0026-895X/92/010177-08$03.00/0

MOLECULAR PHARMACOLOGY, 41:177-184

Guanylnucleotide Specificity for Muscarinic Receptor Inhibitory Coupling to Cardiac Adenylate Cyclase

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Received February 21, 1991; Accepted August 19, 1991

SUMMARY

The guanylnucleotide specificity of muscarinic acetylcholine receptor (MR) inhibitory coupling to cardiac adenylate cyclase (AC) was investigated under low $MgCl_2$ (i.e., 0.5 mM) conditions. In purified cardiac sarcolemma, carbachol maximally inhibited AC activity 60% in the presence of GTP. Carbachol-dependent inhibition in the presence of guanosine 5′-*O*-(3-thiotriphosphate (GTPγS) or guanylylimidodiphosphate [Gpp(NH)p] was of lesser magnitude (i.e., 30%) and was evident only during short incubation periods. Of greater interest, carbachol maximally inhibited AC activity in the presence of GDP and guanosine 5′-*O*-(2-thiodiphosphate (GDPβS) by 35 and 60%, respectively. Control studies ruled out transphosphorylation of GDP and GDPβS by nucleoside diphosphate kinase or guanylnucleoside triphosphate contamination as reasons for the inhibitory effects of GDP and GDPβS. Furthermore, isoproterenol stimulated AC in the presence of GTP, GTPγS, and Gpp(NH)p but not in the presence of GDP or GDPβS. Therefore, GDP and GDPβS may serve as agonists on MR-activated G_i but not on β-adrenergic receptor-activated G_s in these membranes. Time course studies revealed that carbachol-dependent inhibition of AC in the presence of either GTP or GDP occurred without a detectable lag period, and this inhibition was rapidly reversed by atropine. In contrast, a 1–2-min lag time was required for carbachol- and GDPβS-dependent inhibition of AC to occur, and inhibition, once developed, was only partially and slowly reversed by atropine. Preincubation of sarcolemma with carbachol and GDPβS, in the absence of ATP or under nonphosphorylating conditions, eliminated the lag time for inhibition of AC activity. Although it is unlikely that GDP and GDPβS have physiological relevance of MR-G_i-AC coupling, these studies provide unique insights into this coupling mechanism in cardiac membranes.

Cardiac AC is coupled to βARs and MRs by G_s and G_i, respectively (1, 2). Although the mechanism for βAR-G_s-AC stimulatory coupling is well understood, relatively less is known about the MR-G_i-AC inhibitory mechanism. After agonist stimulation, activated βARs interact with G_s to accelerate the exchange of bound GDP for GTP in the medium (3, 4). GTP-G_s has been hypothesized to dissociate from βAR, and GTP-G_s may further dissociate into GTP-$G_{s\alpha}$ and β/γ subunits (2). Interaction of GTP-$G_{s\alpha}$ with AC increases catalytic activity, and activation is terminated by hydrolysis of GTP to GDP by the GTPase activity of $G_{s\alpha}$ (3). GDP-$G_{s\alpha}$ then reassociates with the β/γ subunits, and the cycle persists until agonist is removed or receptor desensitization occurs. The exchange of G_s-GDP for the nonhydrolyzable analogues GTPγS and Gpp(NH)p is also accelerated in response to βAR activation. However, GTPγS and Gpp(NH)p are not hydrolyzed by GTPase, and AC remains persistently activated. In cardiac membranes, βAR agonists stimulate AC activity by 2–3-fold in the presence of GTP, GTPγS, and Gpp(NH)p (5, 6).

The mechanism for MR-G_i inhibition of AC is thought to be analogous in many respects to the βAR-G_s coupling mechanism (1, 2). In cardiac sarcolemma and other membranes, MR agonists inhibit AC activity via a heterotrimeric G_i protein, which is uncoupled from MRs by pertussis toxin (1, 2, 7). Activation of MRs stimulates GDP/GTP exchange on G_i (8), and GTP-G_i then inhibits AC activity possibly by two potential mechanisms. In *cyc*$^-$ S49 lymphoma cells, which are devoid of G_s, somatostatin can inhibit AC by a mechanism consistent with direct inhibition of AC by G_i-GTP or $G_{i\alpha}$ (1). Evidence that the β/γ subunits dissociated from activated G_i indirectly inhibit GTP-G_s stimulatory effects on AC has also been presented (2). Both direct and indirect inhibitory effects of G_i on cardiac AC have been proposed (9).

The guanylnucleotide requirements for MR-G_i-AC coupling versus βAR-G_s-AC coupling in cardiac membranes have been difficult to evaluate. MR agonists have been reported to inhibit cardiac AC activity in the presence of GTP but not in the

This research was supported in parts by grants from the Texas Higher Education Coordination Board, Advanced Technology Program, and from the National Institutes of Health (HL 35433).

ABBREVIATIONS: AC, adenylate cyclase; βAR, β-adrenergic receptor; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); Gpp(NH)p, guanylylimidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); HPLC, high performance liquid chromatography; MR, muscarinic receptor; TLC, thin layer chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; G protein, guanine nucleotide-binding protein.

presence of GTPγS and Gpp(NH)p (5, 6). Because MR agonists also stimulate G_i-GTPase activity in cardiac sarcolemma (10, 11), MR stimulation of GTP hydrolysis was proposed to be functionally correlated with inhibitory coupling to AC under physiologically relevant conditions (9). Data against this proposal were provided by Smith and Harden (12), who found that oxotremorine could inhibit cardiac AC activity in the presence of GTPγS under low $MgCl_2$ conditions. GTPγS has also been shown to antagonize receptor-G_i-AC inhibition in a number of other cellular membranes (1), which may account for the variation in effects reported.

This study was undertaken to re-evaluate the guanylnucleotide requirements of MR agonist-mediated inhibition of AC activity in purified cardiac sarcolemma. During this investigation, it was unexpectedly discovered that carbachol inhibited AC activity in the presence of GDP or GDPβS, although isoproterenol had no effect on AC activity in their presence. A preliminary account of these observations has been published (13).

