Reconstitution of the Purified Porcine Atrial Muscarinic Acetylcholine Receptor with Purified Porcine Atrial Inhibitory Guanine Nucleotide Binding Protein†

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ABSTRACT: Purified porcine atrial muscarinic receptor (mAcChR) was reconstituted with purified porcine atrial inhibitory guanine nucleotide binding protein (G_i) in a lipid mixture consisting of phosphatidylcholine, phosphatidylserine, and cholesterol (1:1:0.1 w/w). 5′-Guanylyl imidodiphosphate (0.1 mM) had no effect on the binding of the muscarinic antagonist L-quinuclidinyl benzilate but converted high-affinity carbachol binding sites (K_d equal to 1 μM) in the reconstituted preparation to the low-affinity state (K_d equal to about 100 μM). Steady-state kinetic measurements of GTPase activity showed that the turnover number was increased from 0.19 min^{-1} in the presence of the muscarinic antagonist L-hyoscyamine to 2.11 min^{-1} for the agonist carbachol. The affinity of G_i for GDP was reduced by about 50-fold upon interaction with the carbachol–mAcChR complex, and the observed rate constant for GDP dissociation was increased by 38-fold from 0.12 to 4.5 min^{-1}. Thus, the increase in steady-state GTPase activity observed for muscarinic agonists is largely, if not exclusively, due to the increase in GDP dissociation from G_i—probably the rate-limiting step in the steady-state mechanism. Carbachol-stimulated GTPase was sensitive to ADP-ribosylation of the reconstituted G_i by pertussis toxin, but the high-affinity agonist binding was uncoupled only when the reconstituted preparation was treated with pertussis toxin in the presence of GTP and the agonist acetylcholine. These results suggest that association with the mAcChR protects G_i from ADP-ribosylation by pertussis toxin.

The muscarinic acetylcholine receptor (mAcChR)[1] belongs to the class of neurotransmitter receptors in which signal transduction is mediated by guanine nucleotide binding regulatory proteins. Receptors that stimulate adenylyl cyclase have been shown to interact through G_s, while receptors that inhibit the enzyme, such as the mAcChR, appear to be coupled through G_i (Stryer & Bourne, 1986). In atrial tissue, G_i activated by mAcChRs has also been shown to function as a G_K (Pfaffinger et al., 1985; Breitweisser & Szabo, 1985; Yatani et al., 1987; Logothetis et al., 1987; Codina et al., 1987) to activate inward-rectifying potassium channels. An analogous system can be found in vertebrate photoreceptors where light-activated rhodopsin stimulates cGMP phosphodiesterase via G_T (Fung, 1983; Stryer & Bourne, 1986).

The guanine nucleotide binding regulatory proteins are heterotrimers with differing α subunits (α_i = 41 kDa, α_s = 45 kDa, α_o = 39 kDa) and similar β (35 kDa) and γ (5–10 kDa) subunits (Northup et al., 1983; Bokoch et al., 1984; Codina et al., 1984; Hildebrandt et al., 1984; Stryer & Bourne, 1986). It is thought that light-activated rhodopsin or agonist-bound receptors activate the G protein by catalyzing the exchange of bound GDP for GTP (Cassel & Selinger, 1978; Brandt & Ross, 1986). The binding of GTP will then uncouple the G protein from the receptor (Rodbell, 1980) or rhodopsin (Fung & Stryer, 1980) as well as causing the dissociation of the heterotrimers into α plus $\beta\gamma$ subunits (Northup et al., 1983; Katada et al., 1984a,b). The activation of the G protein results in an enhancement of G protein mediated GTPase activity. The β-adrenergic receptor–agonist complex binding to G_s is thought to induce a conformational change in G_s that increases the rate of GDP release and GTP association (Cassel & Selinger, 1978; Brandt & Ross, 1986).

The interactions of purified brain G_i and mAcChRs resolved from guanine nucleotide binding proteins (Florio & Sternweiss, 1985) as well as purified brain mAcChR and purified G_i (Haga et al., 1985, 1986; Kurose et al., 1986) have been demonstrated in reconstituted systems. These studies have shown that the mAcChR–agonist complex was capable of stimulating the GTPase activity of G_i and that association with G_i results in high-affinity agonist binding to the mAcChR. ADP-Ribosylation of G_i prior to reconstitution (Haga et al., 1986) prevented the purified brain components from interacting in the reconstituted system. ADP-Ribosylation of G_i also uncouples the mAcChR from inhibition of adenylyl cyclase in heart tissue (Martin et al., 1985).

The purpose of this paper is to characterize the interaction of purified porcine atrial mAcChR and G_i in a reconstituted system. Since the heart and brain mAcChRs are known to be different gene products (Kubo et al., 1986a,b; Peralta et

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[1] Abbreviations: mAcChR, muscarinic acetylcholine receptor; buffer A, 10 mM HEPES, 0.1 M NaCl, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF, pH 7.4; buffer B, 10 mM HEPES, 50 mM NaCl, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF, pH 7.4; buffer C, 25 mM imidazole, 0.1 M NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF, pH 7.4; PC, soybean L-α-phosphatidylcholine; PS, bovine brain L-α-phosphatidyl-L-serine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; L-QNB, the L isomer of quinuclidinyl benzilate; G_s, the stimulatory guanine nucleotide binding protein; G_i, the inhibitory guanine nucleotide binding protein; G_T, transducin; GppNHp, 5′-guanylyl imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; IAP, islet-activating protein (pertussis toxin); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; BHT, butylated hydroxytoluene; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; PEG, poly(ethylene glycol); TLC, thin-layer chromatography; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; SDS, sodium dodecyl sulfate.

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al., 1987) and the sequence homology of brain and heart G_i is as yet unknown, these studies should provide additional information regarding the similarity and possible differences in mAcChR–effector interactions in different tissues.

