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Thermodependence of Guanine Nucleotide-Activated Rat Cardiac Adenylate Cyclase Activity

Effect of Cholera Toxin Pretreatment

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SUMMARY

At 37°, maximal activation of rat cardiac adenylate cyclase with GTP and nonhydrolyzable GTP analogues decreased in the following order: guanosine 5′-*O*-(3-thio)triphosphate > guanosine 5′-*O*-(β-γ-imido)triphosphate [Gpp(NH)p] > guanosine 5′-*O*-(β-γ-methylene)triphosphate > GTP ≥ 0. The K_{act} values of these nucleotides were 0.3 μM, 1.1 μM, 1 μM, and 1 μM, respectively. At 25°, K_{act} values were the same but maximal adenylate cyclase activities were reduced by 50–60%. Cholera toxin pretreatment of cardiac membranes induced a marked increase in adenylate cyclase activity in the presence of GTP with no change in the characteristics of adenylate cyclase activation by GTP analogues. Stimulation by NaF was reduced by 40% in comparison to native membranes. Arrhenius plots of adenylate cyclase activity in native membranes were linear below 31°, the energy of activation (E_a) being 10.9 ± 0.6 kcal/mole under basal conditions or in presence of GTP and around 18 kcal/mole in the presence of the three nonhydrolyzable GTP analogues or of NaF. Similar data were obtained with cholera toxin-pretreated membranes with the exception of the E_a value of GTP-stimulated adenylate cyclase activity, which increased to 17.1 ± 0.7 kcal/mole. The addition of (±)-isoproterenol, glucagon, secretin, or VIP in the presence of either GTP or Gpp(NH)p increased enzyme activity, but E_a values were the same as those observed when using guanine nucleotides alone. These data suggest that the E_a value of adenylate cyclase activity was lower when the hydrolysis of GTP was possible in rat cardiac membranes (i.e., when the adenylate cyclase system was in dynamic equilibrium between active and inactive states) than when regulatory site(s) were occupied with a nonhydrolyzable GTP analogue, this lower E_a value in the presence of GTP reflecting a lower organization of the system.

INTRODUCTION

Adenylate cyclase is a multicomponent, membrane-bound enzyme (1) consisting of at least three functional entities: hormone receptor(s) located at the outer face of plasma membranes, and a catalytic unit (C) and a guanine nucleotide-regulatory protein (G) located at the inner face so that the activity of the system is regulated by hormones and by guanine nucleotides (2, 3). In addition, the interaction between the three entities is sensitive to the properties of the phospholipid bilayer, since an alteration in phospholipid composition and/or physicochemical properties by exogenous lipid (4–7) or by mutation (8) greatly influences the kinetics of adenylate cyclase when studied as a function of temperature.

The over-all activation sequence can be described by three temperature-dependent steps (3):

$$R^\circ + G \rightleftharpoons R^\circ G$$

$$R^\circ G + \text{guanine nucleotide} \rightleftharpoons R^\circ + G^*$$

$$G^* + C \rightleftharpoons G^* C$$

where R° is the hormone-receptor complex; G and G^* represent the guanine nucleotide-regulatory protein in, respectively, ground and activated states; and C is the catalytic unit. In the present paper, we investigated the temperature dependence of the last step, i.e., the interaction between the two components located at the inner face of rat cardiac membranes, the guanine nucleotide regulatory protein G being activated by GTP or by nonhydrolyzable GTP analogues, before and after chol-

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era toxin pretreatment or in the presence of the peptide hormones glucagon and secretin or the *beta*-adrenergic agonist (±)-isoproterenol.

EXPERIMENTAL PROCEDURES

Chemicals

GTPγS,[3] Gpp(NH)p, and Gpp(CH_2)p were purchased from Boehringer (Mannheim, Federal Republic of Germany). (±)-Isoproterenol, GTP, and ATP Grade 1 were obtained from Sigma Chemical Company (St. Louis, Mo.). Cholera toxin was from Schwarz/Mann (Orangeburg, N. Y.). Natural porcine secretin was prepared by Dr. V. Mutt (Karolinska Institutet, Stockholm, Sweden); porcine glucagon was provided by Novo Industri (Ets. Couvreur, Brussels, Belgium). [α-^{32}P]ATP and cyclic [8-^{3}H]AMP, obtained from the Radiochemical Centre (Amersham, Bucks., England), had specific radioactivities of 10–20 Ci/mmole and 27 Ci/mmole, respectively.

