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5'-Guanylylimidodiphosphate-Activated Adenylate Cyclase of Cardiac Sarcolemma Displays Higher Affinity for Magnesium Ions

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SUMMARY

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Activation of heart sarcolemmal adenylate cyclase by 5'-guanylylimidodiphosphate [Gpp(NH)p] resulted in enhanced affinity of the enzyme for Mg^{2+}. The nucleotide increased the apparent affinity of adenylate cyclase for Mg^{2+} 3-fold in the absence of isoproterenol, and 5-fold in its presence. At various Mg^{2+} concentrations, the nucleotide (with and without isoproterenol) did not alter the apparent K_m (0.10 ± 0.02 mM) for ATP. Activation of adenylate cyclase by Gpp(NH)p showed temperature dependence. The nucleotide failed to activate the enzyme below 30° but enhanced enzyme activity 3-7-fold between 30° and 37°. The synergistic influence of isoproterenol and nucleotide resulted in 7-12-fold higher enzyme activity, depending on the Mg^{2+} concentration. Previous incubation of sarcolemma with or without isoproterenol in the absence of Gpp(NH)p above 15° led to a rapid decline in enzyme activity, whereas prior incubation in the presence of Gpp(NH)p not only prevented the decay but also enhanced both basal and isoproterenol-sensitive enzyme activity; the degree of activation depended on the prior incubation temperature. Treatment of sarcolemmal membranes with phospholipase A markedly reduced basal enzyme activity and activity in the presence of NaF, Gpp(NH)p, and Gpp(NH)p plus isoproterenol. The impairment was more pronounced in the case of enzyme activity stimulated by NaF and by Gpp(NH)p plus isoproterenol. Adenylate cyclase stimulated by NaF or by Gpp(NH)p in the presence of isoproterenol was relatively more resistant to phospholipase C treatment. Exposure of sarcolemmal membranes to extremely low concentrations of trypsin (ratio of trypsin to sarcolemmal protein, 0.0008-0.001) significantly enhanced basal and Gpp(NH)p-stimulated enzyme activity, but treatment with higher concentrations of trypsin (ratio of trypsin to sarcolemmal protein, 0.008-0.04) resulted in complete loss of enzyme activity. These observations suggest that activation of adenylate cyclase by Gpp(NH)p involves a conformational change in the enzyme that results from the interaction with Gpp(NH)p, presumably at the nucleotide binding site. Isoproterenol facilitates the nucleotide-induced transition in the enzyme. The conformational change is temperature-dependent. An altered enzyme configuration displays increased affinity for Mg^{2+} at the metal binding site.

INTRODUCTION

Recent studies in our laboratory (1) have

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shown that adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in highly purified heart sarcolemmal membranes is modestly sensitive to catecholamines and that the *beta* adrenergic amine sensitivity is markedly enhanced by the guanyl nucleotides GTP and 5'-guanylylimidodiphosphate, a nucleotide phosphohydrolase-resistant analogue of GTP (2). These observations are consistent with numerous studies implicating an obligatory if not essential role for guanyl nucleotides in the modulation of adenylate cyclase activity in a variety of tissues, including cardiac muscle (3). Gpp(NH)p[3] has been found to activate adenylate cyclase in several eukaryotic cells irrespective of the hormone receptors coupled to these systems, or even in the absence of functionally coupled receptors (4–8). Presumably, both GTP and Gpp(NH)p act by binding to a common site on adenylate cyclase termed the "nucleotide regulatory site," which apparently is distinct from the catalytic site. The exact mechanism(s) by which guanyl nucleotides exert a regulatory influence on the hormonal sensitivity of adenylate cyclase is not yet clearly understood, although several recent reports have emphasized the complex nature of molecular events involved in the process (9–11). We report here several aspects of the interaction between Gpp(NH)p and sarcolemmal adenylate cyclase in the presence and absence of a *beta* adrenergic agonist, isoproterenol. The influence of temperature and of membrane lipid- and protein-perturbing agents on these interactions is also described. A preliminary report of some of these findings has appeared (12).

METHODS

Preparation of cardiac sarcolemmal membranes. Highly purified sarcolemmal membranes were prepared from heart ventricular tissue of female guinea pigs (280–400 g) following a method recently developed in this laboratory (13, 14).

[3] The abbreviations used are: Gpp(NH)p, 5'-guanylylimidodiphosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

Determination of adenylate cyclase activity. Sarcolemmal membranes (30–60 μg of protein) were incubated at 30° in the "standard" adenylate cyclase reaction mixture (0.15 ml), containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 8 mM theophylline, 20 mM creatine phosphate, 50 μg of creatine kinase, 1 mM cyclic 3',5'-AMP, and 0.2 mM [α-^{32}P]ATP (20–30 cpm/pmole). The reaction was started by the addition of labeled substrate to the assay tubes following preliminary incubation at 30° for 5 min with the rest of the assay components. Gpp(NH)p and/or isoproterenol,[4] when added, were present during the preliminary incubation period. The reaction was stopped by placing the tubes in a boiling water bath for 3 min after the addition of 0.02 ml of 0.5 M sodium acetate (pH 4). The remaining procedure was the same as described earlier by Sulakhe *et al.* (15). Changes made in the assay protocol are specified in the legends to individual figures.

It was established in several experiments that under these assay conditions the rate of cyclic AMP formation was linear with respect to time of incubation. The preliminary incubation (5 min, 30°) prior to addition of substrate effectively abolished a short lag period (2–3 min) that was otherwise observed before the onset of stimulation of adenylate cyclase by Gpp(NH)p. The kinetic data reported here refer to the linear reaction rate. The data were taken from a representative experiment, and similar findings were obtained in at least three experiments using separate membrane preparations.

Determination of membrane protein. Protein was determined by the method of Lowry *et al.* (16), with bovine serum albumin as standard.

Materials. [α-^{32}P]ATP (10–17 Ci/mmole) was purchased from New England Nuclear, Montreal. Unlabeled ATP, cyclic AMP, creatine phosphate, creatine kinase, theophylline, trypsin, trypsin inhibitor, snake venon (*Naja naja*), phospholipase C, DTT, alumina, bovine serum albumin, and DL-isoproterenol were obtained from

[4] Solutions of isoproterenol and creatine kinase were made just prior to their use.

Sigma Chemical Company; 5'-guanylylimidodiphosphate, from ICN Pharmaceuticals; and disposable columns (0.7 × 4 cm), from Bio-Rad. All other reagents were of the highest purity available from British Drug Houses, Montreal, or P-L Biochemicals, Milwaukee. Solutions were prepared, and all glassware and plastic ware were thoroughly rinsed, with deionized, glass-distilled water.