Materials and Methods

[^{35}S]GTPγS (1000 Ci/mmol), [γ-^{32}P]GTP (20–30 Ci/mmol), and [α-^{32}P]GTP (800 Ci/mmol) were purchased from New England Nuclear. L-[^{3}H]QNB (46 Ci/mmol) was purchased from Amersham. Cholesterol, PC (type III-s), PS (bovine brain), CHAPS, digitonin, carbachol, acetylcholine, L-hyoscyamine, GTP, GppNHp, acetylcholinesterase (type V-S), succinic thiokinase, pyruvate kinase (type VII), and activated charcoal were purchased from Sigma. GDP and GTPγS were purchased from Boehringer Mannheim. The purity of GTP and GTPγS was determined by thin-layer chromatography using a solvent of 1-propanol/NH_4OH/H_2O (6:3:1). When necessary, these nucleotides were purified by applying them to DEAE-Bio-Gel A and eluting with a 0–0.5 M LiCl gradient. Pertussis toxin was from List Biological Laboratories and was stored in 50 mM sodium phosphate/0.25 M NaCl, pH 7.0, at 100 μg/mL. mAcChR (10–15 nmol of L-[^{3}H]QNB/mg) was purified from porcine atria (Peterson et al., 1984). G_i (1.4–8 nmol of [^{35}S]GTPγS/mg of protein) was purified as a byproduct of the mAcChR purification procedure by sequential chromatography on DEAE-Sephacel, Ultragel ACA-34, and octyl-Sepharose, followed by sucrose gradient centrifugation in cholate buffers (details will be published elsewhere).

Reconstitution of mAcChR and mAcChR–G_i. PC/PS (1:1), plus 5% w/w cholesterol, stored in toluene/ethanol (1:1), plus 0.02% BHT was rotovapped to dryness and resuspended in 25 mM imidazole, 0.1 M NaCl, 1 mM EDTA, and 8.4 mM CHAPS, pH 7.4, to a final concentration of 1.2 mg of total lipid/mL. The suspension was sonicated to clarity at 0 °C under argon. DTT (1 mM) and $MgCl_2$ (5 mM) were then added. The lipid–detergent solution (330 μL) was transferred to a 10-mL polycarbonate centrifuge tube. mAcChR alone (10 pmol in 10–20 μL of 25 mM imidazole, 1 mM EDTA, 0.08% digitonin, and 0.016% sodium cholate, pH 7.4) or mAcChR plus G_i (50 pmol in 30–40 μL of 20 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 75 mM sucrose, and 0.8% sodium cholate, pH 8.0) was added to the lipid–detergent mixture along with acetylcholine to 50 μM. The final lipid concentration was 1 mg/mL, and the final CHAPS concentration was 7 mM in a volume of 0.4 mL. This mixture was incubated on ice for 5 min and then diluted slowly with ice-cold buffer C to a volume of 8 mL. Lipid was then precipitated by adding 2 mL of 50% PEG 8000 containing 5 mM $MgCl_2$ and 0.1 M NaCl to yield a final PEG concentration of 10%. This solution was incubated on ice for 1 h followed by centrifugation at 4 °C in a Beckman Ti 75 rotor at 250000g for 75 min. The resulting pellet was resuspended in 0.25 mL of buffer A or buffer C. Typical binding site recoveries after reconstitution were 30–40% for both mAcChR and G_i. Estimation of recovered protein [see Rosenbaum et al. (1987)] showed that about 100% of the protein was recovered with the pellet. This indicated that inactive G_i and mAcChR were also present in the reconstituted system.

Preparation of [α-^{32}P]GDP. [α-^{32}P]GDP was prepared from the treatment of [α-^{32}P]GTP with succinic thiokinase. One volume of [α-^{32}P]GTP (usually 160 pmol in 12.5 μL of 10 mM Tricine, pH 7.6) was added to 1 volume of solution containing 0.2 M Tris–acetate, pH 8.0, 100 μM coenzyme A, 20 mM sodium succinate, 10 mM $MgCl_2$, and 0.4 unit/mL succinic thiokinase. This reaction was allowed to proceed for 30 min at room temperature after which EDTA was added to 7.5 mM and the sample was diluted into 0.5 mL of H_2O and heated at 100 °C for 2 min. The sample was then applied to a 4 × 1 cm DEAE-Bio-Gel A column which had been equilibrated with 5 mM ammonium bicarbonate, pH 7.5. A 5–500 mM linear gradient of ammonium bicarbonate was used to elute the column at 0.1 mL/min. Fractions which contained radioactivity and eluted at the same position as cold GDP were pooled, concentrated under vacuum, applied to a 15 × 0.75 cm column of Bio-Gel P-2 preequilibrated in H_2O, and eluted with H_2O at 0.1 mL/min. The fractions which contained radioactivity were lyophilized at least twice to remove any residual ammonium bicarbonate. Purity of the [α-^{32}P]GDP was monitored by thin-layer chromatography on Merk Silica Gel 60 F254 plates using a solvent of 6:3:1 1-propanol/NH_4OH/H_2O, and exposing the TLC plate to X-ray film. At least 90% of the radioactivity migrated with GDP. This material was stored at −20 °C in H_2O until used.

Ligand Binding. mAcChR was quantitated in terms of L-[^{3}H]QNB binding sites using the DEAE filter disk assay (Peterson & Schimerlik, 1984). All binding assays for reconstituted material were performed in buffer B using 125 μL of sample. Nonspecific binding was determined in the presence of 10 μM L-hyoscyamine or 0.1 M carbachol. Total mAcChR concentration was determined by using 20 nM L-[^{3}H]QNB plus or minus 10 μM L-hyoscyamine or lipids without mAcChR for nonspecific binding.

[^{35}S]GTPγS binding was performed as described by Northup et al. (1982). Detergent-solubilized G_i was incubated for 60 min at 32 °C in buffer A supplemented with 25 mM $MgCl_2$, 0.1% Lubrol PX, and 1 μM [^{35}S]GTPγS (3–8 Ci/mmol). Nonspecific binding was determined in the absence of protein and was less than 0.5% of the total radioactivity. Reconstituted G_i was assayed in buffer A. In some instances, the reaction mix was diluted into wash buffer (10 mM Hepes, 0.1 M NaCl, and 25 mM $MgCl_2$) plus 100 μM GTP to further reduce nonspecific binding.

The determination of bound [α-^{32}P]GDP was done in a similar manner except that the reaction mixture (50 μL) was diluted into ice-cold wash buffer containing 0.5 mM GDP and immediately filtered and washed with 2 mL of buffer. The time from dilution to the end of the final wash was about 7 s.

For Scatchard plots and titration curves, duplicate aliquots of the reaction mixture were removed to determine the total radioligand present.

In experiments to determine the binding of nonlabeled GTP, a GTP-regenerating system was included so that GDP, produced by the GTPase activity of G_i, would not accumulate. The regenerating system consisted of buffer B plus 5 mM KCl, 1 mM phosphoenolpyruvate, and 70 ng/mL pyruvate kinase.