Experimental Procedures

Materials. cAMP, ATP (disodium salt), alamethicin, atropine sulfate, carbachol chloride, GTP (lithium salt), GDP (disodium salt), GDPβS (trilithium salt), GTPγS (tetralithium salt), Gpp(NH)p (tetralithium salt), *l*-isoproterenol, *dl*-propranolol, and UDP (sodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO). GDPβS was also obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). [α-^{32}P]ATP (25 Ci/mmol) was from ICN (Irvine, CA), and [^{3}H]cAMP (30 Ci/mmol) was from New England Nuclear (Wilmington, DE). [^{3}H]GDP (10–20 Ci/mmol) and [^{3}H]GTP (10–20 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). PEI cellulose F TLC plates with fluorescent indicator were obtained from Merck.

Preparation of sarcolemmal membranes. Sarcolemmal membranes of high purity were prepared from canine ventricles by our recently described procedure (14). Sarcolemma was suspended in 10 mM HEPES, pH 7.4, 2.0 mM $MgCl_2$, 0.5 mM dithiothreitol, frozen with liquid nitrogen, and stored at −80° for up to 2 months without loss of AC activity.

Measurement of AC activity. AC activity in sarcolemmal membranes was assayed in a final volume of 100 μl in medium A, containing 0.5 mM $MgCl_2$, 50 mM HEPES, pH 7.4, 0.3 mM dithioerythritol, 100 mM NaCl, 0.3 mM KCl, 0.02 mM cAMP, 2.5 μg of alamethicin, 50–100 μM [α-^{32}P]ATP (0.5 μCi), 1.0 mM NaN_3, and 25 μM ouabain. Unless otherwise indicated, reactions were started by the addition of 2–2.5 μg of sarcolemmal membrane protein, and tubes were incubated for 1–5 min at 30°. UDP or ADP was included when required to prevent conversion of GDP to GTP by nucleoside diphosphate kinase activity (see below). Various drugs and nucleotides were added as indicated in Results. AC activity was linear for 5 min at 30° under these conditions. A limited number of studies were performed in medium B, which was the same as medium A except that an ATP-regenerating system composed of 5 mM creatine phosphate and 2.5 units of creatine phosphokinase was included. Reactions were stopped with 0.1 ml of 40 mM ATP, 2% sodium dodecyl sulfate, and 0.01 μCi of [^{3}H]cAMP, and 0.8 ml of water was added to each tube at room temperature. Fractions were decanted into 1-ml Dowex columns, and cAMP was separated by method C, as described by Salomon *et al.* (15). Four milliliters of the eluent from the alumina columns were counted in 8.0 ml of Ecolume. Recovery was approximately 60%. Protein was determined by the method of Bradford (16). Each assay point was performed in duplicate or triplicate, and the standard deviation of the mean of triplicate determinations was usually <5%. Basal AC activity ranged from 300 to 500 pmol/mg/min in different membrane preparations and, therefore, data from different experiments were normalized to 400 pmol/mg/min, to facilitate comparison of agonist and guanylnucleotide effects.

Nucleoside diphosphate kinase. Nucleoside diphosphate kinase activity was measured in medium A (see above), in a final volume of 50 μl. Various concentrations of [^{3}H]GDP (0.5 μCi) were incubated for 1–5 min at 30°, in the presence and absence of UDP or ADP. Reactions were stopped with 0.01 ml of 5% sodium dodecyl sulfate. Aliquots (2 μl) were spotted on PEI cellulose F TLC plates (0.25-mm thick), with 1 μl of 2 mM GMP, GTP, and GDP as carriers. GTP, GDP, and GMP standards were examined separately. Plates were developed for 10 cm at room temperature in 2 N acetic acid with 0.5 M LiCl or 0.75 M KH_2PO_4, at pH 4.3. Development in either solution completely separated GMP, GDP, and GTP. Spots were detected with UV light, scraped, and counted in 8 ml of Ecolume. The metabolism of [^{3}H]GTP was examined under the same conditions.

Nucleotides were routinely screened for relative purity by the TLC procedure used above. The purity of nucleotides was occasionally analyzed by reverse phase HPLC (Waters μ-Bondapak C18 column, eluted with 100 mM KH_2PO_4, pH 6.0, and 3% methanol) and strong anion exchange HPLC (Whatman Partisil 10 column, eluted with 25–100 mM KH_2PO_4, pH 5.5). The results from HPLC analyses were in agreement with the TLC methods. All of the chromatographic procedures described above effectively separated GTP, GDP, GDPβS, UDP, and GMP.

Results

Nucleoside diphosphate kinase activity. In initial studies, the metabolism of GDP and GTP under AC assay conditions was examined. In the cardiac sarcolemma used here, [^{3}H]GDP was found to be rapidly converted to [^{3}H]GTP (i.e., 75% within 5 min at 30°) in the presence of 100 μM ATP in medium A, which does not contain an ATP-regenerating system (Fig. 1). This indicated that these membranes possess nucleoside diphosphate kinase activity, which donates a phosphate to GDP from ATP (17). In agreement with Jakobs and Wieland (18), GDP could be protected against phosphorylation by inclusion of UDP in the medium as a substrate competitor for nucleoside diphosphate kinase (Fig. 1). In the presence of 400 μM UDP, conversion of GDP to GTP was ≤5% after a 5-min incubation. Therefore, all further studies with GDP and AC activity were done in the presence of 400 μM UDP in medium A. It was also

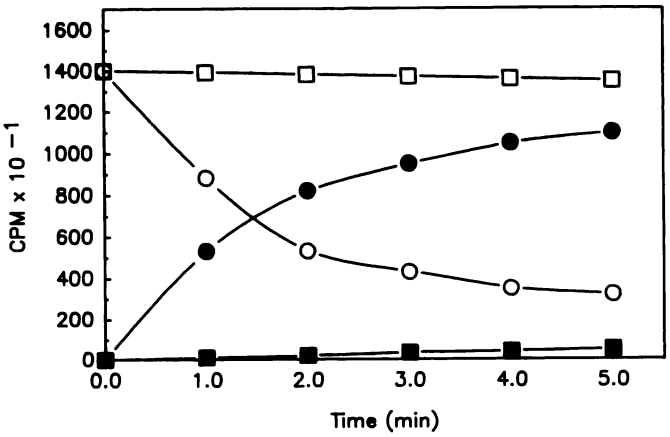

Fig. 1. Nucleoside diphosphate kinase activity in cardiac sarcolemmal membranes. Membranes were incubated at 30° with 250 nM [^{3}H]GDP. Shown are [^{3}H]GDP in the absence (○) and presence (□) of 400 μM UDP and [^{3}H]GTP synthesized from [^{3}H]GDP in the absence (●) and presence (■) of 400 μM UDP. Results are the means of triplicate determinations and are representative of five separate experiments.

determined that [^{3}H]GTP did not change by more than 5% after a 5-min incubation under these conditions.

