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Synergistic Activation of Adenylate Cyclase by Guanylyl Imidophosphate and Epinephrine†

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ABSTRACT: A kinetic analysis of the synergistic activation of turkey erythrocyte adenylate cyclase by 1-catecholamines and guanylyl imidodiphosphate (Gpp(NH)p) is described. We have found that the role of the catecholamine hormone is to facilitate the activation of the enzyme by the guanyl nucleotide according to the following mechanism:

$$R \cdot E + G \rightleftharpoons R \cdot EG$$

$$R \cdot EG + H \rightleftharpoons HR \cdot EG \xrightarrow{k} HR \cdot E''G$$

where R is the receptor, E the enzyme, G the guanyl nucleotide effector, and H the hormone. The binding steps are fast and reversible but the conversion of the inactive enzyme E to its active stable form (E″) occurs with a rate constant of $k = 0.7$ min^{-1}. This step is essentially irreversible in the presence of high Gpp(NH)p concentrations. In the absence of β-agonist (1-catecholamine) and at low free Mg^{2+} concentrations, the activation of the enzyme is insignificant. At high Mg^{2+} concentration the conversion of E to E″ occurs slowly in the absence of hormone, probably by another pathway. Thus, the presence of a guanyl nucleotide at the allosteric site is obligatory but not sufficient to induce the conversion of the inactive enzyme to its active form. The process of enzyme activation requires both Gpp(NH)p and hormone and under these conditions is essentially irreversible. The permanently active enzyme is stable in the absence of hormone and Gpp(NH)p and its high catalytic activity is stable for many hours. However, hormone and ATP induce a conversion of the high activity to the low activity form. Thus, it seems that both the process of enzyme activation by Gpp(NH)p and its reversal are hormone dependent. Both processes are blocked by the β-blocker propranolol.

Recently it was demonstrated that GTP[1] plays a key role as a regulatory ligand in the action of hormone-activated adenylate cyclases (Rodbell et al., 1971a,b; Londos et al., 1974). It has also been shown that the GTP analogues Gpp(NH)p and to a lesser extent $Gpp(CH_2)p$ are superior to GTP in stimulating hormone-activated adenylate cyclases (Londos et al., 1974, and references therein). It was suggested that the activation of adenylate cyclases is brought about by the synergistic action of Gpp(NH)p and hormone, namely that the activation of adenylate cyclase by hormone and Gpp(NH)p when present together have a greater combined stimulatory effect than the sum of their individual effects. This synergistic effect is seen with GTP analogues stable to phosphotransferase reactions such as Gpp(NH)p and $Gpp(CH_2)p$. Rodbell and his group demonstrated that the role of the hormone is to facilitate the rate of adenylate cyclase activation by guanine nucleotides (Rodbell et al., 1974) in a number of adenylate cyclases from different sources. More recently, Rodbell and his group proposed a generalized model for the interrelationships between hormone and guanine nucleotides in their action as activators of adenylate cyclases (Salomon et al., 1975; Lin et al., 1975; Rendell et al., 1975).

Similar to many other hormone-stimulated adenylate cyclases (Londos et al., 1974), it was demonstrated that 1-epinephrine stimulated adenylate cyclase from nucleated erythrocytes is also activated by Gpp(NH)p (Bilezikian and Aurbach, 1974; Schramm and Rodbell, 1975; Pfeuffer and Helmreich, 1975). We have studied in some detail the interaction between the hormone binding site and the guanyl nucleotide regulatory site to gain a better understanding on the

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[1] Abbreviations used are: Gpp(NH)p, guanylyl imidodiphosphate; GTP, GDP, guanosine tri- and diphosphate; ATP, adenosine triphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