RESULTS

Influence of Gpp(NH)p added to adenylate cyclase reaction mixture on apparent affinity of enzyme for Mg^{2+}, Mn^{2+}, and ATP. In several experiments, adenylate cyclase activity was determined as a function of $MgCl_2$ concentration (0.25–50 mM) in the absence and presence of Gpp(NH)p (10 μM) and/or isoproterenol (50 μM) in the assay medium. It was observed that stimulation of enzyme activity by Gpp(NH)p in the presence and absence of isoproterenol was relatively higher at lower concentrations of $MgCl_2$ (not shown). For example, at 1 mM $MgCl_2$ (ATP, 0.19 mM) Gpp(NH)p-stimulated activity was 3–4 times higher than the basal (unstimulated) level, whereas at higher concentrations of $MgCl_2$ (7.5, 10, 15, and 50 mM) the increase was 2-fold or less. In the presence of isoproterenol the nucleotide increased the enzyme activity 6–9-fold at 1 mM $MgCl_2$ and about 3-fold at higher $MgCl_2$ concentrations. Qualitatively similar findings were noted with a higher (1 mM) ATP concentration. These results suggested that Gpp(NH)p, in both the absence and presence of isoproterenol, caused an increase in the apparent affinity of the enzyme for Mg^{2+}. In these experiments total ATP was held constant whereas total $MgCl_2$ was varied in the assay medium. This situation complicated the interpretation of the data, since concentrations of magnesium (free as well as that complexed with ATP) and of free ATP were being varied simultaneously. We therefore decided to study the dependence of adenylate cyclase on free Mg^{2+} at fixed $[MgATP]^{2-}$ concentrations. The apparent affinity of the basal enzyme for $[MgATP]^{2-}$ was found to be 0.1 ± 0.02 mM (mean ± standard error of three experiments) when the free Mg^{2+} concentration was either 2.5 or 10 mM. Furthermore, Gpp(NH)p (10 μM) in the absence and presence of isoproterenol (50 μM) failed to produce any significant effect on the apparent K_m for $[MgATP]^{2-}$ (results not shown).

Following activation by Gpp(NH)p and isoproterenol, the enzyme displayed an altered dependence on Mg^{2+} concentration at both 0.2 and 1.0 mM $[MgATP]^{2-}$ (Fig. 1A). The concentrations of Mg^{2+} required for half-maximal velocity are shown in Fig. 1B. Using 2 × K_m and 10 × K_m concentrations of $[MgATP]^{2-}$, the half-maximal velocity of the unstimulated reaction occurred at 3.1 mM Mg^{2+}, whereas the activated enzyme required 0.92 and 0.38 mM Mg^{2+}, respectively, for half-maximal velocity. At either substrate concentration, Mg^{2+} increased the V_{max} of the reaction with the unstimulated as well as the activated enzyme. The basal enzyme was saturated between 10 and 15 mM Mg^{2+}, whereas the activated enzyme was saturated between 2 and 4 mM and between 6 and 8 mM Mg^{2+}, when the $[MgATP]^{2-}$ concentrations were 0.2 and 1.0 mM, respectively.

Gpp(NH)p in the absence of isoproterenol also caused a similar qualitative change in the dependence of the enzyme on Mg^{2+} (Fig. 2). The effect of varying the concentration of Gpp(NH)p in the absence and presence of isoproterenol (50 μM) is shown in the inset of Fig. 2. It is apparent from these data that Gpp(NH)p induced a change in the enzyme that was reflected in its altered dependence on Mg^{2+} as well as in the V_{max}. The results shown in Figs. 1 and 2 were obtained when the reaction was carried out at 37°. However, similar results were observed when the reaction was carried out at 30° (not shown). That the degree of activation of the enzyme (evidenced by the V_{max}) and the requirement for Mg^{2+} (at half-maximal velocity) are inversely related to each other is suggested by the data shown in the inset of Fig. 2.

The nucleotide-induced positive shift in the affinity of adenylate cyclase for Mg^{2+} and its further enhancement by isoproterenol were also observed when the rate of

