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Reconstitution of Muscarinic Receptor-Mediated Inhibition of Adenylyl Cyclase

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SUMMARY

Inhibition of bovine brain calmodulin-sensitive adenylyl cyclase was examined in a system consisting of the reconstituted purified porcine atrial muscarinic acetylcholine receptor, the purified inhibitory guanine nucleotide-binding protein (G_i), and the partially purified stimulatory guanine nucleotide-binding protein·adenylyl cyclase complex. Under conditions where G_i existed mainly as the G_i·GDP complex, adenylyl cyclase was selectively preactivated with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS). Addition of carbachol formed the receptor·carbachol complex, which catalyzed the exchange of GDP bound to G_i for GTPγS, initiating G_i-mediated inhibition of adenylyl cyclase. Adenylyl cyclase activated by calcium plus calmodulin was more sensitive to inhibition by carbachol than either unstimulated adenylyl cyclase or adenylyl cyclase activated by GTPγS or forskolin. Studies using the resolved subunits of G_i showed that the βγ subunit could inhibit adenylyl cyclase activated by GTPγS or calcium plus calmodulin, as well as the unactivated enzyme. The α subunit of G_i inhibited adenylyl cyclase only when adenylyl cyclase was activated by calcium plus calmodulin. Possible explanations for these results are discussed.

Activation of mAcChRs in several tissues including heart (1) and brain (2) results in a decrease in cAMP levels. In atrial tissue, as in several other cell types (3), the acetylcholine-induced attenuation of cAMP levels is mediated by pertussis toxin-sensitive G_i and/or G_o. This is not true for all cell lines, because mAcChRs regulate cAMP levels in 1321N1 astrocytoma cells by activating a calmodulin-sensitive phosphodiesterase in a pertussis toxin-insensitive manner (4).

Of the five mAcChR subtypes whose coupling to physiological effector systems has been characterized (5–8), the M2 and M3 mAcChRs [the nomenclature used in this manuscript is that of Peralta *et al.* (9)] appear to couple more tightly to adenylyl cyclase inhibition than to stimulation of inositol phospholipid metabolism. Northern analysis (9) has indicated that both of these subtypes are found in the brain, suggesting that they may play a role in the regulation of adenylyl cyclase activity in that tissue.

Regulation of adenylyl cyclase by G proteins requires that the G proteins are first activated by their respective receptors. The hormone-receptor complex increases the dissociation rate constant of GDP bound to the α subunit of the G protein, allowing GTP to bind. The activated G protein heterotrimer apparently dissociates into α and βγ subunits, which are involved in the regulation of adenylyl cyclase activity. The $G_{s\alpha}$·GTP complex stimulates adenylyl cyclase activity. Stimulation is terminated when GTP hydrolyzes to GDP and the G_s subunits reassociate (10).

Recently, several laboratories have examined the effects of isolated G protein subunits on adenylyl cyclase activity, using reconstituted (11–14) or detergent-solubilized preparations (15, 16). Three mechanisms have been proposed to account for G_i-mediated inhibition of adenylyl cyclase. The first is that inhibition of adenylyl cyclase by the βγ subunits of pertussis toxin-sensitive G proteins is predominantly due to mass action equilibria favoring reassociation of the G_s heterotrimer (12, 17). A second potential mechanism (15, 16, 18) is that $G_{i\beta\gamma}$ inhibits adenylyl cyclase by either binding to the protein directly or, in the case of calmodulin-sensitive adenylyl cyclase, competing for the calcium·calmodulin complex. The third mechanism suggests that $G_{i\alpha}$·GTPγS competes directly with $G_{s\alpha}$ for a binding site on adenylyl cyclase (15). Inhibition of forskolin-stimulated adenylyl cyclase activity in S49 Cyc⁻ cells by $G_{i\alpha}$·GTP was thought to occur by direct binding to the protein, but the effect was small (19).

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ABBREVIATIONS: mAcChR, muscarinic acetylcholine receptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; buffer A, 20 mM Tris·HCl, pH 8, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride; buffer B, 25 mM imidazole, 0.1 M NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride; G_s, the stimulatory guanine nucleotide-binding protein; G_i, the inhibitory guanine-nucleotide binding protein; G_o, a similar guanine nucleotide binding protein of unknown function; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; G protein; guanine nucleotide-binding protein; QNB, quinuclidinyl benzilate.

Adenylyl cyclase, G_s, and stimulatory hormone receptors have been successfully coreconstituted into liposomes (20–22). These studies defined the minimum requirement for hormone-induced activation of adenylyl cyclase. It has been more difficult to prepare a reconstituted system in which the adenylyl cyclase is modulated by inhibitory receptor proteins. It was not possible to demonstrate photoreceptor-initiated inhibition of adenylyl cyclase in a reconstituted system consisting of the enzyme plus G_s, G_i or transducin, and rhodopsin, because both G_i and G_s were activated under the experimental conditions used (23).

The interactions between mAcChR and G_i in a reconstitution system consisting of purified atrial mAcChR and purified atrial G_i have recently been described (24). In this study, the calmodulin-sensitive adenylyl cyclase·G_s complex from bovine brain was added to the mAcChR-G_i reconstitution system. Conditions were found such that adenylyl cyclase could be activated by G_s and/or calmodulin, while remaining sensitive to inhibition by mAcChR-activated G_i. It was also possible to test the effects of resolved subunits of porcine atrial G_i on the activity of the unstimulated adenylyl cyclase and adenylyl cyclase activated by G_s or calmodulin.