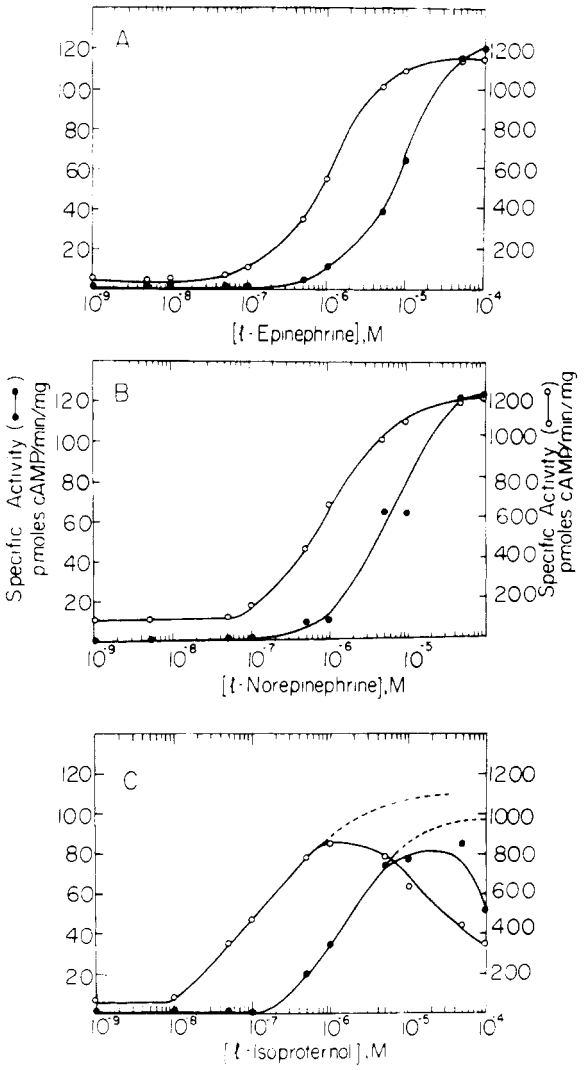

FIGURE 1: Activation of adenylate cyclase by agonists in the absence and in the presence of Gpp(NH)p. Enzyme activity was measured as described earlier (Steer and Levitzki, 1975a) but in the presence of 10^{-4} M Gpp(NH)p. Isoproterenol at high concentrations was found to be inhibitory (Figure 1C) both in the presence and in the absence of Gpp(NH)p. The dotted lines represent the theoretical behavior in the absence of inhibition at high ligand concentration. (● — ●) No Gpp(NH)p, (○ — ○) with Gpp(NH)p.

nature of their interaction. On the basis of our findings we propose a detailed model describing the interaction between the catecholamine hormone and the guanine nucleotide effector.

Materials and Methods

All chemicals used were of analytical grade. The concentration of nucleotides was determined spectrophotometrically. Gpp(NH)p was obtained from Boeheringer (Mannheim, W. Germany). [α^{32}P]ATP was obtained from Radiochemical Centre, England, and [^{3}H]propranolol was prepared by the Israel Atomic Energy, Negev Research Center, as described earlier (Levitzki et al., 1974; Atlas et al., 1974). [^{3}H]-Gpp(NH)p was obtained from ICN Pharmaceuticals Inc., Calif., U.S.A.

All solutions were prepared in Corning double-distilled water. Plasma membranes were prepared from turkey erythrocytes as described earlier (Steer and Levitzki, 1975a). These membranes were the source of β-receptor activated adenylate cyclase, the activity of which was assayed according to Salomon et al. (1974). Assays were linear up to 40 min at 37 °C and were conducted for 20-min duration.

