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Regulation of GDP and GTP Binding in Cardiac Sarcolemma by Muscarinic Receptor Agonists

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SUMMARY

Regulation of GTP and GDP binding and GTPase activity of cardiac sarcolemmal guanine nucleotide-binding proteins was investigated. In purified sarcolemmal membranes, carbachol and a variety of other muscarinic receptor (MR) agonists induced increases in [^{3}H]GTP, [γ-^{32}P]GTP, and [^{3}H]GDP binding to relatively high affinity sites. Carbachol-dependent GTP and GDP binding changes were maximal within 5 sec at 30° and thereafter remained at steady state. Carbachol increased GTP binding to two sites with apparent K_{app} values of 50 nM and 250 nM and GDP binding to a single site with a K_{app} of 100 nM. *N*-Ethylmaleimide attenuated carbachol-dependent GDP and GTP binding, tentatively identifying the binding sites as G_i and/or G_o. Further studies showed that [^{3}H]GDP and [^{3}H]GTP bound to G_i/G_o in the presence of carbachol rapidly exchanged with GTP and GDP in the medium. In membranes preincubated with carbachol and [γ-^{32}P]GTP or carbachol and [^{3}H]GDP, postaddition of atropine resulted in complete hydrolysis of [γ-^{32}P]GTP bound to G_i/G_o, to unlabeled GDP and $^{32}P_i$, by GTPase, within 10 sec, whereas [^{3}H]GDP remained bound. This study also showed that bound [^{3}H]GDP did not exchange with GDP or GTP in the absence of an MR agonist. Under identical conditions, atropine reversed adenylate cyclase (AC) inhibition by carbachol and GTP or GDP in 5–10 sec. MR agonists appear to increase the rate of dissociation of GDP from G_i/G_o, which results in rapid GTP turnover on these sites by a combination of GTPase and GDP/GTP exchange reactions. Furthermore, MR-G_i/G_o may be tightly coupled during AC inhibition, so that GTP hydrolysis as well as MR-G_i/G_o uncoupling may be required to reverse AC inhibition.

AC is coupled to MRs by G_i and possibly G_o in cardiac myocytes (1, 2). In the presence of guanylnucleotides, MR agonists attenuate AC activity by 30–60% in sarcolemmal membranes (3–6), and inhibition is uncoupled by pertussis toxin or NEM treatment (2, 7). Cardiac AC activity is inhibited by MR agonists both in the presence and in the absence of βAR agonists. Therefore, activated G_i or $G_{i\alpha}$ may directly interact with AC to promote inhibition (3, 5), and/or the β/γ subunits released from G_i during MR activation may bind with $G_{s\alpha}$ and thus indirectly attenuate βAR agonist stimulation of AC activity (1, 8).

Many key aspects of the molecular mechanism of MR-G_i-AC coupling have been described. MR agonists stimulate GTPase activity on G_i in cardiac membranes (9) or in liposomes reconstituted with purified porcine atrial MR and G_i (10). Activation of MRs by agonists stimulates the rate of [^{3}H]GDP release and GTP exchange on G_i (10), in analogy to studies showing that receptor agonists stimulate GDP release and GTP exchange on G_s (11, 12) or transducin (13). Steady state binding of G_i-GTP may then be maintained by G protein-GTPase hydrolysis and GDP/GTP exchange reactions. After receptor inactivation (i.e., due to agonist removal), GTP on G_s or G_i is hydrolyzed to GDP, and this hydrolysis reaction is widely considered to be responsible for termination of the receptor-G protein-mediated response (8, 11). In adipocyte membrane prelabeled with [^{3}H]GTP, receptor agonists stimulate [^{3}H]GDP release from G_i (14), indicating that GTP had been hydrolyzed to GDP on G_i. However, our recent studies demonstrated that carbachol could also inhibit cardiac AC activity by 35–60% in the presence of GDP or GDPβS (15). This inhibition could be reversed by atropine (15), which cannot be accounted for by G_i-GTPase hydrolysis. These observations suggested that the mechanism of MR-G_i-AC coupling may be unusual in cardiac sarcolemma, which prompted additional lines of investigation.

In this study, an assay was developed that enabled quantitative measurement of MR agonist-dependent [^{3}H]GDP and [^{3}H]GTP or [γ-^{32}P]GTP binding and exchange on G_i/G_o in these membranes. MR regulation of GTPase and AC activities could also be evaluated under identical conditions. These results show that MR agonists reduce the affinity of G_i for guanylnucleotides, with a resultant increase in the rates of

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ABBREVIATIONS: AC, adenylate cyclase; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); Gpp(NH)p, guanylylimidodiphosphate; MR, muscarinic receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; G protein, guanine nucleotide-binding protein; QNB, quinuclidinyl benzilate; NEM, *N*-ethylmaleimide.

GTP and GDP exchange, as well as GTP hydrolysis, on G proteins. Therefore, GTPase may play a role in promoting GTP turnover on G proteins, in addition to reversing AC inhibition due to MR agonists and GTP.

Experimental Procedures

Materials. [α-^{32}P]ATP (25 Ci/mmol) was obtained from ICN (Irvine, CA), and [^{3}H]AMP (30 Ci/mmol) was from New England Nuclear (Wilmington, DE). [^{3}H]GDP (10–20 Ci/mmol), [^{3}H]GTP (10–20 Ci/mmol), and [γ-^{32}P]GTP (10 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Other chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO). GDPβS was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Preparation and NEM treatment of cardiac sarcolemma. Sarcolemmal membranes were prepared from canine cardiac ventricular tissue by a procedure recently described in detail (16). Purified sarcolemma was suspended in 10 mM HEPES, pH 7.4, 2.0 mM $MgCl_2$, 0.5 mM dithiothreitol, frozen in liquid nitrogen, and stored at −80° without loss of guanylnucleotide binding or AC activity. The sarcolemmal preparations consisted of approximately 75–80% sealed right-side-out and 20–25% unsealed or inside-out vesicles. Vesicles were made permeable to guanylnucleotides with alamethicin, added at a ratio of 1 μg:1 μg of sarcolemmal protein. In some studies, permealized membranes were incubated 30 min at 5° with NEM, and the reaction was stopped by the addition of 5 mM dithiothreitol (final concentration). Protein was assayed by the method of Bradford (17).