Experimental Procedures

Materials. Porcine atrial mAcChR (specific activity, 10 nmol of L-[^{3}H]QNB bound/mg of protein) was purified as described by Peterson *et al.* (25) and atrial G_i (specific activity, 8 nmol of [^{35}S]GTPγS bound/mg of protein) was purified as a by-product of the receptor preparation (24). Sodium dodecyl sulfate-polyacrylamide gel analysis of the mAcChR and G_i preparations gave results similar to those previously published (Ref. 24, Fig. 1), showing a single band for the mAcChR and three bands with apparent molecular weights of 41,000, 35,000, and 10,000 corresponding to the $G_{i\alpha}$, $G_{i\beta}$, and $G_{i\gamma}$ subunits. The bovine brain calmodulin-sensitive adenylyl cyclase·G_s complex was prepared in the absence of guanine nucleotides and purified about 100-fold by DEAE-Sephacel and calmodulin-Sepharose chromatography, as described by Yeager *et al.* (26). The enzyme had a specific activity of 10 nmol of cAMP/min/mg of protein when assayed in the presence of 10 mM $MnCl_2$. The major contaminating protein in this preparation has been reported to be a calmodulin-activated phosphodiesterase (Ref. 26, Fig. 6, *lane F*). Bovine brain calmodulin was a generous gift from Dr. S. R. Anderson, Department of Biochemistry and Biophysics, Oregon State University. The sources of all other reagents were previously described (24).

The subunits of G_i were separated by a modification of the procedure described by Katada *et al.* (27). G_i (50 μg) was incubated for 2 hr at 32° in a final volume of 250 μl containing 10 μM GTPγS, 10 mM Na·HEPES, pH 7.4, 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% (v/v) Lubrol PX. The activated protein was diluted 20-fold with cold buffer A containing 0.1 M NaCl and 0.25% (w/v) cholate and was applied to a 10-ml column of heptyl-agarose (Bio-Rad Affigel 10 reacted with excess heptylamine) equilibrated with the same buffer at 4°. The column was eluted with 20 ml of buffer A plus 0.1 M NaCl and 0.25% (w/v) cholate, followed by a 60-ml gradient from 0.25% (w/v) cholate, 0.25 M NaCl, to 1.2% (w/v) cholate, 0.025 M NaCl, in buffer A. $G_{i\alpha}$ did not bind to the column and was eluted before the application of the gradient, whereas $G_{i\beta\gamma}$ was eluted at about 0.8% (w/v) cholate.

Reconstitution. mAcChR (20–30 pmol) and G_i (30–50 pmol) were reconstituted as previously described (24). Briefly, both proteins were added to a mixture of 7 mM CHAPS, 50 μM acetylcholine, and 1 mg/ml lipid (1:1:0.1, w/w, phosphatidylcholine/phosphatidylserine/cholesterol), diluted 25-fold with buffer B, and precipitated by addition of polyethylene glycol 8000 to a final concentration of 10% (w/v). After incubation for 1 hr at 4°, the mixture was centrifuged at 250,000 × *g* for 75 min. The pellet, containing lipid plus mAcChR and G_i, was resuspended in 250 μl of buffer B. The adenylyl cyclase·G_s complex (64 μg/ml in 50 mM Tris·HCl, pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% Tween 20) was diluted 10-fold with either lipids containing the mAcChR and G_i or control lipids that were prepared as described above but without addition of mAcChR. GDP was then added to a final concentration of 167 μM and the mixture was incubated on ice for 10 min before initiation of adenylyl cyclase assays.

Reconstitution of adenylyl cyclase with G_i subunits. Lipids containing resolved G_i subunits were prepared as described above for the G_i·mAcChR reconstitution, using either 30 pmol of $G_{i\alpha}$ or 1.36 μg (about 28 pmol) of $G_{i\beta\gamma}$ subunit. The recovery of $G_{i\alpha}$ was about 24%, as determined by [^{35}S]GTPγS binding, and the final concentration in the adenylyl cyclase assay was 7.8 nM. The recovery of $G_{i\beta\gamma}$ was determined to be 43% by electrophoresis on 12% acrylamide gels (28), where proteins were visualized by silver staining (29). The final concentration of $G_{i\beta\gamma}$ in the adenylyl cyclase assays was 0.63 μg/ml, equal to about 13 nM.

Adenylyl cyclase assays. Fifteen microliters of the reconstituted preparation were added to 25 μl of a solution containing 200 μM EGTA, 140 mM NaCl, 7 mM $MgCl_2$, 480 nM GTPγS, 2 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride, and the reaction was initiated by addition of 10 μl of a solution containing 1 mg/ml bovine serum albumin, 50 mM HEPES, pH 7.4, 250 μM cAMP, 25 mM theophylline (to inhibit the calmodulin-activated phosphodiesterase contaminant in the adenylyl cyclase preparation), 2.5 mM ATP, and 3×10^6 cpm of [α^{32}P]ATP (120 cpm/pmol). The reaction was allowed to proceed for 1 hr at 32° and [α^{32}P]cAMP was then isolated as described by Salomon (30), using [^{3}H]cAMP to quantitate recoveries.

In experiments involving calcium·calmodulin stimulation of adenylyl cyclase, 100 nM calmodulin and 200 μM $CaCl_2$ were substituted for 200 μM EGTA (see above). When adenylyl cyclase was activated by $G_{s\alpha}$·GTPγS, the Tween 20-solubilized G_s·adenylyl cyclase preparation was preincubated with 8 μM GTPγS at 32° for 30 min before addition to the solution containing reconstituted mAcChR, G_i, and GDP. No additional GTPγS was added for these experiments and the final GTPγS concentration was 240 nM, as in the controls. All assays contained a final adenylyl cyclase concentration of 1.9 μg/ml, a residual Tween 20 concentration of 0.003% (v/v), and 2.5% (v/v) ethanol (theophylline was dissolved in ethanol). Control experiments showed that the rate of cAMP formation was linear as a function of time for 60 min in the absence of G_i. In the presence of G_i, a burst phase (about 10 min) was observed, corresponding to the rate of cAMP formation in the absence of G_i, followed by a linear rate of cAMP formation for the remainder of the 60-min time course. The final concentrations of mAcChR and G_i are indicated in the figure legends.