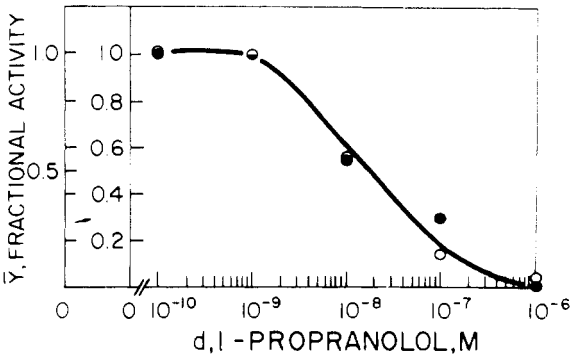

FIGURE 2: The inhibition of adenylate cyclase activation by propranolol. One milligram of membrane protein was incubated with 1.0×10^{-4} l-epinephrine and 1.0×10^{-4} M Gpp(NH)p, 1.0 mM $MgCl_2$, 1.0 mM EDTA, and 0.05 Tris-HCl, pH 7.4, at 37 °C, for 40 min in a final volume of 1.0 ml. Different concentrations of propranolol were added to the incubation mixture as shown in the figure. After the incubation period, 10.0 ml of 0.05 M Tris-HCl, pH 7.4, containing 2 mM $MgCl_2$ and 1.0 mM EDTA were added and the enzyme was centrifuged at 23 000g for 10 min. The pellet was resuspended in 10.0 ml of the same buffer and recentrifuged. The process was repeated four times. The enzyme was then assayed in the absence of added hormone or Gpp(NH)p (basal activity). The maximal activity obtained for the fully activated enzyme in the absence of added epinephrine or propranolol was 1250 pmol of cAMP mg^{-1} min^{-1} The same enzyme had a specific activity of 500 pmol of cAMP mg^{-1} min^{-1} when assayed in the presence of 1.0×10^{-4} M epinephrine. (● — ●) Basal activity, (○ — ○) epinephrine activity.

Results

Activation of Adenylate Cyclase by Hormone and Gpp(NH)p. The activation curve of adenylate cyclase by l-catecholamines is shifted to lower agonist concentrations, by one order of magnitude, in the presence of Gpp(NH)p. This effect is shown in Figure 1. This effect was reported earlier by other workers (Schramm and Rodbell, 1975; Pfeuffer and Helmreich, 1975) and constitutes the basic finding. The maximal values for the fully stimulated enzyme is 1200 pmol of cAMP min^{-1} mg^{-1} for all full agonists. These activities are stable for many hours.

Dependency on Gpp(NH)p Concentration. It was found that under assay conditions the behavior described in Figure 1 is such that full activation occurs with Gpp(NH)p concentrations above 3×10^{-5} M. Thus, under these assay concentrations the synergistic activation of adenylate cyclase by hormone and Gpp(NH)p is maximal. Maximal activation can also be obtained at 3×10^{-6} M Gpp(NH)p in the presence of 10^{-4} M l-epinephrine if prior to the assay the enzyme is preincubated with these ligands at these concentrations for 10 min at 37 °C in 0.05 M Tris-HCl, pH 7.4, containing 1.8 mM $MgCl_2$ and 1.0 mM EDTA.

Preactivation of Adenylate Cyclase and Its Inhibition by Propranolol. When the enzyme is preactivated by l-epinephrine and Gpp(NH)p and the ligands are then washed thoroughly, enzyme activity remains stable in the absence of hormone or Gpp(NH)p for many hours. This finding was reported earlier for the frog erythrocyte adenylate cyclase (Schramm and Rodbell, 1975). The preactivation process can be inhibited by propranolol (Figure 2). Furthermore, propranolol only stops the activation process but does not reverse it. Thus, if propranolol is added during the process of activation the activity exhibited by the enzyme is the one achieved at the instant of propranolol addition. When propranolol is added to the preactivated enzyme no inhibition of the cyclase activity