Studies with guanylnucleoside triphosphates. The guanylnucleoside triphosphate requirements for carbachol inhibitory and isoproterenol stimulatory effects on sarcolemmal AC were first evaluated. In the absence of guanylnucleotides, carbachol or isoproterenol had no effect on AC activity (Table 1), suggesting that the sarcolemma was not significantly contaminated with endogenous guanylnucleotides. GTP, GTPγS, or Gpp(NH)p stimulated AC activity in the absence of receptor agonists, and activity was further stimulated 1.6–2.5-fold in the presence of isoproterenol. In this sarcolemma, carbachol inhibited AC activity by 60%, 31%, and 26% in the presence of GTP, GTPγS, and Gpp(NH)p, respectively (Table 1). In the presence of GTP, carbachol inhibited AC activity by 60% in the presence and absence of isoproterenol. These studies were performed in the presence of 0.5 mM $MgCl_2$, which accounts for the relatively high level of carbachol/GTP induced inhibition (12). The inhibitory effects of carbachol were antagonized by 10 μM atropine, an MR antagonist, and the stimulatory effects of isoproterenol were antagonized by 5 μM propranolol, a βAR antagonist, in the presence of GTP, Gpp(NH)p, or GTPγS (data not shown).

The concentration dependencies of GTP, GTPγS, and Gpp(NH)p both to stimulate AC activity and to support MR-G_i inhibitory coupling were further examined. GTP alone stimulated AC activity by 25–50% at ≥25 nM GTP (Fig. 2), whereas higher concentrations of GTP (i.e., 100–200 nM) were required for the expression of carbachol-dependent inhibition. The changes in AC activity dependent on carbachol and GTP were obtained by subtraction of the curves in Fig. 2 and are replotted in Fig. 3. The apparent IC_{50} for GTP obtained was approximately 40 nM.

In the absence of agonists, GTPγS or Gpp(NH)p stimulated AC activity with relatively high affinities. Half-maximal activation occurred in the presence of 3 nM GTPγS (Fig. 4) and 50–75 nM Gpp(NH)p (data not shown). Carbachol-dependent inhibition of AC activity was approximately 30% in the presence of 10 nM GTPγS, and a plot of the carbachol-dependent changes showed that the IC_{50} for GTPγS was approximately 3 nM (Fig. 3). The IC_{50} of Gpp(NH)p to support carbachol-dependent inhibition of AC activity was approximately 75–100 nM (data not shown). Collectively, these observations are supportive of previous studies in cardiac membranes (5, 6, 12) and serve as important controls to show that the βAR-G_s-AC and MR-G_i-AC regulatory mechanisms function as expected in the sarcolemma used here.

Studies with guanylnucleoside diphosphates. Studies

TABLE 1

Effects of carbachol and isoproterenol on cardiac sarcolemmal AC activity in the presence of guanylnucleotides

Reactions were started by the addition of sarcolemma, and incubations were for 5 min at 30° in medium A containing 400 μM UDP.

Guanylnucleotide	AC activity		
	Control	+10 μM Carbachol	+10 μM Isoproterenol
	pmol/mg/min		
None	400 ± 21	403 ± 11	410 ± 8
GTP (1 μM)	552 ± 13	220 ± 15	1375 ± 42
GTPγS (100 nM)	1050 ± 11	719 ± 18	1680 ± 58
Gpp(NH)p (500 nM)	776 ± 35	574 ± 40	1862 ± 56
GDP (500 nM)	835 ± 26	517 ± 46	926 ± 32
GDPβS (10 μM)	880 ± 38	528 ± 32	885 ± 17

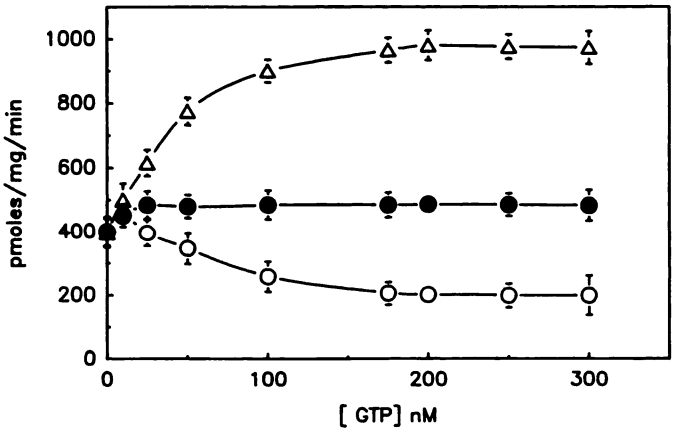

Fig. 2. GTP concentration dependence of AC activity with no additions (●), 10 μM isoproterenol (△), or 10 μM carbachol (○). Incubations were at 30° in medium A containing 400 μM UDP. Results are representative of five to eight separate experiments, and each *point* is the mean ± standard deviation of triplicate determinations.

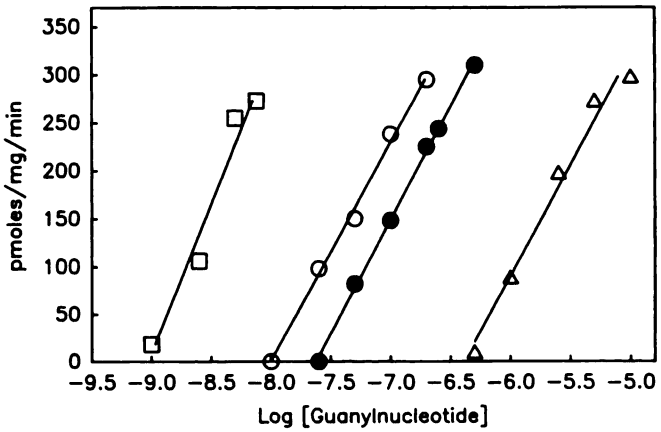

Fig. 3. Guanylate nucleotide concentration dependence of carbachol-mediated inhibition of AC activity with GTP (○), GTPγS (□), GDP (●), or GDPβS (△). Data points were obtained from Figs. 2, 4, 5, and 7 and represent the difference in AC activity measured in the presence and absence of carbachol.