GTP and GDP binding. The final composition of the medium used for the measurement of [^{3}H]GDP, [^{3}H]GTP, or [γ-^{32}P]GTP binding was 100 mM NaCl, 0.5 mM $MgCl_2$, 0.3 mM dithiothreitol, 1.0 mM NaN_3, 25 μM ouabain, and 50 mM HEPES, pH 7.4. Various concentrations of labeled guanylnucleotides, MR agonists, and atropine were included as indicated. To initiate binding, 25 μl of sarcolemmal suspension (5–6 μg) were added to 75 μl of medium that had been prewarmed at 30° for 10 sec. To reduce low affinity or background binding, 100 μM each of ADP and ATP was included in the [^{3}H]GTP binding studies and 200 μM UDP was included in [^{3}H]GDP binding studies. In dissociation or exchange studies, sarcolemma was preincubated for 10 sec in the same medium with carbachol, at a final volume of 80 μl, and 20 μl of control buffer or various agents were subsequently added. After a 5–60-sec incubation period at 30°, 80 μl of suspension were pipeted on Whatman GF/C filters under mild suction. The filters were immediately washed three times with 3 ml of 25 mM Tris·HCl, pH 7.6, at a flow rate of 1 ml/sec. Membrane protein recovery by the filter was ≥90%. Filters were suspended in 8.0 ml of Ecolume (ICN) in scintillation vials and counted. The assay variation of triplicates was approximately 5% of the mean under optimal conditions. In other studies, the binding was stopped by the addition of 1.0 ml of 25 mM Tris·HCl, pH 7.6, with 50 μM atropine, and tubes were centrifuged for 10 min at 5° in a 1.5-ml microfuge tube, at approximately 12,000 rpm. The pellet was washed twice by this procedure, and the pelleted membranes were suspended with 0.5 ml of the same buffer and counted.

GTPase activity. GTPase activity was assayed under the conditions used above for the GDP or GTP binding assay. Membranes were incubated for 10–60 sec at 30°, in the presence of 100 nM [γ-^{32}P]GTP (0.1 μCi/100 μl), and reactions were stopped with 0.9 ml of cold 5% activated charcoal in 20 mM phosphoric acid (9). After 10 min, the tubes were centrifuged at 2500 × g, and 0.5 ml of the supernatant was counted for $^{32}P_i$. MR agonist-stimulated activity was estimated by subtraction of counts obtained in the presence and absence of 10 μM carbachol. Carbachol increased $^{32}P_i$ approximately 50% above background, with an error of <5% of the means. Carbachol-stimulated $^{32}P_i$ release was linear for at least 60 sec at 30° in the presence of 100 nM GTP.

AC activity. Aliquots of suspended sarcolemma (2–2.5 μg) were added to 100 μl (final volume) of medium containing 100 mM NaCl, 0.5 mM $MgCl_2$, 0.3 mM dithiothreitol, 1.0 mM NaN_3, 25 μM ouabain, 0.3 mM KCl, 0.12 mM cAMP, 50 mM HEPES, pH 7.4, 100 μM [α-^{32}P]ATP (0.5 μCi), and 400 μM UDP. Various concentration of GDP, GTP, MR agonists, and atropine were added where indicated. UDP was added to prevent conversion of GDP to GTP by nucleoside diphosphate kinase (15). cAMP production was linear for 5 min under these conditions. For shorter time course assays (20–80 sec), the usual concentration of sarcolemma was doubled and the amount of [α-^{32}P]ATP was tripled. AC activity was determined by the method of Salomon *et al.* (18), as described in the accompanying paper (15). The standard deviation of triplicate determinations was usually <5% of the mean.

Estimation of MR Density. Sarcolemmal membranes (4–5 μg) were added to tubes containing (in a final volume of 1.0 ml) 100 mM NaCl, 0.5 mM $MgCl_2$, 0.3 mM dithiothreitol, 1.0 mM NaN_3, 25 μM ouabain, 0.3 mM KCl, 50 mM HEPES, pH 7.4, and 1.0 nM [^{3}H]QNB (Amersham). After 60 min at 25°, the suspensions were filtered on GF/C membranes (Whatman) under mild suction and were washed three times with 25 mM Tris·HCl, pH 7.6. Nonspecific binding obtained in the presence of 10 μM atropine was subtracted from the total binding. The density of MRs ranged from 4 to 6 pmol/mg of protein in different lots of sarcolemma.

Results

Time courses of GDP and GTP binding. Carbachol induced a rapid increase in [^{3}H]GDP and [^{3}H]GTP binding to cardiac sarcolemma (Figs. 1 and 2). As shown below, carbachol increased GTP or GDP binding to high affinity sites in sarcolemma, which correspond to G_i and possibly G_o. Generally, it has been difficult to measure receptor agonist-stimulated binding to GTP and GDP in membranes, due to high background or nonspecific binding. The success of the binding assay used here was dependent on inclusion of high micromolar concentrations of nonguanylnucleotides such as ADP and ATP or UDP in the assay medium, to reduce background binding by 80% (Table 1), as well as permealization of the vesicular membranes with alamethicin, to allow immediate access of GTP or GDP to G proteins. As a result of these modifications, carbachol

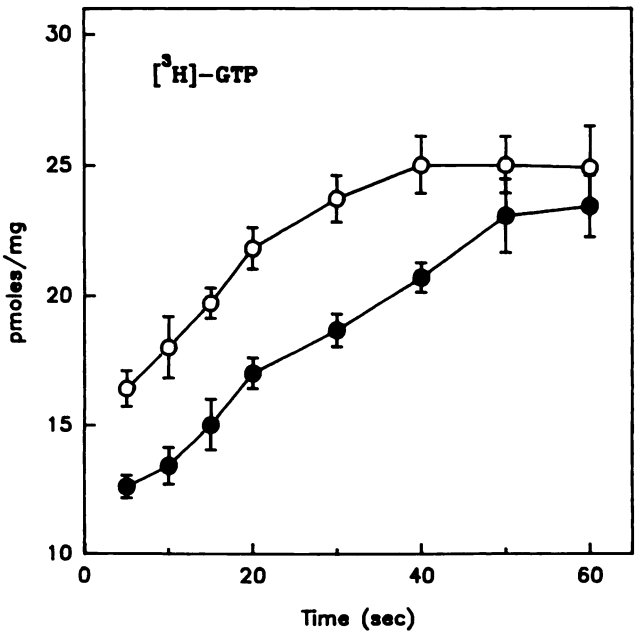

Fig. 1. Time courses of [^{3}H]GTP binding to cardiac sarcolemma in the absence (●) or presence (○) of 10 μM carbachol. The concentration of GTP was 100 nM. The data are presented as the means ± standard deviations of triplicate determinations and are representative of at least five separate experiments.

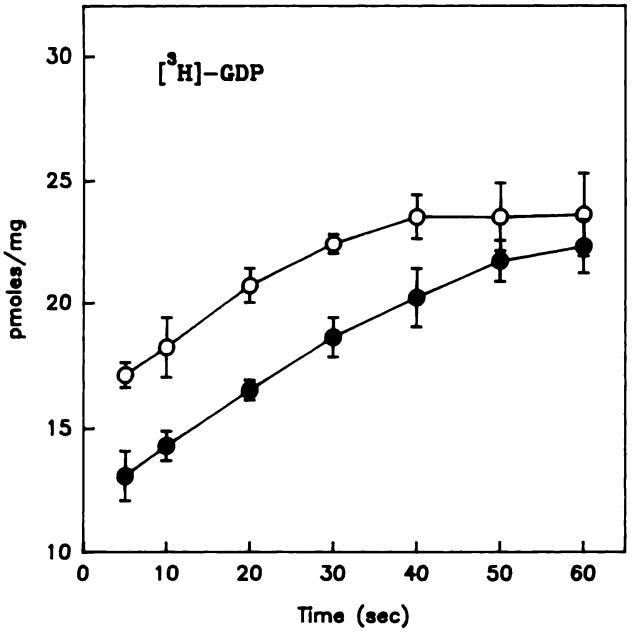

Fig. 2. Time courses of [^{3}H]GDP binding to cardiac sarcolemma in the absence (●) or presence (○) of 10 μM carbachol. The concentration of GDP was 100 nM. The data are presented as the means ± standard deviations of triplicate determinations and are representative of at least four separate experiments.