Methods

Preparation of cardiac membranes. The procedure of Snyder and Drummond (9), described for rabbit heart, was adapted to rat tissue with few modifications. The heart was dissected out, rinsed at room temperature with isotonic sodium chloride, and minced with scissors. All subsequent operations were performed at 4°. The tissue was suspended in 20 ml of 20 mM Tris-HCl containing 2 mM dithioerythritol and 5 mM $MgCl_2$ (pH 7.5), and homogenized with a glass-Teflon homogenizer. After a 2-fold dilution with the same buffer, the homogenate was filtered through two layers of medical gauze and centrifuged at 520 × *g* for 10 min. The pelleted membranes were washed once with 40 ml of the homogenization buffer. The resulting pellet was resuspended in 20 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 5 mM $MgCl_2$. An equal volume of 20 mM Tris-HCl buffer (pH 7.5) enriched with 0.25 M sucrose, 2 mM dithioerythritol, and 2.5 M KCl was added dropwise. The final mixture was stirred for 1 hr in a cold room. The final suspension was centrifuged at 37,000 × *g* for 10 min, and the resulting pellet was washed three times with a 20 mM Tris-HCl buffer (pH 7.5) enriched with 0.25 M sucrose and 2 mM dithioerythritol. The membranes were finally resuspended in an adequate volume of the last buffer in order to obtain a concentration of approximately 6 mg of protein per milliliter. Protein was assayed by the method of Lowry *et al.* (10).

Adenylate cyclase assay. Adenylate cyclase activity was determined by the conversion of [α-^{32}P]ATP into cyclic [^{32}P]AMP. The standard assay medium contained the following final concentrations: 0.5 mM [α-^{32}P]ATP, 5 mM $MgCl_2$, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM cyclic AMP, 1 mM theophylline, 30 mM Tris-HCl, and an ATP-regenerating system that consisted of 10 mM phospho(enol)pyruvate and pyruvate kinase (30 μg/ml). The concentrations of further additions are mentioned in the legends to the figures. The pH of the final medium was adjusted to 7.4 at 37°. The kinetics of adenylate cyclase activity was determined as follows. Each glass tube contained 0.25 ml of the standard assay medium equilibrated at the desired temperature. The incubation was initiated by adding 0.05 ml of the membrane suspension (around 3 mg of protein per milliliter). After 2, 4, 6, 8, and 10 min, 0.05-ml aliquots of the incubation medium were removed and added to 0.5 ml of a 0.5% sodium dodecyl sulfate solution containing 1.5 mM ATP, 0.5 mM cyclic AMP, and cyclic [8-^{3}H]AMP (20,000 cpm in order to determine nucleotide recovery). Cyclic AMP was separated from ATP by two successive chromatographies according to the method of Salomon *et al.* (11). Under our experimental conditions, the kinetics was found to be linear and was not affected by the moderate increase in the pH of the incubation medium at lower temperatures (from 7.4 at 37° to 7.7 at 17°).

Cholera toxin pretreatment. The cholera toxin treatment developed for turkey erythrocyte membranes (12) was used as described in ref. 13. Cholera toxin (100 μg in 0.1 ml) was preactivated for 15 min at 37° in 4 ml of cytosol from human erythrocytes, and diluted 1:1.5 in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 0.6 mM dithioerythritol. After this preincubation, an equal volume of cardiac membranes (6 mg of protein per milliliter), suspended in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) containing 2 mM NAD^+, 4 mM ATP, 10 mM phospho(enol)pyruvate, pyruvate kinase (16 units/ml), and soybean trypsin inhibitor (0.05 mg/ml), was added. After incubation for 10 min at 30°, the reaction was stopped by centrifugation at 37,000 × *g* for 5 min at 2°. The pellet was rinsed out, resuspended in 10 ml of 20 mM Tris-HCl buffer (pH 7.5) enriched with 0.25 M sucrose and 2 mM dithioerythritol, and frozen. After thawing, the washing procedure was repeated twice in the presence of 2% bovine serum albumin and twice without bovine serum albumin. The washed membranes were finally homogenized in the last buffer at the final concentration of 6 mg of protein per milliliter. Control membranes underwent the complete procedure without cholera toxin addition.

RESULTS

The stimulation of rat cardiac adenylate cyclase with the *beta*-adrenergic agonist (±)-isoproterenol or with a polypeptide hormone (glucagon or secretin) acting through distinct receptors (14, 15) in the presence of either GTP or Gpp(NH)p provoked higher enzyme activity than that observed when the guanine nucleotide was used alone (Fig. 1). However, this combined stimulation was unable to modify the E_a observed with GTP alone (10.9 ± 0.6 kcal/mole below 31°) or with Gpp(NH)p alone (17.5 ± 0.8 kcal/mole) (Table 1; Fig. 1).