Binding assays. L-[^{3}H]QNB and [^{35}S]GTPγS binding were performed for the reconstituted proteins as described by Tota *et al.* (24). In order to determine L-QNB and GTPγS binding under the conditions of the adenylyl cyclase assays, minor modifications were made. When L-[^{3}H]QNB binding was performed in a 50-μl volume at high ionic strength, aliquots were removed and diluted 2.5-fold with 10 mM sodium phosphate, 1 mM EDTA, pH 7.4, buffer immediately before being applied to DE81 discs. [^{35}S]GTPγS binding was assayed by substituting 240 nM [^{35}S]GTPγS for unlabeled nucleotide and filtering 15-μl samples.

Results

Before reconstitution with the mAcChR and G_i, the response of the reconstituted adenylyl cyclase preparation to several agents known to stimulate the enzyme was determined (Table 1). Addition of forskolin or calcium·calmodulin caused a 4-fold stimulation of adenylyl cyclase activity relative to control values, whereas the G_s-stimulated activity was about twice that of the control. To ensure that these conditions gave maximal adenylyl cyclase stimulation, higher concentrations of GTPγS or calcium·calmodulin were also tested. Increasing GTPγS

TABLE 1

Stimulation of adenylyl cyclase activity

The adenylyl cyclase·G_s complex was added to liposomes and assayed as described in Experimental Procedures. The liposomes contained no mAcChR, G_i, or muscarinic ligands. Measurements shown are mean ± standard deviation of triplicate determinations. Values in parentheses are the mean ± standard deviation of the results of *n* experiments.

Conditions	Activity	Activity relative to control
	pmol of cAMP/hr/50 μl	
100 μM EGTA (control)	9.4 ± 0.2	1.0 ± 0.0
100 μM EGTA + GTPγS-preactivated G_s	18.0 ± 0.9	1.9 ± 0.1 (1.8 ± 0.1, *n* = 3)
100 μM $CaCl_2$ + 50 nM calmodulin	38.1 ± 1.7	4.1 ± 0.2 (3.9 ± 0.2, *n* = 4)
100 μM EGTA + 100 μM forskolin	39.7 ± 1.5	4.2 ± 0.2 (*n* = 1)
GTPγS-preactivated G_s + 100 μM $CaCl_2$ + 50 nM calmodulin	70.5 ± 1.9	7.5 ± 0.3 (8.5 ± 0.9, *n* = 3)

concentrations from 8 to 193 μM gave no additional stimulation, whereas increasing calmodulin from 50 to 250 nM increased adenylyl cyclase activity by only 3%. Increasing calcium concentrations by 5-fold reduced enzymatic activity by 40% (data not shown). A combination of calcium·calmodulin- and G_s-mediated stimulation gave more than an additive response (7.5-fold versus 6-fold). These results suggested that the biochemical properties of adenylyl cyclase were retained under the conditions used for reconstitution experiments.

The relative concentrations and order of addition of GDP and GTPγS were carefully manipulated to reduce background inhibition of adenylyl cyclase by free G_i and to selectively activate G_i coupled to the mAcChR in the presence of G_s. When 50 μM GDP was added to the reconstituted adenylyl cyclase·G_s complex before 8 μM GTPγS, stimulation of the adenylyl cyclase by G_s was not observed. Thus, prior addition of 50 μM GDP effectively blocked binding of GTPγS to G_s.

Based on the previously determined affinities of GTPγS and GDP for free G_i (24), only about 16% of the G_i was expected to bind GTPγS at the above concentrations of GTPγS and GDP. The data in Fig. 1A showed that the reconstituted mAcChR-G_i preparation (11.8 nM G_i) bound 2.00 ± 0.15 nM [^{35}S]GTPγS (17% occupancy of the total [^{35}S]GTPγS binding sites) in the presence of *l*-hyoscyamine and final concentrations of [^{35}S]-GTPγS and GDP of 240 nM and 50 μM, respectively. Similar results (2.5 ± 0.8 nM [^{35}S]GTPγS bound, 21% occupancy of the total available [^{35}S]GTPγS sites) were found when adenylyl cyclase was reconstituted with the mAcChR and G_i (Fig. 1B). In control experiments omitting G_i, [^{35}S]GTPγS binding was less than 60 pM. Thus, most of the bound label could be attributed to the reconstituted G_i, and the addition of adenylyl cyclase to the mAcChR-G_i preparation did not appear to affect the interaction of G_i with GDP and GTPγS.

Activation of G_i by the mAcChR·carbachol complex decreases the affinity of G_i for GDP by about 50-fold but does not affect the binding of GTPγS (24). Therefore, in the presence of carbachol, the binding of [^{35}S]GTPγS should increase. As predicted, when carbachol was used instead of *l*-hyoscyamine (Fig. 1), the amount of [^{35}S]GTPγS bound to G_i increased about 2-fold. Taken together, these results suggest that 1) it was possible to selectively activate G_i while inhibiting G_s by addition of GDP before GTPγS and 2), by using the appropriate ratio of GDP to GTPγS, it was possible to increase the signal to noise ratio by preferentially activating that population of G_i that couples to the carbachol·mAcChR complex.