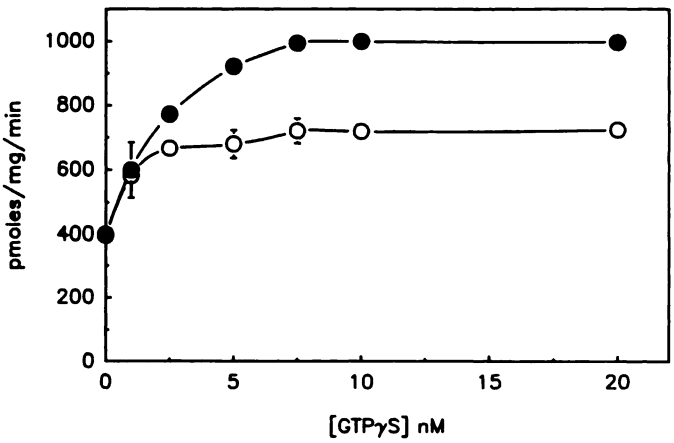

Fig. 4. GTPγS concentration dependence of AC activity in the absence (●) or presence (○) of 10 μM carbachol. Incubations were for 5 min at 30° in medium A with 400 μM UDP. Results are representative of two separate experiments, and each *point* represents the mean ± standard deviation of triplicate determinations.

with guanylnucleoside diphosphates surprisingly revealed that carbachol could inhibit AC activity by 40–60% in the presence of GDPβS (Fig. 5). Of further interest, GDPβS alone stimulated AC activity by 2–3-fold. In the absence of carbachol, AC activity was maximally stimulated at approximately 200 nM GDPβS. An Eadie-Hofstee replot of the stimulation data from Fig. 5 allowed estimation of an EC_{50} for GDPβS of 40 nM (Fig. 6). By comparison, 20–40-fold higher concentrations of GDPβS were required to support carbachol-dependent inhibition of AC activity. Optimal inhibition by carbachol was obtained with 5–10 μM GDPβS (Fig. 5), and the apparent IC_{50} of GDPβS estimated from a replot of data from Fig. 5 was 2 μM (Fig. 3). Further studies showed that carbachol and GDPβS could maximally inhibit AC activity up to 60% at longer incubation times (see Fig. 10). In contrast, isoproterenol had no effect on AC activity in the presence of 10 μM GDPβS (Table 1; Fig. 5). HPLC and TLC analyses revealed that GDPβS was not contaminated with guanylnucleoside triphosphates, although GMP was present at ≤5%. Furthermore, carbachol had no effect on AC activity in the presence of 10 μM GMP (data not shown).

Both GDPβS stimulation and carbachol/GDPβS-dependent inhibition of AC activity were observed in three separate lots of GDPβS. AC activity was stimulated 2–3-fold by GDPβS from all three lots, with an EC_{50} of approximately 40 nM.

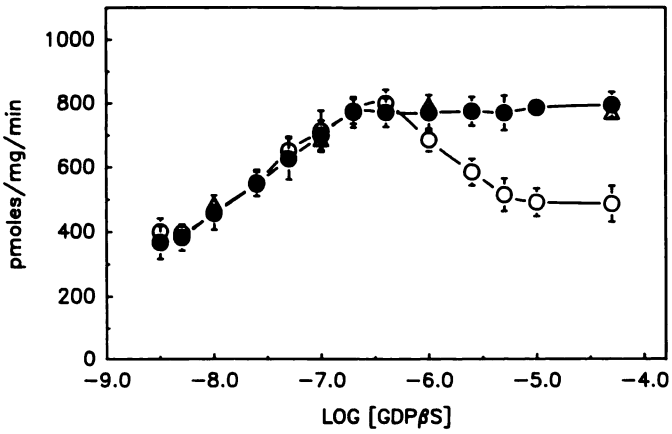

Fig. 5. GDPβS concentration dependence of AC activity with no additions (●), 10 μM carbachol (○), or 10 μM isoproterenol (△). Results are representative of at least five separate experiments, and each *point* is the mean ± standard deviation of triplicate determinations.

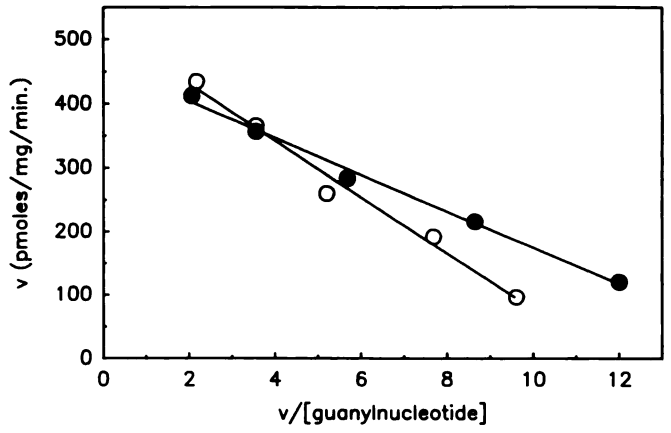

Fig. 6. Activation of AC activity by GDP (●) and GDPβS (○). Results, plotted according to the Eadie-Hofstee method, are from Figs. 5 and 7 and represent changes induced by these nucleotides.

However, the maximal inhibition achieved by 2 μM carbachol and 10 μM GDPβS was significantly different (i.e., 16, 28, and 40%) between lots. Because these differences could not be accounted for by chemical impurities, it is suggested that the variation in carbachol-dependent inhibition may be attributable to differences in the ratios of diastereoisomers in GDPβS. GDPβS and other thio-substituted nucleotides form diastereoisomers during chemical synthesis, and usually only one isomer is active (19, 20). These observations would imply that only one isomer of GDPβS may support inhibition, whereas both isomers can equally stimulate AC activity.

Studies to determine whether GDP *per se* could support MR inhibitory coupling to AC were experimentally more difficult because of sarcolemmal nucleoside diphosphate kinase activity (Fig. 1). However, by using short incubation times (i.e., 5 min) and low membrane concentrations (1.5–2.5 μg/100 μl) and by including 400 μM UDP in the assay medium, conversion of GDP to GTP was limited to <5%. Under these conditions, GDP alone stimulated AC activity approximately 2-fold (Fig. 7), with an EC_{50} of 25 nM (Fig. 6). In the presence of 250 nM GDP, carbachol maximally inhibited AC activity by 35–40% (Fig. 7), and the IC_{50} for GDP was approximately 90 nM (Figs. 3 and 7). In contrast, AC activity was stimulated <10% by isoproterenol and 25–300 nM GDP (Fig. 7), compared with 100–200% in the presence of GTP (Fig. 2). HPLC and TLC analyses showed that GDP preparations occasionally contained traces of GMP (≤5%) but never GTP.