We focused our attention, therefore, on the guanine nucleotide-regulatory protein (*G*). Our preparations of KCl-pretreated rat cardiac membranes showed linear kinetics of adenylate cyclase stimulation with various guanine nucleotides for at least 10 min at both 37° (Fig. 2A) and 25° (Fig. 2B). Linearity of the enzyme stimulation was also observed in the presence of hormones or

[3] The abbreviations used are: GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; Gpp(NH)p, guanosine 5′-*O*-(β-γ-imido)triphosphate; Gpp(CH_2)p, guanosine 5′-*O*-(β-γ-methylene)triphosphate; E_a, energy of activation.

TABLE 1

E_a of basal, guanine nucleotide-, and fluoride-stimulated adenylate cyclase activity in rat cardiac membranes: effects of (±)-isoproterenol and of cholera toxin pretreatment

Arrhenius plots were determined at five temperatures (from 17° to 37°; see Fig. 5). Values are means ± standard error of the mean of three experiments.

Activator (at saturating concentration)	E_a	
	Control membranes	Cholera toxin-pretreated membranes
	kcal/mole	
Basal	10.9 ± 0.6[a]/0.5 ± 0.2[b]	10.8 ± 0.6[a]/0.5 ± 0.2[b]
30 μM GTP	10.9 ± 0.6[a]/0.5 ± 0.2[b]	17.1 ± 0.7
30 μM GTPγS	17.6 ± 0.7	18.0 ± 0.7
30 μM Gpp(NH)p	17.5 ± 0.7	19.0 ± 0.8
30 μM Gpp(CH_2)p	17.7 ± 0.8	18.3 ± 0.7
30 μM GTP + 10^{-5} M (±)-isoproterenol	11.0 ± 0.8	ND[c]
30 μM Gpp(NH)p + 10^{-5} M (±)-isoproterenol	17.6 ± 0.6	ND[c]
10 mM NaF	20.0 ± 0.8	19.2 ± 0.8

[a] Below 31°.
[b] Above 31°.
[c] Not determined.

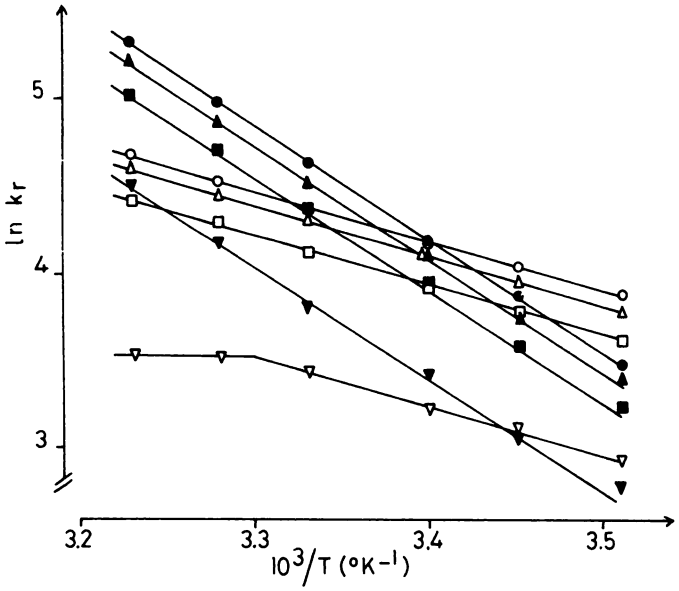

FIG. 1. *Temperature dependence of rat cardiac adenylate cyclase activity stimulated with 2 × 10^{-5} M GTP alone (▽), 1 × 10^{-5} M Gpp(NH)p alone (▼), and with 2 × 10^{-5} M GTP (open symbols) or 1.10^{-5} Gpp(NH)p (closed symbols) in the presence of 1 × 10^{-5} M isoproterenol (○, ●), 3 × 10^{-6} M glucagon (△, ▲), or 3 × 10^{-7} M secretin (□, ■)*

The results are representative of three separate experiments. In a few experiments at least 11 points were used to confirm the existence or absence of a break in the Arrhenius plots. The initial rate constant of cyclic AMP formation (k_r) was expressed as picomoles of cyclic AMP per minute per milligram of protein.

NaF and at all other temperatures tested (data not shown).