The adenylyl cyclase activity of the reconstituted preparation containing the adenylyl cyclase·G_s complex, mAcChR, and G_i was determined after activation of the enzyme by G_s, calcium·calmodulin, forskolin, or calcium·calmodulin plus G_s, under

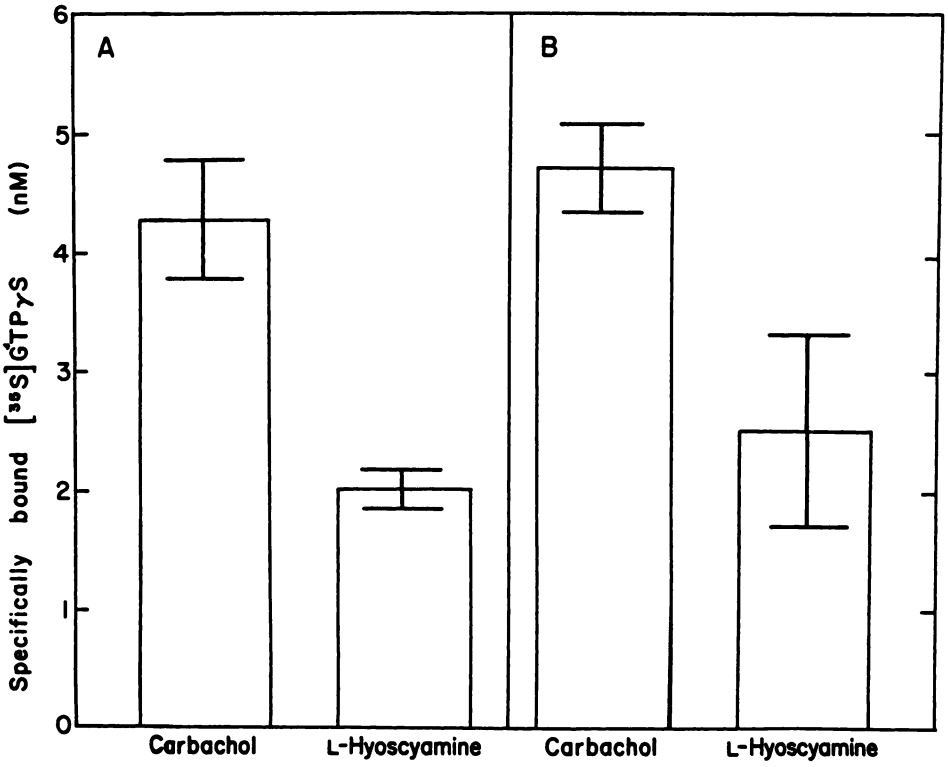

Fig. 1. [^{35}S]GTPγS binding to liposomes containing 5.4 nM mAcChR and 11.8 nM G_i. A, Adenylyl cyclase·G_s complex was added to liposomes and [^{35}S]GTPγS binding was performed as described in Experimental Procedures. Conditions were similar to an adenylyl cyclase assay, except unlabeled ATP was used. B, No adenylyl cyclase·G_s or Tween 20 was added. Samples contained either 2 mM carbachol or 1 μM *l*-hyoscyamine. *Error bars*, standard deviation of triplicate determinations.

TABLE 2

Muscarinic agonist-mediated inhibition of adenylyl cyclase activity

The adenylyl cyclase·G_s complex was added to liposomes and assayed as described in Experimental Procedures. Final concentrations of mAcChR and G_i were 6.9 and 10.8 nM, respectively. Assays were performed in the presence of either 1 μM *l*-hyoscyamine or 2 mM carbachol. Measurements shown are the mean ± standard deviation of triplicate determinations. Inhibition was calculated by dividing the activity in the presence of carbachol by the activity in the presence of *l*-hyoscyamine and subtracting the result from 1. The value was then multiplied by 100 to express it as a percentage. Values in parentheses are the mean ± standard deviation of the results of *n* experiments.

Conditions	Activity			Inhibition
	l-Hyoscyamine	Carbachol	Difference	
	pmol of cAMP/hr/50 μl			%
100 μM EGTA (control)	6.2 ± 0.3	5.1 ± 0.1	1.1 ± 0.3	17.5 ± 4.6 (*n* = 1)
100 μM EGTA + GTPγS-preactivated G_s	15.0 ± 0.6	13.7 ± 0.2	1.3 ± 0.6	8.8 ± 3.8 (10.9 ± 4.4, *n* = 3)
100 μM $CaCl_2$ + 50 nM calmodulin	24.2 ± 0.4	16.9 ± 0.2	7.3 ± 0.4	30.3 ± 1.3 (32.3 ± 4.4, *n* = 3)
100 μM EGTA + 100 μM forskolin	34.1 ± 1.4	32.4 ± 1.2	1.7 ± 1.8	5.1 ± 5.2 (*n* = 1)
100 μM $CaCl_2$ + 50 nM calmodulin + GTPγS-preactivated G_s	60.4 ± 0.8	50.8 ± 2.4	9.6 ± 2.5	15.9 ± 4.1 (14.0 ± 2.8, *n* = 2)