Other control studies (data not shown) were performed to investigate potential competitive interactions between UDP and GDP for G_i. The IC_{50} values for GDP-dependent inhibition of AC activity in the presence of carbachol were identical in the presence of 100, 200, and 400 μM UDP. In studies using 100 or 200 μM UDP, it was necessary to reduce the incubation time to 2.5 min and the sarcolemmal protein concentration to 1.5 μg/100 μl to prevent significant conversion of GDP to GTP. Concentrations of UDP less than 100 μM were ineffective, whereas concentrations of UDP greater than 400 μM slightly suppressed the effects of GDP and GDPβS on AC activity, presumably by competition for binding to G_i. Conversion of UDP to UTP could not account for the carbachol/GDP-

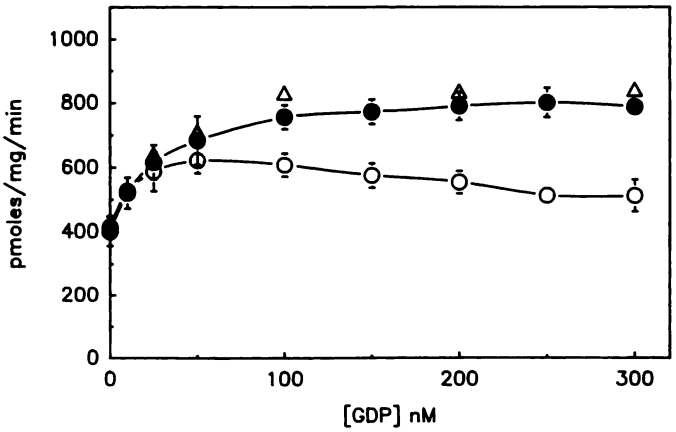

Fig. 7. GDP concentration dependence of AC activity with no additions (●), 10 μM carbachol (○), or 10 μM isoproterenol (△). *Points* represent the means and standard deviations of triplicate determinations and are representative of at least seven separate experiments. Incubations were in medium A containing 400 μM UDP, for 5 min at 30°.

dependent inhibition, because carbachol had no effect on AC activity in the presence of 200 μM UTP.

Time course and atropine reversal studies. To further examine the mechanism of MR inhibitory coupling to cardiac AC, time course and atropine reversal studies were performed. In the presence of 200 nM GTP or GTP and 10 μM carbachol, cAMP production was linear for 5 min at 30° (Fig. 8). This finding is in agreement with others (6, 9, 12) that MR agonist- and GTP-dependent inhibition of cAMP production occurs without a measurable lag period. In AC assay medium B (i.e., containing an ATP-regenerating system), linearity of cAMP production was maintained for up to 20 min at 30° in the presence of GTP, with or without carbachol (data not shown). In membranes preincubated for 1 min with GTP and carbachol, after addition of atropine (to displace carbachol from MRs) the rate of cAMP production was increased to the same rate obtained in membranes not preincubated with carbachol (Fig. 8). The slopes of the curves in membranes incubated with or without carbachol were parallel from 1 to 5 min after atropine addition, indicating that atropine caused a complete reversal of carbachol- and GTP-dependent inhibition in <1 min. In control studies, atropine had no effect on AC activity in the absence of carbachol. cAMP production was also linear for 5 min in the presence of GDP or carbachol and GDP, indicating that the inhibition was rapid, as evidenced by the lack of a detectable lag period (Fig. 9). Atropine also caused reversal of inhibition due to carbachol and GDP within ≤60 sec in these membranes. The observation that inhibition did not increase with time in the presence of carbachol and GDP further argues against phosphorylation to GTP as a mechanism for the GDP inhibitory effects.

In contrast to the aforementioned studies with GTP or GDP, carbachol- and GDPβS-dependent inhibition of cAMP production was delayed by 1–2 min (Fig. 10). Afterwards, cAMP production was linear from 2 to 15 min. In membranes preincubated for 2.5 min with carbachol and GDPβS, to allow for maximal inhibition, atropine produced no change in the rate of cAMP production after a 2.5-min postincubation (Fig. 10),

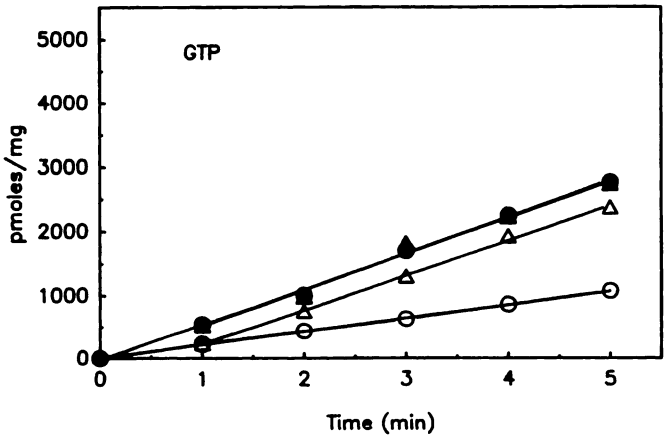

Fig. 8. Time courses of inhibition and atropine reversal of cardiac AC activity by GTP and carbachol. Sarcolemma was incubated in the presence of 250 nM GTP (●) or 250 nM GTP and 10 μM carbachol (○). For atropine reversal experiments, sarcolemma was preincubated for 1 min with 250 nM GTP (▲) or 250 nM GTP and 10 μM carbachol (△) and further incubated with 50 μM atropine. Results are representative of three separate experiments, and data points are the means of triplicate determinations. Incubations were at 30° in medium A, in the presence of 400 μM UDP.