Dose-effect curves of activation at 37° (Fig. 3A) showed that maximal activations with GTPγS, Gpp(NH)p, and Gpp(CH_2)p corresponded to, respectively, 3.5-, 3-, and 2-fold increases in basal activity, on an average. The concentrations necessary for half-maximal activation of the cyclase (K_{act}) derived from dose-effect curves were 0.3, 1, and 1 μM with GTPγS, Gpp(NH)p, and Gpp(CH_2)p, respectively. GTP exerted only a slight effect, in agreement with previous reports (14, 16), the K_{act} value being approximately 1 μM (a value confirmed by competition experiments, when using GTPγS as activator; data not shown). These experiments, when repeated at 25°, gave similar K_{act} values (Table 2), but V_{max} values were decreased by 50–60% (Fig. 3B).

The apparent K_m for ATP-Mg^{2+} (0.18 ± 0.03 mM) of the catalytic unit of rat cardiac adenylate cyclase was the same at 37° and 25°, and was not influenced by the presence of guanine nucleotides (data not shown). On the basis of this preliminary evidence, we felt justified in interpreting our subsequent data on the thermodependence of adenylate cyclase activity, at fixed concentrations of guanine nucleotides and ATP, in terms of only variations in initial enzyme velocity.

Arrhenius plots of adenylate cyclase activity displayed a break at around 31° when tested under basal conditions or in the presence of 2.10^{-5} M GTP. The E_a was 10.5 kcal/mole below 31° and 0.5 kcal/mole above that temperature (Figs. 1 and 5A). By contrast, adding 10 mM NaF or the three nonhydrolyzable analogues of GTP, GTPγS, Gpp(NH)p, and Gpp(CH_2)p, at a saturating 30 μM concentration (Fig. 3) provoked a linearization of Arrhenius plots and a change in their slopes (Fig. 5A), yielding significantly higher E_a values (around 18 kcal/mole) (Table 1). Higher E_a values were also observed when (±)-isoproterenol, glucagon, or secretin was offered in combination with Gpp(NH)p (around 18 kcal/mole) rather than GTP (around 11 kcal/mole) (Table 1; Fig. 1).

To study further the difference in E_a values of adenylate cyclase activation with GTP and GTP analogues, rat cardiac membranes were pretreated with NAD^+ in the presence of cholera toxin in order to inhibit the GTPase activity supposedly associated with the guanine nucleotide-regulatory protein (*G*) (17, 18).

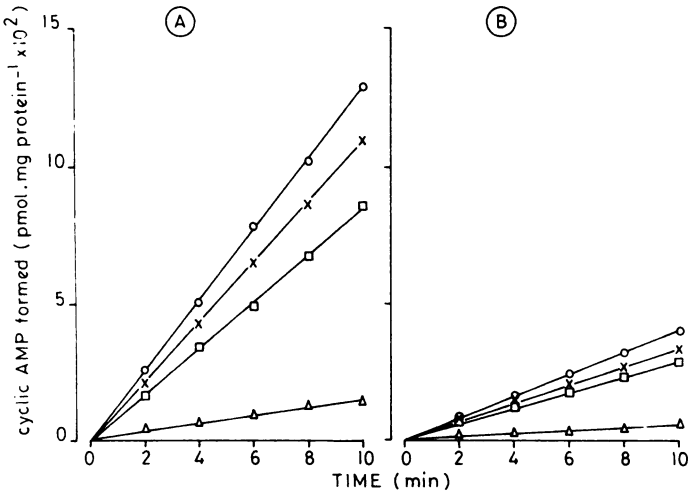

FIG. 2. *Time course of rat cardiac adenylate cyclase stimulation with 2 × 10^{-5} M GTP (△), 3 × 10^{-5} M Gpp(CH_2)p (□), 3 × 10^{-5} M Gpp(NH)p (×), and 3 × 10^{-5} M GTPγS (○) at 37° (A) and 25° (B)*

Assay conditions were as described under Experimental Procedures.

