tuček *et al.*, 1990; Proška and Tuček, 1994; Guo *et al.*, 1995; Jakubík *et al.*, 1995b, 1997; Lazareno and Birdsall, 1995). We found recently that several allosteric modulators of muscarinic receptors (i.e., alcuronium, gallamine, and strychnine) not only modify the affinity of the receptors for the agonists and antagonists but also influence the interaction between the receptors and the G proteins (Jakubík *et al.*, 1996). In experiments on CHO cell lines stably transfected with individual subtypes of muscarinic receptors, allosteric modulators had agonist-like effects on the synthesis of cAMP and of inositol phosphates in the cells. These effects could not be blocked by the orthosteric muscarinic antagonist QNB and could not be induced in cells that had not been transfected with the genes for muscarinic receptors. In certain cases, the direction of the effect of allosteric modulators on the synthesis of second messengers varied depending on the density of muscarinic receptors in the cell line used.

In the current work, we investigated whether the interaction between muscarinic receptors and a G protein also can be affected by the allosteric modulator alcuronium in a simplified system consisting of artificial lipid membranes incorporating purified M_2 receptors and purified G_o proteins and whether it depends on the stoichiometric ratio between the densities of the M_2 receptors and the G_o proteins (R/G_o ratio). The effects of carbachol (an orthosteric agonist) and atropine (an orthosteric antagonist) were investigated in parallel with those of alcuronium. Receptor-mediated increases in the binding of [^{35}S]GTPγS served as the indicator of G protein

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ABBREVIATIONS: ABT, 3-[2′-aminobenzhydryl-oxy]tropane; CHO, Chinese hamster ovary; GTPγS, guanosine-γ-thiotriphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; QNB, quinuclidinyl benzilate; R/G_o ratio, ratio between the densities of muscarinic receptors and G_o proteins.

activation. The results that we obtained indicate that empty receptors can themselves activate the G_o proteins and that there are substantial differences among the effects of the agonist, the allosteric modulator, and the antagonist. The effects of the allosteric modulator vary depending on the R/G_o ratio. The "active state" of muscarinic receptors induced by the allosteric modulator is different from the "active state" induced by the orthosteric agonist.

Experimental Procedures

Materials. Agarose (Sepharose 4-B) and DEAE-Sephadex were from Pharmacia Fine Chemicals (Uppsala, Sweden), Ultrogel AcA-34 was from Sepracor/IBF (Villeneuve la Garenne, France). Hydroxylapatite was from Sigma Chemical (St. Louis, MO). DEAE-Toyopearl 650S was from Toyo Soda MFG (Tokyo, Japan). Digitonin, phosphatidylcholine (from egg yolk), and phosphatidylinositol (L-α-phosphatidyl-D-*myo*-inositol-4-phosphate from bovine brain) were from Wako Pure Chemicals (Osaka, Japan). [^{3}H]QNB (79 Ci/mmol) and [^{35}S]GTPγS (1 Ci/μmol) were from DuPont-NEN (Boston, MA). ABT was synthesized (Haga and Haga, 1983), and ABT-agarose gel (Haga and Haga, 1985) and heptylamine Sepharose (Shaltiel, 1974) were prepared as described.

Purification of muscarinic M_2 receptors. Receptors were obtained by solubilization of membranes of Sf9 cells transiently expressing human m2 gene (Rinken *et al.*, 1994; Guo *et al.*, 1995). The membranes were treated with 1% digitonin and 0.1% sodium cholate, and muscarinic receptors were isolated by single-step affinity chromatography on ABT-agarose. After elution with atropine, they were concentrated on hydroxylapatite columns as described previously (Haga and Haga, 1985). The purity of the final preparation was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Isolation of G proteins. Crude membranes were prepared from porcine brains and their integral proteins were solubilized with 1% sodium cholate. G_o and G_i proteins were isolated together by chromatography on DEAE-Sephacel, Ultrogel AcA 34, and heptylamine-Sepharose (Haga *et al.*, 1986). DEAE-Toyopearl columns were used to separate the G_o from the G_i protein as described previously (Haga *et al.*, 1986), except that 0.7% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate was substituted for Lubrol PX in all solutions applied. The purity of G_o protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blots (negative reaction with monoclonal antibodies against G_i, G_q, G_{11}, G_{16}, and G_s, kindly provided by Dr. J. Novotný).

Reconstitution of M_2 receptors and G proteins in lipid vesicles. The procedure was modified from that described by Shiozaki and Haga (1992). The lipid mixture was obtained by mixing 4 volumes of a solution of cholesterol hemisuccinate (10 mg/ml in methanol), 48 volumes of a solution of phosphatidyl choline (20 mg/ml in chloroform), and 48 volumes of a solution of phosphatidyl inositol (20 mg/ml in chloroform). The solvents were evaporated with a stream of nitrogen so as to obtain a thin layer of lipids on the wall of the test tube. Then, the lipids were emulsified to form vesicles in a solution of 20 mM K-HEPES, pH 8.0, 1 mM EDTA, 160 mM NaCl, and 1% sodium cholate by 30-min sonication at 4°. Vesicles were mixed with a suitable amount of purified M_2 receptors and separated on a column with 2 ml of Sephadex G-50. A suitable amount of G_o protein was added to them, and the mixture was incubated on ice for 60 min, after which it was diluted as requested.

