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Stable GDP analog-induced inactivation of G_i proteins promotes cardiac adenylyl cyclase inhibition by guanosine 5′-(βγ-imino) triphosphate and muscarinic acetylcholine receptor

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Abstract

Low concentrations of GDP and its stable analog guanosine 5′-*O*-(2-thio)diphosphate (GDPβS) have been shown to stimulate adenylyl cyclase activity in canine cardiac sarcolemmal membranes independent from a phosphate transfer reaction. The mechanism of this stimulation was further examined. The stable GTP analog guanosine 5′-(βγ-imino)triphosphate (Gpp(NH)p) increased basal adenylyl cyclase activity and inhibited forskolin-stimulated activity with EC_{50} (half-maximal effective concentration) values of 0.7 μmol/l and 10 nmol/l, respectively. In the presence of GDPβS (5 μmol/l), which increased basal activity by about 150%, addition of Gpp(NH)p inhibited adenylyl cyclase activity by up to 50% with an EC_{50} value of 40 nmol/l. Activation of cardiac muscarinic acetylcholine receptors by carbachol amplified this Gpp(NH)p-induced inhibition of GDPβS-stimulated adenylyl cyclase activity. The stimulatory effect of GDPβS and the inhibitory effect of Gpp(NH)p on GDPβS-stimulated adenylyl cyclase activity were both attenuated by increasing the Mg^{2+} concentration or substituting Mn^{2+} for Mg^{2+} in the assay. Furthermore, both effects were strongly reduced or abolished upon pretreatment of the sarcolemmal membranes with a low concentration of the SH reagent *N*-ethylmaleimide (10 μmol/l). These results suggest that the stimulatory effect of GDPβS on basal adenylyl cyclase activity in canine cardiac sarcolemmal membranes is caused by inactivation of G_i proteins, which are then rendered susceptible to activation by Gpp(NH)p and inhibitory receptors.

Keywords: Adenylyl cyclase; G protein; Guanine nucleotide; Muscarinic receptor; Myocardium

1. Introduction

Adenylyl cyclase of cardiac sarcolemmal membranes is dually regulated by stimulatory and inhibitory receptors [1–3]. Both receptor effects are mediated by the activation of distinct guanine nucleotide-binding regulatory proteins (G proteins), termed G_s and G_i for stimulation and inhibition of adenylyl cyclase, respectively. Activation of a G protein-coupled receptor by an agonist catalyzes the release of GDP from and binding of GTP to the α subunit of the interacting G protein. Binding of GTP is followed by a further conformational change of the G protein, leading to the dissociation of the G protein βγ dimer from the GTP-liganded α subunit. At this step, the G protein is in its active confirmation and either the GTP-bound α subunit and/or the free βγ dimer then regulate activities of responsive effectors such as adenylyl cyclase. The α subunit carries an intrinsic GTPase activity which hydrolyzes the bound GTP to GDP. The GDP-bound α subunit is inactive in terms of effector regulation and has an increased affinity for βγ dimers. Hence, the subunits reassociate and may be reactivated by a receptor. These reaction steps are thought to be common to all members of the G protein family, including G_i and G_s [4–6].

We have recently reported that basal adenylyl cyclase activity of canine cardiac sarcolemmal membranes is not only increased by GTP and its stable analog, guanosine 5′-(βγ-imino)triphosphate (Gpp(NH)p), but that the guanine nucleoside diphosphate GDP can also stimulate basal enzyme activity [7]. This GDP-dependent stimulation of cardiac adenylyl cyclase, which occurs at submicromolar concentrations (EC_{50} value of about 0.1 μmol/l GDP), was not affected by inhibiting phosphotransfer reactions. Furthermore, the GDP analog, guanosine 5′-*O*-(2-thio)diphosphate (GDPβS), mimicked the effect of GDP to stim-

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ulate cardiac adenylyl cyclase activity at low concentrations, with an EC_{50} value of about 0.4 μmol/l [7]. Thus, the guanine nucleoside diphosphates GDP and GDPβS, generally considered to keep G proteins in their inactive state and/or to competitively inhibit G protein activation by GTP and its analogs, increased instead of inhibiting cardiac adenylyl cyclase activity. Since cardiac adenylyl cyclase is dually regulated by G_s and G_i proteins, as is the enzyme activity in most other cell types, the observed adenylyl cyclase stimulatory actions of the guanine nucleoside diphosphates are most likely due to an 'inhibitory' action on G_i proteins, eventually resulting in adenylyl cyclase stimulation. The present study was performed to characterize this effect and thus to understand the unexpected finding of GDP stimulation of adenylyl cyclase. It is shown here that stimulation of canine cardiac sarcolemmal adenylyl cyclase by GDPβS is potently inhibited by the stable GTP analog Gpp(NH)p, that this inhibition is amplified by the muscarinic acetylcholine receptor agonist carbachol, and that both stimulation of adenylyl cyclase by GDPβS and inhibition of this activity by Gpp(NH)p are strongly reduced by agents interfering with G_i protein function.