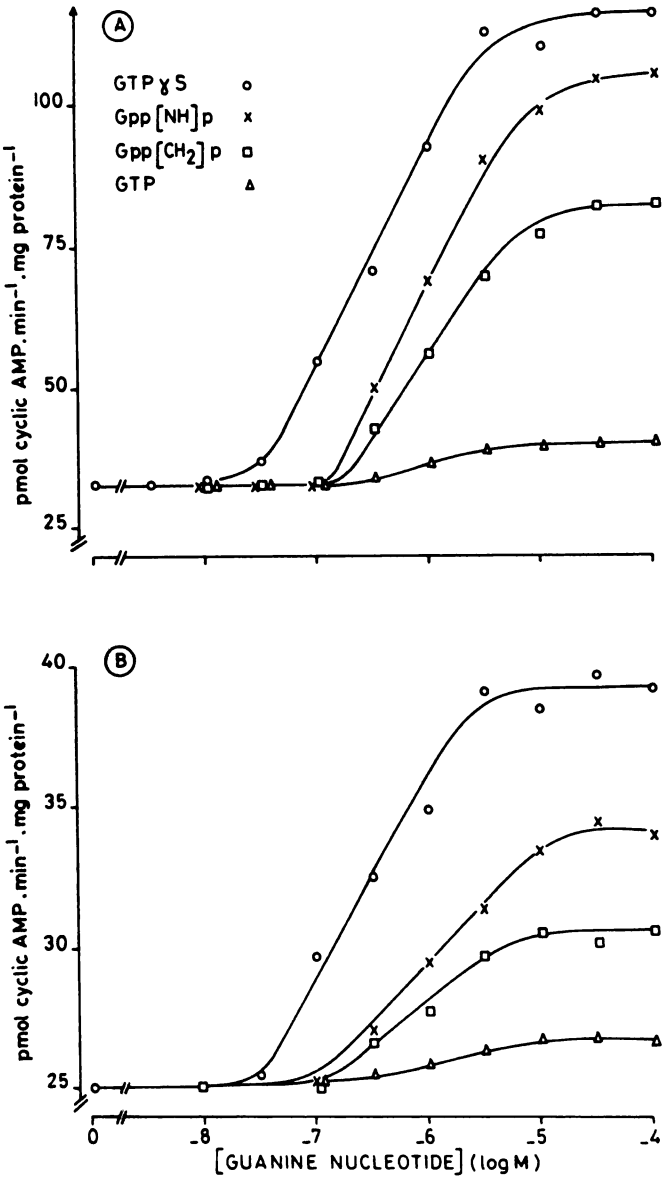

FIG. 3. *Dose-response curves of rat cardiac adenylate cyclase activation at 37° (A) and 25° (B) by GTPγS (○), Gpp(NH)p (×), Gpp(CH₂)p (□), and GTP (△)*

The results are representative of three separate experiments.

TABLE 2

K_{act} values for the activation of rat cardiac adenylate cyclase by four guanine nucleotides at 37° and 25°: effects of cholera toxin pretreatment

Results are the means ± standard error of the mean of three experiments

Activator	K_{act}			
	Control membranes		Cholera toxin-pretreated membranes	
	37°	25°	37°	25°
	μM		μM	
30 μM GTP	1.0 ± 0.2	ND[a]	0.8 ± 0.1	0.8 ± 0.2
30 μM GTPγS	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.2
30 μM Gpp(NH)p	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.2
30 μM Gpp(CH₂)p	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.3

[a] Activities were too low to be determined with precision.

The main effect of the cholera toxin pretreatment (Fig. 4) was to increase drastically (3- to 4-fold) the activity of adenylate cyclase in the presence of GTP, in line with an expected inhibition of GTPase activity. V_{max} at saturating GTP concentration was now the same as that obtained with GTPγS at the same concentration (compare Fig. 4 with Fig. 3A and B). In addition, K_{act} values with GTP were the same as those in control membranes and were identical at 25° and 37° (Table 2). K_{act} and V_{max} values for the nonhydrolyzable guanine nucleotides were also unchanged. The activity of adenylate cyclase stimulated with 10 mM NaF was reduced in cholera toxin-pretreated membranes: 160 pmoles of cyclic AMP produced per minute per milligram of protein as compared with 252 pmoles of cyclic AMP produced per minute per milligram of protein in control membranes.

In cholera toxin-pretreated membranes, the effects of temperature (Fig. 5A and B) on basal and nonhydrolyzable guanine nucleotide-stimulated adenylate cyclase activity were not modified but Arrhenius plots were now linear in the presence of GTP, with an E_a value of 18 kcal/mole (Table 1), comparable to that obtained with nonhydrolyzable guanine nucleotide analogues in both cholera toxin-pretreated and native membranes.