TABLE 1

Effects of nonguanylnucleotides on [^{3}H]GDP binding to cardiac sarcolemma in the presence and absence of carbachol

Incubation was for 10 sec at 30°, with or without μM carbachol. Other conditions were standard.

Nonguanylnucleotides	[^{3}H]GDP bound		Change
	Control	+Carbachol	
	pmol/mg		%
None	68 ± 11	69 ± 6	
100 μM UDP	12.1 ± 0.3	15.8 ± 0.4	31
200 μM UDP	11.9 ± 0.6	15.7 ± 0.6	32
400 μM UDP	12.4 ± 0.4	16.4 ± 0.3	32
800 μM UDP	12.2 ± 0.2	14.6 ± 0.4	19
100 μM ATP + 100 μM ADP	12.6 ± 0.4	16.7 ± 0.5	32
200 μM ATP + 200 μM ADP	12.2 ± 0.6	15.9 ± 0.4	30

and other MR agonists reproducibly increased binding of nanomolar concentrations of [^{3}H]GDP and [^{3}H]GTP by 30–40% above background. The carbachol-dependent changes could be quantified relatively accurately, because the range of error in triplicate determinations was routinely 5% during 5–30-sec incubations. Carbachol-dependent increases in GDP and GTP binding were completely blocked by 50 μM atropine. Carbachol-stimulated [^{3}H]GDP or [^{3}H]GTP binding could also be measured by a centrifugation and washing procedure (see Experimental Procedures). The [^{3}H]GDP bound in response to MR stimulation remained tightly bound after repeated washing (data not shown).

Carbachol induced maximal changes in GDP or GTP binding within 5 sec at 30°, although carbachol-independent or background binding increased with time (Figs. 1 and 2). The time-dependent increase in background binding decreased the signal to noise ratio of carbachol-dependent changes and, therefore, 10–30-sec incubations were used when possible. The carbachol-dependent changes in GDP and GTP binding may actually decrease after 40 sec, but this possibility could not be reliably assessed by this method.

Carbachol-dependent and -independent [^{3}H]GTP binding was linear with sarcolemmal protein concentration from 2.5 to 10 μg/100 μl (Fig. 3). The percentage increase in binding ranged from 30 to 40% between lots of sarcolemmal membranes but was very constant within aliquots from the same preparation. An identical linear relationship between [^{3}H]GDP bound and sarcolemmal protein concentration was also found (data not shown). The intermembrane variation was found to correspond to differences in membrane purity, as determined from [^{3}H]QNB binding studies. In these membranes, the density of MRs ranged from 4 to 6 pmol/mg, which corresponds closely to the MR density in canine sarcolemma purified by a slightly different procedure (9).

The possibility of potential interactions between UDP, ADP, and ATP used to decrease background binding and carbachol-dependently bound GDP was investigated. In the presence of 100 nM [^{3}H]GDP, background binding was the same in the presence of 200–800 μM UDP (Table 1). Carbachol-dependent GDP binding was also unchanged in the presence of 100–400 μM UDP but was significantly depressed at 800 μM UDP. In these membranes (15), ≥400 μM UDP also slightly suppressed carbachol/GDP-dependent inhibition of AC activity, presumably by competition for binding to G_i. Carbachol-dependent changes in [^{3}H]GDP binding were identical in the presence of 100 μM each of ADP and ATP or 200 μM UDP. Therefore, GDP binding was not dependent on ATP and, furthermore, ADP and ATP did not significantly compete for carbachol-dependently bound GDP. From thin layer chromatography analysis (15), it was determined that the concentrations of [^{3}H]GDP (in the presence of UDP) or [^{3}H]GTP (in the presence of ATP) did not change more than 5% during the incubation periods used here.

Guanylnucleotide concentration dependence studies. Both carbachol-dependent and -independent GTP binding in-

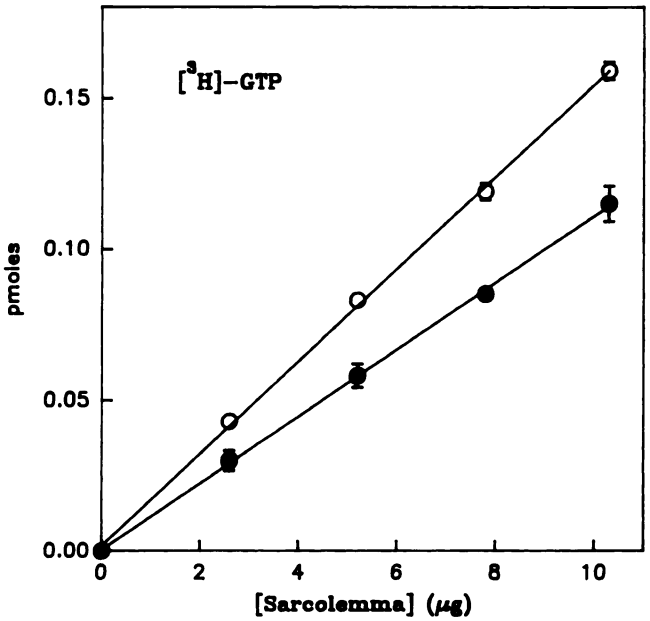

Fig. 3. Relationship between [^{3}H]GTP binding and sarcolemmal protein concentration in the absence (●) or presence (○) of 10 μM carbachol. The concentration of [^{3}H]GTP was 100 nM, and the incubation time was 10 sec at 30°. Results are representative of two separate experiments.

creased as the concentration of [^{3}H]GTP was varied from 10 to 500 nM (Fig. 4). Carbachol-dependent changes in GTP binding were undetectable at 10 nM GTP but increased gradually from 25 to 500 nM GTP. The reliability of carbachol-dependent binding measurements declined at ≥500 nM GTP, because of the high background binding. A plot of the carbachol-dependent GTP binding (Fig. 4, *inset*), obtained from subtraction of the curves, was biphasic. In the higher affinity range, GTP binding was half-maximal at approximately 50 nM, and the binding capacity was 5–6 pmol/mg. In the lower affinity binding range (i.e., 150–500 nM GTP), carbachol increased binding an additional 10 pmol/mg, with an apparent K_{app} of approximately 250–300 nM GTP. Essentially identical results were obtained using either [^{3}H]GTP or [γ-^{32}P]GTP as the binding ligands, thus indicating that bound GTP and not GDP was being measured. Because bound GTP can be hydrolyzed to GDP (see below), the K_{app} values may actually correspond to the K_m values for G_i or G_o GTPases (10).

