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Muscarinic Receptor-Mediated Inhibition of GDP-Activated Adenylyl Cyclase Suggests a Direct Interaction of Inhibitory Guanine Nucleotide-Binding Proteins and Adenylyl Cyclase

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SUMMARY

To differentiate the effects of GDP and GTP on adenylyl cyclase regulation, adenylyl cyclase in canine sarcolemmal membranes was studied under conditions where only 3–12% of added GDP was converted to GTP by membrane-associated nucleoside diphosphate kinase. Adenylyl cyclase was stimulated up to 180% by GDP at 7-fold lower concentrations than required for stimulation by GTP (GDP half-maximal activation, 120 nM; GTP half-maximal activation, 830 nM). Transphosphorylation of GDP to GTP was blocked completely by the addition of 3 mM UDP. However, UDP did not affect GDP-mediated adenylyl cyclase activation, and guanosine 5′-*O*-(2-thiodiphosphate) had the same effect on adenylyl cyclase activity as did GDP, indicating that GDP-mediated stimulation of adenylyl cyclase was not due to transphosphorylation of GDP to GTP. Carbachol inhibited GDP-stimulated adenylyl cyclase activity even without addition of GTP; however, this inhibition was clearly dependent upon the endogenous formation of GTP. Half-maximal adenylyl cyclase inhibition by carbachol required the addition of either 330 nM GDP or 25 nM GTP. Taking into account a 3–12% conversion of GDP to GTP by membrane-associated nucleoside diphosphate kinase, sufficient GTP was generated from GDP to support receptor-mediated inhibition of adenylyl cyclase. In addition carbachol-mediated adenylyl cyclase inhibition in the presence of GDP, but not GTP, was blocked completely by 3 mM UDP. In conclusion, GDP-activated adenylyl cyclase could be inhibited by carbachol in the presence of GTP concentrations that were 34-fold below the concentrations needed for GTP-mediated activation of stimulatory guanine nucleotide-binding proteins. In addition, at low GTP concentrations carbachol reduced adenylyl cyclase to levels below "basal" activity (activity in the absence of guanine nucleotides). Although indirectly, these results suggest that carbachol-mediated inhibition of adenylyl cyclase may be independent of G_s activity and possibly due to direct interaction of inhibitory guanine nucleotide-binding proteins and adenylyl cyclase.

Cardiac cAMP synthesis underlies dual regulation by stimulatory and inhibitory signal transduction. Binding of stimulatory and inhibitory receptor agonists catalyzes the respective G protein (i.e., G_i and G_s) activation by increasing the release of GDP and binding of GTP to the α subunit (α_s and α_i). Binding of GTP leads to a transition into the active G protein, which is associated with subunit dissociation. The inactivation of G proteins is thought to be due to the intrinsic GTPase activity of the α subunit (1).

Whereas hormonal activation of AC is mediated by a direct interaction of the activated α_s-GTP with the cyclase catalyst, the mechanism of AC inhibition remains controversial (1). Studies with purified subunits of G_i demonstrated an inhibitory effect of the $\beta\gamma$ dimer but not of the α_i subunit. From these results it has been suggested that $\beta\gamma$ dimers of G_i associate with α_s and thereby promote inhibition of hormone- and GTP-stimulated adenylyl cyclase (2). This concept of indirect AC inhibition by $\beta\gamma$ dimers has been questioned by studies with the plant diterpene forskolin, a direct activator of AC. Hormonal inhibition of forskolin-stimulated AC is not impaired in S49 *syc*$^-$ cells (3), which are deficient in G_s. However activation by forskolin is sensitive to an interaction of AC with G_s (4). Therefore, in cells not deficient in G_s hormonal inhibition of forskolin-stimulated AC does not rule out indirect inhibition by reassociation of $\beta\gamma_i$ with α_s.