2. Materials and methods

2.1. Materials

Alamethicin, *N*-ethylmaleimide (NEM) and carbachol were purchased from Sigma (Deisenhofen, Germany). ATP, Gpp(NH)p and GDPβS were from Boehringer Mannheim (Mannheim, Germany). [α-^{32}P]ATP was from DuPont-New England Nuclear (Bad Homburg, Germany).

2.2. Purification of sarcolemmal membranes

Purified sarcolemmal membranes were prepared from beagle hearts according to Jones et al. [8]. Membranes were frozen in liquid nitrogen and stored at −80°C. Adenylyl cyclase activity was stable over a period of six months when stored under these conditions.

2.3. Measurement of adenylyl cyclase activity

Adenylyl cyclase activity of canine cardiac sarcolemmal membranes was determined in the absence of a nucleoside triphosphate-regenerating system by measuring the conversion of [α-^{32}P]ATP to [^{32}P]cAMP, according to Jakobs et al. [9]. The assay volume was 100 μl containing 0.1 mmol/l ATP with $(2–5) \times 10^5$ cpm of [α-^{32}P]ATP, 3 mmol/l $MgCl_2$ (unless otherwise indicated), 0.1 mmol/l cAMP, 1 mmol/l EDTA, 0.5 mmol/l dithiothreitol, 0.05 mg bovine serum albumin, and 75 mmol/l Tris-HCl (pH 7.6). The membranes (2.5–3 μg protein) were preincubated with alamethicin for 20 min at 4°C at a 1 : 1 ratio (w/w) to unmask latent adenylyl cyclase activity. This peptide ionophore increases the accessibility of substrates to the adenylyl cyclase in sealed sarcolemmal vesicles without affecting the functional coupling to receptors [8]. Omission of alamethicin revealed lower overall adenylyl cyclase activity, however, the effects of guanine nucleotides were not altered. The adenylyl cyclase reaction was started by the addition of membrane protein and continued for 10–15 min at 37°C. Under these conditions, adenylyl cyclase activity was linear with time (1–20 min) and protein (0.1–3 μg). Protein was determined, using the Bradford-Bio-Rad dye-binding assay.

2.4. NEM pretreatment of sarcolemmal membranes

When appropriate, and concurrent with alamethicin treatment, membranes were pretreated with NEM (10 μmol/l final concentration) for 15 min on ice. The alkylation reaction was stopped by the addition of 4 mmol/l dithiothreitol. Control membranes for these experiments received dithiothreitol only.

2.5. Statistical analysis

All results are expressed as means ± standard deviation of at least three separate experiments with triplicate determinations. To compare the mean values of multiple groups, repeated measure one-way ANOVA was carried out followed by post-tests if significant. Comparisons between two groups were carried out using paired *t* tests.

3. Results

Basal adenylyl cyclase activity in canine cardiac sarcolemmal membranes was increased by the stable GTP analog Gpp(NH)p by maximally 160% ($P < 0.01$). Half-maximal and maximal activation was observed at 0.7

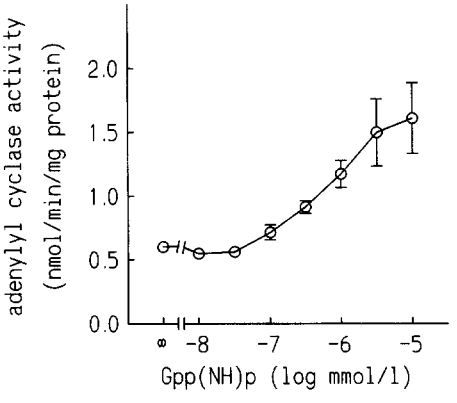

Fig. 1. Stimulation of basal cardiac adenylyl cyclase activity by Gpp(NH)p. Basal adenylyl cyclase activity of canine cardiac sarcolemmal membranes was determined at the indicated concentrations of Gpp(NH)p. Values are the means ± standard deviation (SD) of four separate experiments.