The number of receptors incorporated into vesicles was determined in saturation binding experiments with increasing concentrations of [^{3}H]QNB, using Whatman glass fiber filters (GF/B) for the separation of the radioligand bound to vesicles. Data from the [^{3}H]QNB saturation experiments indicated the presence of a homogeneous population of binding sites, with K_d values in the range of 330–400 pM for all vesicle preparations. The amount of G protein incorporated into vesicles was determined according to the binding of [^{35}S]GTPγS at its single saturating concentration of 1 μM, again using Whatman GF/B filters for the separation of vesicle-bound radioactivity. These determinations were performed in the absence of GDP and in the presence of Mg^{2+}, ensuring virtually irreversible [^{35}S]GTPγS binding to all guanyl nucleotide binding sites (K_d = 11–26 nM; Shiozaki and Haga, 1992).

Three kinds of reconstituted vesicles were used in most experiments, in which the ratio between the molar concentration of muscarinic receptors (as determined by [^{3}H]QNB binding) and of G_o protein (as determined by [^{35}S]GTPγS binding) (i.e., the R/G_o ratio), was close to 1:100, 10:100, or 50:100. A fourth kind of vesicles also was used when required that contained only muscarinic receptors but no G proteins (10:0), and the fifth kind of vesicles contained only G proteins but no receptors (0:100). An even broader scale of R/G_o ratios was applied in experiments described in Fig. 2.

Kinetics of [^{35}S]GTPγS binding. For measurements of [^{35}S]GTPγS binding to G proteins in reconstituted vesicles, the vesicles were preincubated at 30° in 150 μl of a medium consisting of 10 mM $MgCl_2$, 1 mM dithiothreitol, and the investigated muscarinic ligand. After 15 min (or 30 min, where indicated), [^{35}S]GTPγS and GDP were added to final concentrations of 50 nM and 1 μM, respectively, and the incubation was continued in a final volume of 200 μl as needed. The binding was arrested by the addition of 500 μl of a stopping solution consisting of 50 mM Tris·HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, and 0.1 mM GTP, and the radioactivity bound to vesicles was separated on Whatman GF/B filters. In experiments designed to compare the effect of various concentrations of the same muscarinic ligand on [^{35}S]GTPγS binding (i.e., to obtain concentration-response curves), [^{35}S]GTPγS association was arrested at a suitable time during the phase of linear association, namely after 10 min in experiments with most ligands, after 5 min in experiments with carbachol at the 50:100 R/G_o ratio, or after 30 min in experiments with atropine and alcuronium at the 1:100 and 10:100 R/G_o ratios.

Treatment of data. Data in figures and tables are mean ± standard error of three experiments performed with incubation in duplicate. Values of K_d (equilibrium dissociation constant for the binding of a ligand to a single population of binding sites or to an undefined number of populations), K_{low}, and K_{high} (equilibrium dissociation constants for the binding of a ligand to two populations of binding sites displaying low and high affinity, respectively), f_{high} (fraction of binding sites displaying high affinity and expressed as percentage of the total number of the binding sites), K_i (equilibrium dissociation constant of an inhibitor), B_{max} (maximum binding capacity), EC_{50} (concentration of a ligand producing one half of maximum effect), and k_{obs} (apparent association rate constant) were computed by nonlinear regression according to accepted conventions (Limbird, 1986; Hulme, 1992; Jakubík and Tuček, 1994a, 1994b; Jakubík *et al.*, 1995a, 1995b). Apparent association rate constants (k_{obs}) for the binding of [^{35}S]GTPγS to G proteins in liposomes were obtained by fitting the equation

$$B_t = B_{eq} \times (1 - e^{-k_{obs} \times t}),$$

where B_t is binding at time t, and B_{eq} is binding at equilibrium.

In concentration-response curves of Figs. 3 and 5, data on the binding of [^{35}S]GTPγS in the presence of muscarinic ligands were expressed as multiples of the binding in their absence, and the concentration-response curves were obtained by fitting the equation

$$B_L = B_0 + \frac{(B_{max} - B_0) \times [L]^{n_H}}{EC_{50}{}^{n_H} + [L]^{n_H}}$$

where B_0 describes the binding in the absence of a muscarinic ligand, B_L indicates the binding in the presence of a muscarinic ligand at concentration [L], and B_{max} corresponds to the extrapolated value for the binding in the presence of an infinitely high concentration of the

muscarinic ligand. The maximum change of [^{35}S]GTPγS binding induced by a ligand (E_{max} in Tables 1 and 2) corresponded to B_{max}/B_0.

Results

Effects of carbachol, atropine, and alcuronium on [^{35}S]GTPγS association with reconstituted vesicles at different R/G_o. The time course of the association of [^{35}S]GTPγS with G proteins in vesicles with three different R/G_o ratios is shown in Fig. 1. Characteristics of the vesicles used and the numerical values computed from experiments in Fig. 1 are summarized in Table 1. The effects of muscarinic ligands on the rates of [^{35}S]GTPγS association were investigated in additional separate experiments on a broader scale of R/G_o ratios (Fig. 2).

It can be seen from Table 1 that the binding of [^{35}S]GTPγS occurred not only to vesicles reconstituted with receptors and G_o proteins, but also to vesicles that did not contain any receptors (0:100 ratio). In the absence of receptors, carbachol, alcuronium, and atropine had no effect on the rate of [^{35}S]GTPγS binding. The inclusion of receptors (with no accompanying ligand) into the membranes enhanced the rate of [^{35}S]GTPγS binding, with k_{obs} values changing from 0.026 min^{-1} at an R/G_o ratio of 0:100 to 0.128 min^{-1} at an R/G_o ratio of 50:100 (Table 1). The rate of [^{35}S]GTPγS association was regularly accelerated (compared with control samples) by carbachol. However, at the highest R/G_o ratio of 300:100, the rate of [^{35}S]GTPγS association in the absence of the agonist reached the same high value as that which could be maximally induced by carbachol (Fig. 2).