DISCUSSION

Rat cardiac adenylate cyclase activity exhibited a break in Arrhenius plots at around 31° when tested in the absence or presence of added GTP, so that the energy of activation was 0.5 kcal/mole above 31° and 10.9 kcal/mole below 31° (Table 1). Similar data have been re-

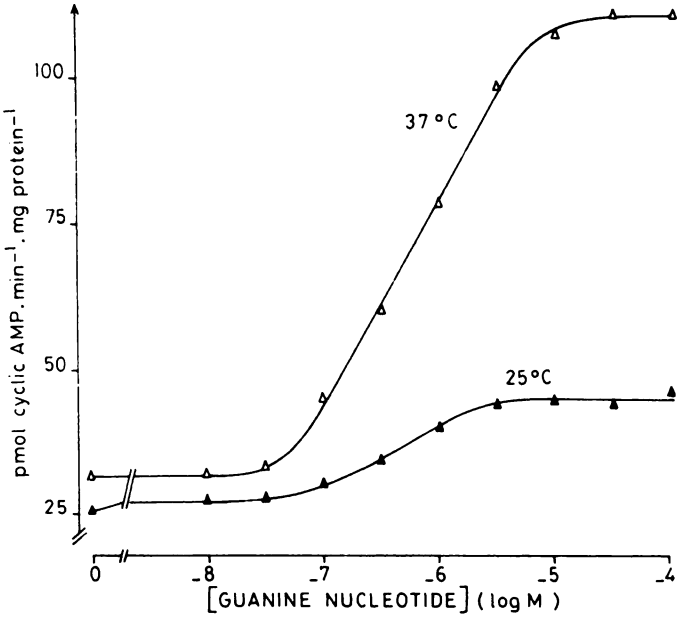

FIG. 4. *Dose-response curves of GTP stimulation of cholera toxin-pretreated rat cardiac adenylate cyclase at 37° (open symbols) and 25° (closed symbols)*

The results are representative of experiments performed with three different preparations of cholera toxin-pretreated cardiac membranes. The basal activity and the activity after stimulation with nonhydrolyzable nucleotides of these cholera toxin-pretreated membranes were identical with those observed in native membranes (Fig. 3A and B) and in membranes subjected to the same pretreatment without cholera toxin addition.

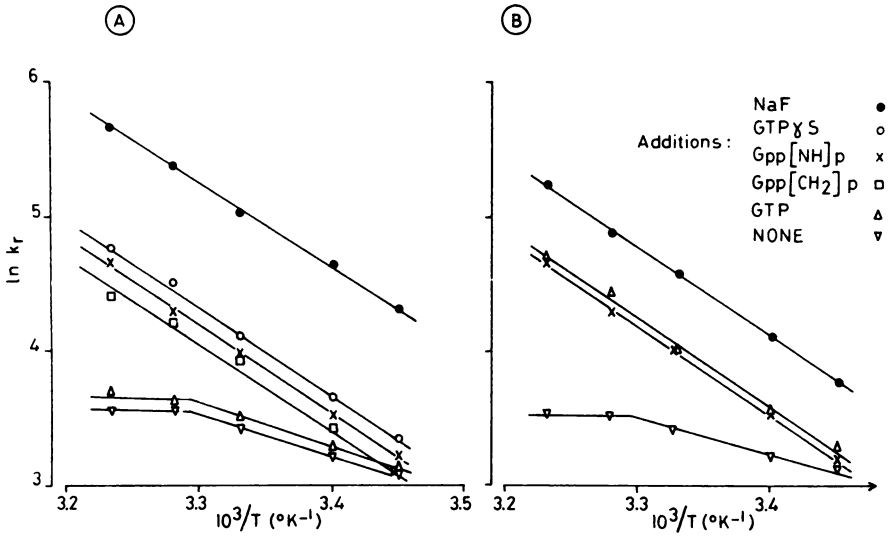

FIG. 5. *Temperature dependence of native (A) and cholera toxin-pretreated (B) rat cardiac adenylate cyclase activity*

A. In native membranes, basal activity (▽) was compared with that observed after activation with 3×10^{-5} M GTPγS (○), 3×10^{-5} M Gpp(NH)p (×), 3×10^{-5} M Gpp(CH_2)p (□), 3×10^{-5} M GTP (△), or 10^{-2} M NaF (●). Each point represents the mean of three separate experiments.

B. After cholera toxin pretreatment, basal adenylate cyclase activity (▽) was compared with that observed in the presence of 2×10^{-5} M Gpp(NH)p (×), 2×10^{-5} M GTP (△), or 1×10^{-2} M NaF (●). Each point represents the mean of three separate experiments. For the sake of simplicity, Arrhenius plots in the presence of GTPγS and Gpp(CH_2)p were not reported as they gave identical results in control and cholera toxin-pretreated membranes.