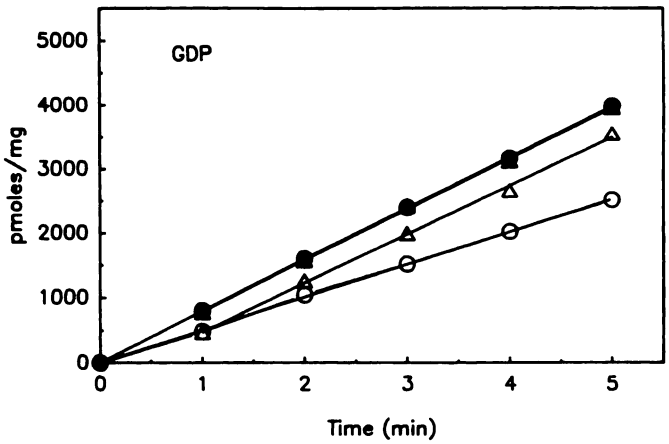

Fig. 9. Time courses of inhibition and atropine reversal of cardiac AC activity by GDP and carbachol. Sarcolemma was incubated in the presence of 250 nM GDP (●) or 250 nM GDP and 10 μM carbachol (○). For atropine reversal experiments, sarcolemma was preincubated for 1 min with 250 nM GDP (▲) or 250 nM GDP and 10 μM carbachol (△) and further incubated with 50 μM atropine. Results are representative of three similar experiments, and data points are the means of triplicate determinations. Incubations were at 30° in medium A in the presence of 400 μM UDP.

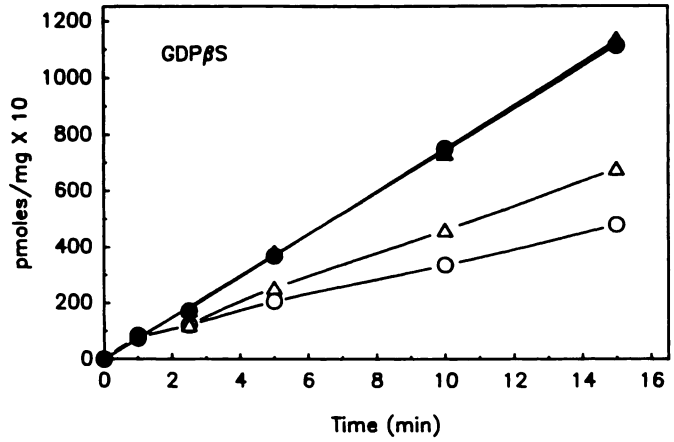

Fig. 10. Time courses of inhibition and atropine reversal of cardiac AC activity by GDPβS and carbachol. Sarcolemma was incubated in the presence of 10 μM GDPβS (●) or 10 μM GDPβS and 10 μM carbachol (○). For atropine reversal, sarcolemma was preincubated for 2.5 min with 10 μM GDPβS (▲) or 10 μM GDPβS and 10 μM carbachol (△) and further incubated with 50 μM atropine. Results are representative of three similar experiments, and *points* are the means of triplicate determinations. Incubations were at 30° in medium B containing 200 μM UDP.

although the rate of cAMP production gradually increased thereafter. It was calculated that carbachol- and GDPβS-dependent inhibition of AC activity was reversed only 40% by atropine after 15 min. The possibility that the lag period associated with carbachol- and GDPβS-dependent inhibition of AC activity was a result of phosphorylation of GDPβS to GTPβS was examined. For these studies, membranes were preincubated for 3 min in the presence of GDPβS, with or without carbachol, before the AC assay was started with ATP. This preincubation was performed in the absence of a nucleoside triphosphate or an ATP-regenerating system and in the presence of 200 μM UDP, to eliminate the possibility of phosphorylation of GDPβS. In membranes preincubated with GDPβS or GDPβS and carbachol, cAMP production was linear from 0 to 5 min, indicating that the lag period for inhibition

was eliminated (Fig. 11). These observations suggest that GDPβS in the presence of carbachol was able to interact with G_i during the preincubation period, to yield an optimal inhibitory state, without a requirement of ATP or a prior phosphorylation reaction. Using a slight modification of this protocol, membranes were preincubated for 3 min with GDPβS and the AC assay was started with carbachol and ATP. As a result, carbachol- and GDPβS-dependent inhibition of cAMP production was again characterized by a 1–2-min delay (Fig. 11). This experiment provided two important observations. Firstly, a lag period would not have been obtained if GDPβS was converted to a species capable of supporting carbachol-dependent inhibition during the preincubation period and, secondly, GDPβS cannot interact with G_i to promote inhibition in the absence of a MR agonist.

In the presence of GTPγS, there was a nonlinear increase in cAMP with time (Fig. 12). Carbachol inhibited cAMP production by 25–30% during the first 3–10 min of the incubation period, and thereafter the relative inhibition diminished with time to 10% at 20 min. Similar results were obtained with 500 nM Gpp(NH)p, with or without carbachol, except that inhibition by carbachol was undetectable after 10–15 min of incubation (data not shown).

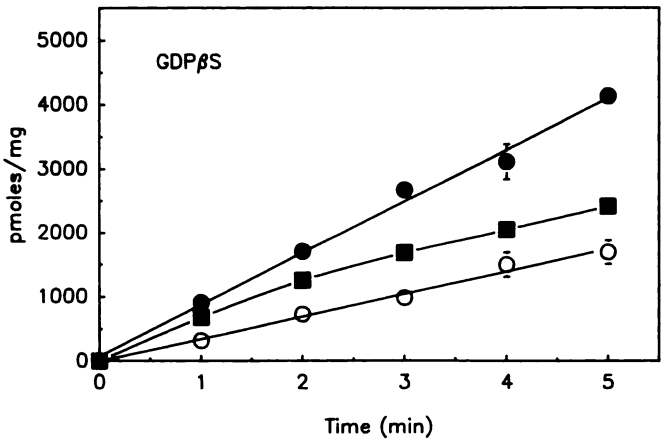

Fig. 11. Effects of preincubation of cardiac sarcolemma with GDPβS, with or without carbachol, on the time course of AC activity. Membranes were preincubated for 3 min at 30° in the presence of 10 μM GDPβS (●) or 10 μM GDPβS and 10 μM carbachol (○) before the AC assay was started with ATP, or sarcolemma was preincubated for 3 min with 10 μM GDPβS before the AC assay was started with 10 μM carbachol and ATP (■). Results are representative of two similar experiments, and each *point* represents the mean ± standard deviation of triplicate determinations.