The rate of [^{35}S]GTPγS association was not affected by 100 nM atropine and, as long as atropine was present, remained virtually the same at all R/G_o ratios between 0:100 and 50:100, although it became augmented at R/G_o ratios of 100:100 and 300:100. Because the rate of [^{35}S]GTPγS association was increased by increases in the R/G_o ratios in control experiments (i.e., in the absence of any muscarinic ligand), it seemed that atropine decelerated [^{35}S]GTPγS association at R/G_o ratios of 30:100 and higher (Figs. 1 and 2 and Table 1).

Compared with control samples, alcuronium enhanced the rate of [^{35}S]GTPγS association at R/G_o ratios of 0.3:100 and 1:100 (Figs. 1 and 2 and Table 1). Increasing R/G_o ratios to higher values had much less enhancing effect on the rates of [^{35}S]GTPγS association in the presence of alcuronium than it had in the absence of muscarinic ligands. As a result, the rate of [^{35}S]GTPγS association was faster in the presence of alcuronium than in control samples at low R/G_o ratios and slower than in control samples at high R/G_o ratios.

The concentration dependence of the effects of carbachol, alcuronium, and atropine on the rates of [^{35}S]GTPγS binding to reconstituted vesicles with different R/G_o ratios is shown in Fig. 3 and relevant numerical data are summarized in Table 1. The notable features of Fig. 3 include the opposite effects of alcuronium at the 1:100 and 50:100 R/G_o ratios and the higher relative inhibition of [^{35}S]GTPγS association rate by atropine at the higher R/G_o ratio.

Effects of carbachol, alcuronium, and atropine on [^{35}S]GTPγS association with reconstituted vesicles at different densities of receptors and G_o proteins. Data in the previous section were obtained in experiments in which the concentration of the G_o proteins in the reconstituted vesicles was kept constant and the concentration of receptors was varied. The observed variations in the binding rates were presented as a consequence of changes in the ratio of receptors to G proteins. It might be argued that the rates of [^{35}S]GTPγS association were influenced by changes in receptor densities in the membranes rather than by changes in R/G_o ratio. To check such an alternative interpretation, experiments were performed in which the R/G_o ratio was kept at a constant level of 10:100 and the total amounts of receptors and G_o proteins per incubation tube also were kept constant, but the amounts of the lipid mixture (and thus the numbers of vesicles and the areas of the membranes available for receptor and G protein incorporation) used for reconstitution were different. Three different sets of tubes were used. In those with the "1 × Basic" densities, the densities of receptors and G_o proteins in liposomal membranes were the same as they had been in the experiments described in Figs. 1–3 and in Table 1 using the 10:100 R/G_o ratio. In the tubes

TABLE 1

[^{35}S]GTPγS binding to reconstituted vesicles containing M_2 receptors and G_o proteins at different concentration ratios, with the concentration of G_o held constant

Values were derived from the experiments shown in Figs. 1 and 3. They are mean ± standard error of three experiments performed with incubations in duplicate.

	R/G_o ratio expected				
	1:100	10:100	50:100	10:0	0:100
B_{max} for [^{3}H]QNB (fmol/tube)	4.20 ± 0.04	38.2 ± 0.3	199 ± 3	38.5 ± 0.3	
Overall concentration of [^{3}H]QNB binding sites (pM)	21	191	995	192	0
B_{max} for [^{35}S]GTPγS (fmol/tube)	444 ± 3	441 ± 8	436 ± 4		452 ± 6
Overall concentration of [^{35}S]GTPγS binding sites (pM)	2220	2205	2180	0	2260
R/G_o obtained	0.0097 ± 0.0001	0.089 ± 0.001	0.454 ± 0.008		
k_{obs} (min^{-1}) without muscarinic ligand	0.029 ± 0.001	0.044 ± 0.000	0.128 ± 0.001		0.026 ± 0.000
k_{obs} (min^{-1}) in the presence of					
1 mM carbachol	0.173 ± 0.002	0.179 ± 0.001	0.195 ± 0.003		0.027 ± 0.000
10 μM alcuronium	0.040 ± 0.000	0.043 ± 0.001	0.045 ± 0.001		0.026 ± 0.000
100 nM atropine	0.028 ± 0.000	0.029 ± 0.000	0.031 ± 0.000		0.027 ± 0.000
E_{max} (fold over basal) for					
Carbachol	3.27 ± 0.04	2.32 ± 0.00	1.30 ± 0.00		
Alcuronium	1.28 ± 0.01		0.50 ± 0.01		
Atropine		0.81 ± 0.01	0.37 ± 0.00		
EC_{50} (μM) for					
Carbachol	8.06 ± 0.18	16.62 ± 0.12	25.72 ± 0.23		
Alcuronium	1.760 ± 0.014		0.651 ± 0.011		
Atropine		0.0016 ± 0.0000	0.0010 ± 0.0000		

designated as "0.1 × Basic" or "5 × Basic," the densities of receptors and G_o proteins were 10-fold lower or 5-fold higher, respectively, than for the "1 × Basic" concentrations. The R/G_o ratio always remained at the 10:100 level.