GDP has recently been suggested to be an activator of AC, acting independently of G_s (5–7). Studies using GDP may be hampered by the activity of membrane-associated NDPK, which phosphorylates GDP to GTP in the presence of phosphate donors. In the present study, the effects of GDP on AC activity and inhibition by the muscarinic receptor agonist carbachol were studied under conditions where only 3–12% of added GDP was converted to GTP by NDPK. It is shown that GDP is an activator of AC, acting independently from a transphosphorylation to GTP. Muscarinic receptor-mediated inhi-

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; NDPK, nucleoside diphosphate kinase; AC, adenylyl cyclase; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); G_s and G_i, "stimulatory" and "inhibitory" guanine nucleotide-binding proteins, respectively.

bition of basal and GDP-activated AC could be demonstrated, possibly indicating a direct interaction of G_i with the AC.

Experimental Procedures

Materials. Alamethicin, carbachol, isoproterenol, and forskolin were purchased from Sigma (Deisenhofen, Germany). All nucleotides, creatine kinase, and creatine phosphate were obtained from Boehringer (Mannheim, Germany), and GDPβS was purchased from Boehringer and Sigma. [α-^{32}P]ATP was obtained from NEN Du Pont (Bad Homburg, Germany). [^{3}H]GDP was purchased from Amersham-Buchler (Braunschweig, Germany). Polyethyleneimine-cellulose F plates were obtained from Merck (Darmstadt, Germany).

Purification of sarcolemmal membranes. Purified sarcolemmal membranes were prepared from beagle dog hearts according to the method of Jones *et al.* (8). Membranes were frozen in liquid nitrogen and stored at −80°. AC activity did not decrease over a period of 6 weeks when membranes were stored under these conditions.

Measurement of AC activity. AC activity was determined by measuring the conversion of [α-^{32}P]ATP to [^{32}P]cAMP according to the method of Jakobs *et al.* (9). The assay volume was 100 μl, containing 0.1 mM ATP with 2 × 10^5 cpm of [α-^{32}P]ATP, 3 mM $MgCl_2$, 0.1 mM cAMP, 1 mM EDTA, 0.5 mM dithiothreitol, 0.05 mg of bovine serum albumin, and 75 mM Tris·HCl, pH 7.6. The effect of GTP on AC activity (see Figs. 2A, 3A, and 5A) was measured in the presence of creatine phosphate (5 mM) and creatine kinase (0.025 mg). In the absence of creatine phosphate and creatine kinase, GTP concentrations needed for AC activities increased about 2-fold. Alamethicin was added to unmask latent AC activity, in a 1:1 (w/w) ratio with regard to sarcolemmal protein. Alamethicin, a peptide ionophore, increases the accessibility to substrates of the active sites of enzymes inside sealed sarcolemmal vesicles without affecting the functional coupling of these enzymes to the corresponding receptors (8). The reaction was started by the addition of [α-^{32}P]ATP/ATP/$MgCl_2$ and continued for 20 min at 37°. The membranes (2.5–3 μg of protein) were preincubated with alamethicin for 20 min at room temperature. Under these conditions, AC activity was linear with time (1–20 min) and protein (0.1–3 μg). Carbachol-mediated effects on AC activity were completely blocked by the addition of atropine (10 μM). Protein was determined using the Bradford Bio-Rad dye-binding assay.

NDPK. Membrane-associated NDPK activity was determined, using [^{3}H]GDP as a substrate, under conditions identical to those used in the measurement of AC activity, except that [α-^{32}P]ATP was omitted. Reactions were stopped by the addition of 5 μl of 10% sodium dodecyl sulfate. Aliquots of 10 μl (in 2-μl steps) were spotted on polyethyleneimine-cellulose F thin layer chromatography plates. Ten microliters of GTP/GDP/GMP (10 mM) were run in parallel, detected under UV light, and used as markers. Chromatography was performed with a 1:1 mixture of 2 M formic acid and 1 M LiCl, for 30 min at room temperature. Reaction products (GTP, GDP, and GMP) were located under UV light, scraped off, and counted in 4 ml of liquid scintillation mixture cocktail (Aquasure; NEN Du Pont) after 2 hr of preincubation.