GTPase. GTPase activity was determined as described in Sunyer et al. (1984) with the following alterations. Vesicles were diluted with buffer A to a final concentration between 1 and 4 nM [^{35}S]GTPγS sites. Muscarinic ligands were added as described in the text and allowed to equilibrate for 5 min at 32 °C. The GTPase reaction was initiated by adding [γ-^{32}P]GTP and incubating at 32 °C for the desired amount of time. The reaction (50 μL) was quenched by the addition of 0.25 mL of an ice-cold suspension of activated charcoal (5% w/v) in 20 mM sodium phosphate (pH 2.3). This mixture was placed on ice for 5 min and then centrifuged at 15000g for 15 min at 4 °C. The ^{32}P in 0.1 mL of the clear supernatant was measured by Cerenkov counting. Nonenzymatic hydrolysis was subtracted from all determinations. The time

course of the GTPase activity was linear under the conditions in which the GTP concentration dependence was performed.

ADP-Ribosylations. Pertussis toxin was activated by incubating with 10 mM DTT and 50 μM ATP at 32 °C for 15 min. Reconstituted G_i in buffer A was ADP-ribosylated by diluting into an assay mixture to give final concentrations of 5 μM thymidine, 2 mM NAD, and about 0.4 μg/mL activated pertussis toxin. This resulted in a 2-fold dilution of vesicles. The reaction was allowed to proceed for 30 min at 32 °C and was then diluted at least 20-fold into buffer A or B before subsequent experiments were executed.

Data Analysis

Titration curves were fit to eq 1, using Marquardt's algorithm as described by Duggleby (1984). In eq 1, $\bar{Y}$ equaled

$$\bar{Y} = \frac{[L]}{K}\left(\frac{F_1}{1 + [I]/K_1 + [L]/K} + \frac{F_2}{1 + [I]/K_2 + [L]/K}\right) \quad (1)$$

the fractional saturation of the protein by radioligand, and [L] and [I] are the free radioligand and inhibitor concentrations, respectively. F_1 and F_2 are the fractions of binding sites having high affinity and low affinity for inhibitor with dissociation constants K_1 and K_2, respectively, and K is the overall dissociation constant for the radioligand. Data were normalized according to eq 2 where $\bar{Y}_0$ was the fractional saturation in the absence of inhibitor.

$$\% \text{ specific bound} = (\bar{Y}/\bar{Y}_0) \times 100 \quad (2)$$

Since K appeared to vary slightly depending on the preparation, its value was calculated for each experiment by using the law of mass action and the concentration of bound protein, the concentration of free protein, and [L] determined in the absence of inhibitor.

Steady-state GTPase kinetics were analyzed by using Marquardt's algorithm (Duggleby, 1984) by a two-component fit to the observed steady-state velocity.

$$v = \frac{F_1 V_{m1}[S]}{K_{m1} + [S]} + \frac{F_2 V_{m2}[S]}{K_{m2} + [S]} \quad (3)$$

In eq 3, v is the observed steady-state velocity at GTP concentration [S]; F_1 and F_2 equal the fractions of enzyme having a maximum velocity of V_{m1} and V_{m2} and a Michaelis constant of K_{m1} and K_{m2}, respectively. Turnover numbers for V_{m1} and V_{m2} were obtained by dividing each maximal velocity by the concentration of [^{35}S]GTPγS binding sites.

Kinetic analysis of [α-^{32}P]GDP dissociation was done by using nonweighted least-squares fitting to either eq 4 or eq 5 where $y(t)$ equaled the specifically bound ligand at time t,

$$y(t) = A_1 e^{-t/\tau_1} \quad (4)$$

$$y(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (5)$$

A_i values are the amplitudes, and τ_i^{-1} values are the reciprocal relaxation times for the exponential decay processes. For eq 5, the slow phase was analyzed after the decay of the fast kinetic phase. The fast phase was then evaluated after subtraction of the slower component.

Results

Purified porcine atrial muscarinic mAcChR reconstituted in a molar ratio of 1:5 with purified porcine atrial G_i (SDS gel shown in Figure 1) was able to bind L-[^{3}H]QNB in a concentration-dependent manner showing a single class of binding sites having a dissociation constant of 297 ± 26 pM in the absence of GppNHp and a dissociation constant of 292 ± 12 pM in the presence of 100 μM GppNHp. That the binding of L-QNB to the mAcChR was not affected by the presence of 0.1 mM GppNHp was in agreement with findings on reconstituted porcine brain muscarinic receptor and G_i (Haga et al., 1986). Figure 2A shows the carbachol displacement of L-[^{3}H]QNB bound to mAcChR reconstituted without G_i. The titration curve was analyzed by assuming one class of carbachol binding sites, and the resulting dissociation constant (122 μM) was not significantly different from the low-affinity carbachol binding site for membrane-bound and detergent-solubilized atrial mAcChR (see Discussion). However, when mAcChR was reconstituted with G_i (Figure 2B), there appeared a class of high-affinity carbachol binding sites which were sensitive to 0.1 mM GppNHp. Treatment of the reconstituted mAcChR and G_i with 0.1 mM GppNHp caused an apparent uncoupling of the two proteins since the mAcChR bound carbachol as if it were reconstituted without G_i. The average K_d value (n = 4) for the high-affinity binding site was 1.1 ± 0.5 μM, and 44% ± 11% of the L-[^{3}H]QNB binding sites were sensitive to guanine nucleotides.

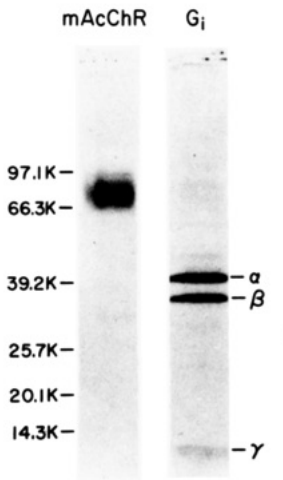

FIGURE 1: SDS–polyacrylamide gel of purified porcine atrial mAcChR and purified porcine atrial G_i used for reconstitution studies. Samples and molecular weight markers (phosphorylase *a*, M_r 97 114; bovine serum albumin, M_r 66 296; aldolase, M_r 39 210; chymotrypsinogen A, M_r 25 666; soybean trypsin inhibitor, M_r 20 095; and lysozyme, M_r 14 314) were electrophoresed on an 8–18% linear acrylamide gradient gel using the discontinuous buffer system of Laemmli (1970) and visualized by silver staining (Wray et al., 1981).