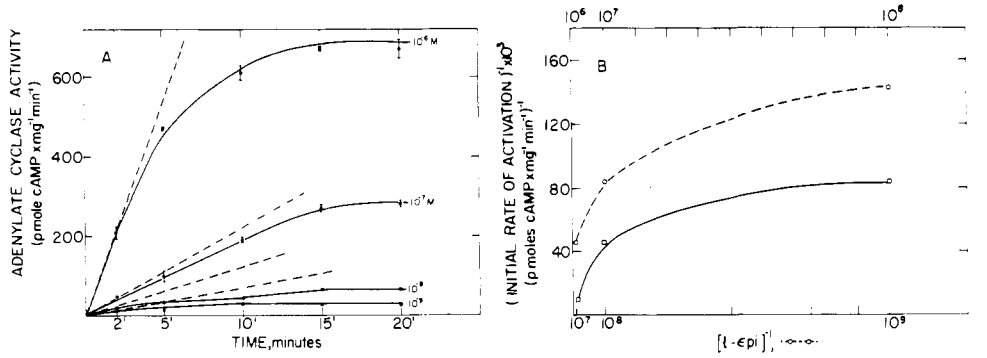

FIGURE 3: Rate of adenylate cyclase activation by Gpp(NH)p as a function of 1-epinephrine. 1.0 mg of plasma membranes was incubated at 37 °C with Gpp(NH)p 1.0×10^{-4} M, in 0.05 M Tris-HCl, pH 7.4, containing 2.0 mM $MgCl_2$, 1.0 mM EDTA in a final volume of 1.0 ml, with increasing concentrations of 1-epinephrine as depicted in the figure. 2×10^{-5} M propranolol (final concentration) was added to stop the activation process at different time points and then samples were washed thoroughly and assayed for adenylate cyclase basal activity. The specific activity of the adenylate cyclase as a function of incubation time is plotted in A. From the initial slopes the initial velocity, v, for enzyme activation at each epinephrine concentration was obtained. In part B the double-reciprocal plot of (initial rate)$^{-1}$ vs. [Epi]$^{-1}$ is shown. The lower abcissa in B [1-Epi]$^{-1}$ covers the range of 10^{-9} M to 10^{-7} M 1-epinephrine and the data points are shown on the full line. The upper abscissa [Epi]$^{-1}$ covers the range of 10^{-8}–10^{-6} M 1-epinephrine and the data points fall on the dotted line that is in fact a continuation of the full line. It can be seen that the values for the (Initial Rate)$^{-1}$ for 10^{-8} and 10^{-7} M appear in the two sections of the graph. It can be seen that over the range of 10^{-9}–10^{-6} M epinephrine strong concavity downward appears in the double-reciprocal plot.

is observed. This finding is identical whether or not the hormone and the Gpp(NH)p are removed prior to propranolol addition. It is clear that such an experiment demonstrates qualitatively the fact that propranolol inhibits epinephrine action but this experiment cannot be used to calculate the inhibition constant for propranolol. This property of propranolol is useful in the study of the kinetics of enzyme activation by hormone and Gpp(NH)p, since addition of propranolol can stop the activation process at any time point without causing any inhibition.

Kinetics of Gpp(NH)p Activation as a Function of Hormone Concentration. When the adenylate cyclase preparation was preincubated with hormone and Gpp(NH)p, prior to its assay, it was found that the enzyme is irreversibly converted to its stimulated form. The rate of this conversion depends on hormone concentration in the incubation mixture. Thus, when the enzyme is preincubated in the presence of 10^{-4} M Gpp(NH)p and increasing 1-catecholamine concentrations the specific activity of the enzyme increases as a function of time (Figure 3). Each time point represents the time at which the activation process was stopped by the addition of excess propranolol and then the enzyme thoroughly washed by centrifugation, as described in the legends to Figures 2 and 3. At each time point the enzyme was assayed in the absence of added ligands thus exhibiting the true activity at the time of propranolol addition.

The activation rate of Gpp(NH)p saturated enzyme (GE) by hormone was analyzed according to the following scheme:

$$R \cdot EG + H \underset{}{\overset{K_H}{\rightleftharpoons}} HR \cdot EG \xrightarrow{k} HR \cdot E''G \qquad (1)$$

where K_H describes the hormone-receptor dissociation constant and k the rate of conversion of the enzyme to its active state E″. This rate, v, is given by:

$$v - v_0 = \frac{k[E_0][H]}{K_H + [H]} \qquad (2)$$

where v is the observed rate of enzyme activation by Gpp(NH)p alone and E_0 is the concentration of enzyme. The data in Figure 3A are analyzed in Figure 3B by plotting the double-reciprocal plot $1/v$ vs. $1/[H]$. The extrapolated value on the double-reciprocal plot should yield the maximal rate of