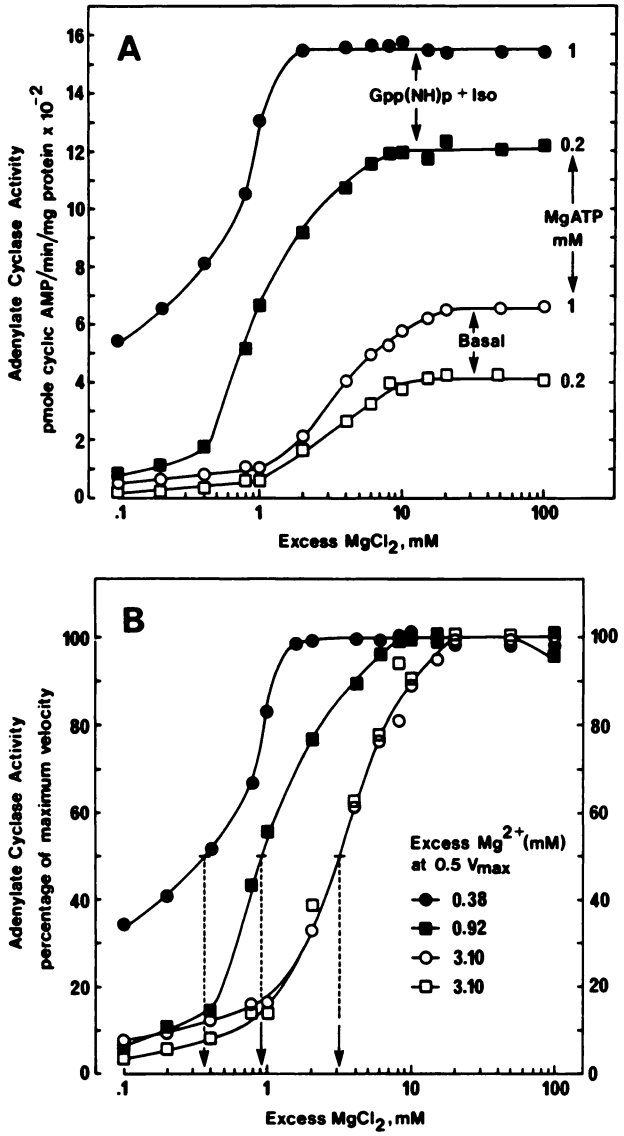

FIG. 1. *Dependence of sarcolemmal adenylate cyclase on free magnesium ions*

Adenylate cyclase activity was determined under standard assay conditions (see METHODS), except that incubation prior to addition of substrate was carried out for 10 min and the assays were conducted at 37°. The concentrations of $[MgATP]^{2-}$ and free Mg^{2+} are indicated in the figure. When present, Gpp(NH)p was 10 μM and isoproterenol (IsO) was 50 μM; under these conditions, maximal stimulation of the cyclase was observed (also see inset of Fig. 2). A. Plot of velocity as a function of excess (free) $MgCl_2$ concentration. B. Plot of percentage of maximum velocity as a function of excess $MgCl_2$ concentration. ○ and □, basal activity; ● and ■, Gpp(NH)p- plus isoproterenol-stimulated activity. Enzyme assays were also carried out using 0.6 mM $[MgATP]^{2-}$ under these conditions, and the results obtained were essentially similar (not shown). The apparent affinity of the cyclase for $[MgATP]^{2-}$ was found to be 0.10 ± 0.02 mM; hence the experimental V_{max} values obtained at 0.2 mM $[MgATP]^{2-}$ and 1 mM $[MgATP]^{2-}$ represent 66.6% and 90.9%, respectively, of the theoretical V_{max}. In Fig. 1B the respective experimental V_{max} values were set to represent 100%. In this way, the concentration of free Mg^{2+} needed at half-maximal velocity as well as at saturation was evaluated for basal and fully activated enzyme. Essentially similar data were obtained when assays were performed at 30° (not shown).

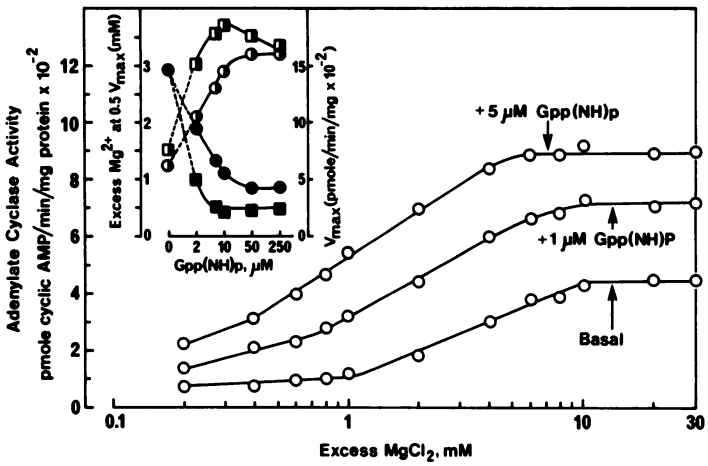

FIG. 2. *Influence of varying concentrations of Gpp(NH)p on dependence of sarcolemmal adenylate cyclase on free magnesium ions*

Adenylate cyclase activity was determined at 37° as described in the legend to Fig. 1 either in the absence of Gpp(NH)p, with various concentrations of Gpp(NH)p, or with various concentrations of Gpp(NH)p plus 50 μM isoproterenol. $[MgATP]^{2-}$ was held constant at 1 mM. The concentrations of Gpp(NH)p and free $MgCl_2$ were varied as shown. The main figure is a plot of velocity as a function of free $MgCl_2$ concentration in the absence of Gpp(NH)p (basal) and at two fixed concentrations of Gpp(NH)p. The inset shows maximal velocity (right ordinate) at different concentrations of Gpp(NH)p in the absence (◑) as well as in the presence (◨) of isoproterenol. Also shown in the inset is the requirement of excess (free) Mg^{2+} (left ordinate) for half-maximal velocity at different concentrations of Gpp(NH)p in the absence (●) and presence (■) of isoproterenol.

cyclic AMP formation was measured at various Mg^{2+} concentrations and with different $[MgATP]^{2-}$ concentrations in the adenylate cyclase assay medium (not shown). In these experiments, isoproterenol alone caused only a 30–40% increase in the basal enzyme activity, in agreement with our previous work (1).

Mn^{2+}, at very low concentrations (0.25–1 mM), is known to be a more potent activator of adenylate cyclase than is Mg^{2+} (17). When Mn^{2+} was substituted for Mg^{2+}, qualitatively similar findings were obtained concerning the effect of Gpp(NH)p. In this case the apparent affinity of the basal enzyme for Mn^{2+} was 0.7 ± 0.1 mM, and in the presence of Gpp(NH)p and isoproterenol the apparent K_a was decreased to 0.2 ± 0.05 mM.

Effect of varying concentrations of Gpp(NH)p on adenylate cyclase activity. In another set of experiments adenylate cyclase activity, with and without isoproterenol (50 μM), was measured as a function of various concentrations (0.001–100 μM) of Gpp(NH)p in the assay medium. At lower concentrations (below 0.1 μM) the nucleotide (with and without isoproterenol) did not stimulate adenylate cyclase activity (not shown). In fact, modest inhibition (15–20%) of basal enzyme activity was noticed, an observation consistent with our previously reported findings (18). Between 0.1 and 100 μM, Gpp(NH)p enhanced both basal enzyme activity and the activity in the presence of isoproterenol. Half-maximal stimulation of enzyme activity was observed with 2 μM Gpp(NH)p in the absence of isoproterenol and with 0.5 μM Gpp(NH)p in the presence of isoproterenol (not shown).[5] This was seen at three different concentrations (1, 2.5, and 10 mM) of $MgCl_2$ in the assay.

Influence of incubation temperature on stimulation of adenylate cyclase by

[5] In other experiments, it was noted that 50 μM isoproterenol caused maximal stimulation of the enzyme in the absence of Gpp(NH)p. Since the extent of stimulation by isoproterenol without Gpp(NH)p was only moderate (up to 40%), the affinity of the receptor-enzyme complex for isoproterenol could not be reliably assessed.

Gpp(NH)p. In these experiments, stimulation of adenylate cyclase by Gpp(NH)p with and without isoproterenol was studied as a function of incubation temperature. The experiments were performed with two different $MgCl_2$ concentrations. The stimulation of adenylate cyclase by Gpp(NH)p in the presence and absence of isoproterenol displayed significant temperature dependence (Fig. 3). Gpp(NH)p alone failed to activate the enzyme below 30° but increased the activity 3–7-fold between 30° and 37° when the $MgCl_2$ concentration was 2.5 mM (Fig. 3, left). At a higher $MgCl_2$ concentration (10 mM) in the assay, the nucleotide produced modest inhibition of enzyme activity at low temperatures (0–24°) (Fig. 3, right), whereas between 30° and 40° the nucleotide stimulated the enzyme activity. When both Gpp(NH)p and isoproterenol were present, stimulation of the enzyme activity was detectable at 15°. With 2.5 mM $MgCl_2$ in the assay (Fig. 3, left), the nucleotide and isoproterenol together augmented the enzyme activity 12-fold at 37°, but only 7-fold with 10 mM $MgCl_2$ (Fig. 3, right). To a large extent, the difference in stimulation at the two $MgCl_2$ concentrations was due to the differential response to the cation of the basal and the nucleotide (with and without isoproterenol)-stimulated enzyme.