Coupling of the mAcChR to G_i was also demonstrated by the ability of carbachol to increase the GTPase activity associated with G_i (Figure 2C). The apparent dissociation constant for carbachol-stimulated GTPase activity (2.1 μM) was in good agreement with the GppNHp-sensitive high-affinity carbachol binding site seen in the titration curves (Figure 2B). The carbachol-induced increase in GTPase was blocked by 10 μM hyoscyamine. GTPase activity in the presence of L-hyoscyamine was slightly lower than in the absence of ligands (76% ± 11%, n = 4). The presence of acetylcholinesterase during the GTPase reaction did not alter the inhibition due to the antagonist; thus, contamination by acetylcholine from the reconstitution was unlikely.

The dependence of the conversion of low-affinity muscarinic agonist binding to the high-affinity state on G_i concentration was determined by reconstitution of the mAcChR (10 pmol) with varying amounts of G_i (10–200 pmol). The amount of 0.1 mM GppNHp-sensitive high-affinity binding was maximal when the G_i and mAcChR were reconstituted in a ratio of 3:1 to 5:1. Similarly, as the ratio of G_i to mAcChR was increased, the mAcChR-stimulated GTPase decreased to a stable plateau at a 4–6-fold ratio of G_i to mAcChR. At much higher ratios

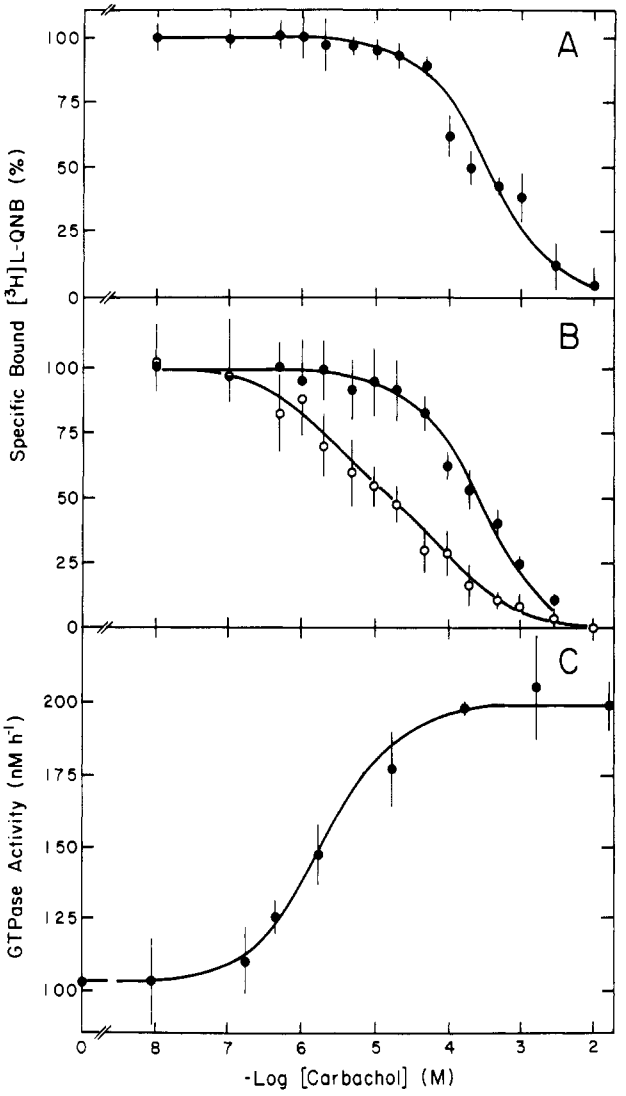

FIGURE 2: Carbachol titration of specifically bound L-[^{3}H]QNB and mAcChR-stimulated GTPase. (A) Reconstituted mAcChR alone was diluted in buffer B to a concentration of 141 pM in L-[^{3}H]QNB sites. Samples were added to tubes containing the indicated final concentration of carbachol, and after 30 min at 32 °C, 550 pM L-[^{3}H]QNB was added. After 2 h, specific binding was determined as described under Materials and Methods. Data points represent the average ± SD of triplicate determinations. The data were fit to eq 1, holding F_1 at 1.0 to give a K_d for carbachol of 122 ± 11 μM and using a K_d of 305 pM for L-QNB. (B) Reconstituted mAcChR and G_i were diluted into buffer B in the presence (●) or absence (○) of 0.1 mM GppNHp and titrated with carbachol as described above. The titration in the absence of GppNHp contained 73 pM L-[^{3}H]QNB sites and 350 pM [^{35}S]GTPγS sites. The data were fit to eq 1 by using a K_d for L-QNB of 460 pM. Analysis gave F_1 equal to 0.53 ± 0.04, K_1 equal to 1.0 ± 0.3 μM, F_2 equal to 0.47 ± 0.04, and K_2 equal to 66.5 ± 11.7 μM. In the presence of GppNHp, the curve was fit to eq 1, holding F_1 at 1.0 and using 93 pM L-[^{3}H]QNB total binding sites and a K_d for L-QNB equal to 431 pM. The K_d for carbachol was calculated to equal 121.6 ± 10.3 μM. (C) Samples were preincubated in buffer A for 5 min at 32 °C with the indicated final carbachol concentrations. The GTPase reaction was initiated by adding [γ-^{32}P]GTP to a concentration of 500 nM and incubating for an additional hour. Samples (50 μL containing 4.8 nM [^{35}S]-GTPγS sites and 0.82 nM L-[^{3}H]QNB sites) were assayed for ^{32}P released as described under Materials and Methods. The data points represent the average ± SD of triplicate determinations and were fit to the law of mass action to give an apparent dissociation constant of 2.1 ± 0.3 μM for carbachol.