Within the range of three different receptor and G protein densities used at the constant R/G_o ratio of 10:100, the rates of [^{35}S]GTPγS association were stable both in the absence of muscarinic ligands and in the presence of 100 nM atropine or 10 μM alcuronium (Fig. 4 and Table 2). The k_{obs} values computed from experiments with 1 mM carbachol (Table 2) indicate that the rate of [^{35}S]GTPγS association was slightly but significantly faster under the "5 × Basic" conditions (i.e., if the densities of receptors and G proteins were high). Concentration-response curves shown in Fig. 5 confirm that the inhibitory effect of atropine on [^{35}S]GTPγS association was virtually the same at the three concentrations of receptors and G proteins examined and that various concentrations of alcuronium had little effect under the conditions used in this set of experiments.

Inhibition of [^{3}H]QNB binding to reconstituted vesicles by carbachol. It generally is assumed that receptors that are associated with a G protein display higher affinities for their agonists and that this can be seen in agonist-versus-antagonist competition binding curves. To obtain information about this aspect of our system, we performed competition binding experiments (carbachol versus [^{3}H]QNB) on vesicles in which the R/G_o ratios were 1:100, 10:100, 50:100, or 10:0. As shown in Fig. 6 (*left*), the curves describing the inhibition of [^{3}H]QNB binding by carbachol differed in their steepness and their left-to-right position depending on the R/G_o ratio. The higher the relative density of receptors (compared with that of G proteins), the more to the right and the steeper were the binding curves, suggesting that the proportion of receptors coupled to G_o ($[RG]/[R_{total}]$) was the highest in the vesicles with the 1:100 R/G_o ratio and the lowest in the vesicles without G proteins. The fraction of the high affinity binding sites (presumably reflecting the proportion of receptor/G protein complexes) computed from the binding data diminished from 73% at the 1:100 R/G_o ratio to 16% at the 50:100 R/G_o ratio.

As shown in the Fig. 6 (*right*), changes in the density of receptors in the liposomes had no effect on the shape of the competition binding curves of carbachol versus [^{3}H]QNB when the R/G ratio was kept constant.

TABLE 2

[^{35}S]GTPγS binding to reconstituted vesicles containing M_2 receptors and G_o proteins at a fixed molar ratio of 10:100 but at different final concentrations in liposomal membranes

Values were derived from the experiments shown in Figs. 4 and 5. They are mean ± standard error of three experiments performed with incubations in duplicate.

	Receptor and G_o protein concentration		
	0.1 × Basic	1 × Basic	5 × Basic
R/G_o ratio expected	10:100	10:100	10:100
B_{max} for [^{3}H]QNB (fmol/tube)	41.25 ± 0.23	38.2 ± 0.3	38 ± 0
Overall concentration of [^{3}H]QNB binding sites (pM)	206	191	190
B_{max} for [^{35}S]GTPγS (fmol/tube)	438 ± 5	454 ± 7	436 ± 3
Overall concentration of [^{35}S]GTPγS binding sites (pM)	2190	2270	2180
R/G_o ratio obtained	0.094 ± 0.002	0.084 ± 0.002	0.087 ± 0.001
k_{obs} (min^{-1}) without muscarinic ligand	0.042 ± 0.000	0.044 ± 0.000	0.045 ± 0.001
k_{obs} (min^{-1}) in the presence of			
1 mM carbachol	0.177 ± 0.002	0.178 ± 0.002	0.185 ± 0.000
10 μM alcuronium	0.042 ± 0.000	0.042 ± 0.000	0.044 ± 0.001
10 nM atropine	0.030 ± 0.000	0.029 ± 0.000	0.029 ± 0.000
E_{max} (fold over basal) for			
Carbachol	2.63 ± 0.06	2.35 ± 0.04	2.07 ± 0.06
Atropine	0.80 ± 0.00	0.77 ± 0.01	0.75 ± 0.01
EC_{50} (μM) for			
Carbachol	14.72 ± 0.17	16.85 ± 0.06	20.85 ± 0.60
Atropine	0.0019 ± 0.0001	0.0016 ± 0.0000	0.0013 ± 0.0000

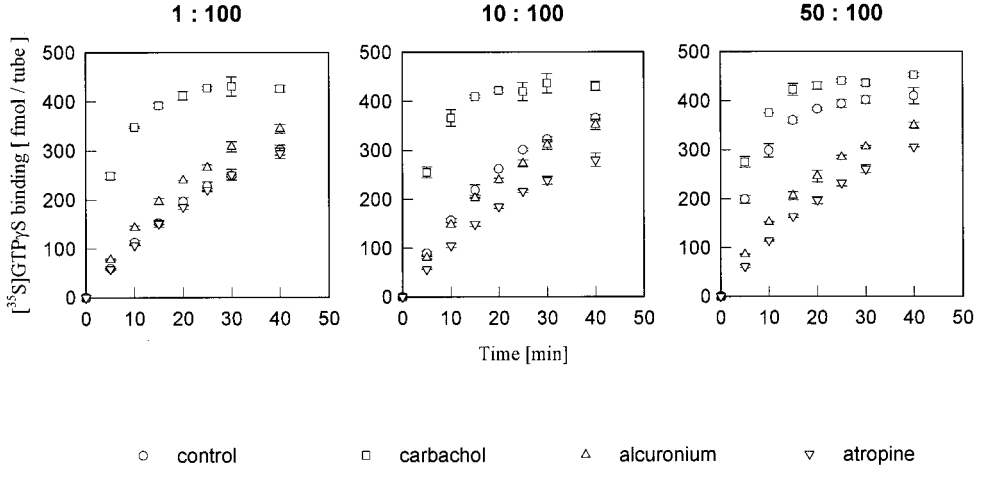

Fig. 1. Effects of 1 mM carbachol, 10 μM alcuronium, and 100 nM atropine on the time course of [^{35}S]GTPγS binding to lipid vesicles containing the M_2 receptors and the G_o protein at nominal ratios of 1:100, 10:100, and 50:100. *Abscissa*, time after the addition of [^{35}S]GTPγS. *Ordinate*, fmol of [^{35}S]GTPγS bound/incubation tube. Note that the content of G_o/incubation tube was the same in all three graphs, whereas the content of receptors increased (*left graph* < *middle graph* < *right graph*). See Table 1 for numerical characteristics of the incubation conditions and of the results.