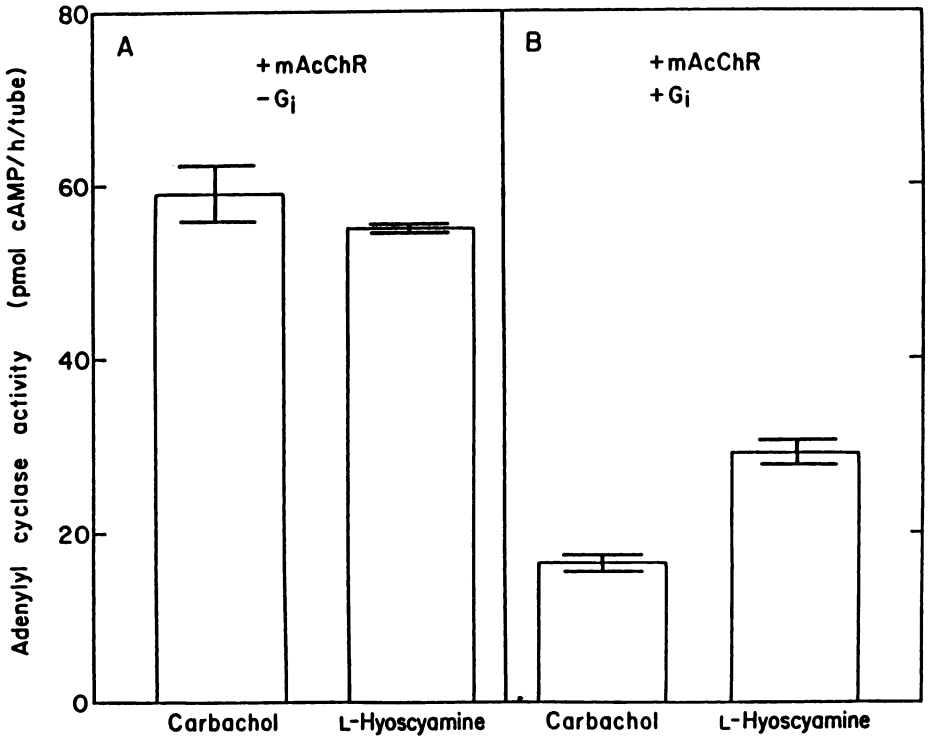

Fig. 2. Requirement of G_i for mAcChR-mediated inhibition of adenylyl cyclase. Adenylyl cyclase·G_s complex was added to liposomes and stimulated by calcium and calmodulin, as described in Experimental Procedures. Liposomes contained either 11.3 nM mAcChR (A) or 5.8 nM mAcChR and 12 nM G_i (B). Assays contained either 1 μM *l*-hyoscyamine or 2 mM carbachol. *Bars*, standard deviation of triplicate determinations.

conditions where the mAcChR was occupied by either the agonist carbachol or the antagonist *l*-hyoscyamine (Table 2). The enzymatic activity observed in the presence of the antagonist (Table 2, column 1) was lower than that found for adenylyl cyclase·G_s added to control lipids (Table 1, column 1). Because these two experiments were done at the same time under identical conditions, the difference was attributed to the fraction of G_i that was estimated to be activated by GTPγS in the absence of a muscarinic agonist (17–21%) (Fig. 1). Replacing *l*-hyoscyamine with carbachol (Table 2, column 2) resulted in a further decrease in the adenylyl cyclase activity. The reduction in activity was most pronounced for the calcium·calmodulin-stimulated adenylyl cyclase (30%), followed by the nonstimulated (17%) and G_s-plus calcium·calmodulin-stimulated enzyme (16%). The G_s- or forskolin-stimulated adenylyl cyclase appeared to be less sensitive to inhibition resulting from the muscarinic agonists. Differences in activity for the unstimulated and G_s-stimulated enzyme were significant at the $p < 0.05$ level. Although the difference in the activity of the forskolin-stimulated adenylyl cyclase in the presence of carbachol, compared with *l*-hyoscyamine, was similar to or slightly larger than that found for the control and G_s-stimulated adenylyl cyclase, its value compared with the observed signal was much smaller. Thus, the difference was not statistically significant.

In order to further analyze the effects of muscarinic agonists on adenylyl cyclase activity in the reconstituted preparation, the method of activation of the enzyme that showed the greatest inhibition after carbachol addition was chosen. Thus, the remaining experiments were done with the calcium·calmodulin-stimulated adenylyl cyclase. Control experiments for this prep-

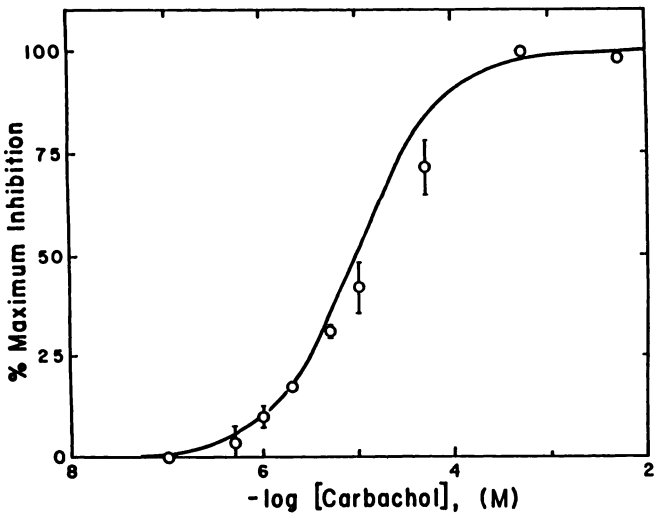

Fig. 3. Dependence of mAcChR-mediated adenylyl cyclase inhibition on carbachol concentration. Calcium·calmodulin-stimulated adenylyl cyclase was measured as described in Experimental Procedures, in the presence of the indicated concentrations of carbachol. The mAcChR concentration was 6.5 nM and the G_i concentration was 11.3 nM. *Data points*, average of duplicate determinations; *bars*, range. The data were fit assuming one class of carbachol binding sites, giving an EC_{50} of 9.5 ± 0.6 μM. *Curve through the data points*, the theoretical curve calculated using the fitted EC_{50} for carbachol. The maximum adenylyl cyclase activity was 44.0 ± 2.3 pmol/hr/50 μl and, at the maximum carbachol concentration, the activity was 28.7 ± 0.1 pmol/hr/50 μl.