Carbachol-dependent [^{3}H]GDP binding was characterized by a threshold of approximately 50 nM, and maximal binding was achieved at 200–250 nM GDP (Fig. 5). The plot of the carbachol-dependent changes in GDP binding was monophasic (Fig. 5, *inset*). A Scatchard plot of these data was linear, and an apparent K_{app} (or K_d) for GDP of 110 nM was obtained (data not shown). The capacity of carbachol-dependent GDP binding was 5–6 pmol/mg, which corresponds to the capacity of the high affinity GTP binding site described above. The possibility that carbachol increased GDP binding to a second lower affinity site could not be ruled out. For instance, the high background binding achieved at >500 nM GDP could potentially mask binding, and/or GDP bound to a lower affinity site could be removed during filtration and washing (see Experimental Procedures).

MR agonist specificity. Studies were undertaken to further characterize the carbachol-dependent GTP and GDP G protein binding sites in sarcolemma. At 10 or 100 μM concentrations, carbachol, oxotremorine, methacholine, acetylcholine, bethanechol, and arecoline produced maximal increases in [^{3}H]GDP or [^{3}H]GTP binding (Table 2). These agonists also produced maximal inhibitory changes in AC activity in the presence of GDP or GTP. Pilocarpine, a partial agonist, produced significantly smaller changes in GDP and GTP binding and AC activity. The parallel effects on binding and AC inhibition by these agonists suggest that MR agonists may stimulate binding of GTP and GDP to G_i or G_o associated with AC. G_p or G_z would be unlikely candidates for MR agonist-dependent GTP or GDP binding sites, because oxotremorine, an M2 agonist, does not stimulate polyphosphoinositide phospholipase C in heart (19, 20). Furthermore, the IC_{50} values for GTP or GDP to support carbachol-dependent inhibition of AC activity (15)

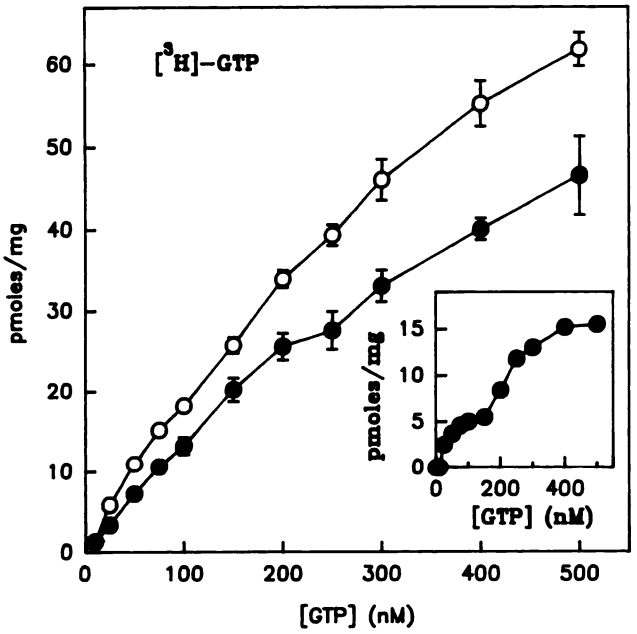

Fig. 4. Concentration-dependence curves of [^{3}H]GTP binding to cardiac sarcolemma in the absence (●) or presence (○) of 10 μM carbachol. The carbachol-dependent changes in [^{3}H]GTP binding were obtained by subtraction of these curves and are plotted in the *inset*. The incubation time was 10 sec at 30°. The results are representative of at least six experiments.

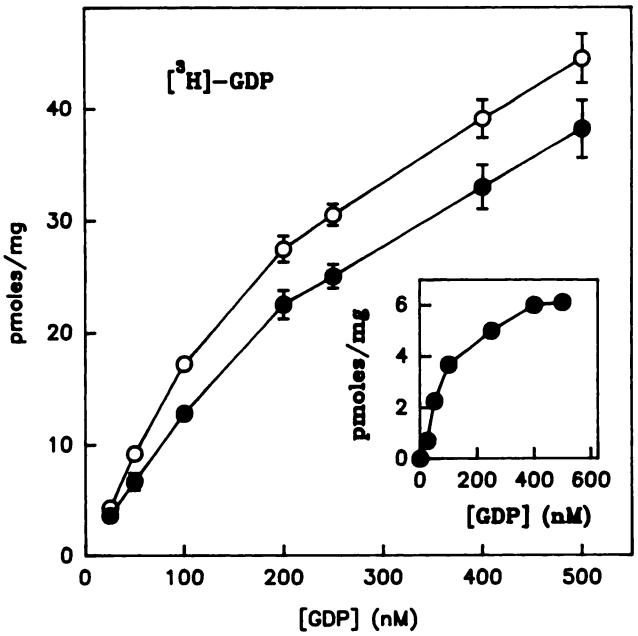

Fig. 5. Concentration-dependence curves of [^{3}H]GDP binding to cardiac sarcolemma in the absence (●) or presence (○) of 10 μM carbachol. The carbachol-dependent changes in [^{3}H]GDP binding were obtained by subtraction of these curves and are plotted in the *inset*. The incubation time was 10 sec at 30°. The results are representative of at least four separate experiments.

TABLE 2

Effects of MR agonists on GDP and GTP binding and AC activity in cardiac sarcolemma

Results are from three separate experiments and values represent the mean ± standard error of nine determinations. Other conditions were standard.

Agonist (100 μM)	Changes (+) in binding[a]		Changes (−) in AC activity[b]	
	[^{3}H]GDP (100 nM)	[^{3}H]GTP (100 nM)	GDP (500 nM)	GTP (500 nM)
	pmol/mg		*pmol/mg/min*	
Carbachol	3.6 ± 0.6	4.6 ± 0.5	308 ± 12	325 ± 16
Methacholine	3.5 ± 0.7	4.6 ± 0.4	325 ± 16	313 ± 11
Arecoline	3.8 ± 0.8	4.9 ± 0.6	317 ± 16	308 ± 9
Acetylcholine[c]	3.2 ± 0.8	4.7 ± 0.4	317 ± 18	297 ± 19
Oxotremorine	3.3 ± 0.5	4.7 ± 0.6	300 ± 12	305 ± 14
Bethanechol	3.0 ± 0.6	4.4 ± 0.5	267 ± 16	277 ± 13
Pilocarpine	2.1 ± 0.6	3.2 ± 0.5	217 ± 11	235 ± 12

[a] Incubation time was 10 sec at 30°.
[b] Incubation time was 5 min at 30°.
[c] Physostigmine (15 μM) was included with acetylcholine.

corresponded very closely to the K_{app} values for carbachol-induced binding of GTP (to the higher affinity site) and GDP (Figs. 4 and 5). For both processes, the IC_{50} or K_{app} values for GTP and GDP were approximately 40 and 100 nM, respectively. The function associated with the lower affinity GTP binding site is unknown, although it also is likely a subtype of G_i or G_o.