μmol/l and 5 μmol/l Gpp(NH)p, respectively (Fig. 1). The diterpene forskolin (5 μmol/l) stimulated cardiac adenylyl cyclase 13-fold (from 1.26 ± 0.2 to 18.31 ± 1.4 nmol/min per mg protein). Gpp(NH)p-mediated inhibition of forskolin-stimulated enzyme activity was evident at much lower concentrations of Gpp(NH)p than those required for stimulation of basal activity. Half-maximal and maximal inhibition (40%, $P < 0.01$) of forskolin-stimulated activity was observed at about 10 nmol/l and 100 nmol/l Gpp(NH)p, respectively. The stable GDP analog, GDPβS, increased basal adenylyl cyclase activity in canine cardiac sarcolemmal membranes by 150% (Fig. 2, $P < 0.01$), with half-maximal and maximal effects occurring at about 0.4 μmol/l and 5 μmol/l, respectively (data shown in reference 7). Co-addition of Gpp(NH)p did not result in further stimulation, instead Gpp(NH)p potently reduced GDPβS (5 μmol/l)-stimulated adenylyl cyclase activity (Fig. 2). At concentrations up to 1 μmol/l, Gpp(NH)p now reduced enzyme activity by 50% ($P <$ 0.01), with half-maximal inhibition occurring at about 40 nmol/l Gpp(NH)p. At higher concentrations, the inhibitory effect of Gpp(NH)p decreased and eventually, at a concentration of 100 μmol/l, was no longer observed.

Increasing the free magnesium concentration in the assay from 2 mmol/l to 5 or 10 mmol/l enhanced basal adenylyl cyclase activity by 190% and 360%, respectively (Fig. 3A). The absolute increase in basal enzyme activity caused by the two guanine nucleotides, GDPβS (5 μmol/l) and Gpp(NH)p (1 μmol/l), also increased. When expressed relative to basal activity, Gpp(NH)p-stimulated activity was not altered by increasing the concentration of free Mg^{2+} from 2 mmol/l to 5 mmol/l, and was reduced from 170 to 125% stimulation at 10 mmol/l free Mg^{2+} (Fig. 3B). On the other hand, GDPβS stimulation of adenylyl cyclase activity was much more sensitive to increases in Mg^{2+} concentration. Relative to basal activity, GDPβS-stimulated enzyme activity decreased from

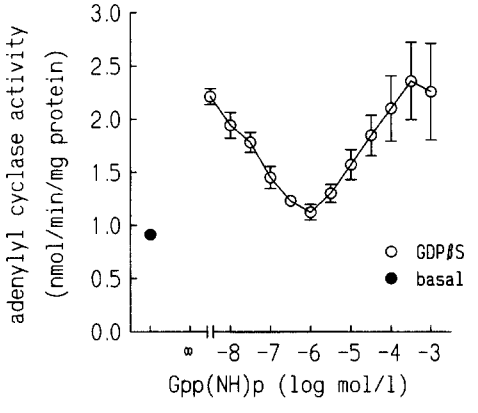

Fig. 2. Influence of Gpp(NH)p on cardiac adenylyl cyclase activity in the presence of GDPβS. Adenylyl cyclase activity of canine cardiac sarcolemmal membranes was determined in the presence of 5 μmol/l GDPβS and the indicated concentrations of Gpp(NH)p. Values are the means ± SD of three separate experiments.