Discussion

Artificial membranes reconstituted with purified G protein-coupled receptors and G proteins have been fruitfully used for investigations of receptor/G protein interactions (for a review, see Birnbaumer and Birnbaumer, 1995). Although many aspects of these interactions remain controversial (Chidiac and Wells, 1992), two points have been generally accepted:

First, agonist-liganded receptors diminish the affinity of G proteins for GDP and stimulate the dissociation of GDP from them (Brandt and Ross, 1986; Tota *et al.*, 1987; Florio and Sternweis, 1989; Haga *et al.*, 1989; Shiozaki and Haga, 1992).

Second, agonist-liganded receptors stimulate the binding of GTP or its unhydrolyzable analogue [^{35}S]GTPγS to G proteins, and this is mainly due to the receptor-induced decrease in the binding of GDP. In studies of reconstituted systems containing G_i and G_o, the addition of GDP was necessary to reveal the effect of agonists on [^{35}S]GTPγS binding (Florio and Sternweis, 1989; Ikegaya *et al.*, 1990; Shiozaki and Haga, 1992; but see Kurose *et al.*, 1986, and Freissmuth *et al.*, 1991a), whereas the addition of GDP was not necessary in systems containing G_s (Cerione *et al.*, 1985; Brandt and Ross, 1986; Freissmuth *et al.*, 1991b) or G_q (Nakamura *et al.*, 1995). The meaning of these differences is not clear, and their analysis is difficult in view of the tight binding of GDP to G proteins (Ferguson *et al.*, 1986).

Our data offer several new insights with regard to G_o protein activation by unliganded muscarinic receptors and by receptors associated with an agonist, antagonist, or allosteric modulator, to the importance of the R/G_o ratio, and to the likely spacial organization of receptors and G proteins in liposomal membranes. The following are the most important findings.

Empty muscarinic receptors increase the apparent rate of [^{35}S]GTPγS binding to G_o proteins. When present at a high concentration (R/G_o ratio = 300:100), empty receptors were able to activate the G_o proteins to the same degree as the agonist-liganded receptors (Fig. 2). Similar

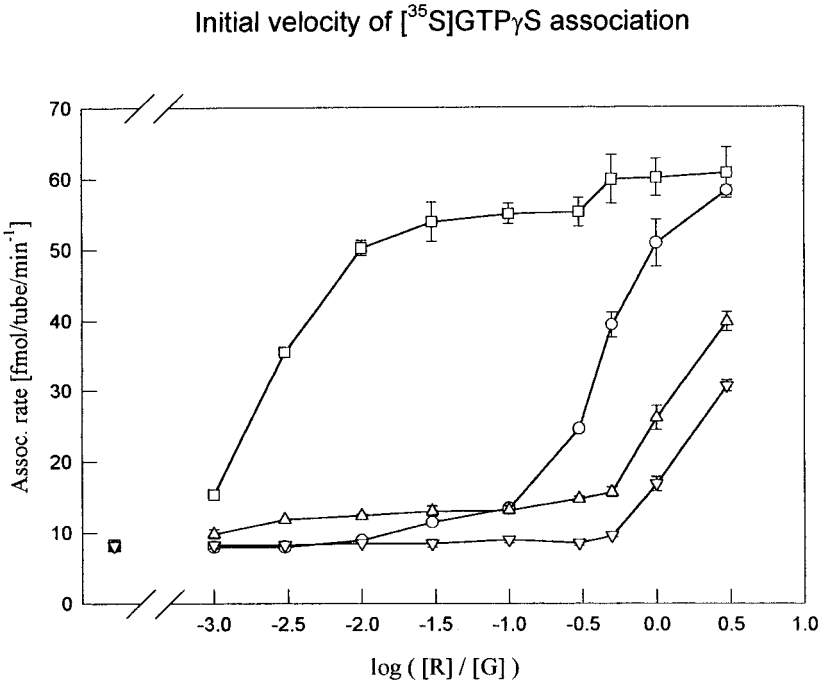

Fig. 2. Initial velocity of [^{35}S]GTPγS binding to reconstituted vesicles with different R/G_o ratios. Vesicles were preincubated in the control medium (○) or in the presence of 100 μM carbachol (■), 10 μM alcuronium (△), or 0.1 μM atropine (▽). After 30 min, [^{35}S]GTPgS (50 nM) was added simultaneously with 1 μM GDP, and [^{35}S]GTPγS binding was determined at time 0 and at three time points within the linear phase of radioligand association. *Abscissa*, log R/G_o ratio. *Ordinate*, rate of [^{35}S]GTPγS association (fmol/tube/min).