NEM treatment. It has been reported that NEM uncouples MR-G_i interactions by alkylating G_i or G_o (7, 8, 21). Preincubation of sarcolemma for 30 min at 5° with 150 μM NEM completely blocked carbachol-induced inhibition of AC activity in the presence of GTP, without affecting isoproterenol stimulatory effects on AC (Table 3). NEM also abolished carbachol-dependent increases in both GDP and GTP binding (Fig. 6). The effect of NEM was concentration dependent, and NEM was maximally effective at 175–300 μM. Preincubation of membranes with 175 μM NEM also blocked carbachol-dependent binding in the presence of 400 nM [^{3}H]GTP (data not shown). Therefore, NEM treatment blocks carbachol-stimulated binding of [^{3}H]GTP to high and low affinity sites (see Fig. 4). The NEM sensitivity of the carbachol-dependent guanylnucleotide binding sites further identifies these sites as G_i and possibly G_o. It was also apparent that NEM treatment resulted in a dose-dependent decrease in carbachol-independent binding and, therefore, sulfhydryl groups may modulate binding to these sites.

TABLE 3

Effects of NEM on carbachol- and isoproterenol-dependent changes in cardiac sarcolemma AC activity

Membranes were preincubated for 30 min at 5°, with or without 150 μM NEM. Incubations were for 5 min at 30° in the presence of 500 nM GTP.

Agonists	AC activity	
	Control	+NEM
	pmol/mg/min	
None	522 ± 23	182 ± 7.5
10 μM Carbachol	190 ± 25	176 ± 8.0
1 μM Isoproterenol	1184 ± 51	485 ± 6.6
10 μM Carbachol + 1 μM isoproterenol	482 ± 28	453 ± 14

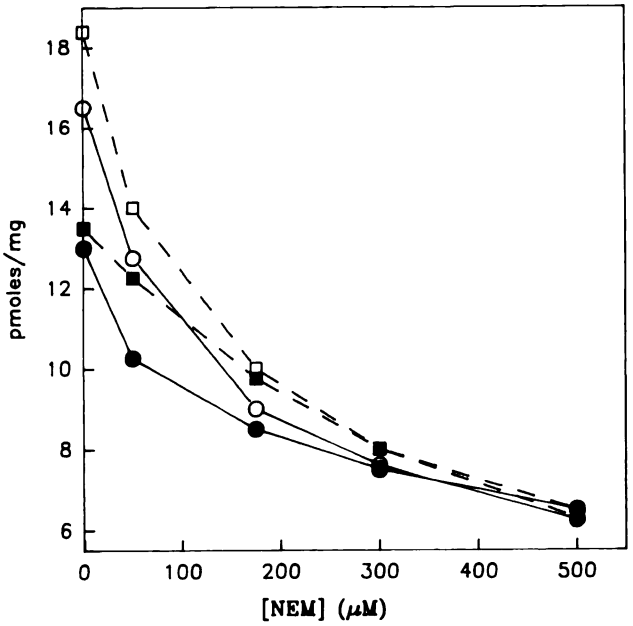

Fig. 6. Effect of pretreatment of cardiac sarcolemma with NEM on [^{3}H]GDP and [^{3}H]GTP binding. [^{3}H]GDP binding curves were obtained in the absence (●) or presence (○) of 10 μM carbachol. [^{3}H]GTP binding curves were obtained in the absence (■) or presence (□) of 10 μM carbachol. The incubation time for the binding assay was 10 sec, and the concentrations of GDP and GTP were 100 nM. Other details are indicated in Experimental Procedures. The data points represent the means of triplicate determinations, and similar results were obtained in three separate experiments. The standard deviations of the means were approximately 10%.

GDP and GTP exchange. In sarcolemma preincubated with carbachol and 200 nM [^{3}H]GDP, the addition of unlabeled GTP, GDP, or GDPβS caused an 80–90% loss of carbachol-dependently bound [^{3}H]GDP within 10 sec (Table 4). In membranes prelabeled with carbachol and 100 nM [^{3}H]GTP, postincubation with these guanylnucleotides also decreased carbachol-dependently bound [^{3}H]GTP, but the loss was of smaller magnitude than with [^{3}H]GDP. Presumably, the ability of guanylnucleotides to exchange more effectively for GDP versus GTP reflects the higher affinity of the binding site for GTP (Figs. 4 and 5). GDPβS, which is shown in the accompanying paper to inhibit AC activity in the presence of carbachol (15), also exchanged with [^{3}H]GDP. The inability of GMP, ATP, ADP, or UDP to exchange with labeled GTP or GDP provides further evidence that carbachol promotes GDP and GTP binding to selective G protein sites. These observations suggest that MR activation induces changes in G_i/G_o to increase the rate of exchange of GTP and GDP on G_i/G_o in these membranes. In the absence of GTP, MR agonists likely promote rapid GDP exchange on G_i without hydrolysis. In the presence of [^{3}H]GTP, MR activation would presumably increase GDP/[^{3}H]GTP exchange, and then bound [^{3}H]GTP would be hydrolyzed to [^{3}H]GDP by G_i-GTPase (see below); the cycle would be repeated as long as MR agonist was present (see below).

Studies were then undertaken to determine the fate of carbachol-dependently bound GDP or GTP after MRs had been inactivated with atropine. In sarcolemma preincubated for 10 sec in the presence of 5 μM carbachol and 100 nM [^{3}H]GTP or [^{3}H]GDP, the addition of 50 μM atropine did not cause a loss of carbachol-dependently bound [^{3}H]GDP or [^{3}H]GTP after a 30-sec postincubation (Fig. 7). Although it cannot be ascertained by this result, [^{3}H]GTP was likely hydrolyzed to [^{3}H]

TABLE 4

Ability of various nucleotides to exchange for [^{3}H]GDP and [^{3}H]GTP bound to carbachol-dependent sites in cardiac sarcolemma

Sarcolemma was preincubated for 10 sec at 30° with 100 nM [^{3}H]GTP or 200 nM [^{3}H]GDP, with or without 10 μM carbachol. The indicated additions were made after the 10-sec preincubation, and tubes were further postincubated for 10 sec before the binding assay was stopped by filtration. The data represent the mean ± standard deviation of quadruplicate determinations from a single experiment. Essentially identical results were obtained in three separate experiments.