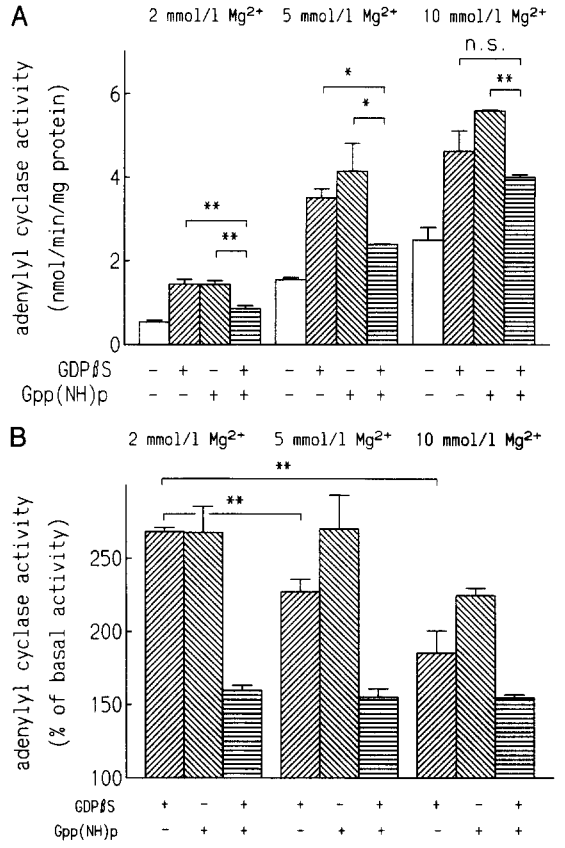

Fig. 3. Influence of Mg^{2+} on cardiac adenylyl cyclase stimulation and inhibition by GDPβS and Gpp(NH)p. Adenylyl cyclase activity of canine cardiac sarcolemmal membranes was determined in the absence and presence of GDPβS (5 μmol/l), Gpp(NH)p (1 μmol/l) or the combination of these two nucleotides with either 2 mmol/l, 5 mmol/l or 10 mmol/l free Mg^{2+}, as indicated. Absolute enzyme activities are given in A, while in B activities in the presence of GDPβS −, Gpp(NH)p − and GDPβS + Gpp(NH)p are shown relative to the respective basal activities measured at the different Mg^{2+} concentrations. Values are the means ± SD of three separate experiments where ** and * indicate $P < 0.01$ and 0.05, respectively.

170% stimulation at 2 mmol/l Mg^{2+} to only 120% at 5 mmol/l free Mg^{2+} ($P < 0.01$), and at 10 mmol/l free Mg^{2+} GDPβS increased adenylyl cyclase activity by only 85% ($P < 0.01$ compared to relative increase at 2 mmol/l Mg^{2+}). Inhibition of adenylyl cyclase activity in the presence of GDPβS by Gpp(NH)p decreased from 40% at 2 mmol/l Mg^{2+} to 13% at 10 mmol/l Mg^{2+} ($P < 0.01$). Adenylyl cyclase activity measured in the presence of both GDPβS and Gpp(NH)p remained at the same level compared to basal activity, i.e. 55–60% higher, at all concentrations of free Mg^{2+} studied. Thus, the relative stimulatory effects of GDPβS and inhibition of GDPβS-stimulated adenylyl cyclase activity by Gpp(NH)p were reduced to the same extent by increasing the free Mg^{2+} concentration.

When the $MgCl_2$ in the assay was replaced with $MnCl_2$ (total concentration 1.5 mM), basal activity increased by 240% (Fig. 4). Under these conditions, GDPβS-mediated

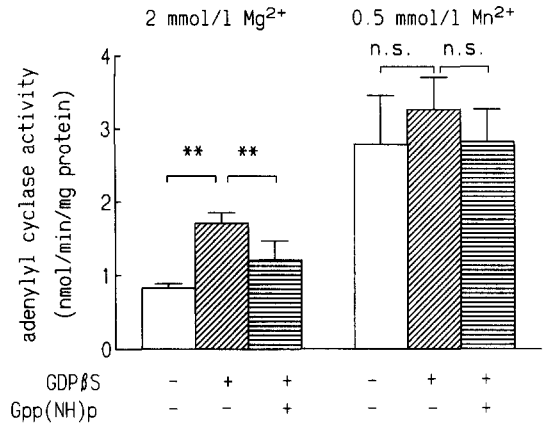

Fig. 4. Effects of Mn^{2+} on cardiac adenylyl cyclase stimulation and inhibition by GDPβS and Gpp(NH)p, respectively. Adenylyl cyclase activity was determined in the absence and presence of GDPβS (5 μmol/l) or GDPβS (5 μmol/l) plus Gpp(NH)p (1 μmol/l) as indicated with either 2 mmol/l free Mg^{2+} or 0.5 mmol/l free Mn^{2+}. Values are the means ± SD of five separate experiments where * * indicates $P < 0.01$.