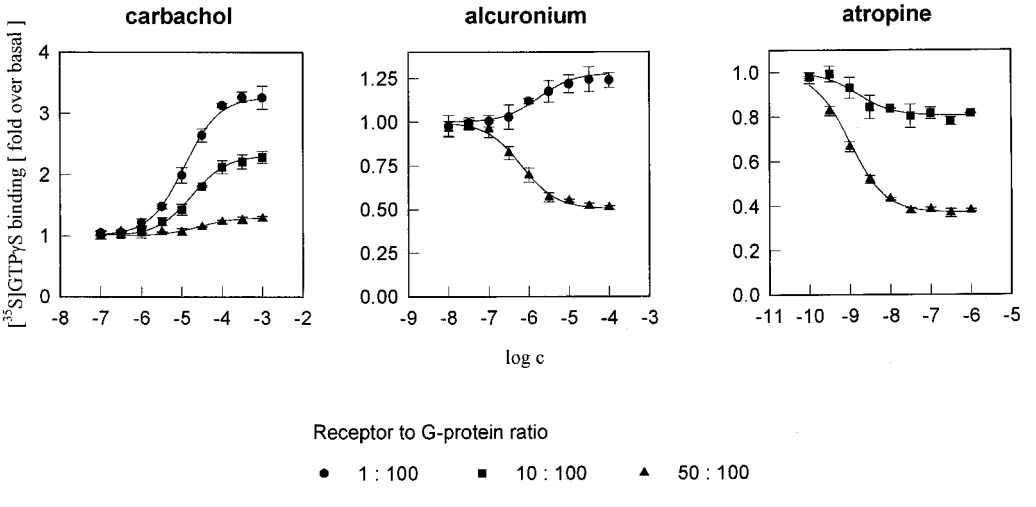

Fig. 3. Concentration-response curves for the effects of carbachol, alcuronium, and atropine on the binding of [^{35}S]GTPγS to lipid vesicles containing the M_2 receptors and the G_o protein at the ratios of 1:100, 10:100, and 50:100. *Abscissa*, $\log_{10}$ of the concentration of the muscarinic ligand (M). *Ordinate*, fold change in the amount of [^{35}S]GTPγS bound, compared with the situation in the absence of any muscarinic ligand. See the text for the duration of incubation and Table 1 for numerical characteristics of the incubation conditions and of the results.

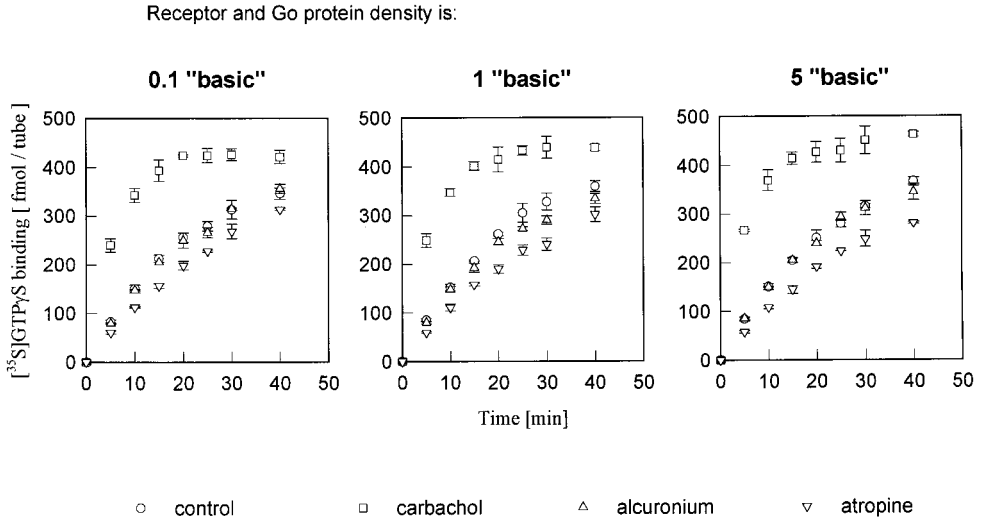

Fig. 4. Effects of 1 mM carbachol, 10 μM alcuronium, and 100 nM atropine on the time course of [^{35}S]GTPγS binding to lipid vesicles containing the M_2 receptors and the G_o protein at the same ratio of 10:100 but at three different concentrations. Reconstituted vesicles had been obtained in such a way that the same amounts of receptors and 10-fold higher amounts of G_o protein had been incorporated into different volumes of the vesicle emulsion. Consequently, the densities of both receptors and G_o were 10- and 50-fold higher (*middle and right*, respectively, compared with the *left*). Under the "1 Basic" conditions (*middle*), the densities of receptors and G_o proteins corresponded to those in the 10:100 samples used in Figs. 1 and 3. See Table 2 for numerical characteristics of the incubation conditions and of the results.