Adenylate cyclase activity following incubation of sarcolemmal membranes at various temperatures with and without Gpp(NH)p and/or isoproterenol. In a series of experiments, sarcolemmal membranes were incubated for 10 min at different temperatures with and without Gpp(NH)p and/or isoproterenol. This preliminary incubation was carried out in the absence as well as the presence of $MgCl_2$ (5 mM). The incubated membranes were then washed to remove free nucleotide and isoproterenol and were assayed for enzyme activity at 30° in the absence of Gpp(NH)p and isoproterenol. As shown in Fig. 4, the adenylate cyclase activity of sarcolemmal membranes previously incu-

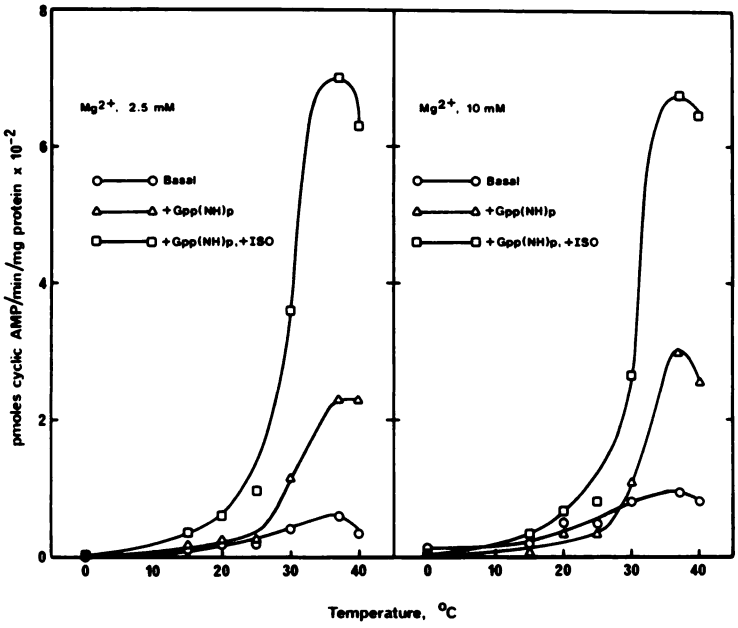

FIG. 3. *Effect of incubation temperature on stimulation of sarcolemmal adenylate cyclase by Gpp(NH)p*

Sarcolemmal membranes (33 μg of protein) were incubated in the standard adenylate cyclase reaction mixture at various temperatures with two fixed $MgCl_2$ concentrations as indicated. The reaction was started by the addition of [α-^{32}P]ATP (final concentration, 0.21 mM) after a 5-min preliminary incubation and was terminated 10 min following the addition of substrate. The concentrations of Gpp(NH)p and isoproterenol (ISO), when present, were 2 μM and 50 μM, respectively.

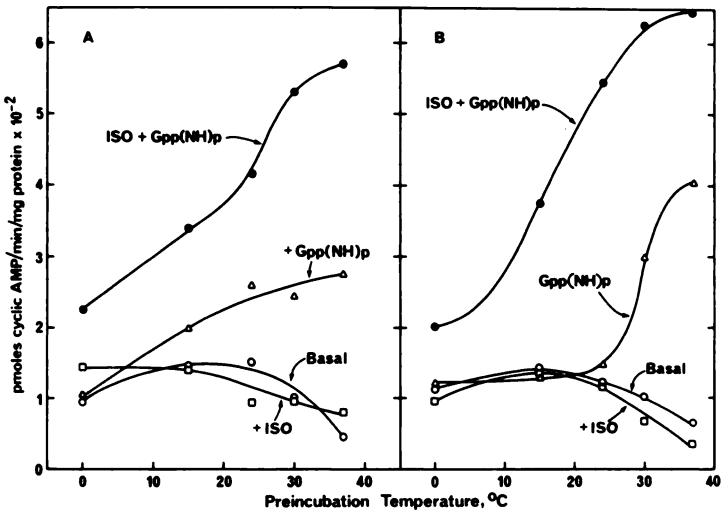

FIG. 4. *Adenylate cyclase activity in sarcolemmal membranes previously incubated at different temperatures in the presence and absence of Gpp(NH)p and/or isoproterenol*

Sarcolemmal membranes (200 μg of protein) were first incubated in 10 mM Tris-HCl (pH 7.5) containing 2 mM DTT and 10% sucrose (sucrose-TD buffer) for 10 min at different temperatures as indicated. This preliminary incubation was carried out with and without 10 μM Gpp(NH)p and/or 50 μM isoproterenol (ISO) in the absence (A) as well as the presence (B) of $MgCl_2$ (5 mM). The volume of the preliminary incubation mixture was 0.3 ml. Then the tubes were chilled on ice and centrifuged at 40,000 × *g* for 15 min to obtain the membrane pellet. The pellets were washed by resuspension in sucrose-TD buffer and recentrifugation, and were finally suspended in 0.2 ml of sucrose-TD buffer. Adenylate cyclase activity was determined at 30° under standard assay conditions (see METHODS) with 0.82 mM [α-^{32}P]ATP in the absence of Gpp(NH)p and isoproterenol.

bated at temperatures above 15° with and without isoproterenol decreased progressively with increasing preliminary incubation temperature whether the preliminary incubation system contained $MgCl_2$ or not. After preliminary incubation at 37° in the absence and presence of isoproterenol, the enzyme activity declined by 40–60%. Although a noticeable increase in activity occurred when preliminary incubation with Gpp(NH)p was carried out at 15° in the absence of Mg^{2+}, activity was increased only at a higher temperature (24°) with Mg^{2+} in the preliminary incubation system. However, maximal activation of the enzyme by the nucleotide with and without isoproterenol was attained if the preliminary incubation medium (37°) contained Mg^{2+}.