Results

NDPK. During the first 5 min of incubation, the amount of [^{3}H]GTP derived from [^{3}H]GDP (1 μM) increased rapidly. After 5 min 12% of total [^{3}H]GDP was converted to [^{3}H]GTP. Thereafter, the amount of [^{3}H]GTP decreased slowly (Fig. 1A). Variation of [^{3}H]GDP concentration had only slight effects on the proportion of GTP formed (Fig. 1B). The proportion of [^{3}H]GMP formed under these conditions increased with increasing [^{3}H]GDP concentrations, from 4% at 0.1 μM to 15% at 10 μM [^{3}H]GDP. The addition of 300 μM UDP resulted in a 55% decrease in GTP formation, and no conversion of GDP to GTP could be detected in the presence of 3000 μM UDP.

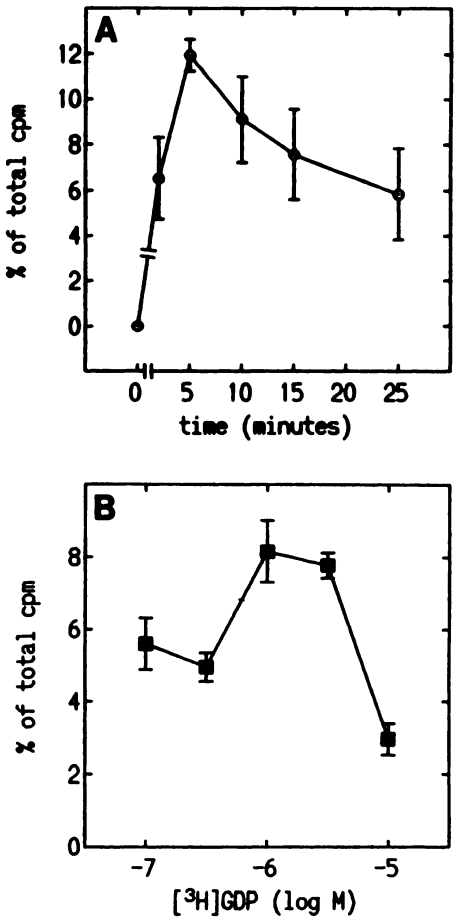

Fig. 1. Influence of incubation time and substrate concentration on the amount of [^{3}H]GTP formed from [^{3}H]GDP in cardiac sarcolemmal membranes. A, Membranes were incubated with 1 μM [^{3}H]GDP under "AC assay" conditions for the indicated time intervals. B, Membranes were incubated for 20 min with the indicated concentrations of [^{3}H]GDP as in A. Values are expressed as percentage of [^{3}H]GTP in total radioactivity recovered (i.e., sum of [^{3}H]GTP, [^{3}H]GDP, and [^{3}H]GMP) and are given as means ± standard deviations of triplicate determinations.

Influence of guanine nucleotides on AC activity. GTP stimulated AC up to 190%, and half-maximal activation was obtained at 826 ± 160 nM GTP (Figs. 2A and 3A). Isoproterenol stimulated AC in the presence of 1 μM GTP up to 240% (Table 1). GTP at 1 μM increased forskolin-stimulated AC activity up to 124% (Table 1).

The dose-response curve for GDP-mediated AC stimulation showed a plateau at concentrations between 0.5 and 2 μM GDP. If GDP concentrations were further increased to 100 μM, a second phase of stimulation could be observed (Fig. 2B). Taking into account a 3–12% conversion of exogenous GDP to GTP, only the first AC activation phase, with a maximum at about 1 μM, can be regarded as a true GDP effect, because GTP concentrations up to 0.1 μM had almost no stimulatory effect on AC (Fig. 3A). GDP stimulated AC up to 180%, and half-maximal activation was obtained at 121 ± 27 nM (Figs. 2B and 3B). Isoproterenol stimulated AC in the presence of GDP (1 μM) up to 110%. GDP increased forskolin-stimulated AC up to 170% (Table 1).

GDPβS at concentrations up to 1 μM stimulated AC activity as did GDP but did not show the biphasic dose-response effect seen with GDP (Fig. 2C). GDPβS stimulated AC up to 210%, with half-maximal activation at 191 ± 22 nM (Figs. 2C and 3C).