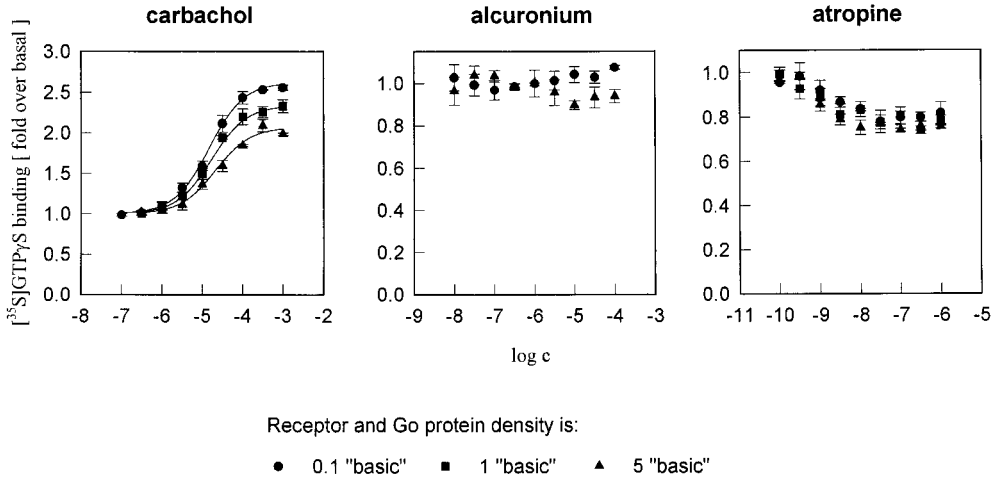

Fig. 5. Concentration-response curves for the effects of carbachol, alcuronium, and atropine on the binding of [^{35}S]GTPγS to lipid vesicles reconstituted with the M_2 receptors and the G_o protein at a fixed ratio of 10:100 and at three different receptor and G_o protein concentrations. *Abscissa*, $\log_{10}$ of the concentration (M) of muscarinic ligand. *Ordinate*, fold change of the amount of [^{35}S]GTPγS bound compared with the situation in the absence of the muscarinic ligand. The arrangement of experiments was the same as in Fig. 4. See the text for the duration of incubation and Table 2 for numerical characteristics of the incubation conditions and of the results.

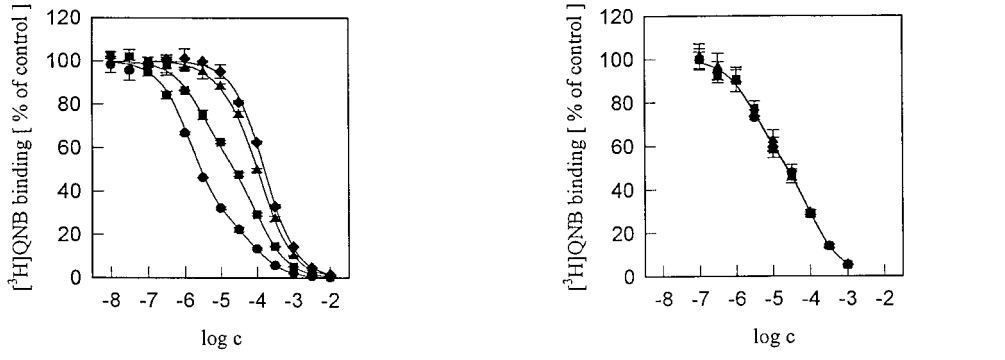

Fig. 6. Inhibition of [^{3}H]QNB binding to reconstituted vesicles by carbachol. *Abscissa*, $\log_{10}$ of the concentration (M) of carbachol. *Ordinate*, [^{3}H]QNB binding in the presence of carbachol, expressed as percentage of the binding in its absence. *Left*, inhibition of [^{3}H]QNB binding to vesicles with different R/G_o ratios. These vesicles corresponded to those used in Figs. 1 and 3 and Table 1. *Right*, inhibition of [^{3}H]QNB binding to vesicles with a constant R/G_o ratio of 10:100 but with three different concentrations of receptors and G_o proteins, corresponding to those used in Figs. 3 and 4 and Table 2. The "1 Basic" concentrations of receptors and G_o proteins (*right*) corresponded to the concentrations in the samples denoted as 10:100 (*left*). See Table 3 for computed characteristics of carbachol binding.

effects of empty A_1 adenosine and β-adrenergic receptors have been noted (Schütz and Freissmuth, 1992).

Atropine prevents or diminishes the agonist-independent stimulatory effects of muscarinic receptors on [^{35}S]GTPγS binding. However, increases in the rate of [^{35}S]GTPγS binding could be observed even in the presence of atropine if the R/G_o ratio was raised above 50:100 (Fig. 2). It seems that the effect of atropine can be viewed as an increase of the EC_{50} concentration of receptors required for G_o activation.

Carbachol increases the apparent rate of [^{35}S]GTPγS binding to G_o proteins and its effect varies with the R/G_o ratio. Although the enhancement of [^{35}S]GTPγS binding by muscarinic agonist was expected (Kurose *et al.*, 1986; Florio and Sternweis, 1989; Hilf *et al.*, 1989; Shiozaki and Haga, 1992; Lazareno and Birdsall, 1993; Nakamura *et al.*, 1995), several features of the current data merit attention:

First, the apparent rate of carbachol-stimulated [^{35}S]GTPγS binding approaches a maximum when the R:G_o ratio reaches 1:100 and is changed little by further increases in the relative density of receptors (Figs. 1 and 2, Table 1). Although the ceiling in the amount of [^{35}S]GTPγS bound is given by the quantity of G_o available for binding, it is not clear what determines the ceiling in the rate of [^{35}S]GTPγS binding at increasing receptor concentrations.

Second, carbachol brings about a leftward shift of the curve describing how the rate of [^{35}S]GTPγS association depends on the concentration of receptors, and its effect can be viewed as a decrease in the EC_{50} concentration of receptors required for G_o activation.

Third, the concentration of carbachol necessary for its half-maximal effect on [^{35}S]GTPγS binding (EC_{50} of carbachol) increases with increasing R/G_o ratios (Table 1). This can be explained by changes in the proportion of receptors that are physically associated with G proteins (Fig. 6 and Table 3). Although the absolute number of RG complexes increases with increasing R/G_o ratios, the proportion of coupled receptors (RG as a percentage of R_{total}) decreases, and this is the likely cause of the increases in the EC_{50} values. A very small increase of the EC_{50} values for carbachol also was observed when the R/G_o ratios were kept constant, but the densities of receptors and G proteins were augmented (Table 2). In this case, the above explanation cannot be applied, and we are uncertain as to the likely reason.