From the above studies it was established that exposure of sarcolemmal membranes to Gpp(NH)p (with and without isoproterenol) at 30° or 37° leads to marked activation of membrane-bound adenylate cyclase. Such activation of the enzyme following incubation with Gpp(NH)p is consistent with our previous observations (1) and those of others (8, 10). It was of interest, therefore, to examine the properties of the enzyme following activation by Gpp(NH)p. The following studies were carried out using membrane preparations that had been incubated with and without Gpp(NH)p and/or isoproterenol at 30° for 10 min and then washed extensively.

Apparent affinity of adenylate cyclase for Mg^{2+} following activation by Gpp(NH)p. In a series of experiments adenylate cyclase activity was determined as a function of $MgCl_2$ concentration (0.25–10 mM) using sarcolemmal membranes that had first been incubated at 30° for 10 min in the absence and presence of Gpp(NH)p (10 μM) and/or isoproterenol (50 μM). During the subsequent incubation Gpp(NH)p and isoproterenol were absent from the reaction mixture. The stimulation of enzyme activity following activation by

Gpp(NH)p (with and without isoproterenol) was markedly higher at lower $MgCl_2$ concentrations compared with nonactivated membrane preparations (not shown). Analysis of the data by a Lineweaver-Burk plot showed that prior activation of adenylate cyclase by Gpp(NH)p (with and without isoproterenol) resulted in enhanced affinity of the enzyme for Mg^{2+}. Data from a typical experiment are shown in Fig. 5. It can be seen that Gpp(NH)p, in the process of activating adenylate cyclase, enhanced the affinity of the enzyme for Mg^{2+} 3-fold in the absence of isoproterenol and 5-fold in its presence. Prior incubation with Gpp(NH)p increased the V_{max} 28% in the absence of isoproterenol and more than 200% in the presence of the *beta* adrenergic agonist. These results are similar to those obtained when the nucleotide was present in the adenylate cyclase reaction mixture (Figs. 1 and 2).

Effects of phospholipase A and phospholipase C treatment of sarcolemma on stimulation of adenylate cyclase by Gpp(NH)p. Since membrane lipids are known to play an important role in the process by which hormones stimulate adenylate cyclase (19–21), it was of interest to ascertain whether treatment of membranes with lipid-perturbing agents would affect Gpp(NH)p activation of adenylate cyclase. Sarcolemmal membranes were treated with various concentrations of phospholipases A and C, and the membranes were pelleted by centrifugation, washed, and then assayed for enzyme activity in the absence and presence of Gpp(NH)p and/or isoproterenol. Enzyme activity was also measured in the

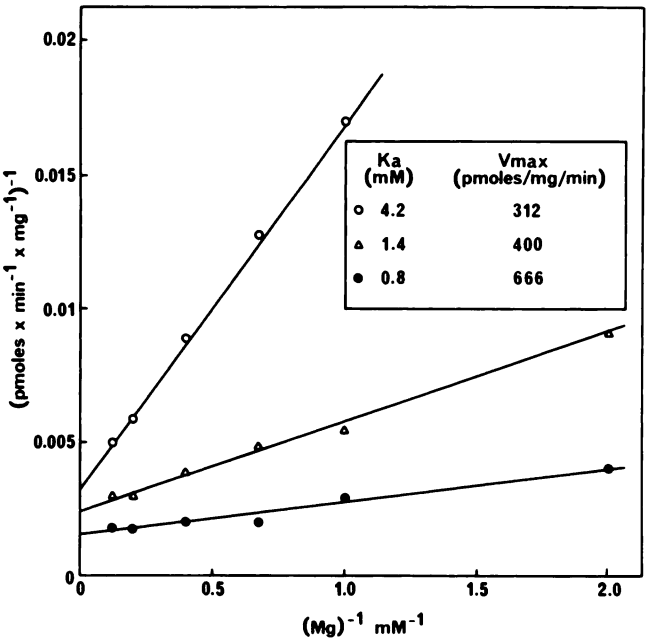

FIG. 5. *Adenylate cyclase activity in Gpp(NH)p-activated and nonactivated sarcolemmal membranes: Lineweaver-Burk plot of enzyme activity as a function of $MgCl_2$ concentration*

Sarcolemmal membranes (600 μg of protein) were first incubated in 10 mM Tris-HCl buffer (pH 7.5) containing 2 mM DTT and 10% sucrose (sucrose-TD buffer) at 30° for 10 min in the presence and absence of 10 μM Gpp(NH)p and/or 50 μM isoproterenol. The volume of this preliminary incubation mixture was 0.3 ml. Then the tubes were chilled on ice, and the membranes were collected after centrifugation and washing as described in the legend to Fig. 4. Adenylate cyclase activity was determined at 30° under standard assay conditions (see METHODS) with various concentrations (0.5–10 mM) of $MgCl_2$ and 0.16 mM [α-^{32}P]ATP in the absence of Gpp(NH)p and isoproterenol. ○, previously incubated without Gpp(NH)p and isoproterenol; △, previously incubated with Gpp(NH)p; ●, previously incubated with Gpp(NH)p and isoproterenol.

presence and absence of NaF. The results are shown in Fig. 6. Phospholipase A treatment resulted in marked reduction of adenylate cyclase activity. Basal, Gpp(NH)p-stimulated, and Gpp(NH)p- plus isoproterenol-stimulated activity declined progressively with increasing concentrations of phospholipase A. At all concentrations employed, the reduction in Gpp(NH)p- plus isoproterenol-stimulated activity was greater than the decrease in basal activity or in the activity stimulated with Gpp(NH)p alone. The extent of reduction in NaF-stimulated activity remained the same (80%) at all concentrations of phospholipase A. All forms of enzyme activity were relatively more resistant to phospholipase C than to phospholipase A treatment. Thus higher concentrations of phospholipase C, compared with phospholipase A, were needed to produce significant reductions in enzyme activity. At the highest concentration employed (ratio of phospholipase C to sarcolemmal protein, 0.2), both basal and Gpp(NH)p-stimulated activities were inhibited to the same extent (approximately 40%). Gpp(NH)p- plus isoproterenol-stimulated activity was reduced by as much as 30%, and NaF-stimulated activity was essentially unaffected by phospholipase C treatment of membranes.