(>20-fold), mAcChR-stimulated GTPase was seen to decrease further. A ratio of G_i to mAcChR of 5:1 was therefore used for all reconstitution experiments. In order to more completely examine the coupling of the mAcChR to G_i in terms of the

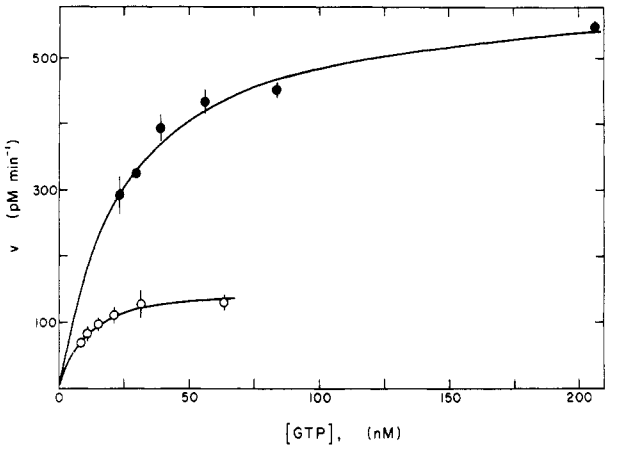

FIGURE 3: Dependence of GTPase activity of G_i reconstituted with mAcChR on GTP concentration. Reconstituted material (0.8 nM [^{35}S]GTPγS binding sites and 0.2 nM L-[^{3}H]QNB binding sites) in buffer A was incubated with either 2 mM carbachol (●) or 10 μM L-hyoscyamine (○) for 5 min at 32 °C, and the GTPase reaction was started by adding [γ-^{32}P]GTP and allowed to continue for 15 min. Values represent the mean ± standard deviation of triplicate determinations. Data were analyzed by a direct fit to the Michaelis–Menton equation to give a K_m of 9.79 ± 1.42 nM and a V_{max} of 154 ± 8 pM min^{-1} for L-hyoscyamine-treated vesicles and a K_m of 25.5 ± 1.3 nM and a V_{max} of 612 ± 11 pM min^{-1} for carbachol-treated vesicles. Alternatively, data were analyzed according to eq 3 as described under Results assuming 30% interactive G_i. The theoretical curve from this analysis was virtually superimposable over the curve drawn in the figure.

muscarinic agonist-stimulated GTPase, a GTP concentration dependence of the GTPase activity of G_i was done in the presence of either saturating carbachol or saturating L-hyoscyamine. As seen in Figure 3, treatment of the reconstituted system with carbachol resulted in an apparent 4-fold increase of V_{max} and a 2.6-fold increase in the K_m for GTP. The apparent turnover numbers in this experiment were 0.19 and 0.76 min^{-1} for the L-hyoscyamine and carbachol-treated preparations, respectively.

In order to more fully elucidate the properties of the G_i coupled to the mAcChR, a series of ligand binding and kinetic studies were initiated. The first probe used was the non-hydrolyzable GTP analogue GTPγS. Because of the possibility of interference from bound GDP (Ferguson et al., 1986; M. R. Tota et al., unpublished results), we did not directly examine the association rate constant of GTPγS. The equilibrium dissociation constant for [^{35}S]GTPγS was determined in the presence of 2 mM carbachol (332 ± 32 pM) or L-hyoscyamine (454 ± 70 pM). These binding studies showed that GTPγS bound to a homogeneous class of sites and its dissociation constant was not strongly affected by muscarinic ligands within the range of concentrations examined.

The dissociation constant of GDP was, however, strongly affected by muscarinic ligands (Figure 4). When incubated with L-hyoscyamine, the displacement of [^{35}S]GTPγS by GDP could be fit by assuming one class of binding sites for GDP (K_d = 7 nM). Incubation with carbachol produced a shallower titration curve which was fit by assuming two classes of binding sites for GDP. Thus, the presence of carbachol had produced a population of G_i with a low affinity for GDP (32%, K_d = 499 nM) with no significant change in the remaining population (K_d = 12.7 nM).

In order to determine to what extent the change in GDP affinity caused by carbachol was due to a change in the dissociation rate of GDP, the release of [α-^{32}P]GDP was measured in the presence of a muscarinic agonist and antagonist (Figure 5). The results indicated a single phase of GDP

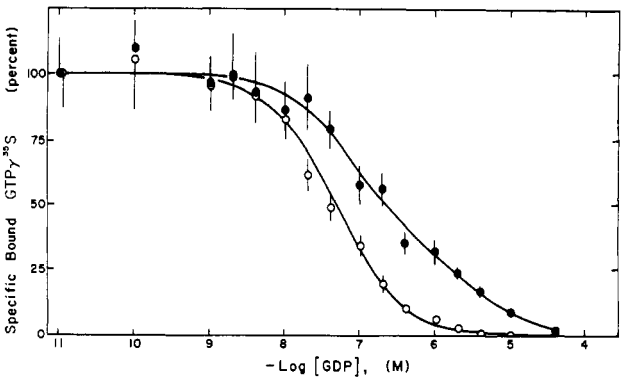

FIGURE 4: GDP titration of specifically bound [^{35}S]GTPγS. The reconstituted preparation containing approximately 31 pM L-[^{3}H]QNB binding sites and 170 pM [^{35}S]GTPγS sites in buffer A was supplemented with either 2 mM carbachol (●) or 10 μM L-hyoscyamine (○). Samples were then added to tubes containing the indicated final concentrations of GDP and incubated for 15 min at 32 °C. [^{35}S]-GTPγS was then added to a final concentration of 1.6 nM and the incubation continued for an additional 2 h at which time samples were filtered as described under Materials and Methods. Data for carbachol-treated vesicles were analyzed by using eq 1 and a K_d of 280 pM for [^{35}S]GTPγS and 188 pM for the total number of GTPγS sites. A fit to eq 1 gave $F_1 = 0.68 \pm 0.04$, $K_1 = 12.7 \pm 2.1$ nM, $F_2 = 0.32 \pm 0.04$, and $K_2 = 499 \pm 86$ nM. The analysis for L-hyoscyamine-treated vesicles assumed one class of binding sites with a K_d of 277 pM for [^{35}S]GTPγS and 164 pM in GTPγS binding sites. The dissociation constant for GDP was calculated to equal 7.0 ■ 0.3 nM from the fit to eq 1 fixing F_1 at 1.0.

release from antagonist-treated material with a τ^{-1} of 0.1 min^{-1}. In the presence of carbachol, there was a rapid phase of GDP release ($\delta A_1/\delta A_t = 27\%$, $\tau_1^{-1} = 4.5$ min^{-1}) followed by a slower phase ($\delta A_2/\delta A_t = 63\%$, $\tau_2^{-1} = 0.13$ min^{-1}) which was similar to the rate of GDP release in the presence of L-hyoscyamine. Although the fast phase was poorly defined, a value could be estimated that was 33–44 times faster than the slow phase of GDP release. The change in K_d for GDP could therefore be explained by a change in the dissociation rate for GDP.