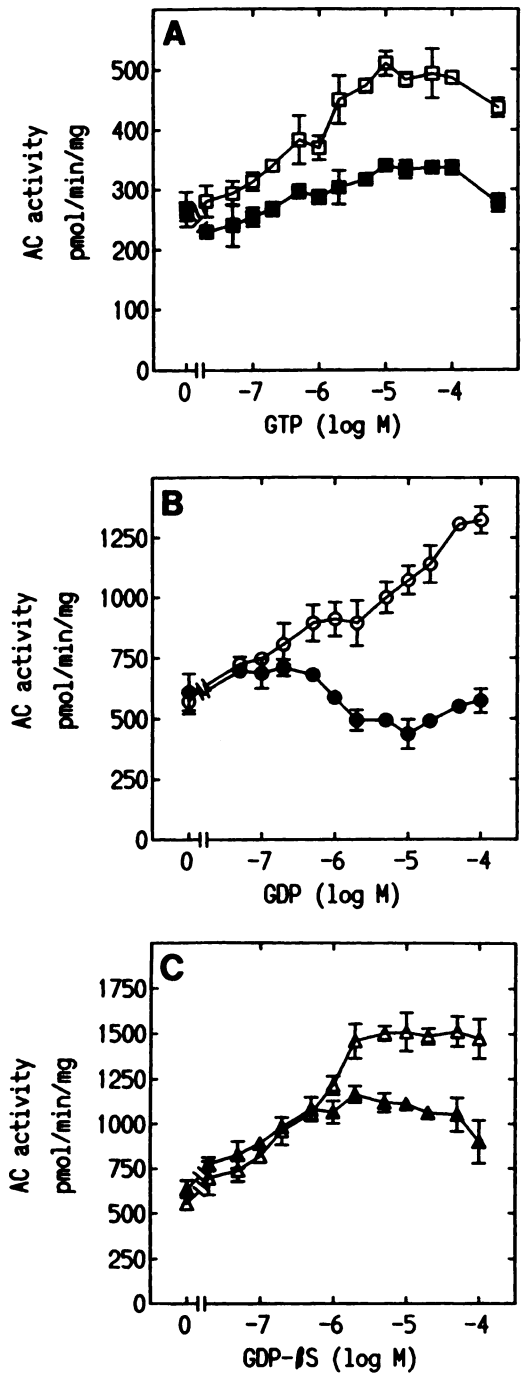

Fig. 2. Influence of guanine nucleotides on cardiac AC activity and inhibition by carbachol. AC activity was determined at increasing concentrations of GTP (A), GDP (B), and GDPβS (C) in the absence (*open symbols*) and in the presence of 1 mM carbachol (*filled symbols*). Values are the means of triplicate determinations.