aration showed that the inhibitory effects caused by carbachol were blocked by prior addition of the muscarinic antagonist *l*-hyoscyamine. A cyclase assay containing 4.9 nM mAcChR and 14.3 nM G_i had an activity of 27.0 ± 0.9 pmol of cAMP/hr/50 μl in the presence of 2 mM carbachol, 34.0 ± 1.6 pmol of cAMP/hr/50 μl in the presence of 100 μM *l*-hyosyamine, and 33.7 ± 0.4 pmol of cAMP/hr/50 μl when 100 μM *l*-hyoscyamine was added before the addition of 2 mM carbachol. The effect of carbachol in this experiment was less pronounced than in other assays (Table 2, Figs. 2 and 3) and may be a result of higher G_i and lower mAcChR concentrations. A second control experiment was done to ensure that minor contaminants in the adenylyl cyclase or mAcChR preparations were not responsible for mediating guanine nucleotide-dependent inhibition of the calcium·calmodulin-stimulated adenylyl cyclase (Fig. 2). The results of this experiment clearly showed that no inhibition was observed after addition of carbachol unless G_i was present in the reconstituted system (Fig. 2B). Thus, both G_i and the carbachol·mAcChR complex were required in order to inhibit the calcium·calmodulin-stimulated adenylyl cyclase.

Agonist activity for the inhibition of calcium·calmodulin-activated adenylyl cyclase was then related to the affinity of carbachol for the mAcChR, determined by competition versus L-[^{3}H]QNB. The EC_{50} for carbachol inhibition of calcium·calmodulin-activated adenylyl cyclase (Fig. 3) was 9.5 ± 0.6 μM, whereas the dissociation constant for the high (K_1) and low (K_2) affinity agonist sites equaled 19 ± 10 μM and 4.1 ± 0.7 mM, respectively (Fig. 4). These results suggested that the high affinity agonist binding sites correlated with the mAcChR subpopulation that mediates carbachol-induced inhibition of calcium·calmodulin-stimulated adenylyl cyclase activity.

The effects of G_i subunits on adenylyl cyclase activity were investigated by addition of the enzyme and GTPγS to lipids containing either $G_{i\alpha}$·GTPγS or $G_{i\beta\gamma}$. The mAcChR was not included in these experiments, because the agonist receptor was expected to be uncoupled from guanine nucleotide-activated G_i (10, 24). The results shown in Table 3 suggest that, at the concentration used, the βγ subunit inhibited G_s-stimulated, calmodulin-stimulated, or basal adenylyl cyclase activity by about 25% but $G_{i\alpha}$ only inhibited calmodulin-stimulated adenylyl cyclase (20%).

Discussion

The adenylyl cyclase preparation used for this study could be stimulated by G_s, calcium·calmodulin, and forskolin. That calmodulin and forskolin were more effective than G_s in their ability to activate adenylyl cyclase agreed with previous studies (26).

In order to study the inhibition of the adenylyl cyclase·G_s

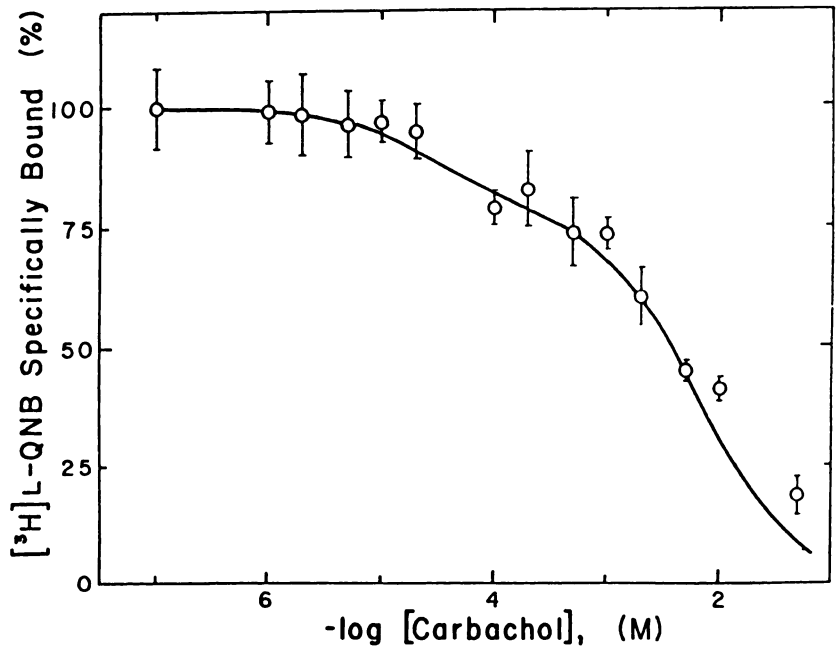

Fig. 4. Carbachol titration of L-[^{3}H]QNB binding sites. L-[^{3}H]QNB binding was performed in the presence of varying carbachol concentrations using the same buffer conditions as in Fig. 3, with the exception that labeled ATP was replaced by unlabeled ATP, the reaction was allowed to proceed for 20 min instead of 1 hr, and no guanine nucleotides were added. The samples containing 11 nM G_i and 4.7 nM L-QNB sites were diluted and immediately applied to DE81 paper, as described in Experimental Procedures. The data were fit by nonlinear regression analysis, as described previously (24), using a K_d for L-QNB of 12.1 nM, determined by Scatchard analysis under similar conditions (data not shown). The fitted parameters were F_1 = 0.22 ± 0.03, K_1 = 19 ± 10 μM, F_2 = 0.78 ± 0.03, and K_2 = 4.1 ± 0.7 mM.

TABLE 3

Inhibition of adenylyl cyclase by G_i subunits

The adenylyl cyclase·G_s complex was added to liposomes containing the indicated G_i subunits, as described in Experimental Procedures. Values shown are the mean ± standard deviation of triplicate determinations. Values in parentheses are the mean ± standard deviation of the results of *n* experiments.