Since 30% of the [^{35}S]GTPγS sites were affected by muscarinic agonists, it was necessary to reevaluate the carbachol-stimulated GTPase data assuming that 30% of the G_i was responsive to carbachol and the remaining protein hydrolyzed GTP with a rate similar to that found in the presence of L-hyoscyamine. The concentration dependence of the steady-state velocity of GTP hydrolysis in the presence of carbachol was then analyzed as the sum of two simultaneous reactions (see Figure 3 and eq 3) where 30% of the total enzyme appeared to interact with the mAcChR. This gave a K_m for GTP equal to 31 ± 4 nM and a turnover number of 2.1 ± 0.1 min^{-1} for the carbachol-stimulated GTPase. In three experiments where the ratio of G_i/mAcChR was between 5:1 and 4:1, the average value of the carbachol-stimulated turnover was 2.17 ± 0.07 min^{-1}. This value was independent of slight variations in the ratio of G_i and mAcChR recovered after reconstitution, and the value of 30% obtained from the two GDP binding experiments was used without corrections in other experiments where the ratio of the two proteins was not exactly the same. Therefore, carbachol promoted an actual 11-fold increase in turnover number rather than the observed 4-fold stimulation.

The effectiveness of guanine nucleotides in altering the binding of L-[^{3}H]QNB in the presence of 100 μM carbachol could be related to their affinity for G_i in the presence of agonist. The apparent K_d for GTPγS was poorly defined (K_{app} = 2.7 ± 3.4 nM); however, the value for GDP was similar to the thermodynamic dissociation constant obtained from competition binding with [^{35}S]GTPγS (K_{app} = 557 ± 207 nM),

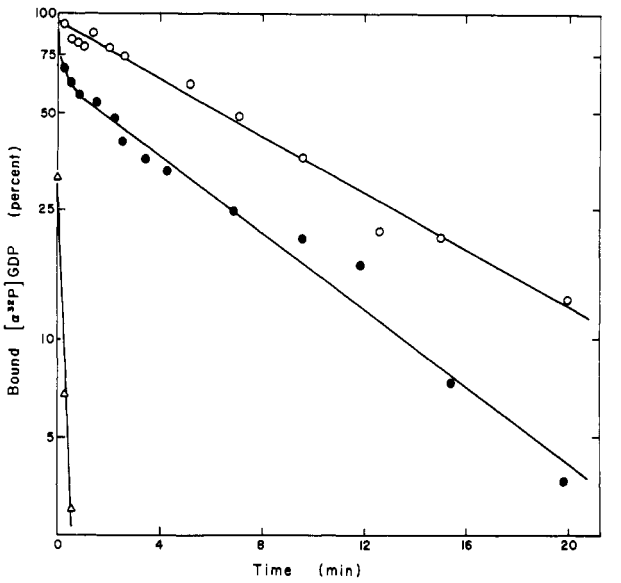

FIGURE 5: Time course for [α-^{32}P]GDP dissociation. Reconstituted mAcChR (360 pM in L-[^{3}H]QNB binding sites) and G_i (2 nM in [^{35}S]GTPγS sites) were incubated in buffer A at 32 °C in the presence of 100 nM [α-^{32}P]GDP for 30 min. The dissociation reaction was initiated by adding unlabeled GDP to a final concentration of 100 μM plus either 2 mM carbachol (●) or 10 μM L-hyoscyamine (○). Aliquots were removed at the time indicated and filtered as described under Materials and Methods. L-Hyoscyamine data were analyzed by a least-squares fit to a single exponential (eq 4) which gave an ordinate intercept of 94% ± 3% bound and a rate constant of 0.100 ± 0.004 min^{-1}. The time course for dissociation in the presence of carbachol contained two kinetic phases and was analyzed according to eq 5. The slow phase ($\delta A_2/\delta A_t = 63\% \pm 4\%$; $\tau_2^{-1} = 0.136 \pm 0.001$ min^{-1}) was analyzed by a least-squares fit using data obtained after 1 min. The fast phase was then evaluated after subtraction of the slow phase ($\delta A_1/\delta A_t = 27\% \pm 8\%$; $\tau_1^{-1} = 4.5 \pm 0.8$ min^{-1}).

and the apparent dissociation constant for GTP (K_{app} = 33 ± 5 nM) was similar to the K_m value obtained from the GTP concentration dependence of the carbachol-stimulated GTPase activity. The order of potentcy was GTPγS > GTP > GDP where GTP was about 17-fold more potent than GDP in eliciting a change in carbachol binding.

The effect of ADP-ribosylation on muscarinic agonist-stimulated GTPase was then determined for the reconstituted preparation. A solution containing 0.13 pmol of L-[^{3}H]QNB sites and 0.61 pmol of [^{35}S]GTPγS sites in 8 μL was ADP-ribosylated as described under Materials and Methods in a total reaction volume of 20 μL. The IAP-treated mixture was diluted 20-fold into buffer A and assayed for GTPase activity with 500 nM [γ-^{32}P]GTP and either 2 mM carbachol or 10 μM L-hyoscyamine (assays were performed in triplicate). When NAD was omitted, 67.2 ± 0.2 nM ^{32}P was released in 1 h in the presence of carbachol while 25.6 ± 3.4 nM was released in the presence of L-hyoscyamine. When 2 mM NAD was included, the amount of ^{32}P released was 26.0 ± 3.2 and 21.6 ± 4.4 nM for carbachol- and L-hyoscyamine-treated samples, respectively. Thus, pertussis toxin plus NAD attenuated most, if not all, of the agonist-stimulated GTPase. Neither the nonstimulated GTPase nor the amount of [^{35}S]-GTPγS sites was affected by ADP-ribosylation (data not shown). A similar treatment of reconstituted material had no effect on the ability of carbachol to displace L-[^{3}H]QNB or on the ability of 0.1 mM GppNHp to alter carbachol binding (Figure 6A,B). The ADP-ribosylation experiment was then repeated in the presence of acetylcholine and GTP. Prior to titration curve experiments, acetylcholine was removed from the system by treatment with acetylcholinesterase, and the majority of the GTP should have been hydrolyzed to GDP