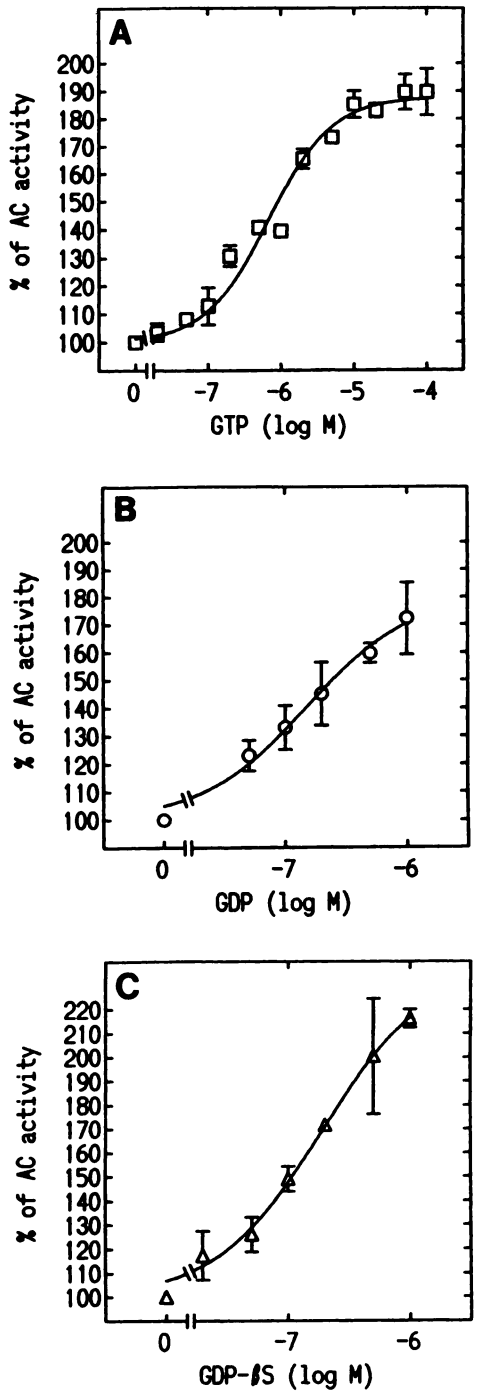

Fig. 3. Stimulation of cardiac AC by guanine nucleotides. AC activity was determined at the indicated concentrations of GTP (A), GDP (B), and GDP-βS (C). Values are the means ± standard deviations of three separate experiments.

Isoproterenol did not stimulate AC in the presence of GDPβS (1 μM). GDPβS increased forskolin-stimulated AC up to 165% (Table 1).

UDP reduced basal AC activity dose dependently, down to 50% at 3 mM UDP (from 393 ± 78 to 187 ± 41 pmol/min/mg). At a concentration that completely suppressed the formation of GTP, UDP (3 mM) had no effect on the stimulation of AC by GDP, GDPβS, or GTP (Fig. 4).

Influence of guanine nucleotides on the inhibition of AC activity. Carbachol (1 mM) had no effect on AC activity in the absence of guanine nucleotides. Addition of GTP resulted in up to 40% inhibition of AC activity by carbachol (half-maximal inhibition at 25 ± 12 nM GTP) (Figs. 2A and 5A). At low GTP concentrations, carbachol reduced AC activity to levels below basal activity (activity in the absence of guanine nucleotides) (Fig. 5A). UDP (up to 3 mM) did not impair carbachol-mediated inhibition of AC in the presence of GTP (1 μM) (Fig. 6). Addition of GDP resulted in up to 50% inhibition of AC activity by carbachol (half-maximal inhibition at

TABLE 1

Influence of guanine nucleotides on receptor-mediated AC stimulation and inhibition and forskolin-mediated enzyme activation

The concentrations used were 100 μM, 5 μM, and 1 mM for isoproterenol, forskolin, and carbachol, respectively. Values are the means ± standard deviations of three separate experiments.

Addition	Ac activity			
	None	GDP (1 μM)	GDPβS (1 μM)	GTP (1 μM)
	pmol/min/mg			
None	320 ± 22	543 ± 106	557 ± 94	413 ± 82
Isoproterenol	320 ± 59	600 ± 149	533 ± 154	993 ± 367
Forskolin	1830 ± 374	3055 ± 719	3015 ± 629	2260 ± 496
Carbachol	353 ± 19	367 ± 98	500 ± 71	253 ± 57

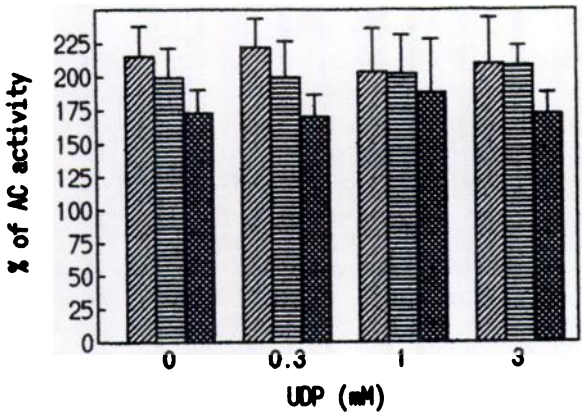

Fig. 4. Influence of UDP on the stimulation of cardiac AC activity by guanine nucleotides. AC activity was determined in the presence of 1 μM GDP (▨), 5 μM GDPβS (▩), and 10 μM GTP (■) at the indicated concentrations of UDP. Values are given as percentage of stimulation of basal AC activities (activity in the absence of guanine nucleotides) at the indicated UDP concentration and are the means ± standard deviations of three separate experiments.