In both panels, k_r (the initial rate constant of cyclic AMP formation) was expressed as picomoles of cyclic AMP per minute per milligram of protein.

ported with other adenylate cyclase activities (4, 19, 20).

The low activation energy above the break may conceivably (20) be due to (a) thermal denaturation, (b) parallel or successive reactions with distinct kinetic characteristics, (c) enzyme *trans*-conformation between two forms in equilibrium, or (d) diffusional effects due to the membranous localization of the enzyme. Hypothesis a does not appear to hold, since we verified that an 8-min preincubation of cardiac membranes in the assay medium exerted no effect on enzyme activity and that no break in activity occurred in the presence of a hormone. Hypothesis b can also be eliminated on theoretical grounds (20): in parallel reactions, the Arrhenius plot should be concave upward (21), whereas in successive reactions the variation in activation energy should be at least 200 kcal (21). Hypotheses c and d may involve a regulation by lipid movements of membrane-bound enzymes in general (22) and of adenylate cyclase in particular (4–6, 23–25). Alterations in membrane fluidity due to changes in temperature or lipid composition may indeed affect basal and activated adenylate cyclase activity. In turkey erythrocytes, it has been concluded that the coupling between hormone receptor and adenylate cyclase is a diffusion-controlled process (5). Furthermore, breaks in Arrhenius plots of adenylate cyclase activity have been associated with transitions in the inner or the outer monolayer of the membrane (23, 24). In this respect, the spin-label study by Gordon *et al.* (26) has demonstrated the existence of a thermotropic lipid phase separation in rat heart plasma membranes with a high temperature onset (32°). In conclusion, as Arrhenius plots in the present study were linear in the presence of GTP plus a hormone, the break under basal conditions and in the presence of GTP may only be explained by a thermotropic lipid phase separation in the inner face of rat heart plasma membranes.

Guanine nucleotides exerted distinct effects on the E_a of rat cardiac adenylate cyclase, according to their chemical nature. The use of the nonhydrolyzable guanine nucleotides Gpp(CH_2)p, Gpp(NH)p, and GTPγS required an E_a of 18 kcal/mole, whereas the E_a value with GTP alone or with GTP in the presence of hormone was only 11 kcal/mole (Table 1; Fig. 1). As in other systems (2), guanine nucleotides are required in rat cardiac membranes to couple the receptor (when occupied by a hormone agonist) to the regulatory protein (G) and the catalytic unit (C) (15, 16). The addition of hormonal stimulants such as (±)-isoproterenol, glucagon, or secretin, in the presence of either GTP or Gpp(NH)p, increased the reaction rate constant k_r at all temperatures tested, without affecting the E_a value characteristic of the guanine nucleotide utilized (Fig. 1). This finding suggests that the chemical structure of the four guanine nucleotides utilized was the sole determinant responsible for different E_a values, depending on the effect of these nucleotides on the regulatory protein ($G \rightarrow G^*$) and on the subsequent activation of the catalytic unit.

The difference in E_a values with GTP and its nonhydrolyzable analogues suggests that the energy required for the synthesis of cyclic AMP was lower when the hydrolysis of GTP by the regulatory protein (G) was possible. An indirect argument in favor of this hypothesis is that the E_a value with NaF, obviously nonhydrolyzable but acting also on protein G (2), was as high as that observed with nonhydrolyzable guanine nucleotides (Table 1).

To test this hypothesis more directly, rat cardiac membranes were pretreated with cholera toxin. This pretreatment is known to inhibit the GTPase activity of the guanine regulatory protein (G) in several systems (17, 18, 27). This was apparently also the case in rat cardiac membranes (compare Fig. 4 with Fig. 3A and B). Arrhenius plots in the presence of GTP showed an E_a value of 17.1 kcal/mole that was now comparable to that obtained with the nonhydrolyzable analogues of GTP.

The thermodynamic significance of E_a might be further analyzed when examining the relative contribution of ΔS and ΔG, based on the transition state theory (28). This theory states that the enthalpy of activation (ΔH^+) can be calculated from the equation $\Delta H^+ = E_a - RT$, where R is the gas constant (1.99 kcal/mole) and T the absolute temperature (in °K). The free energy of activation (ΔG^+) can also be calculated from the equation $\Delta G^+ = -RT \ln k_r + RT \ln (kT/h)$, where k_r is the rate constant of the reaction (here defined in picomoles of cyclic AMP per minute per milligram of protein), k is Boltzmann's constant, and h is Planck's constant. Substitution of ΔG^+ and ΔH^+ by their values in