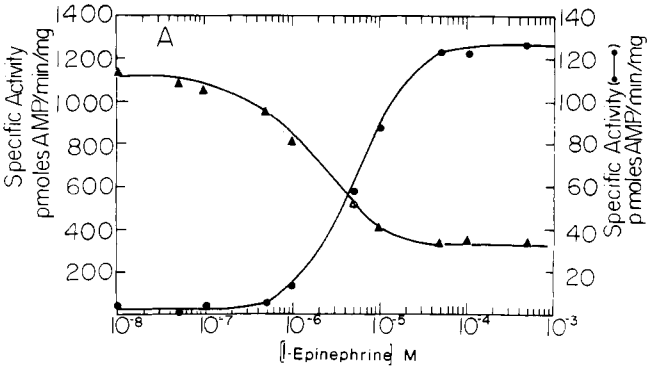

FIGURE 4: The reversibility of Gpp(NH)p activation. One milligram of plasma membranes was incubated for 40 min at 37 °C with Gpp(NH)p 10^{-4} M, or Gpp(NH)p 10^{-4} M + 1-epinephrine in 10^{-4} M in the presence of 0.8 mM $MgCl_2$ in 0.05 M Tris-HCl, pH 7.4, in a final volume of 1.0 ml. Then the membranes were washed thoroughly (dilution factor $1:10^8$) and assayed for adenylate cyclase activity in the presence of increasing 1-epinephrine concentrations in the assay. Preincubation conditions: (○ — ○), no additions, (▲ — ▲) 1×10^{-4} M Gpp(NH)p and 1×10^{-4} 1-epinephrine.

enzyme activation $V_{max} = k \times E_0$ (Figure 3B). Assuming that 1 mg of enzyme protein contains about 1 pmol of enzyme, one can calculate a value of 0.7 min^{-1} for k. The assumption that 1 mg of membrane protein contains 1 pmol of enzyme is based on the finding that 1 mg of membrane protein contains 1 pmol of receptors (Levitzki et al., 1974, 1975; Atlas et al., 1974), and on the assumption that the receptor to enzyme stoichiometry is 1:1 (Levitzki et al., 1974, 1975). The deviation of the double-reciprocal plot from linearity will be discussed under the Discussion.

The Reversibility of the Gpp(NH)p Activation. When the enzyme is activated by Gpp(NH)p and 1-epinephrine to the permanently active state and assayed in the absence of any regulatory ligand, the specific activity is found to be the highest. When the assay is conducted in the presence of 1-epinephrine the activity is reduced by 50–65% (Figure 4). The extent of this hormone-induced inhibition increases as a function of hormone concentration (Figure 4).

When the fully-activated enzyme is washed with 1-epinephrine and assayed in the presence of 1-epinephrine the enzyme possesses 35–50% activity of the maximal activity

TABLE I: Reversibility of Gpp(NH)p Activation.[a]

Preincubation	Washings	Assay: Basal[b] (pmol of cAMP min^{-1} mg^{-1})	Assay: 10^{-4} M l-Epinephrine	Assay: 10^{-4} M Epinephrine + 10^{-4} M Gpp(NH)p
Gpp(NH)p + l-epinephrine	Buffer	1096 ± 50	599 ± 40	1100 ± 50
Gpp(NH)p + l-epinephrine	10^{-4} M l-Epinephrine	528 ± 35[c]	538 ± 35	-
Gpp(NH)p + l-epinephrine	10^{-4} M l-Epinephrine then buffer	1130 ± 50	649 ± 65	-

[a] One milligram of membrane-bound enzyme was preincubated at 37 °C for 40 min in the presence of 1×10^{-4} M Gpp(NH)p and 1×10^{-4} M l-epinephrine in 0.05 M Tris-HCl, pH 7.4, containing 2 mM $MgCl_2$ and 1 mM EDTA in a final volume of 1.0 ml. The membranes were then diluted in 10.0 ml of 0.05 M Tris-HCl, pH 7.4, containing 2 mM $MgCl_2$, 1 mM EDTA, and the added ligands as specified in the table. This mixture was centrifuged at 23 000*g* for 10 min. The pellet was resuspended in 10.0 ml of the same buffer and recentrifuged. This procedure was repeated four times. The enzyme was then assayed under three different conditions as specified in the table. [b] No hormone added in the assay. [c] Assay in the presence of 10^{-4} M l-epinephrine.