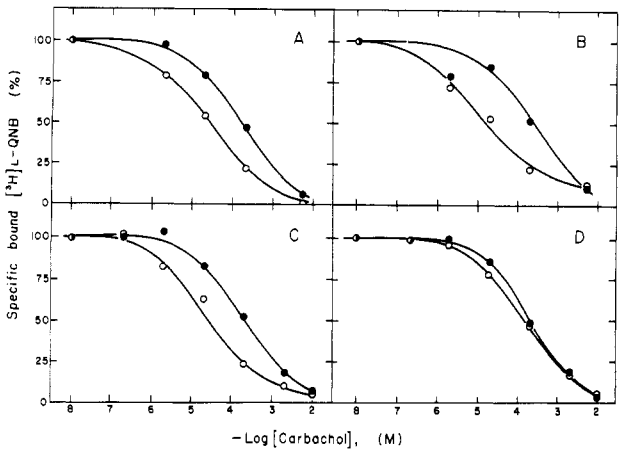

FIGURE 6: Effect of ADP-ribosylation on binding of carbachol to mAcChR. mAcChR and G_i were ADP-ribosylated as described under Materials and Methods with (B, D) or without (A, C) NAD. (C) and (D) were supplemented with 500 nM GTP and 5 μM acetylcholine and after a 30-min incubation were allowed to incubate 5 additional min at 32 °C with 0.76 unit mL^{-1} acetylcholinesterase. Reaction mixtures were then diluted into buffer B to give an L-[^{3}H]QNB site concentration of 72 pM and a GTPγS site concentration of 334 pM for (A) and (B) and 78 pM L-[^{3}H]QNB sites and 400 pM GTPγS sites for (C) and (D). Carbachol titration of specifically bound L-[^{3}H]QNB was performed in the presence (●) or absence (○) of 100 μM GppNHp. Lines through the data were drawn by eye and have no theoretical basis.

by the activated G_i. Any residual GTP and GDP was diluted below a concentration (less than 2 nM) in which they were effective in uncoupling the proteins. Under these conditions, the binding of carbachol to the mAcChR was decreased, and there was no further effect of 0.1 mM GppNHp (compare panels C and D of Figure 6).

Discussion

The data presented above demonstrated that porcine atrial mAcChR effectively coupled to porcine atrial G_i in the reconstituted systems. The criteria for coupling were (1) the ability of muscarinic ligands to alter the interaction of guanine nucleotides with G_i, (2) the 10-fold stimulation of steady-state GTP hydrolysis by the muscarinic agonist carbachol, and (3) the appearance of a guanine nucleotide sensitive high-affinity carbachol binding by the mAcChR. Treatment of the coupled system with 0.1 mM GppNHp resulted in an apparent uncoupling of the two proteins. After treatment with the nucleotide, the receptor interacted with carbachol in a manner similar to mAcChR reconstituted without G_i. mAcChR reconstituted alone had a homogeneous low-affinity binding as seen in Figure 2A. It should be noted that there was some variability between receptor preparations and that mAcChR reconstituted alone or mAcChR and G_i treated with 0.1 mM GppNHp did not always show a homogeneous low-affinity carbachol binding site. This heterogeneity with respect to carbachol binding has been previously observed both in detergent-soluble preparations (Peterson & Schimerlik, 1984) and for the reconstituted brain mAcChR (Haga et al., 1986), and has not yet been adequately explained. Although a fraction of the mAcChR high-affinity sites occasionally remained in the high-affinity state in the presence of saturating GppNHp, guanine nucleotide sensitive high-affinity agonist binding was observed for all reconstituted preparations. The average dissociation constant for carbachol binding to the GppNHp-sensitive high-affinity site was 1.1 ± 0.5 μM, and 44% ± 11% of the L-[^{3}H]QNB sites were sensitive to quanine nucleotides. This maximum value of mAcChR that can be coupled to G_i agrees with that found for the brain mAcChR reconstituted with brain G_i (40–50%; Haga et al., 1986); however, in the atrial system, only a 3-fold excess of G_i over receptor was necessary to see the maximum effect as opposed to a 20-fold excess for the brain system. The reasons for this difference are not known but may be due to the different structures of the brain and heart mAcChRs (Kubo et al., 1986a,b; Peralta et al., 1987) or to the differences in reconstitution procedures. The K_d of carbachol for the coupled receptor (1.1 μM) agreed quite well with the K_{app} for the carbachol-stimulated GTPase activity (K_{app} = 2.1 ± 0.3 μM). This was further evidence that the high-affinity carbachol binding site was interacting with G_i. This dissociation constants for carbachol binding to the low-affinity (K_L equal to 66–120 μM) and high affinity (K_H equal to 1 μM) agonist sites agreed with the values found previously in this laboratory for the membrane-bound mAcChR (K_H equal to 0.1–0.8 μM, K_L equal to 30–70 μM; Schimerlik & Searles, 1980; Peterson & Schimerlik, 1984) although the assay conditions were somewhat different (10 mM phosphate) than those used in these experiments. The affinity of L-QNB for the reconstituted receptor preparation was, however, lower by 7–10-fold than found previously (41 pM; Schimerlik, 1986). Whether this was due to differing ionic conditions, alterations in the receptor lipid environment compared to native membranes, or alterations in the receptor conformation during reconstitution is not known.

The mAcChR could also be uncoupled from G_i by treatment with other guanine nucleotides. The order of potency for this effect was GTPγS > GTP > GDP. The ability of GDP to uncouple the mAcChR from G_i has also been noted by others (Haga et al., 1986). A K_{app} was determined for each of these nucleotides, and the K_{app} for GDP (557 nM) agreed with the dissociation constant of GDP from receptor-activated G_i (500 nM). A K_{app} for GTP was determined to be 33 nM. It was somewhat difficult to rigorously interpret this value, since even though there was a GTP-regenerating system included, GTP would be converted to GDP on G_i. However the value obtained was similar to the K_m of GTP for the carbachol-activated G_i (31 nM). Determination of the K_{app} for GTPγS was also not straightforward. Because of the low K_d (about 0.4 nM from Scatchard analysis), certain assumptions had to be made in terms of free ligand. Since both carbachol and L-hyoscyamine-treated mAcChR–G_i had about the same high-affinity binding for GTPγS, all of the G_i present was assumed to participate equally in binding GTPγS for the calculation of free nucleotide. This gave a value 2.7 ± 3.4 nM for an estimation of K_{app} which was almost an order of magnitude greater than the estimated K_d. However, the value for K_{app} is not well-defined and may reflect the inaccuracies arising from the use of an inappropriate G_i concentration. In any case, it was clear that GTPγS was more effective than GTP in uncoupling the receptor. Also, both GTP and GDP uncouple the receptor by binding to the activated agonist mAcChR–G_i complex.