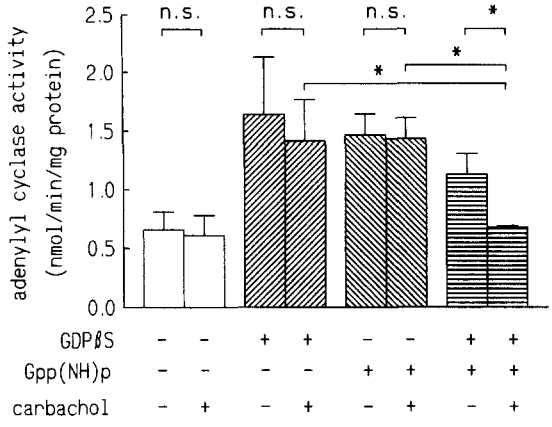

Fig. 6. Inhibition of cardiac adenylyl cyclase activity by Gpp(NH)p and carbachol in the presence of GDPβS. Adenylyl cyclase activity of canine cardiac sarcolemmal membranes was determined in the absence and presence of carbachol (10 μmol/l) and either GDPβS (5 μmol/l), Gpp(NH)p (1 μmol/l) or the combination of GDPβS (5 μmol/l) and Gpp(NH)p (1 μmol/l) as indicated. Values are the means ± SD of three separate experiments where * indicates $P < 0.05$.

stimulation of adenylyl cyclase activity was reduced by 85%. Inhibition of GDPβS-stimulated adenylyl cyclase activity by Gpp(NH)p was also reduced and no more significant.

Pretreatment of canine cardiac sarcolemmal membranes for 15 min with the SH reagent, NEM (10 μmol/l), caused an increase of 30% in basal adenylyl cyclase activity, measured in the absence of guanine nucleotides (Fig. 5, $P < 0.01$). The relative increase in adenylyl cyclase activity caused by GDPβS (5 μmol/l) was reduced in membranes pretreated with NEM (160 and 70% stimulation for control and NEM-treated membranes, respectively, $P < 0.01$). However, absolute values of GDPβS-stimulated activity did not differ significantly. Most obviously, whereas Gpp(NH)p (1 μmol/l) reduced adenylyl cyclase activity in the presence of GDPβS (5 μmol/l) by 35% in control sarcolemmal membranes, this inhibition was virtually abolished in membranes pretreated with the alkylating SH reagent (Fig. 5).

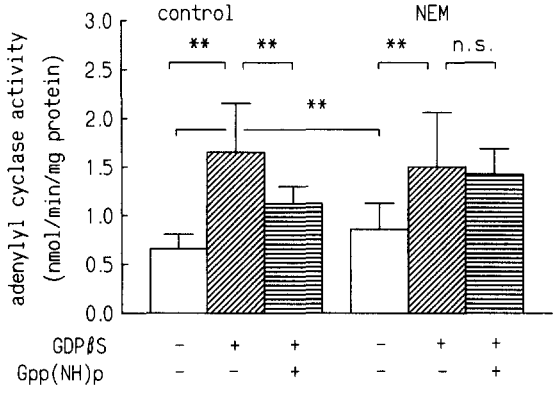

Fig. 5. Influence of NEM pretreatment on regulation of cardiac adenylyl cyclase activity by GDPβS and Gpp(NH)p. Canine cardiac sarcolemmal membranes were pretreated for 15 min on ice with or without (control) 10 μmol/l NEM, followed by addition of 4 mmol/l dithiothreitol to stop the alkylation reaction. In these pretreated membranes, adenylyl cyclase activity was determined in the absence and presence of GDPβS (5 μmol/l) or GDPβS (5 μmol/l) plus Gpp(NH)p (1 μmol/l) as indicated. Values are the means ± SD of five separate experiments where * * indicates $P < 0.01$.

Addition of the muscarinic acetylcholine receptor agonist carbachol (10 μmol/l) to purified canine cardiac sarcolemmal membranes had no effect on adenylyl cyclase activity in the absence of added guanine nucleotides (Fig. 6). Furthermore, carbachol did not inhibit adenylyl cyclase activity stimulated by Gpp(NH)p (1 μmol/l) alone, and only marginally inhibited adenylyl cyclase stimulated by GDPβS (5 μmol/l). In contrast, in the presence of GDPβS (5 μmol/l), inhibition of adenylyl cyclase induced by Gpp(NH)p (1 μmol/l) was increased from 30% to 60% when carbachol (10 μmol/l) was added ($P < 0.05$). The effect of carbachol on adenylyl cyclase activity in the presence of guanine nucleotides was blocked by the muscarinic receptor antagonist atropine (100 μM) and by pretreatment of the sarcolemmal membranes with pertussis toxin and NAD (data not shown).