Sarcolemmal membranes treated with phospholipase A and phospholipase C were

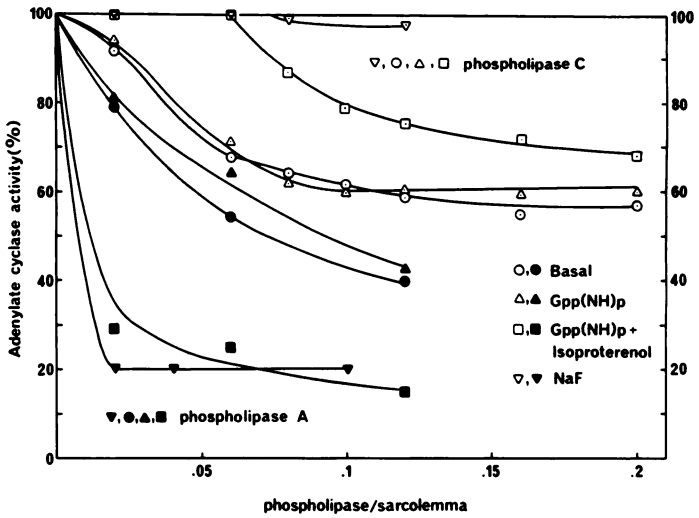

FIG. 6. *Effects of Gpp(NH)p and NaF on adenylate cyclase activity in phospholipase A- and phospholipase C-treated sarcolemma*

For phospholipase A treatment, sarcolemmal membranes (1 mg of protein) were incubated at 30° for 10 min in 10 mM Tris-HCl (pH 7.5) containing 2 mM DTT, 10% sucrose, 1 mM $CaCl_2$, and various concentrations of snake (*Naja naja*) venom (treated at 70° for 15 min to destroy proteinase activity). The reaction was stopped by the addition of EGTA (final concentration, 4 mM). The tubes were chilled on ice, and the membranes were collected by centrifugation and washing as described in the legend to Fig. 4. Sarcolemmal membranes used as a control were processed as above in the absence of snake venom. For phospholipase C treatment, sarcolemmal membranes (1 mg of protein) were incubated at 30° for 10 min in 10 mM Tris-HCl (pH 7.5) containing 2 mM DTT, 10% sucrose, 0.4 mM $CaCl_2$, and various concentrations of phospholipase C. The reaction was stopped by the addition of EGTA (final concentration, 3 mM), and the tubes were chilled on ice. Membranes were collected by centrifugation and washing as described in the legend to Fig. 4. Sarcolemmal membranes, processed as above in the absence of phospholipase C, were used as a control for this experiment. Adenylate cyclase activity was determined at 30° in the standard adenylate cyclase reaction mixture with 38 μg of membrane protein in the presence and absence of 5 mM NaF, 10 μM Gpp(NH)p, and/or 50 μM isoproterenol. Defatted albumin (200 μg) was added to the assay mixture in the case of phospholipase A-treated and the corresponding control membranes. The $MgCl_2$ concentration in the assay was 5 mM, and [α-^{32}P]ATP was 0.34 mM. The data represent percentage enzyme activity compared with control (untreated) membranes.

also used to determine enzyme activity as a function of $MgCl_2$ concentration in the absence and presence of Gpp(NH)p and/or isoproterenol. With phospholipase A-treated membranes, inhibition of enzyme activity [basal, Gpp(NH)p-stimulated, and Gpp(NH)p- plus isoproterenol-stimulated] was observed at all $MgCl_2$ concentrations (Fig. 7A). The impairment in enzyme activity appeared to be due solely to a decrease in V_{max}. Enzyme activation by Gpp(NH)p in the control membranes (with and without isoproterenol) was indeed accompanied by an enhanced affinity of adenylate cyclase for Mg^{2+}, as was observed in other experiments (Figs. 1 and 5).

No significant effect on Gpp(NH)p- plus isoproterenol-stimulated activity was ob-

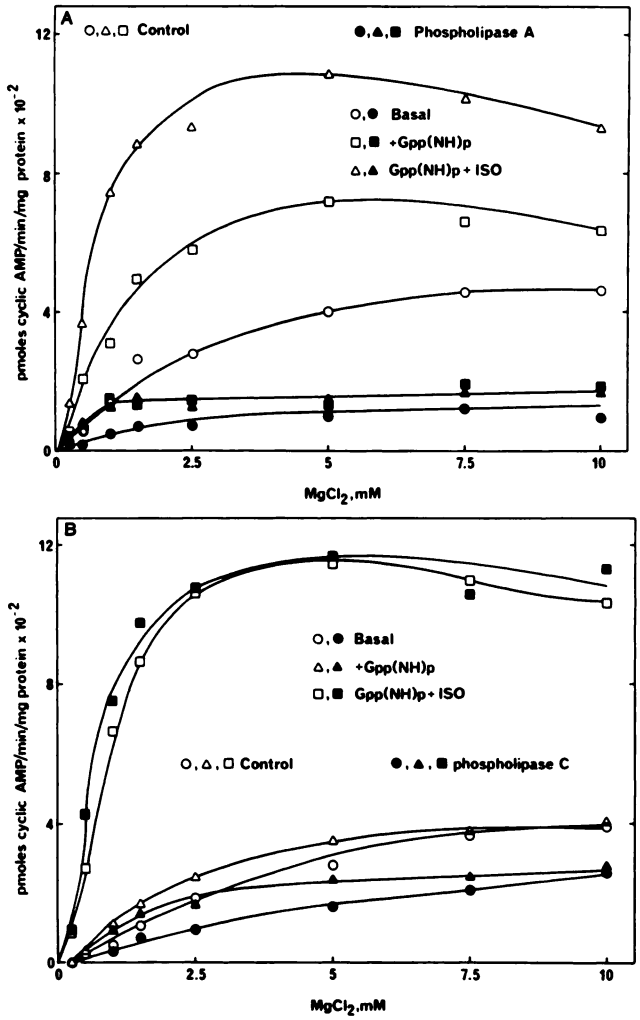

FIG. 7. *Adenylate cyclase activity in phospholipase A (A)- and phospholipase C (B)-treated sarcolemma: effects of Gpp(NH)p as a function of $MgCl_2$ concentration*

Phospholipase A- and phospholipase C-treated and control membranes were prepared as described in the legend to Fig. 6. For phospholipase A treatment, the ratio of phospholipase to sarcolemma was 0.1, and for phospholipase C treatment, the ratio of phospholipase to sarcolemma was 0.07. Adenylate cyclase activity was determined at 30° under standard assay conditions (see METHODS) with 38 μg of membrane protein, 0.32 mM [α-^{32}P]ATP, and various concentrations of $MgCl_2$ in the presence and absence of 10 μM Gpp(NH)p and/or 50 μM isoproterenol (ISO). Defatted albumin (200 μg) was added to the enzyme assay mixture in the case of phospholipase A-treated and the corresponding control membranes.

served when enzyme activity in phospholipase C-treated sarcolemma was measured as a function of $MgCl_2$ concentration (Fig. 7B). Both basal and Gpp(NH)p-stimulated activities were reduced 30–50% and 15–30%, respectively, at various $MgCl_2$ concentrations. The nucleotide-induced shift in the affinity for Mg^{2+} was seen in the phospholipase C-treated membranes.