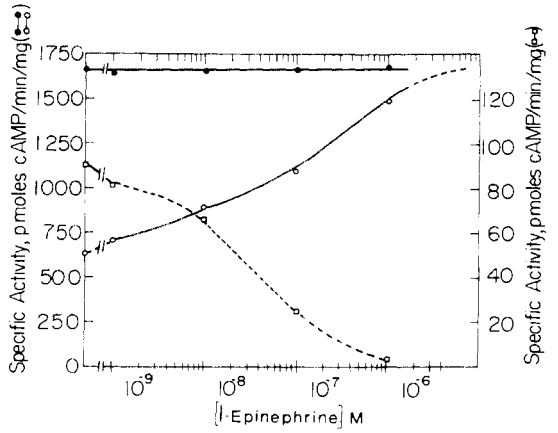

FIGURE 5: The inhibition of the hormone-induced antagonism by propranolol. Plasma membranes were preincubated with 1.0×10^{-4} M Gpp(NH)p + 1.0×10^{-4} M l-epinephrine in the presence of 1.0 mM $MgCl_2$ and 0.05 M Tris-HCl, pH 7.4, for 40 min at 37 °C. Then the membranes were thoroughly washed and assayed for adenylate cyclase activity with increasing propranolol concentrations in the assay, in the absence and in the presence of 5.0×10^{-5} M l-epinephrine. It can be seen that in the absence of l-epinephrine the maximal activity of the high activity form of the enzyme is retained (upper curve) (— ● — ● —). For comparison, the inhibition of nonactivated adenylate cyclase is also shown. (○ — ○) Activated enzyme; (□ — □) nonactivated enzyme.

TABLE II: Effect of Mg^{2+} on Enzyme Activation by Gpp(NH)p and Hormone.[a]

Free $MgCl_2$ (mM)	Basal Act. (pmol of cAMP min^{-1} mg^{-1}): No Epinephrine	Basal Act. (pmol of cAMP min^{-1} mg^{-1}): with Epinephrine in Preincubation
0	16	51 ± 5
0.1	21	860 ± 15
0.5	25	880 ± 30
1.0	26	860 ± 30
2.5	35	-
5.0	88	890 ± 15
5.0[b]	590	890 ± 15

[a] Plasma membranes were thoroughly washed with 0.05 M Tris-HCl, pH 7.4, containing 1 mM EDTA with no $MgCl_2$. Then $MgCl_2$ was added in stoichiometric amounts to neutralize the EDTA to the membrane suspension. These conditions are defined as zero-free Mg^{2+}. The membranes were incubated for 40 min at 37 °C, in the presence of 1×10^{-4} M Gpp(NH)p and 1×10^{-4} M epinephrine as well as in the absence of hormone, with increasing $MgCl_2$ concentration above the EDTA concentration. Then the membranes were washed in the Mg^{2+}-free buffer and assayed for basal activity (no hormone in the assay). All incubation mixtures contained 10^{-4} M Gpp(NH)p. [b] Preincubation of 24 h at 25 °C.

(Table I). When the hormone is removed the specific activity returns to its maximal value (Table I). The hormone inhibition can also be reversed by excess Gpp(NH)p (10^{-4} M Gpp(NH)p, Table I).

The Inhibition of the Hormone Antagonism by Propranolol. When the fully activated enzyme is assayed in the absence of free Gpp(NH)p and in the presence of l-epinephrine with increasing concentrations of l-propranolol the antagonistic effect of the hormone is inhibited progressively (Figure 5).