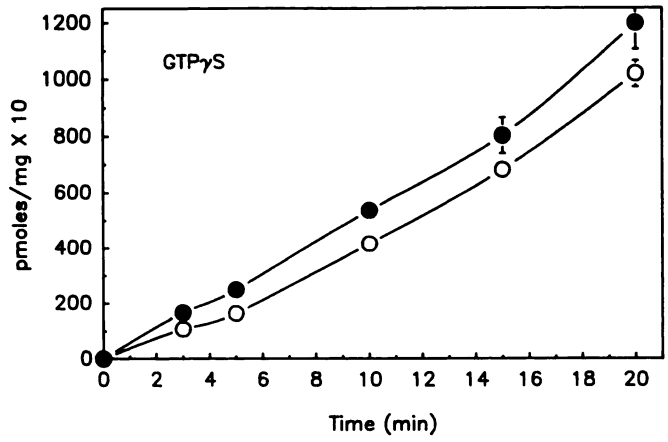

Fig. 12. Time courses of AC activity in the presence of 10 nM GTPγS (●) or 10 nM GTPγS and 10 μM carbachol (○). Results are representative of two similar experiments, and each *point* represents the mean ± standard deviation of triplicate determinations.

Discussion

The results from this investigation confirm and extend our knowledge about the guanylnucleotide specificity of MR inhibitory coupling to cardiac AC. Generally, the results with guanylnucleoside triphosphates on MR inhibitory as well as βAR stimulatory effects on AC activity were confirmatory (5, 6, 9, 12). The findings that GDP and GDPβS can support carbachol-dependent inhibition of cardiac AC activity are novel and of mechanistic interest.

Under the low $MgCl_2$ condition used here, carbachol in the presence of GTP inhibited AC activity up to 60% both in the absence and in the presence of βAR agonists (Table 1). The ability of carbachol to inhibit AC activity in the absence of βAR-G_s stimulation supports the concept that G_i can directly inhibit AC in cardiac sarcolemma. In preliminary studies (13), AC activity was not significantly inhibited by carbachol in the presence of the nonhydrolyzable GTP analogues GTPγS or Gpp(NH)p. This observation was consistent with reports by Watanabe *et al.* (5) and later Jakobs *et al.* (6). However, closer analysis showed that carbachol inhibited AC activity 25–31% in the presence of GTPγS or Gpp(NH)p, during short incubations (i.e., 5–10 min) and in the presence of 0.5 mM $MgCl_2$ (Table 1; Fig. 12). These observations are essentially in agreement with results from a study by Smith and Harden (12), using rat heart membranes. The mechanism(s) underlying the transient nature of inhibition in the presence of Gpp(NH)p or GTPγS are not completely understood, but these analogues have been shown to stimulate G_s directly and thus prevent receptor-mediated inhibition of AC in a number of tissues (1, 12). Regardless of the mechanism, these results do not support the proposal that GTP hydrolysis is required for MR-G_i-AC inhibitory coupling (9).

The finding that carbachol inhibited AC activity in the presence of GDPβS or GDP (Figs. 5 and 7) was surprising. GDPβS and GDP would not be expected to support receptor-G_s or receptor-G_i regulation of AC in most membrane systems, because it is generally believed that only guanylnucleoside triphosphates can activate G proteins (2). Nevertheless, extensive control experiments ruled out the most probable artifactual explanations, such as contamination of GDP or GDPβS preparations with guanylnucleoside triphosphates or, more importantly, conversion of GDP to GTP by nucleoside diphosphate kinase activity. Although nucleoside diphosphate kinase activity was present in this sarcolemma (Fig. 1), phosphorylation of GDP by this enzyme was effectively attenuated 95% with UDP, thus avoiding a serious pitfall in interpretation (17). Because the apparent IC_{50} values of GTP and GDP in the presence of saturating carbachol were only approximately 2-fold different (i.e., 40 and 90 nM, respectively) (Fig. 3), conversion of ≤5% of the GDP to GTP could not account for the inhibitory effects observed here. Furthermore, GDPβS has been shown to be highly resistant to transphosphorylation in a number of membrane systems (19). Lastly, isoproterenol failed to stimulate AC activity in the presence of GDP or GDPβS under essentially identical conditions. The differences in guanylnucleotide specificities of G_s and G_i lead us to hypothesize that G_i may be

unique in cardiac sarcolemma, in that GDP and GDPβS, in addition to GTP, have "agonist" effects on G_i. Accordingly, both guanylnucleoside di- and triphosphates may interact with MR-activated G_i, albeit with varying efficacies, with the formation of an AC-inhibitory form. In cardiac sarcolemma, the rank order of efficacy in supporting carbachol inhibition was GTP ≥ GDPβS > GDP.

It has been reported previously that receptor agonists can stimulate AC activity in the presence of GDP (21) or GDPβS (19), which suggests that GDP and GDPβS have agonist effects on G_s in select membrane systems. These studies (19, 21) were performed under conditions of minimal transphosphorylation of GDP or GDPβS to GTP and, therefore, receptor-G_s-AC coupling was supported by GDP and not by contaminating GTP. GDP was found to have lower efficacy and affinity than GTP on receptor agonist-activated G_s in corpus luteum, NS-20 neuroblastoma, or S49 lymphoma membranes (21), which corresponds to the relative effectiveness of GDP and GTP on cardiac G_i found here. The reason why GDP or GDPβS does not generally have agonist effects on G_i or G_s in membrane systems is unknown, but numerous factors in the membrane environment could potentially dictate guanylnucleotide specificity. The predominant G protein subtype (i.e., $G_{i_{1-3}}$) present, together with conformational constraints imposed by receptors, AC, lipids, or other proteins, may be determinants. Different forms of AC or receptors could also contribute to diversification of G protein-guanylnucleotide interactions (1, 2). As additional possibilities, G proteins may be modified during membrane isolation or their conformations may be dependent on ionic composition or strength.

The agonist effects of GDP or GDPβS on MR-activated G_i, at least in cardiac sarcolemma, mandate that the mechanism for coupling must differ in some way from currently held models (2). It is generally accepted that activation of MRs promotes "opening" of a site on G_i, with the release of bound GDP (1, 2, 8). Subsequently, GTP binds to G_i to promote activation of G_i to an AC-inhibitory form. This could result either from dissociation of G_i into G_i-GTP and β/γ (2) or from a conformationally induced change in G_i without subunit dissociation (22, 23). From the present study, GDP or GDPβS may also bind to MR-activated G_i, converting it to an AC-inhibitory form in cardiac membranes. The time courses of inhibitory changes allowed some insights into the coupling mechanism. For instance, AC inhibition by carbachol and GDP or GTP was immediate (Figs. 8 and 9), whereas carbachol- and GDPβS-dependent inhibition was characterized by a 1–2-min lag period (Fig. 10). The lag period was not associated with phosphorylation of GDPβS, because full inhibition could develop by preincubation of membranes with carbachol and GDPβS in the absence of a phosphate donor (Fig. 11). Furthermore, slow association does not account for the lag period, because GDPβS rapidly exchanges with GTP or GDP on MR-activated G_i in these membranes (24). Therefore, these observations suggest that bound natural guanylnucleotides activate G_i more rapidly than synthetic analogues such as GDPβS, in the presence of an MR agonist. Because of its unique properties, GDPβS may be a useful tool to examine further partial reaction(s) associated with guanylnucleotide modification of G_i and possibly G_s in select systems.