4. Discussion

Studies on different membrane systems, including cardiac membranes, have shown an activation of adenylyl cyclase by GDP and its analog GDPβS, which is apparently independent of transphosphorylation reactions catalyzed by nucleoside diphosphate kinase [7,10–12]. The mechanism of this unexpected stimulation, however, is as yet unclear. G proteins are the only known targets for regulatory effects of guanine nucleotides on adenylyl cyclase activity. Thus, excluding the presence of a novel, but

as yet unidentified GDP-binding protein, the observed stimulatory effects of GDP and GDPβS on cardiac adenylyl cyclase activity are most likely due to an action at G_s or G_i proteins, the G proteins primarily regulating adenylyl cyclase activity [13].

That the observed stimulation of adenylyl cyclase activity by GDP and GDPβS is due to activation of G_s proteins is unlikely for the following reasons. First, it has been shown in many different membrane systems and in reconstitution studies with purified components that only the GTP- or stable GTP analog-liganded G_s protein is capable of stimulating adenylyl cyclase activity, whereas GDPβS is either inactive or inhibitory (or reviews see [4], [5], [13]). Second, phosphorylation of GDP to GTP and subsequent activation of G_s can also be ruled out as the mechanism of adenylyl cyclase activation, since activation by GDP occurred even when transphosphorylation to form GTP was completely prevented [7]. Moreover, GDPβS was equally effective in stimulating cardiac adenylyl cyclase activity, mimicking GDP's stimulatory action at low concentrations [7], despite being known as a poor substrate for phosphate transfer reactions [14,15]. Thus, it seems feasible to assume that stimulation of cardiac adenylyl cyclase activity by GDP and GDPβS is due to an action on the adenylyl cyclase inhibitory G_i proteins rather than to activation of stimulatory G_s proteins. This hypothesis implies that 'basal' adenylyl cyclase activity (i.e. without the addition of any stimulatory or inhibitory agent including guanine nucleotides) in cardiac sarcolemmal membranes is under inhibitory control by G_i proteins, and that GDPβS, by binding to $G_i\alpha$ subunits, relieves this inhibition. Such a hypothesis, furthermore, implies that G_i proteins in cardiac membrane preparations are active, at least in terms of adenylyl cyclase inhibition, in the absence of bound guanine nucleotides.

Several findings presented in this study support the hypothesis that GDPβS stimulation of cardiac adenylyl cyclase is caused by an inhibitory action at G_i proteins. First, the stable GTP analog Gpp(NH)p, which increased basal activity, potently inhibited GDPβS-stimulated adenylyl cyclase activity by up to 50%. The concentrations of Gpp(NH)p necessary for this inhibitory effect were much lower than those required for stimulation of basal adenylyl cyclase activity. Half-maximal inhibition of GDPβS-stimulated enzyme activity was observed at about 40 nmol/l Gpp(NH)p, while a 25-fold higher concentration (1 μmol/l) of Gpp(NH)p was required for half-maximal stimulation of basal activity. The former value (40 nmol/l) is in close agreement with the potency of Gpp(NH)p (10 nmol/l) to inhibit forskolin-stimulated adenylyl cyclase activity. The small, factor 4 difference between these two values is most likely due to the fact that inhibition of forskolin-stimulated activity by Gpp(NH)p was studied in the presence of Gpp(NH)p alone, while in the study on inhibition of GDPβS-stimulated adenylyl cyclase activity by Gpp(NH)p, GDPβS was additionally present at a concentration of 5 μmol/l and would have competed with Gpp(NH)p for binding to G proteins. Thus, in the presence of GDPβS, an adenylyl cyclase inhibitory action of Gpp(NH)p by activation of G_i proteins becomes apparent. The reversal of GDPβS-stimulated adenylyl cyclase inhibition at Gpp(NH)p concentrations greater than 1 μmol/l was likely to be a consequence of Gpp(NH)p binding to, and subsequently activating, G_s proteins.

It has been reported in various membrane systems that inhibitory receptor- and G_i protein-mediated inhibition of adenylyl cyclase is decreased by increasing the free Mg^{2+} concentration [16–18]. Therefore, the influence of various Mg^{2+} concentrations was studied on the adenylyl cyclase stimulatory effect of GDPβS and its inhibition by Gpp(NH)p. The relative stimulatory effect of GDPβS on basal adenylyl cyclase activity and the inhibition of GDPβS-stimulated adenylyl cyclase by Gpp(NH)p were reduced with similar sensitivities by increasing Mg^{2+} concentrations. On the other hand, stimulation of basal enzyme activity by Gpp(NH)p, due to activation of G_s proteins, was clearly less sensitive to increases in Mg^{2+} concentration. This finding suggests that stimulation of basal adenylyl cyclase activity in canine cardiac sarcolemmal membranes by GDPβS and inhibition of this stimulation by Gpp(NH)p are due to an action at a common regulatory site, i.e. G_i proteins.