$$\Delta G^+ = \Delta H^+ - T\Delta S^+ \quad (1)$$

yields

$$\frac{E_a}{RT} = \frac{\Delta S^+}{R} - \ln k_r + \ln \frac{kT}{h} + 1 \quad (2)$$

which relates E_a to both ΔS^+ and k_r and indicates that a change in E_a does not necessarily correspond to a proportional inverse change in the reaction rate constant (20). Considering the case of GTP and Gpp(NH)p-activated membranes, reaction rate constants were identical at the intersections of Arrhenius plots in Fig. 1, i.e., at 18° in the absence of hormone and at about 20–22° in the presence of hormone, when $\ln k_{GTP} = \ln k_{Gpp(NH)p}$. It appears from Eq. 2 that

$$\left(\frac{\Delta S^+}{R} - \frac{E_a}{RT_0}\right)_{GTP} = \left(\frac{\Delta S^+}{R} - \frac{E_a}{RT_0}\right)_{Gpp(NH)p}$$

or

$$\frac{E_{a_{Gpp(NH)p}} - E_{a_{GTP}}}{T_0} = \Delta S^+_{Gpp(NH)p} - \Delta S^+_{GTP}$$

which suggests that the decreased value of E_a when GTP was used in place of Gpp(NH)p reflected a lower ΔS^+ of rat cardiac adenylate cyclase activity (Table 3), i.e., a lower organization of the transition complex.

A simple two-state model of adenylate cyclase activity has been supported experimentally in turkey erythrocytes (29), hepatic plasma membranes (30), and pancreatic plasma membranes (27). The operation of this mechanism implies that a GTP-activated adenylate cyclase is deactivated by the hydrolysis of GTP, this inactivation possibly being mediated by the energy released during this turn-off reaction. In rat cardiac membranes, cholera toxin pretreatment increased the low activity of adenylate cyclase observed in the presence of GTP (compare Fig. 4 with Fig. 3), probably because of an inhibition of GTPase activity. This indirect evidence for GTP hydrolysis in native rat cardiac membranes was in line with more direct data in other systems [including membranes of turkey erythrocytes (31), rat pancreas (32), rat liver (33), and human mononuclear cells (34)], demonstrating directly the existence of low-K_m GTPase activity associated with the adenylate cyclase system.

TABLE 3

Thermodynamic parameters of rat cardiac adenylate cyclase activity

The parameters were calculated by using the E_a values determined from Fig. 1 and given in Table 1. The temperature when $\ln k_{GTP} = \ln k_{Gpp(NH)p}$ was 18° in the absence of (±)-isoproterenol and 22° in its presence.

Activator	ΔH^+	ΔG^+	ΔS^+
	kcal/mole	*kcal/mole*	*e.u.*
Basal	8.9	5.6	10.3
30 μM GTP	8.9	5.6	10.3
30 μM Gpp(NH)p	17.1	5.0	39.0
30 μM GTP + 10^{-5} M (±)-isoproterenol	9.2	5.5	10.4
30 μM Gpp(NH)p + 10^{-5} M (±)-isoproterenol	16.6	5.1	41.0

When considering the present thermodynamic data, it is tempting to suggest that the dynamic equilibrium between inactive and active states in cardiac adenylate cyclase, when G site(s) were transiently occupied by GTP, involved a lesser degree of organization than the situation observed when the same G sites were occupied by a nonhydrolyzable analogue. This hypothesis is supported by preliminary data on cardiac membranes persistently activated with 0.1 mM Gpp(NH)p, then washed extensively to eliminate the activator in excess from the medium. The adenylate cyclase activity of these membranes remained maximal, and the E_a was 16.8 ± 0.6 kcal/mole ($n = 4$), i.e., comparable to that observed when Gpp(NH)p was tested directly on the same membranes (16.8 ± 0.7 kcal/mole). Thus, the cardiac adenylate cyclase system operating with regulatory subunits G occupied with a nonhydrolyzable nucleotide or with GTP after cholera toxin pretreatment (i.e., with a persistent and maximal accumulation of activated catalytic subunits) showed a higher degree of organization than the same system oscillating with a regulatory GTPase cycle closely associated with adenylate cyclase activity.

Finally, the hormonal stimulation of rat cardiac adenylate cyclase, in the presence of GTP, consisted of a higher k_r value than when GTP was used alone, with no alteration of E_a (Fig. 1; Table 1). This may suggest a more rapid operation of the two-state model in the presence of hormones with no further thermodynamic "disorganization" of the system.

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