328 ± 38 nM GDP) (Figs. 2B and 5B). UDP attenuated AC inhibition by carbachol/GDP, and at 3 mM UDP the inhibition of AC by carbachol/GDP was completely abolished (Fig. 4). GDPβS at up to 500 nM did not support AC inhibition by carbachol. Addition of higher concentrations of GDPβS resulted in up to 45% inhibition of AC activity by carbachol (half-maximal inhibition at 3300 ± 360 nM GDPβS) (Figs. 2C and 5C). Unexpectedly, carbachol-mediated inhibition of AC in the presence of high concentrations of GDPβS could not be inhibited by UDP (up to 10 mM UDP, data not shown).

Discussion

Three conclusions may be drawn from the experiments in this paper. 1) Previous reports (5–7) of AC activation by GDP in cardiac membranes are supported. 2) Receptor-mediated AC inhibition in the presence of GDP depends on transphosphorylation of GDP to GTP by NDPK. 3) A receptor-mediated inhibition of basal and GDP-activated AC suggests a direct inhibition of the cyclase catalyst by activated α_i subunits.

Regarding the first conclusion, assessment of GDP-mediated effects on signaling molecules may be hampered by a high activity of NDPK. In this study, similar to a previous report (6), experimental conditions were chosen under which the amount of endogenously formed GTP was limited to 3–12% of the added GDP, depending on incubation time and GDP concentration (Fig. 1). Under these conditions, GDP activated AC

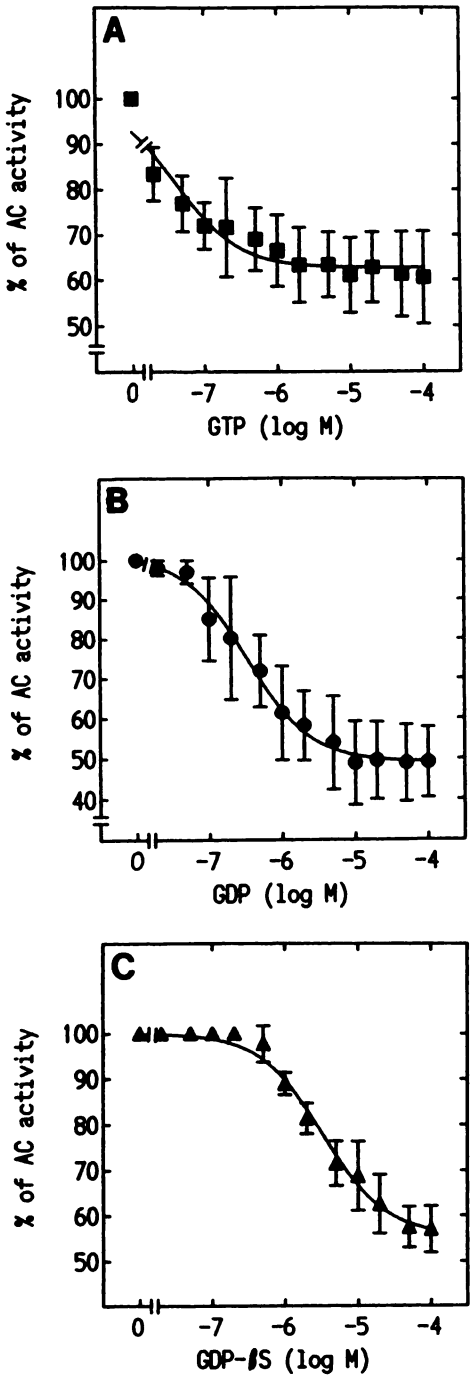

Fig. 5. Influence of guanine nucleotides on the inhibition of cardiac AC activity. AC activity was determined at the indicated concentrations of GTP (A), GDP (B), and GDP-βS (C) in the presence of 1 mM carbachol. Values are the means ± standard deviations from three separate experiments.

at about 7-fold lower concentrations than did GTP (Fig. 3, A and B). The same effect was seen using as an activator GDPβS (Fig. 3C), which is a poor substrate for NDPK in some tissues (10, 11). Isoproterenol had little effect on AC activity in the presence of 1 μM GDP and had no effect in the presence of 1 μM GDPβS, whereas it stimulated AC in the presence of GTP up to 240% (Table 1). UDP (3 mM) suppressed the formation of GTP from GDP completely but had no effect on AC stimulation by GDP and GDPβS (Fig. 4).