Postincubation additions	Carbachol-dependent changes	
	[^{3}H]GTP	[^{3}H]GDP
	pmol/mg	
H_2O	4.6 ± 0.6	5.7 ± 0.9
250 nM GTP	2.7 ± 0.5	1.0 ± 0.8
500 nM GDP	4.3 ± 0.5	1.8 ± 1.1
1000 nM GDP	3.1 ± 0.7	1.0 ± 0.6
10 μM GDPβS	2.3 ± 0.8	0.5 ± 0.5
2 μM GMP	4.5 ± 0.3	5.6 ± 0.9
1 mM ATP	4.3 ± 0.4	5.2 ± 0.9
800 μM ADP	4.2 ± 0.4	5.0 ± 0.6
800 μM UDP	4.3 ± 0.6	4.6 ± 0.7

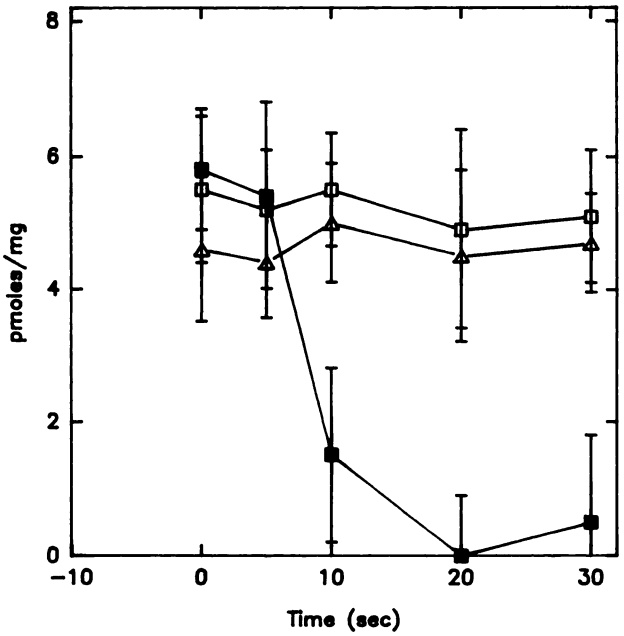

Fig. 7. Fate of carbachol-dependently bound GDP or GTP after MR inactivation by atropine. Sarcolemma was preincubated for 10 sec at 30° with 5 μM carbachol, in the presence of 200 nM [^{3}H]GDP (△), 100 nM [^{3}H]GTP (□), or 100 nM [γ-^{32}P]GTP (■). At time 0, 50 μM atropine was added and the tubes were incubated as indicated. Controls without carbachol were also run under identical conditions (not shown), to determine the levels of carbachol-dependently bound guanylnucleotides, by subtraction. Data points represent the means ± standard deviations of triplicate determinations.

GDP, which remained associated after MR inactivation. In membranes preincubated with [γ-^{32}P]GTP and carbachol, radiolabeling was completely lost within 10–20 sec after atropine addition (Fig. 7). This occurred as a consequence of hydrolysis of [γ-^{32}P]GTP by G_i/G_o-GTPase, leaving unlabeled GDP associated with G_i/G_o. In direct support of this proposal, carbachol was found to stimulate [γ-^{32}P]GTP hydrolysis, as measured by $^{32}P_i$ release, with a specific activity of 35–60 pmol/mg/min in these membranes. This rate of carbachol-dependent GTP-ase activity is sufficient to account for the loss of [γ-^{32}] GTP after MR inactivation. Furthermore, these observations suggest that GDP bound to G_i does not exchange with [γ-^{32}P] GTP in the absence of MR agonists; otherwise, steady state binding of [γ-^{32}P]GTP would have been maintained. To confirm this point, membranes were preincubated for 10 sec with 200 nM [^{3}H]GDP and carbachol, to label G_i/G_o. Then, membranes were subjected to two sequential 10-sec postincubations, the first with water or 50 μM atropine and the second with 500 nM GTP (Table 5). As a result, GTP completely replaced carbachol-dependently bound [^{3}H]GDP if water was added in the first postincubation, confirming that GTP can exchange with bound GDP in the presence of activated MRs. On the other hand, added GTP had no effect on carbachol-dependently bound GDP if atropine was added in the first postincubation to inactivate MRs. These results suggest that GDP dissociates from G_i/G_o at a much slower rate in the absence of MR agonists. Overall, these observations are consistent with other studies showing that receptor agonists increase GTP turnover on G_s or transducin (12–14). This probably occurs secondarily to an agonist-receptor-mediated decrease in the affinity of G_s, transducin, and G_i/G_o for GDP.

TABLE 5

Effect of atropine on GDP/GTP exchange in sarcolemma preincubated with carbachol and [^{3}H]GDP

Sarcolemma was preincubated for 10 sec with 200 nM [^{3}H]GDP, with or without 10 μM carbachol, in a final volume of 80 μl. At 10 sec, 10 μl of H_2O or atropine were added. At 20 sec, 10 μl of H_2O or GTP were added, and the reaction was stopped at 30 sec by filtration. Other conditions were standard.

Postincubation additions		Carbachol-dependently bound [^{3}H]GTP
At 10 sec	At 20 sec	
		pmol/mg
H_2O	H_2O	5.5 ± 1.1
H_2O	500 nM GTP	0.5 ± 0.8
Atropine	H_2O	5.2 ± 1.2
Atropine	500 nM GTP	4.7 ± 1.1

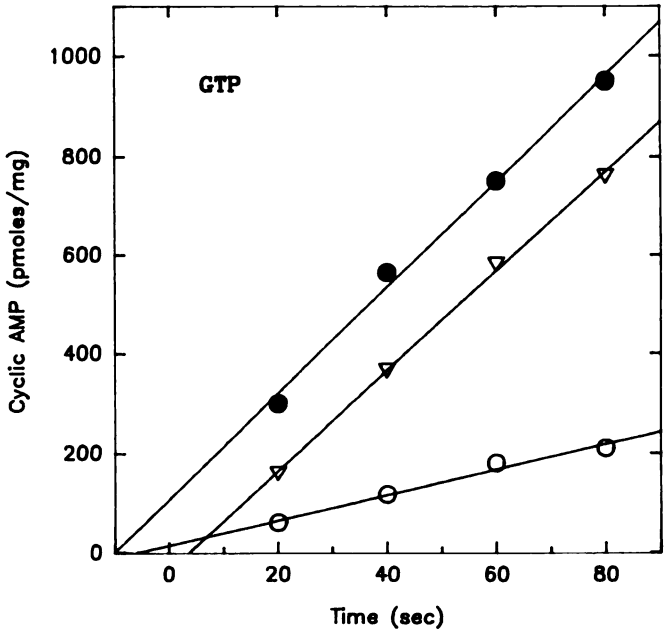

Fig. 8. Reversal of carbachol- and GTP-dependent inhibition of cardiac AC activity by atropine. Shown are time courses of cAMP production in the presence of 500 nM GTP (●) or 500 nM GDP and 5 μM carbachol (○) in which control buffer was added after a 10-sec preincubation and time course of cAMP production in the presence of 500 nM GTP and 5 μM carbachol (▽) in which 50 μM atropine was added after a 10-sec preincubation. Data points represent the means ± standard deviations of triplicate determinations.