Coupling between mAcChR and G_i was also sensitive to ADP-ribosylation by pertussis toxin. As seen for brain system (Haga et al., 1985), ADP-ribosylation interferes with the carbachol-stimulated GTPase. However, ADP-ribosylation using similar conditions did not show any effect on carbachol binding. The mAcChR still demonstrated high-affinity guanine nucleotide sensitive carbachol binding (Figure 6A,B). Addition of GTP and acetylcholine during ADP-ribosylation resulted in a loss of this guanine nucleotide sensitive carbachol binding site. Kurose et al. (1986) also report a similar uncoupling when G_i was ADP-ribosylated before reconstitution.

In the reconstituted system, the ADP-ribosylation must be performed in the presence of acetylcholine and GTP in order to see an effect on carbachol binding, but these conditions were not necessary for seeing an attenuation of carbachol-stimulated GTPase. A possible explanation was that the addition of GTP during the GTPase assay is sufficient to increase ADP-ribosylation efficiency either by further activating the toxin or by stabilizing G_i. Such a phenomenon has been previously noted (Mattera et al., 1986).

Based on IC_{50} values, ATP binds to pertussis toxin about 140 times tighter than GTP (Mattera et al., 1986). Therefore, it seems unlikely that 500 nM GTP would have an effect in the presence of 25 μM ATP. It is also unlikely that the effect was due to additional stabilization of G_i. Without any added guanine nucleotides during ADP-ribosylation, there was still some G_i coupled to the mAcChR. If G_i was denatured during the 0.5-h preincubation with IAP, it would not explain the ability of GppNHp to convert high-affinity agonist sites to lower affinity (Figure 6B). An alternative explanation is that the mAcChR was precoupled to G_i and this protected the associated G_i from ADP-ribosylation. Free G_i was still ADP-ribosylated. The GTPase assay of the ADP-ribosylated sample showed no increase of activity when carbachol was added because all the free G_i was ADP-ribosylated and each precoupled G_i was only able to complete one GTPase cycle before it was also ADP-ribosylated.

The regulation of G_i by agonist-occupied mAcChR was shown by a stimulation of GTPase activity. As seen in Figure 3, there was an apparent 4-fold stimulation of the GTPase activity of carbachol-treated G_i–mAcChR compared to L-hyoscyamine treated, and a 2.6-fold increase in K_m. The K_m (9.8 nM) and V_{max} (0.19 min^{-1}) obtained for L-hyoscyamine-treated reconstituted mAcChR and G_i were similar to the values determined with brain mAcChR and brain G_i (Kurose et al., 1986), determined in the absence of ligand. However, the apparent stimulation observed in the presence of carbachol was greater in the present study. According to the assumption that 30% of the G_i was stimulated by the mAcChR, the GTPase kinetics were reevaluated to give a turnover number of 2.1 min^{-1} for G_i coupled to receptor. This value was similar to the turnover number obtained for the activated catecholamine-stimulated GTPase associated with G_s after similar corrections were performed (Brandt & Ross, 1986). L-Hyoscyamine-treated mAcChR and G_i showed a 24% lower GTPase activity compared to the absence of ligands ($n = 4$). Addition of acetylcholinesterase (PMSF was omitted for this experiment) did not alter these results. Thus, contamination by acetylcholine seems unlikely. The reasons for these observations are not yet known.

It has been suggested that activation of G_s by its associated receptors results in an "open" G protein in which there is an accelerated turnover of quanine nucleotides (Stryer & Bourne, 1986), and a recent study suggested that the agonist stimulation of G_s involves an increase in both the rate of GTP binding and the dissociation of GDP (Brandt & Ross, 1986). In other hormone–G protein systems, agonists were seen to regulate the binding of GTPγS (Brandt & Ross, 1986), and an agonist-induced effect on the apparent association rate of GTPγS has been noted for the reconstituted brain mAcChR and brain G_i (Kurose et al., 1986). In the present study, differences in GTPγS binding between carbachol and L-hyoscyamine treatments were observed (data not shown); however, the effects of bound GDP cannot be ruled out. At 100 nM GTPγS in the presence of L-hyosycamine, the rate of GTPγS binding was similar to the rate of GDP release and was preceded by a burst phase. In the presence of carbachol, the predominant difference was a change in the amplitude of the burst phase which was assumed to represent the fraction of G_i which had no GDP bound or bound GDP with low affinity. When the equilibrium binding of GTPγS was examined, there was no significant difference between incubation with carbachol or L-hyoscyamine. Although there was no change in GTPγS binding, there was a 3.2-fold increase in the K_m of GTP when the GTPase reaction was performed in the presence of carbachol. It is not yet clear if this is due to a difference in the affinity of G_i for GTP or a change in some other intermediate step of the mechanism.

The data presented in Figure 4 demonstrated that agonist stimulation of mAcChR coupled to G_i resulted in a decrease in affinity of GDP for G_i. The change in affinity for GDP (50-fold) could be explained by the change in GDP dissociation rate (38-fold) if the binding of GDP was a simple bimolecular reaction. Such an assumption would predict an association rate between 1.5×10^5 and 2.0×10^5 M^{-1} s^{-1}. This value is within the range of values determined for the association rate of GDP binding to G_o (Higashijima et al., 1987).

The average value for the slow phase of GDP release was about 0.12 min^{-1}, and the turnover of the GTPase reaction in the presence of L-hyoscyamine was 0.19 min^{-1}. The rate-limiting step for the nonstimulated GTPase was therefore the release of GDP. Any stimulation of V_{max} must alter this step, and stimulation caused by agonist-bound mAcChR increases V_{max} to about 2.1 min^{-1}. Therefore, stimulation by the mAcChR causes an increase in GDP release; however, GDP release may be only partially rate limiting in the steady-state mechanism. (The GDP dissociation rate in the presence of carbachol was 4.5 min^{-1}.)

In summary, the atrial mAcChR is able to functionally interact with atrial G_i. The evidence indicates that the proteins are precoupled after reconstitution. Treatment with agonist caused an activation of the GTPase associated with G_i by, at least in part, increasing the dissociation rate of GDP. Guanine nucleotides were able to uncouple the mAcChR from G_i. After uncoupling, the low-affinity form of the receptor for agonists may then combine with another (GDP-bound) G_i. Both rhodopsin (Fung & Styrer, 1980) and the adrenergic receptor (Brandt & Ross, 1986) seem to act catalytically with respect to G protein activation. The findings in this study indicated that if the G_i:mAcChR ratio is about 5:1 and 30% of the G_i is activated, then about 1.5 G_i are activated per mAcChR. Since only 40% of the mAcChR can couple, then three to four G_i's are activated per receptor.

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