Alcuronium affects the binding of [^{35}S]GTPγS to G_o proteins in two directions. Compared with the binding that occurs in the absence of alcuronium, the binding of [^{35}S]GTPγS in the presence of alcuronium is enhanced at low R/G_o ratios (<10:100) and diminished at high R/G_o ratios (>10:100; Figs. 1–3). This point is discussed further.

The effects of carbachol, atropine, and alcuronium on [^{35}S]GTPγS binding are all receptor mediated. The three ligands had no effect when receptors were absent from the system (Table 1).

The kinetics of [^{35}S]GTPγS association with the vesicles are affected very little by changes in the density of receptors and G_o proteins as long as the R/G_o ratio remains constant. The most likely explanation of relevant observations (Fig. 4 and Table 2) is that muscarinic receptors and G_o proteins are not uniformly dispersed in liposomal membranes but rather are clustered in microdomains in which their concentrations remain nearly constant despite variations in the amounts of lipidic membranes in the system. The agglomeration of signaling proteins in microdomains of cell surface membranes has been highlighted by Neubig (1994; see also Huang *et al.*, 1997).

Increases in R/G_o ratios bring about decreases of the fraction of receptors displaying high affinity for the agonist carbachol. The higher the R/G_o ratio, the lower proportion of receptor molecules could be expected to be associated with a G protein. Data in Fig. 6 (*left*) and Table 3 thus support the notion that receptors associated with G proteins (RG complexes) have a higher affinity for the agonist than free receptors. When, however, the densities of receptors and G proteins in liposomal membranes were manipulated by altering the amount of lipid membranes (Fig. 6, *right*; Table 3), no change occurred in the fraction of receptors with a high affinity for the agonist. As pointed out, such behavior of the system can be explained on the assumption that the receptors and G proteins are clustered within microdomains in which their densities remain constant.

Judging from its effects on [^{35}S]GTPγS binding, the allosteric modulator alcuronium can either increase or diminish the activating influence of muscarinic M_2 receptors on G_o proteins, depending on the R/G_o ratio. This corresponds to what had been observed on intact CHO cells expressing the M_1 muscarinic receptors. In cells with a low density of receptors, alcuronium enhanced the production of inositol phosphates, whereas in cells with a high density of receptors (and presumably a high R/G ratio), the production of inositol phosphates was diminished by alcuronium (Jakubík *et al.*, 1996).

Data on the effects of alcuronium are of principal interest. They demonstrate (1) that the interaction between muscarinic receptors and G_o proteins may be influenced by their

TABLE 3

Inhibition by carbachol of [^{3}H]QNB binding to reconstituted vesicles containing M_2 receptors and G_o proteins

Values were derived from experiments shown in Fig. 6. They are mean ± standard error of three experiments performed with incubations in duplicate. Reconstituted vesicles used in A correspond to those characterized in Table 1, whereas those used in B correspond to those characterized in Table 2. Samples with 1 × Basic densities of receptors and G_o proteins in B correspond to the samples with the 10:100 R/G_o ratio in A.

A. Vesicles with different R/G_o ratios				
R/G_o	10:0 (no G_o)	1:100	10:100	50:100
K_{low} (μM)	A single population with K_d of 162 μM	84 ± 1	105 ± 1	140 ± 2
K_{high} (μM)		1.37 ± 0.02	2.92 ± 0.02	11.70 ± 0.10
f_{high} (%)		73 ± 1	45 ± 1	16 ± 0
B. Vesicles with constant R/G_o ratio of 10:100 but with different densities of receptors and G_o proteins				
Densities of receptors and G_o proteins	0.1 × Basic	1 × Basic	5 × Basic	
K_{low} (μM)	104 ± 0	104 ± 0	104 ± 2	
K_{high} (μM)	2.94 ± 0.03	2.95 ± 0.04	3.0 ± 0.0	
f_{high} (%)	44 ± 0	46 ± 1	45 ± 0	

allosteric modulator alcuronium not only in intact cells but also in a reconstituted system and (2) that the activation of receptors induced by alcuronium is not identical with that induced by the classic agonist carbachol.

These observations raise the question of the mechanistic background of the difference between the effects of alcuronium at low and high R/G_o ratios. Presumably, the binding of alcuronium to free receptors has two opposite effects on the ability of the receptors to stimulate [^{35}S]GTPγS binding by G proteins, which occur simultaneously but are differently pronounced at different R/G_o ratios: one supporting [^{35}S]GTPγS binding to G proteins, which prevails at low R/G_o ratios, and another inhibiting [^{35}S]GTPγS binding, which prevails at high R/G_o ratios. Among the functional parameters that might be affected by alcuronium binding to receptors in opposite ways are (1) the rates of receptor/G protein association and dissociation and the resulting affinity of the receptor for the G protein, (2) the velocity of receptor-catalyzed guanine nucleotide exchange, (3) the size and direction of receptor-induced changes in the rates of GDP and, independently, GTP association to and dissociation from the G protein and of the resulting affinities for GDP and for GTP, and others. At least some of the conceivable effects of alcuronium are likely to carry different weight depending on whether the receptor (acting as the catalyst) is saturated with the G protein as its substrate (at a low R/G ratio) or undersaturated (at a high R/G ratio). This consideration applies to situations in which receptors and G proteins interact as independent monomers and bind in a 1:1 ratio. Alternative interpretations can be based on the assumption of an oligomeric arrangement of receptors and G proteins (Chidiac and Wells, 1992; Wreggett and Wells, 1995). Unfortunately, the data we have do not permit identification of the processes involved.

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