The ability of atropine to reverse AC inhibition due to carbachol and GDP or GDPβS would suggest that GTPase is not an absolute requirement for turning off or reversing MR-G_i-AC coupling in the presence of these nucleotides. This, however, does not rule out a GTPase hydrolysis step in the reversal of inhibition by carbachol and GTP and, indeed, our subsequent studies support this possibility (24). We, therefore, speculate that there may be another step involved in the turn-off reaction(s), which is undetectable in studies with GTP and carbachol. For instance, AC-inhibitory GTP-G_i would be dephosphorylated to GDP-G_i as anticipated; however, because GDP-G_i is active in cardiac sarcolemma in the presence of an MR agonist, it may be necessary for active GDP-G_i to revert to an inactive high affinity GDP-binding form, normally present in the resting membrane, to complete reversal. The slow rate of reversal of GDPβS-G_i-mediated inhibition may be related to a much slower rate of reversion of active G_i-GDPβS to an inactive state. In this respect, GDPβS shares some characteristics with GTPγS, which also has quasi-irreversible effects on G_s (1, 2). Other properties of MR-regulated GTPase and GTP or GDP binding to G_i are dealt with more thoroughly in the accompanying report (24).

As an alternative to the possibilities described above, Jakobs and Wieland (18) suggested that GDP could support α_2-adrenergic receptor-G_i-AC inhibition by a different mechanism than proposed here. These workers suggested that GDP bound to G_i-GDP may be phosphorylated by membrane nucleoside diphosphate kinase in response to receptor activation, and G_i-GTP may then inhibit AC. The inhibitory response could then be reversed by GTPase-catalyzed hydrolysis of GTP on G_i. However, in contrast to studies reported here, GDPβS did not support agonist inhibition of AC activity in platelet membranes (18). Therefore, this alternative model would not be applicable to our studies with heart membranes.

Receptor-agonist binding studies further support the hypothesis that GDP may have agonist effects on G_i and G_s. In reconstituted MR and G_i preparations, Florio and Sternweis (25) and Tota *et al.* (8) have shown that GDP, albeit with lower affinity, is as effective as GTP in transforming high affinity agonist binding sites on MR to low affinity sites. GDP also shifts the affinity of MRs for carbachol in purified canine cardiac sarcolemmal membranes (26). Iyengar *et al.* (21) demonstrated that GDP and GTP were equally effective in reducing the affinity of βARs for isoproterenol in a number of membranes. The mechanism associated with these effects of GDP has yet to be explained but may play a key role in the elucidation of receptor-G protein interactions (1).

Although not a focus of this investigation, the stimulatory effects of GDP and GDPβS on sarcolemmal AC activity warrant discussion. It was found here that GDP or GDPβS stimulated AC activity by 2–2.5-fold. Scarpace (27) also reported that GDPβS stimulated AC activity in rat heart membranes. These stimulatory actions were unanticipated, because GDPβS usually has no effect or inhibits AC activity in most membrane systems (1, 2). The stimulation by GDP and GDPβS in cardiac sarcolemma was not associated with effects on G_i, because the EC_{50} values for GDP and GDPβS stimulation were 4–50-fold less than the IC_{50} values for MR-G_i-AC inhibition (Figs. 3 and 6). Stimulation of G_s by GDPβS or GDP would also not be likely and, therefore, we speculate that GDP or GDPβS may act on an unidentified G protein site to stimulate AC. We have recently found that this putative G protein is (i) uncoupled from AC by preincubation for 5 min at 30°, (ii) sensitive to *N*-ethylmaleimide, and (iii) stabilized by 5 mM $MgCl_2$ or guanyl-

nucleotides against heat uncoupling or denaturation.[1] These properties are essentially identical to those of a low molecular mass 23-kDa G protein (28), and the possibility that GDP or GDPβS acts on a member of the low molecular mass G protein family to stimulate AC merits further investigation. We speculate that its functional expression may be highly dependent on the presence of $MgCl_2$ during membrane preparation and storage. Furthermore, $MgCl_2$ may determine the guanylnucleotide specificity, because the stimulatory effects of GDPβS or GDP are observed at 0.5 mM $MgCl_2$ but not at 5 mM $MgCl_2$.[1]

In summary, the results obtained here with guanylnucleoside triphosphates were in general agreement with previous reports and indicate that MR-G_i and βAR-G_s were functionally coupled to AC in purified cardiac sarcolemma. The observations that GDP or GDPβS could support MR agonist-dependent inhibition of AC activity were the most intriguing and indicated that the MR-G_i-AC coupling mechanism in cardiac membranes may be unique. It is proposed that GDP and GDPβS may act as agonists on MR-activated G_i to promote inhibition of cardiac AC. The ability of atropine to reverse carbachol inhibition in the presence of GDP or GDPβS would suggest that GTPase was not required for this process in the presence of guanylnucleoside diphosphates but that an additional step is involved. Although GDP or GDPβS may be capable of supporting MR agonist inhibition under *in vitro* conditions, the predominance of GTP versus GDP, as well as the higher affinity of MR-activated G_i for GTP, suggest that GTP is the physiologically relevant guanylnucleotide in cells. However, studies with GDP or GDPβS provide important insights into the mechanism of MR-G_i-AC coupling that are not attainable in studies with GTP or other guanylnucleoside triphosphates.

Acknowledgments

We would like to thank Nimman Satumtira, M.S, for her excellent technical assistance and Myron Jacobson, Ph.D., Paul Cook, Ph.D., and Michael Martin, Ph.D., for their insightful discussions and reviewing of the manuscript. We also thank Donna Coyle and Paul Loflin for performing the HPLC analyses of guanylnucleotides.

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[1] E. Quist, unpublished observations.

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