Reversal of MR inhibitory coupling to AC by atropine. To further investigate the role of GTPase in terminating MR-G_i inhibition of AC activity, the rates at which atropine reversed carbachol/GDP or carbachol/GTP inhibition of AC activity were more closely examined. In the absence of atropine, cAMP production was linear from 0 to 80 sec in the presence of GTP or carbachol and GTP (Fig. 8), indicating that the maximal inhibition was induced within a few seconds. After the addition of atropine to membranes preincubated for 10 sec with carbachol and GTP (i.e., at time 0), the rate of cAMP production increased to the same rate as in the absence of carbachol (i.e., slopes were parallel between 20 and 80 sec). Extrapolation of the curve obtained in the presence of atropine and carbachol to the point of intersection with the curve obtained with carbachol reveals that atropine produces complete reversal of inhibition within 5–10 sec. Essentially similar findings were found in studies with carbachol and GDP (Fig. 9); however,

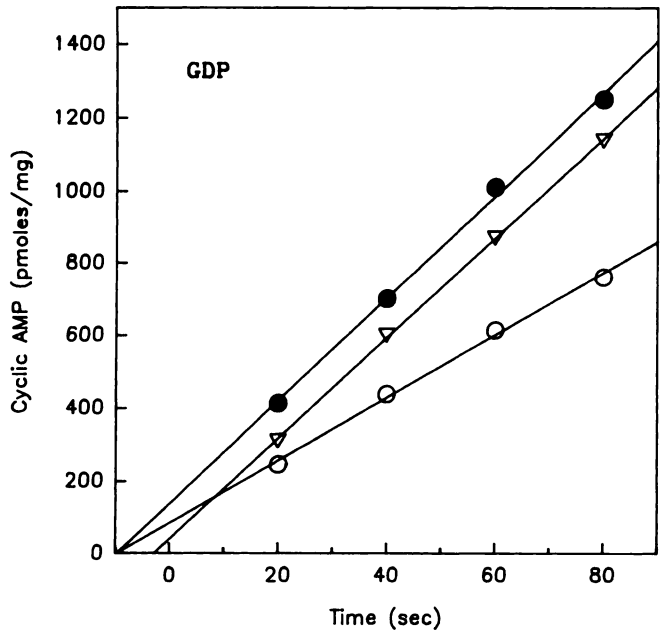

Fig. 9. Reversal of carbachol- and GDP-dependent inhibition of cardiac AC activity by atropine. Shown are time courses of cAMP production in the presence of 500 nM GDP (●) or 500 nM GDP and 5 μM carbachol (○) in which control buffer was added after a 10-sec preincubation period and time course of cAMP production in the presence of 500 nM GDP and 5 μM carbachol (▽) in which 50 μM atropine was added after a 10-sec preincubation. *Points* represent the means of triplicate determinations, and essentially identical results were obtained in two separate experiments.

carbachol-dependent inhibition with GDP was 40%, versus 65% with GTP.

Discussion

In the accompanying study (15), GDP or GDPβS were shown to support MR agonist-dependent inhibition of AC activity in cardiac sarcolemma. These findings suggested that GDP and GDPβS, like GTP, can activate G_i or G_o and that the mechanism of MR-G_i-AC coupling may be unique in these membranes. To more thoroughly investigate this possibility, the properties of MR-regulated GTP or GDP binding on G_i/G_o, as well as G_i/G_o-GTPase and AC activities, were compared here in purified cardiac sarcolemma.

In cardiac membranes, MRs can potentially interact with $G_{i_{1-3}}$ and G_o to inhibit AC activity or "open" potassium channels (1). Evidence reported by Liang and Galper (22) indicated that MRs were coupled to cardiac AC by G_i, although the subtype was not confirmed. MRs may also interact with putative G_p or G_z, which regulate phosphoinositide phospholipase C in heart membranes (16, 23). In cardiac sarcolemmal membranes, carbachol increased GTP and GDP binding to relatively high affinity sites (Figs. 4 and 5). The selectively and affinity of these sites for GDP and GTP further identified these binding sites as G proteins (Table 3). Several observations indicated that carbachol and other MR agonists increased GDP or GTP binding to G_i or G_o sites involved in MR inhibitory coupling to AC, as well as to other G_i or G_o sites (see below). For instance, oxotremorine, which does not stimulate phosphoinositide turnover in heart tissue (19, 20), was as effective as carbachol in promoting GDP or GTP binding to G protein sites on cardiac sarcolemma or in supporting MR-G_i-regulated inhibition of AC activity (Table 2). Furthermore, pilocarpine, a partial MR agonist (24), was only 60% as effective as carbachol in modulating GDP and GTP binding or AC inhibition. Other comparisons revealed that the apparent K_{app} values of carbachol-dependent GTP (high affinity site) and GDP binding (Figs. 4 and 5) and the IC_{50} values of GTP and GDP to support inhibition of AC activity (15) were approximately 50 and 100 nM, respectively. It has been reported previously that NEM pretreatment effectively uncouples MR agonist G_i or G_o coupling in cardiac sarcolemma (7, 21). Here, NEM pretreatment completely blocked carbachol-stimulated GDP and GTP binding (Fig. 6) and carbachol- and GTP-dependent inhibition of AC activity (Table 3). Hilf *et al.* (25) reported that pertussis toxin treatment of porcine atrial sarcolemma also attenuated carbachol-dependent [^{35}S]GTPγS binding to G_i. Carbachol-dependent GTP binding was reduced 50% in canine cardiac sarcolemma incubated for 30 min at 30° with 1.0 mM NAD and 20 μg/ml activated pertussis toxin; however, AC activity was almost completely denatured during this incubation period, thus ruling out comparative analysis of binding and AC inhibition.[1]

Carbachol increased GTP binding to sites with K_{app} values of 50 and 250 nM and with binding capacities of approximately 5 and 10 pmol/mg, respectively (Fig. 4). Because maximal inhibition of AC activity could be obtained in the presence of 100 nM GTP (15), the higher affinity site likely corresponds to a G_i associated with AC inhibition. Carbachol increased GDP binding with a K_{app} of 100 nM and a binding capacity of 5–6 pmol/mg, and this site is likely identical to the high affinity GTP binding site (Fig. 5). This was supported by the observation that GTP can exchange with [^{3}H]GDP bound to this carbachol-regulated site (Table 4). Because the MR density in these sarcolemma was 4–6 pmol/mg, the minimal stoichiometry of MR and G_i in coupling to AC would, therefore, be 1:1. This is consistent with the 1:1 stoichiometry found between α_2-adrenergic receptors and [^{3}H]Gpp(NH)p released from G_i in platelet membranes (26). This stoichiometry suggests that MR-G_i and possibly AC could be tightly coupled in these membranes and that amplification is not required for efficient MR-G_i inhibition of AC, at least at 100 nM GTP. Alternatively, only a fraction of the MRs occupied by carbachol may account for the coupling, and the actual stoichiometry could be higher. Furthermore, carbachol was shown here to increase GTP binding to high and low affinity sites, with a minimal stoichiometry of MR:G_i/G_o of 1:3. Stoichiometries of 1:3 have been found for MR-[^{35}S]GTPγS binding in porcine cardiac sarcolemma (25) and for MR and G_i reconstituted into liposomes (10). Therefore, a single MR can interact with at least three G proteins in sarcolemma membranes or in reconstituted preparations. At this time, the physiological function of the lower affinity GTP binding site reported here remains uncertain, and further studies will be required to determine whether it is a G_i subtype or G_o.