Effect of trypsin treatment of sarcolemma on stimulation of adenylate cyclase by Gpp(NH)p. In a series of experiments, sarcolemmal membranes were exposed to various concentrations of trypsin, centrifuged, washed, and then assayed for adenylate cyclase activity in the presence and absence of Gpp(NH)p, isoproterenol, or NaF (Table 1). Treatment of membranes with very low concentrations of trypsin (1–2 μg) enhanced basal, Gpp(NH)p-stimulated, Gpp(NH)p- plus isoproterenol-stimulated, and NaF-stimulated enzyme activity to varying degrees. Membranes treated with 10 μg of trypsin showed negligible enzyme activity, while no enzyme activity was detectable in membranes treated with 50 μg of trypsin. In additional experiments, the increase in apparent affinity for Mg^{2+} induced by Gpp(NH)p with and without isoproterenol was observed following exposure of membranes to 2 μg of trypsin (not shown).

TABLE 1

Effect of trypsin treatment on adenylate cyclase activity

Sarcolemmal membranes (1.23 mg of protein) were incubated at 24° for 10 min in 10 mM Tris-HCl (pH 7.5) containing 2 mM DTT, 10% sucrose, 0.15 M KCl, and various concentrations of trypsin as indicated; the final volume of the reaction mixture was 1.3 ml. Following this, a 10-fold excess of trypsin inhibitor was added. The tubes were chilled on ice, and membranes were collected by centrifugation and washing as described in the legend to Fig. 4. Membranes carried through the entire procedure in the absence of trypsin served as the control. Adenylate cyclase activity was determined at 30° with 35 μg of membrane protein, 5 mM $MgCl_2$, and 0.34 mM [α-^{32}P]ATP in the absence and presence of either 10 μM Gpp(NH)p 5 mM NaF, or 10 μM Gpp(NH)p plus 50 μM isoproterenol.

Trypsin	Adenylate cyclase			
	Basal	+Gpp(NH)p	+Gpp(NH)p+isoproterenol	+NaF
μg	*pmoles/mg/min* (%)			
0	245 (100)	396 (100)	899 (100)	700 (100)
1	569 (232)	426 (107)	1090 (121)	812 (116)
2	584 (238)	639 (161)	1070 (119)	769 (110)
10	13 (5)	43 (10)	52 (5)	80 (11)
50	0	0	0	0

DISCUSSION

We have shown that sarcolemmal adenylate cyclase exhibits higher affinity for Mg^{2+} following activation by Gpp(NH)p in the presence and absence of isoproterenol. The enhanced affinity for Mg^{2+} seems to reflect alterations in the properties of a metal ion binding site of adenylate cyclase, since no change in the apparent affinity for the substrate, $MgATP^{2-}$, was noticed subsequent to activation of the enzyme by the nucleotide. Contrary to the present observations, Lefkowitz (5) did not detect changes in the affinity of adenylate cyclase for Mg^{2+} in a particulate preparation from canine myocardium when Gpp(NH)p was used as activator. However, Reiss and Katz (22) have recently reported greater stimulation by GTP of isoproterenol-sensitive adenylate cyclase in guinea pig heart sarcolemma, at lower rather than at higher Mg^{2+} concentrations, an observation analogous to the present findings. Similar results have also been reported for the human fat cell enzyme with Gpp(NH)p as activator (23). Our observations support the view that in cardiac sarcolemma activation of adenylate cyclase by guanyl nucleotide(s) involves a conformational change in the enzyme resulting from the interaction with the nucleotide. The observation that isoproterenol, in the presence of nucleotide, further increased the affinity for the cation would imply that the adrenergic agonist facilitates the nucleotide-induced transition. Such stepwise conformational changes may account for higher enzyme activity with Gpp(NH)p alone as well as for the more pronounced activation of the enzyme by nucleotide together with isoproterenol.

In this study maximal basal enzyme activity was obtained when Mg^{2+} concen-

trations were between 10 and 15 mM (Figs. 1 and 2). Recently Alvarez and Bruno (24) observed that 30 mM Mg^{2+} was required for maximal stimulation of the basal activity of the EDTA-treated enzyme preparation from guinea pig heart. In their study, higher concentrations (100 mM) of Mg^{2+} produced slight inhibition, and the level of maximal activity of the basal enzyme varied between 100 and 250 pmoles/min/mg of protein. With the sarcolemmal enzyme, under these conditions, the maximal activity represented 650–800 pmoles/min/mg of protein (see Fig. 1); note that the sarcolemmal enzyme was not treated with EDTA. Despite these differences, the data of Alvarez and Bruno (24) and those described in this study concerning the effect of Gpp(NH)p on the apparent affinity of the cardiac enzyme for Mg^{2+} are in good agreement. However, the results shown in Fig. 1B and Fig. 3A in the paper by Alvarez and Bruno (24) are in conflict. For example, when the MgATP concentration was 1 mM, the velocity of the Gpp(NH)p-activated enzyme was increased about 2-fold when the Mg^{2+} concentration was increased from 3 to 30 mM (Fig. 1B), whereas from the data shown in Fig. 3A it appeared to have decreased.

Alvarez and Bruno (24) suggested that Mg^{2+} interacts with the nucleotide (GTP) regulatory site of adenylate cyclase, whereas we have recently reported (25) that Mg^{2+} regulates binding of Gpp(NH)p at a low-affinity site(s) of heart sarcolemma and that low-affinity sites are likely to be involved in the stimulatory effect of Gpp(NH)p on adenylate cyclase. Taking into account the change in properties of the metal binding site of the cyclase following Gpp(NH)p activation, we proposed (25) that modulation of adenylate cyclase activity by Gpp(NH)p (or GTP) involves interaction between the nucleotide binding site and the metal (Mg^{2+}) binding site of adenylate cyclase.

The temperature dependence for activation of the enzyme by Gpp(NH)p observed in this study is in agreement with recent findings on adipose tissue (8, 23) and kidney (26) adenylate cyclase. These observations suggest that thermal activation is necessary for the nucleotide to effect the proposed conformational change. The appreciable activation of the enzyme detected at temperatures lower than 30° if isoproterenol was present together with the nucleotide implies that the amount of thermal energy needed for the conformational transition may be lower in the presence of hormone.[6] The decline in enzyme activity of membranes incubated above 15° with or without isoproterenol in the absence of Gpp(NH)p suggests that the enzyme is relatively more thermally labile prior to its conformational transition by the nucleotide. Thus the conformational change seems to confer some degree of functional stability on the enzyme at relatively higher temperatures. Recent findings of Lefkowitz and Caron (27) support this view.