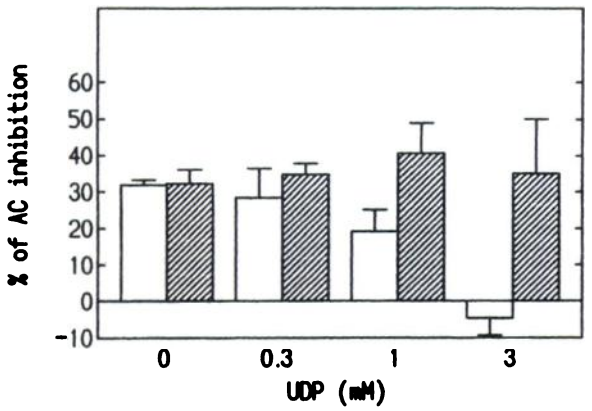

Fig. 6. Influence of UDP on the inhibition of cardiac AC by carbachol with GDP or GTP. AC inhibition was determined in the presence of 1 μM GDP (□) or 1 μM GTP (▨) after the addition of carbachol (1 mM), with the indicated concentrations of UDP. Values are the means ± standard deviations of three separate experiments.

The exact mechanism of GDP-mediated AC stimulation cannot be determined from the results of the present study. The results demonstrate that the stimulatory effect of GDP on AC is independent of a transphosphorylation of GDP to GTP. It cannot be excluded from this study that the effect of GDP on AC activity is due to an interaction with a G protein, and it has not yet been unambiguously proven that α_s-GDP is inactive with regard to AC stimulation. However, it is generally accepted that α_s-GTP is the active form with regard to AC stimulation, and the GTPase activity of the α subunit is considered to be the inactivation mechanism (1). Under these assumptions, an involvement of G_s in the mechanism of GDP-mediated AC stimulation appears very unlikely.

With respect to the second conclusion, although GDP is an activator of AC, receptor-mediated inhibition in the presence of GDP depends on the transphosphorylation of GDP to GTP. In the presence of carbachol, half-maximal inhibition of AC required the addition of either 25 ± 12 nM GTP or 328 ± 42 nM GDP (Fig. 5, A and B). Taking into account a 3–12% conversion of GDP to GTP (Fig. 1), sufficient GTP was generated to support receptor-mediated inhibition. If the conversion of GDP to GTP was suppressed completely by 3 mM UDP, AC inhibition by carbachol/GDP was blocked completely, whereas inhibition by carbachol/GTP was not impaired at all (Fig. 6). In contrast, stimulation of AC by GDP, as mentioned above, was not impaired by 3 mM UDP.

Although GDP and GDPβS stimulated AC at similar concentrations, 10-fold higher concentrations of GDPβS were required to support inhibition of AC by carbachol (Fig. 5C). Unexpectedly, inhibition of AC by carbachol/GDPβS could not be suppressed by UDP. As has been proposed recently by Quist *et al.* (7), the nonphysiological GDP analog, at high concentrations, may be able to substitute for GTP with regard to carbachol-mediated inhibition of AC. However, this effect of GDPβS would be in contrast to the failure of GDP to support carbachol-mediated AC inhibition. Although GDPβS was purchased from two different suppliers (see Experimental Procedures), the GDPβS may have been contaminated with a triphosphate form. Because >100-fold higher concentrations of GDPβS, compared with GTP, were required for AC inhibition, contaminations with GTP of <1% could account for the effect.