Carbachol stimulated maximal or steady state binding of GDP and GTP to G proteins in cardiac sarcolemmal membranes in <5 sec (Figs. 1 and 2). This rate of agonist-stimulated binding corresponds to the rates at which MR agonists stimulate physiological responses in heart cells (1), and thus MRs and G proteins remain efficiently coupled in these isolated

[1] E. Quist, unpublished observations.

membranes. In comparison, receptor agonist-stimulated rates of guanylnucleotide binding to G_i or G_s proteins in reconstituted systems are orders of magnitude slower (10, 12). Presumably, the increase in GDP or GTP binding in response to MR activation occurred as a result of "opening" sites on G_i or G_o, leading to dissociation of resident GDP with subsequent binding of radiolabeled ligand (10, 11). Tota *et al.* (10) showed that MR agonists increased the rate of dissociation of [^{3}H]GDP from purified G_i reconstituted in liposomes with MRs. Furthermore, in washed adipocytes membranes in which G_i had been prelabeled with [^{3}H]GTP, receptor agonists stimulated the release of [^{3}H]GDP (14). Presumably, the increased rate of dissociation reflects a decrease in the affinity of the guanylnucleotide binding site for GDP. In further agreement with studies on receptor regulation of G_s (12) or transducin (13), the GDP or GTP bound to G proteins in cardiac sarcolemma in the presence of MR agonists rapidly exchanged with unlabeled GDP or GTP in the medium (Table 3) and, therefore, a general feature of receptor activation may be to enhance exchange of GDP or GTP on coupled G proteins. In support of this concept, GDP exchange was found to occur in the presence but not in the absence of receptor agonists (Table 5) and, therefore, G_i likely has much higher affinity in the "closed" state. Although both GDP and GTP were shown here to bind to G_i/G_o in response to MR activation, the 2-fold higher affinity of MR-activated G_i/G_o for GTP (Figs. 4 and 5) and the relatively higher concentration of GTP normally found in cells would greatly favor GTP binding in cardiac sarcolemma. It was recently proposed that MR agonists similarly increase guanine nucleotide exchange on G_o in a reconstituted brain MR/G_o preparation, by increasing the rate of dissociation and altering the relative affinities of GTP and GDP so that GTP binding is favored (27).

The studies reported here demonstrate that MR agonists maintain steady state binding of GTP on G_i/G_o in cardiac sarcolemmal membranes by combined GTPase hydrolytic and GDP/GTP exchange reactions. In agreement with Fleming and Watanabe (9), carbachol-stimulated GTPase activity was approximately 35–60 pmol/mg/min in these membranes. This evidence alone indicated that MR activation promotes rapid and continuous turnover of GTP on G_i/G_o, because GTPase *per se* is not modulated by receptor interactions. In strong support, it could be shown directly that [γ-^{32}P]GTP bound to G_i/G_o was rapidly hydrolyzed to GDP and $^{32}P_i$ within 10 sec after MR inactivation by atropine (Fig. 7). Therefore, in the presence of an MR agonist, GTP is sequentially bound to G_i/G_o, hydrolyzed to GDP, and exchanged with GTP in the medium. It is generally assumed that hydrolysis of GTP on G_i or other receptor-regulated G proteins, such as G_s or transducin, is ultimately responsible for turning off or reversing the response after the removal of receptor agonists (8, 11, 13, 28). This hypothesis was further addressed here in cardiac sarcolemma. In these membranes, hydrolysis of G_i/G_o-GTP bound in the presence of an MR agonist and reversal of AC inhibition due to carbachol and GTP were both complete within 10 sec after the addition of atropine (Figs. 7 and 8). Studies here also confirmed that [^{3}H]GDP remained bound to G_i/G_o after MR inactivation (Fig. 7), in agreement with chemical measurements (28). These studies support the proposal that GTP hydrolysis is related to reversal. However, reversal of AC inhibition due to carbachol and GDP (Fig. 9) (or GDPβS) (15) cannot be explained by a GTPase-related mechanism. Because GDP dissociation from G_i/G_o was also ruled out as a possibility (see above) (Table 5), these observations imply that a step distal to GTP hydrolysis may be needed to terminate GDP-G_i inhibitory effects in this system. An obvious possibility would be that MR-activated G_i/G_o reverts back to the form with high affinity for GDP (i.e., normally found in the resting state), as a consequence of agonist removal. This mechanism would further suggest that agonist-MRs are coupling tightly to G_i/G_o during AC inhibition to maintain G_i/G_o in the lower guanylnucleotide affinity or actively exchanging state. In support of this concept, Matesic *et al.* (29) recently reported that detergent-solubilized MRs and G_i remained associated in the presence of receptor agonists and GTP. Such a tightly coupled MR-G_i-AC mechanism would, therefore, compensate for the agonist effects of GDP or GDPβS on G_i in these membranes. In other words, GDP (or GDPβS) agonist effects on G_i would be terminated after MR inactivation. Cassel *et al.* (28) reported a similar phenomenon for G_s coupling to β-adrenergic receptors in parotid membranes. In these membranes, G_s is partially activated by GDPβS and, therefore, added GDPβS could not completely reverse AC stimulation due to isoproterenol and GTP, unless a β-adrenergic receptor antagonist was included. In this same study (28), GDPβS alone effectively reversed inhibition in membrane systems in which G_s was inhibited by GDPβS. Together, these observations indicate that G protein-mediated hydrolysis of GTP to GDP, as well as G protein-receptor uncoupling, may be required to turn off the effects of G proteins on AC in tightly coupled systems.

It is unknown whether the properties of G_i/G_o described here are unique to cardiac sarcolemma and whether the mechanisms described represent a functional adaptation for this tissue. In this respect, MR-G_i may be tightly coupled in cardiac membranes to facilitate control over AC activity. The results of this study also imply that the mechanisms of receptor-G protein coupling may be more diverse than is generally considered. GTPase activity appears to serve roles in supporting rapid GTP turnover on G_i/G_o during MR activation, as well as participating in turning off the inhibitory response of GTP and carbachol. To completely reverse inhibitory effects of carbachol on AC in the presence of GTP or GDPβS and GDP, it is proposed that a MR-G_i uncoupling may also be required in cardiac sarcolemma.

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