Conditions	No G_i Subunits, activity	$G_{i\alpha}$ (7.8 nM)		$G_{i\beta\gamma}$ (13 nM)	
		Activity	Inhibition	Activity	Inhibition
	pmol of cAMP/hr/50 μl	*pmol of cAMP/hr/50 μl*	%	*pmol of cAMP/hr/50 μl*	%
100 μM EGTA (control)	7.7 ± 0.8	7.2 ± 0.1	6.5 ± 9.8 (5.7 ± 1.2, *n* = 2)	5.7 ± 0.1	26.0 ± 7.8 (22.8 ± 5.0, *n* = 2)
100 μM $CaCl_2$ + 50 nM calmodulin	30.8 ± 0.6	24.3 ± 0.6	21.0 ± 3.5 (21.0 ± 7.5, *n* = 3)	23.2 ± 0.9	24.7 ± 3.2 (21.3 ± 4.9, *n* = 2)
100 μM EGTA + GTPγS-preactivated G_s	13.9 ± 0.6	13.9 ± 0.4	0.0 ± 5.2 (−3.4 ± 5.1, *n* = 3)	11.0 ± 0.0	20.8 ± 3.4 (16.2 ± 6.8, *n* = 2)

complex by G_i, it was necessary to find conditions that permitted G_s and G_i to be activated independently of each other but still allowed G_i to be sensitive to changes in guanine nucleotide binding caused by the mAcChR. Under the experimental conditions chosen (50 μM GDP and 240 nM GTPγS), GTPγS did not activate G_s unless it was added before GDP and the activation of G_i by the mAcChR gave maximal effect on adenylyl cyclase activity. Because it was not possible to completely inhibit the binding of GTPγS to G_i, the activity of adenylyl cyclase added to reconstituted muscarinic receptor and G_i plus the antagonist *l*-hyoscyamine was lower than the activity of adenylyl cyclase added to control lipids (compare column one of Tables 1 and 2 and Fig. 2). When the assay was performed in the presence of the muscarinic agonist carbachol instead of an antagonist, a larger fraction of G_i was activated by GTPγS (Fig. 1), resulting in a further decrease in adenylyl cyclase activity (Tables 1 and 2 and Fig. 2B). The carbachol effect was not seen if G_i was omitted (Fig. 2A) or if an excess of the antagonist *l*-hyoscyamine was added before carbachol. The increase in the concentration of G_i activated by GTPγS presumably resulted from a decrease in the affinity of G_i for GDP caused by the mAcChR-carbachol complex (24).

It has been previously demonstrated that detergent-solubilized calcium·calmodulin-sensitive adenylyl cyclase could reconstitute into liposomes (31). This preparation of adenylyl cyclase was functionally reconstituted with the β-adrenergic receptor, and addition of the β-adrenergic agonist isoproterenol increased calmodulin-sensitive adenylyl cyclase activity 2-fold (22). Because an interaction between adenylyl cyclase and reconstituted mAcChR and G_i was observed, it is reasonable to assume that in the experiments described above the adenylyl cyclase was able to reconstitute into liposomes containing the mAcChR and G_i.

The calcium·calmodulin-stimulated adenylyl cyclase was more sensitive to inhibition by G_i than either the forskolin- or GTPγS-activated enzyme. As observed by others (16), the effects of G_i on forskolin- and G_s-stimulated adenylyl cyclase activity were smaller. It seems that this form of adenylyl cyclase is more sensitive to regulation by calcium·calmodulin than by stimulatory hormones.

The mAcChR-mediated inhibition of adenylyl cyclase observed in this reconstituted system (about 30%) was comparable to inhibition caused by muscarinic agonists in native membrane preparations. Developing chick atrial membranes have been reported to show a carbachol-induced inhibition of 26–30% (32). Acetylcholine inhibited basal adenylyl cyclase activity by 30–40% in synaptic plasma membranes from rat striatum (2). GTP- plus isoproterenol-activated adenylyl cyclase was inhibited 17% by methacholine in canine myocardial membranes or 26% in canine myocardial homogenates (33). In the presence of calmodulin and GTP, addition of the adenosine receptor agonist (−)-N^6-phenylisopropyladenosine resulted in a 26% inhibition of adenylyl cyclase activity in rat cerebral cortex membranes (34).

The EC_{50} value obtained from the dependence of adenylyl cyclase inhibition on carbachol concentration (10 μM) (Fig. 3), determined in the presence of 50 μM GDP and 240 nM GTPγS, agreed within a factor of 2 with the dissociation constant of carbachol from the high affinity population of agonist binding sites (20 μM) (Fig. 4), determined in the absence of guanine nucleotides. If inhibition of adenylyl cyclase was proportional to agonist occupancy of the low affinity state of the mAcChR (K_L = 4 mM) (Fig. 4), the calculated receptor occupancy at the EC_{50} [% occupancy = (100) $[L]/K + [L]$] would be about 0.25%. Thus, under conditions where the mAcChR and G_i were in large excess over adenylyl cyclase, only a small fraction of available mAcChRs would have to be occupied to significantly inhibit the enzyme. This notion is consistent with *in vivo* observations that, unlike mAcChR-mediated stimulation of inositol phospholipid metabolism, the inhibition of adenylyl cyclase activity occurs at very low receptor occupancies [i.e., has a large receptor reserve (35, 36)]. If this were the case, the correlation of EC_{50} with the high affinity binding constant for carbachol would be fortuitous. An alternative possibility that cannot be discounted is that, even in the presence of excess guanine nucleotides, a small fraction of the receptor complexes with G_i transiently exist in the high affinity state for agonists. The binding of carbachol to the high affinity form of the receptor would then correlate with the inhibition of enzyme activity. In reconstituted systems containing atrial mAcChRs and G_i (24) or brain mAcChRs and G_i (37), the EC_{50} for carbachol stimulation of the GTPase activity of G_i (at saturating levels of GTP) also agreed with the high affinity agonist binding site determined in the absence of guanine nucleotides. These results suggest that the correspondence between the observed values for high affinity agonist binding and the EC_{50} for a biochemical response in this system are not coincidental and may relate to the overall binding constant for carbachol to a subpopulation of high affinity mAcChRs that are present even under conditions where guanine nucleotides are in excess.