Phospholipase A treatment of sarcolemma resulted in considerable inactivation of adenylate cyclase. The identical patterns of reduction in basal and Gpp(NH)p-stimulated activity following phospholipase A treatment may indicate that the nucleotide regulatory site resides close to the catalytic site. The membrane phospholipid components essential for optimal catalytic function in the basal and nucleotide-activated states of the enzyme may also be similar. Inactivation in the case of Gpp(NH)p- plus isoproterenol-sensitive activity may be ascribed to disruption of phospholipids involved in hormone-receptor interactions and in message transmission from the receptor to the catalytic site. The relatively higher degree of inactivation (resembling additive inhibition) compared with basal or Gpp(NH)p-stimulated activity may be construed to suggest that hormone-receptor interactions are communicated to the catalytic site through the nucleotide regulatory site. The extent of reduction in NaF-stimulated activity in phospholipase A-treated membranes was very similar to that ob-

[6] The following energies of activation (kilocalories per mole) were calculated: with 2.5 mM Mg^{2+} in the assay – basal, 12.3; Gpp(NH)p, 22.5; Gpp(NH)p plus isoproterenol, 26.0; with 10 mM Mg^{2+} in the assay – basal, 12.8; Gpp(NH)p, 22.6; Gpp(NH)p plus isoproterenol, 22.8.

served with Gpp(NH)p and isoproterenol, implicating a role for membrane phospholipids in NaF-mediated activation of adenylate cyclase. Enhancement or complete loss of NaF-stimulated activity, depending on the concentration of phospholipase A, has been described in the case of liver plasma membranes (28). Despite the possible differences in the modes of activation of adenylate cyclase by NaF and hormones (17) it seems likely, as suggested by Harwood and Rodbell (29), that NaF acts at some point on the pathway by which hormonal interaction with the receptor leads to an increase in the catalytic activity of adenylate cyclase. Recently it has also been suggested that stimulation of adenylate cyclase by NaF follows a change in membrane structure induced by NaF (30).

Adenylate cyclase activity in sarcolemma was found to be relatively more resistant to inactivation following phospholipase C treatment. Recent observations of Limbird and Lefkowitz (31) also suggest that higher concentrations of phospholipase C than of phospholipase A are needed to inhibit hormone binding as well as enzyme stimulation by *beta* adrenergic amines in frog erythrocyte membranes. In the present studies, concentrations of phospholipase C capable of inhibiting basal and Gpp(NH)p-stimulated activity failed to reduce Gpp(NH)p- plus isoproterenol-stimulated activity. This may imply that when coupled to hormone-receptor complexes the adenylate cyclase system of sarcolemma has a differential dependence on membrane lipid components. The finding that Gpp(NH)p elicited the change in the apparent affinity for Mg^{2+} even after phospholipase A and C treatments suggests that membrane lipids are not necessary for the proposed conformational change in the enzyme caused by Gpp(NH)p. Rather, membrane lipids are needed for optimal catalytic reactivity.

Although exposure of sarcolemmal membranes to very low concentrations of trypsin markedly increased both basal and Gpp(NH)p-stimulated activity, complete loss of enzyme activity occurred with the use of moderately high concentrations of this proteolytic agent.[7] The enhanced enzyme activity at lower concentrations of trypsin may have resulted either from the exposure of additional catalytic sites that otherwise lay buried in the membrane matrix or from increased accessibility of substrate.

In many studies the activation of the enzyme by Gpp(NH)p has been shown to be an irreversible process (8, 10, 34). Such observations place in question the physiological relevance of the use of Gpp(NH)p in studying regulation of adenylate cyclase. It is conceivable that the natural guanine nucleotide, GTP, interacts with the enzyme reversibly (8, 10). Sevilla *et al.* (11) have recently reported that *beta* adrenergic amines reverse the activation of adenylate cyclase by Gpp(NH)p.

Different models with varying features have been proposed to explain the molecular events governing the activation of adenylate cyclase by guanyl nucleotides and hormones (8–11, 35–37). The cardinal features of some of these models include inhibitory control of enzyme activity by a protonated species of substrate ($HATP^{3-}$) or free ATP (35, 37), a chemical reaction involving the formation of a "nucleotide-derived" enzyme-phosphoryl intermediate (8), and preferential binding of the nucleotide to different energetic states of the enzyme (9). These models were based mainly on experimental data obtained for the hepatic or fat cell enzyme and precluded structural modifications at the metal binding site. The interrelationships

[7] The sodium dodecyl sulfate (0.1%)-polyacrylamide (6%) gel electrophoretic profiles of the lipase-treated and the untreated membranes were identical. Exposure of sarcolemma to trypsin (ratio of trypsin to membrane protein, 1:571), however, was found to degrade two high molecular weight proteins (160,000 and 100,000). Of these, the 100,000 mol wt protein was identified as ATPase, based on phosphorylation experiments (*E*-P, acyl phosphate). The 160,000 mol wt protein is most likely the adenylate cyclase protein present in these membranes, a conclusion based on the recent observations of Johnson *et al.* (32) and those of Neer (33) on particulate adenylate cyclase from brain (32) and kidney (33) (P. J. St. Louis and P. V. Sulakhe, manuscript in preparation).

of Mg^{2+} and Gpp(NH)p described for the hepatic (38) and fat cell (3) enzymes are at variance with the observations on the cardiac enzyme described here and elsewhere (22) as well as on the human fat cell enzyme (23). Arguments against an enzyme-pyrophosphate intermediate have been advanced (34, 39). Furthermore, recent kinetic studies (32, 40) on detergent-dispersed hepatic and cerebellar adenylate cyclase are inconsistent with the mechanism involving inhibition by free ATP. On the other hand, certain evidence strongly suggests a distinct cation binding site on adenylate cyclase (32, 40–42). It is likely that Mg^{2+} interacts at this site by invoking a regulatory influence. It is of further interest that Mg^{2+} can modulate hormonal response, with Mg^{2+} ions affecting the coupling function (26). In view of these observations and the nucleotide-induced shift in the affinity for Mg^{2+} observed in our studies, it is suggested that activation of adenylate cyclase by guanyl nucleotides involves configurational changes affecting the metal binding site of adenylate cyclase.

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