In contrast to the present results, Quist *et al.* (7) suggested that muscarinic receptor-coupled AC inhibition does not require GTP in the presence of GDP. In their study NDPK activity was reduced to a maximum of 5% conversion of GDP to GTP by the addition of 400 μM UDP. The half-maximal nucleotide concentrations for carbachol-mediated AC inhibition were 90 nM and 40 nM for GDP and GTP, respectively. A 5% conversion of GDP to GTP would reveal 10-fold lower GTP concentrations than required for half-maximal AC inhibition. It was concluded that the effect of GDP cannot be due to a transphosphorylation to GTP under these conditions. This conclusion relies upon the premise of an even distribution of GDP, UDP, and the NDPK in the assay medium. UDP, in contrast to GDP, does not bind to G proteins. Therefore, distribution of the two nucleotides in close proximity to G proteins is uneven. An overall suppression of NDPK activity to some extent, where conversion of GDP to GTP is still up to 5%, does not exclude sufficient transphosphorylation in close proximity to G proteins. This effect of an uneven distribution of the two nucleotides would be aggravated if any form of selective association between G proteins and NDPK exists (12). When, as in the present study, transphosphorylation of GDP to GTP was completely suppressed by high concentrations of UDP (3 mM), carbachol-mediated inhibition of GDP-activated AC was blocked completely, whereas inhibition with carbachol/GTP was not impaired at all (Fig. 4). This clearly demonstrates the involvement of a transphosphorylation step in AC inhibition by carbachol/GDP.

Comparing the study of Quist *et al.* (7) and the study presented here, some differences are found, although in both studies sarcolemmal membranes from canine hearts were used. Whereas in the former study a 75% conversion of GDP to GTP was observed after 5 min, in this study only 12% of GDP was converted to GTP in the same period of time. This could be due to differences in membrane purification, which also become apparent in differences in AC activities. Although absolute values of AC activity were similar in the two studies, in the former study only 0.5 mM magnesium was used, compared with 2 mM free magnesium in this study. Therefore, after correction for magnesium concentration, the AC activity of the sarcolemmal membranes used in the former study was severalfold greater than the activities seen in the present study. In addition, whereas the EC_{50} values for the various guanine nucleotides supporting inhibition of AC by carbachol were rather similar in the two studies, the EC_{50} value for GTP-mediated stimulation of AC in the absence of stimulating receptor agonists was about 30-fold higher in the present study. However, with regard to the degree of AC stimulation and the concentration range of the dose-response curve of GTP, the results of the present study are in accordance with the results reported by others (13). The difference in the effect of GTP on basal AC activity may be explained in part by the different magnesium concentrations used in the two studies. In addition, in the study of Quist *et al.* (7) all experiments were done in the presence of 400 μM UDP, which also may have affected the GTP effect on AC.

Regarding the third conclusion, direct inhibition of AC by receptor-G_i systems is difficult to assess, because GTP is required for inhibition and, therefore, an involvement of G_s cannot be ruled out. In the present study, GTP concentrations needed for a G_s-mediated AC activation were 34-fold higher than those required for receptor-mediated inhibition (Figs. 3

and 5). At low GTP concentrations, carbachol reduced AC activity to levels below basal activity (Fig. 5A). In addition, AC could be activated by GDP at concentrations where GTP formation by NDPK was not sufficient to activate G_s, although sufficient GTP was generated to support receptor-mediated inhibition; and thereby a receptor-mediated inhibition of GDP-activated AC could be obtained, i.e., an inhibition in the absence of an AC stimulation by α_s-GTP. According to the present understanding of G protein function, these findings indicate the possibility of a direct interaction of G_i with the AC. Because the approach in this study is indirect, several limitations have to be taken into account. First, α_s *per se* may increase AC activity, and receptor-mediated inhibition in the absence of stimulating amounts of α_s-GTP could be due to an interaction of $\beta\gamma_i$ with α_s. The inhibition of AC to levels below "basal" activity in the presence of low GTP concentrations could be explained in this way. However, the GDP-activated AC was inhibited by carbachol at least to the same extent as the GTP-activated enzyme. If α_s-GTP is considered as the primarily active and α_s-GDP as the primarily inactive form, the inhibition of GDP-activated AC can hardly be attributed to an interaction with α_s. Second, α_s-GDP may be active in the stimulation of AC. Although it is generally believed that the GTPase activity of the α subunit is the mechanism for inactivation of G proteins, this has not been unambiguously proven yet, and inactivation may depend upon reassociation of the subunits.

In summary, GDP is an activator of cardiac AC, acting independently from a transphosphorylation to GTP. Receptor-mediated inhibition of basal and GDP-activated adenylyl cyclase suggests a direct interaction of G_i and AC.

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