The effect of substitution of Mg^{2+} with Mn^{2+} in the assay further suggested an action of GDPβS at G_i proteins. When employed at low concentration, Mn^{2+} preferentially perturbs the G_i protein interaction with adenylyl cyclase [19–21]. Although Mn^{2+} increased the activity of adenylyl cyclase in the absence of added guanine nucleotides, most likely by directly activating the enzyme catalytic subunit, both stimulation of adenylyl cyclase by GDPβS and inhibition of this stimulation by Gpp(NH)p were attenuated.

The SH reagent NEM has a selective effect on G_i proteins when employed at low concentrations and under mild pretreatment conditions, e.g. at 4°C. NEM pretreatment has been shown to cause alkylation of G_i/G_o proteins at two [22] or three [23] distinct sites, one of which is the cysteine residue target for pertussis toxin-catalysed ADP-ribosylation. Since NEM increased basal adenylyl cyclase activity (even in the absence of GTP) it may, in contrast to pertussis toxin, inhibit G_i function at a site distinct from the receptor interaction domain. Despite increased basal activity, the absolute value of adenylyl cyclase activity in the presence of a maximally stimulatory concentration of GDPβS remained constant after NEM pretreatment. Thus, the relative stimulatory effect of GDPβS was strongly reduced from 160% stimulation in membranes pretreated without NEM to only 70% stimulation in NEM-pretreated membranes. Most obviously, the inhibition of GDPβS-stimulated adenylyl cyclase activity by Gpp(NH)p was inhibited after pretreatment of cardiac membranes with the SH reagent. Thus, both GDPβS-in-

duced stimulation of basal adenylyl cyclase activity and Gpp(NH)p-induced inhibition of this stimulated activity were strongly reduced or abolished by NEM treatment, suggesting that a similar target, most likely G_i proteins, are mediating these two effects on cardiac adenylyl cyclase.

Finally, we studied whether GDPβS-stimulated cardiac adenylyl cyclase activity is subject to regulation by the inhibitory muscarinic acetylcholine receptor [1–3]. The receptor agonist carbachol had no effects on adenylyl cyclase activity in the absence of guanine nucleotides, or in the presence of GDPβS or Gpp(NH)p alone. The ineffectiveness of stable GTP analogs to support receptor-mediated inhibition of adenylyl cyclase has been noted previously [24–29] and has led to misleading conclusions [29,30]. In the present study, however, addition of GDPβS discloses an inhibition of adenylyl cyclase by Gpp(NH)p and the muscarinic receptor agonist carbachol. The effectiveness of carbachol plus Gpp(NH)p to inhibit adenylyl cyclase activity only in the presence of GDPβS is interpreted to be due to the receptor-mediated release of GDPβS [31] and subsequent binding of Gpp(NH)p to G_i proteins. This finding indicates that activation of G_i proteins by stable GTP analogs follows commonly described mechanisms. It should be noted that complete reversal of GDPβS-stimulated adenylyl cyclase by Gpp(NH)p alone was not observed, most likely due to its action at both G_i and G_s proteins. However, further addition of carbachol resulted in a full reversal of GDPβS-stimulated adenylyl cyclase activity (Fig. 6). Hence the magnitude of the combined effect of Gpp(NH)p and carbachol exactly correspond to the effects of GDPβS, indicating that the mechanism of GDPβS stimulation of adenylyl cyclase activity is entirely due to the relief of inhibition by G_i proteins.

In conclusion, the data of the present study suggest that the stimulatory effect of GDPβS, and most likely also of GDP, on adenylyl cyclase activity in canine cardiac sarcolemmal membranes is due to an inhibitory interaction at G_i proteins, resulting in a relief of the inhibitory effect of these G proteins on adenylyl cyclase activity. This inhibition seems to be caused by guanine nucleotide-free G_i proteins. Following 'inactivation' by GDPβS, G_i proteins can apparently be reactivated by a GTP analog and by agonist-activated muscarinic acetylcholine receptors.

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