The actual affinity of ligands for the receptor was lower than expected for the reconstituted system (24). This may be due to the residual Tween 20 in the adenylyl cyclase assay. When adenylyl cyclase (in 0.1% Tween 20) was added to the recon-

stituted system, only 6% of the mAcChR binding sites remained after 90 min at 32°; however, in the absence of Tween 20, nearly 100% of the L-QNB binding sites were still present (data not shown). Although these conditions were not optimal for the mAcChr, the carbachol·receptor complex was stable enough to cause GDP dissociation from G_i. As shown in Fig. 2, the presence of adenylyl cyclase (and Tween 20) had a negligible effect on the ability of the receptor to enhance GTPγS binding to G_i.

Because GTPγS binding to G_i is thought to cause dissociation of the protein to give the $G_{i\alpha}$ and $G_{i\beta\gamma}$ subunits (10), the effects of the resolved subunits on adenylyl cyclase activity were examined in the absence of mAcChRs (Table 3). The βγ subunit inhibited calmodulin-stimulated, G_s-stimulated, and unstimulated adenylyl cyclase to about the same degree (25%). These data argue that in this system the βγ subunits must inhibit the enzyme by means other than reduction of the concentration of activated $G_{s\alpha}$. Katada *et al.* (15, 16) reported that the βγ subunits inhibited adenylyl cyclase in detergent solution by binding to the protein and also inhibited the calmodulin-sensitive form by competing with the enzyme for calcium·calmodulin. In the present study it was not possible to distinguish between these two possible mechanisms, but because βγ seemed equally effective at inhibiting unstimulated, G_s-stimulated, and calmodulin-stimulated adenylyl cyclase, the results favor a mechanism in which $G_{i\beta\gamma}$ bound directly to the enzyme.

Although the $G_{i\beta\gamma}$ subunits were equally effective in inhibiting adenylyl cyclase activated by all of the effector systems examined, the $G_{i\alpha}$·GTPγS subunit inhibited only the calcium·calmodulin-stimulated enzyme at the experimental concentration used (7.8 nM). Inhibition of the calmodulin-sensitive adenylyl cyclase by $G_{i\alpha}$·GTPγS has not been previously reported; however, in studies with other adenylyl cyclase preparations $G_{i\alpha}$·GTPγS was found to inhibit only the G_s-stimulated enzyme to a significant degree. Presumably, the inhibition resulted from reduction of the stimulation caused by $G_{s\alpha}$·GTPγS (15). Although there has been a report of $G_{i\alpha}$·GTPγS directly inhibiting adenylyl cyclase in G_s-deficient cyc^- S49 lymphoma cells (38), the amount of inhibition observed was small.

There are several possible explanations to account for the difference in the results found for the inhibitory activity of $G_{i\alpha}$·GTPγS in this study compared with previous work (15). The $G_{i\alpha}$·GTPγS concentration may have been too low to effectively displace $G_{s\alpha}$·GTPγS from adenylyl cyclase, or the dissociation rate of $G_{s\alpha}$·GTPγS may be too slow to be observed in this reconstitution system. Alternatively, $G_{i\alpha}$ may not directly compete with $G_{s\alpha}$ for binding to the calmodulin-sensitive adenylyl cyclase. Another mechanism that cannot be ruled out from these data is that $G_{i\alpha}$·GTPγS and calcium·calmodulin compete for a binding site on adenylyl cyclase. This mechanism may be supported by a recent report that mastoparan can activate G proteins, possibly by mimicking a mastoparan-like structure found in many receptors (39). Mastoparan also binds to calmodulin with high affinity (40). Because $G_{i\alpha}$·GTPγS and calmodulin recognize the same molecule, they may also interact with similar domains on effector proteins. This would explain why $G_{i\alpha}$·GTPγS inhibited only the calcium·calmodulin-activated adenylyl cyclase. Although it is not possible to propose a detailed mechanism, the data in Table 3 do suggest that the calmodulin-stimulated adenylyl cyclase was inhibited by a different mechanism than the G_s-stimulated adenylyl cyclase.

The coupling of the atrial muscarinic receptor with brain calmodulin-sensitive adenylyl cyclase may be physiologically relevant, because M2 receptors are also located in the brain (9). The mechanism for coupling of M2 receptors to calmodulin-sensitive adenylyl cyclase may serve as a model for other mAcChR receptor subtypes (such as M3) and inhibitory receptors such as the adenosine receptor (34) that also couple to calmodulin-sensitive adenylyl cyclase.

In conclusion, this study demonstrated the functional reconstitution of purified atrial mAcChRs and G_i with calmodulin-sensitive adenylyl cyclase. The calcium·calmodulin-stimulated form of adenylyl cyclase showed a larger response to the inhibitory effects of the muscarinic agonist carbachol than the unstimulated, G_s-stimulated or forskolin-stimulated enzyme. Finally, data from reconstitution experiments with resolved G_i subunits suggested that $G_{i\alpha}$ may be able to inhibit the calcium·calmodulin-stimulated adenylyl cyclase by competing with calcium·calmodulin for a binding site on the enzyme.

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