The Activation of Adenylate Cyclase by Gpp(NH)p Alone. It was consistently found that the activation of adenylate cyclase by hormone and Gpp(NH)p depends on the presence of free Mg^{2+} (Table II). It was found that the enzyme is activated very slowly by Gpp(NH)p alone. However, in the presence of 5 mM Mg^{2+} the extent of enzyme activation by Gpp(NH)p alone can reach 70% of the maximal value within 24 h (Table II). In the presence of magnesium concentrations higher than 5 mM, close to 100% activity can be achieved. In the experiment described in Table II, EDTA is present. Similar experiments in which the EDTA is absent show that the preactivation process requires at least 2×10^{-5} M Mg^{2+} in the incubation mixture.

Sequential Activation. When the membranes are incubated at 37 °C for 40 min in the presence of 10^{-4} M Gpp(NH)p in 0.05 M Tris-HCl, 2 mM $MgCl_2$, and 1 mM EDTA, pH 7.4, then thoroughly washed of the free Gpp(NH)p, very low specific activity is attained (50 ± 10 pmol of cAMP mg^{-1} min^{-1}). If the washed membranes are then incubated with l-epinephrine the enzyme is activated to its high activity form (Table III). This experiment indicates that the interaction of Gpp(NH)p with the adenylate cyclase system is hormone independent but is probably an essential step in the process of synergistic activation of the enzyme by the two ligands.

Absence of a GTP Effect. Under assay conditions that apply 2.1 mM ATP and ATP regenerating system (Steer and Levitzki, 1975a), we found no stimulation of the hormone activation by adding GTP. Bilezikian and Aurbach (1974) reported a small increase in the V_{max} of hormone-stimulated adenylate cyclase activity by GTP. When lower concentrations of ATP are used in the adenylate cyclase assay, three- to

TABLE III: Sequential Activation of Adenylate Cyclase by Epinephrine and Gpp(NH)p.[a]

Gpp(NH)p in Incubation (M)	Adenylate Cyclase Act. (pmol min^{-1} mg^{-1})	
	No Additions	In the Presence of 5×10^{-5} *dl*-Propranolol
10^{-7}	140 ± 5	270 ± 10
10^{-6}	272 ± 4	590 ± 13
10^{-4}	373 ± 4	760 ± 40
Control: 10^{-4} M Gpp(NH)p + 10^{-4} M l-epinephrine	860 ± 30	

[a] Membranes (0.20 mg) were incubated in the presence of 0.05 M Tris-HCl, pH 7.4, 2.0 mM Mg^{2+}, 1.0 mM EDTA, and different Gpp(NH)p concentrations as indicated in the table for 1 h at 37 °C in a final volume of 0.2 ml. At the end of the incubation, 10 ml of Tris-Mg-EDTA buffer was added and the mixture was centrifuged at 18 000*g* for 15 min. The pellet was resuspended in 10 ml and recentrifuged. This process was repeated four times. Then the membranes were incubated for 1 h at 37 °C under identical conditions in the presence of 1.0×10^{-4} M l-epinephrine. Samples were then taken to be assayed in the absence and in the presence of 5×10^{-5} M *dl*-propranolol.

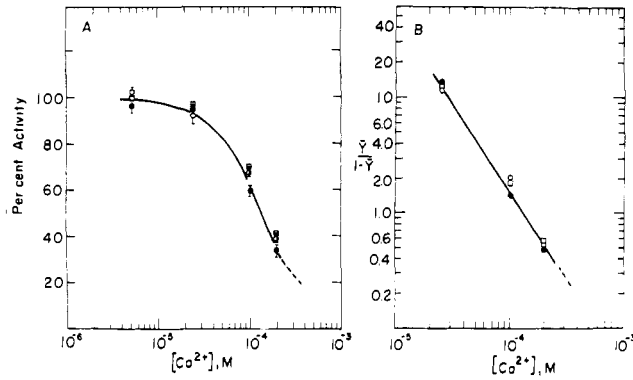

FIGURE 6: Inhibition of adenylate cyclase activity by Ca^{2+}. Membranes were preincubated in the presence of 1.0×10^{-4} M Gpp(NH)p + 1.0×10^{-4} l-epinephrine in 0.05 M Tris-HCl, pH 7.4, containing 2 mM $MgCl_2$ and 1.0 mM EDTA for 40 min at 37 °C. After this period, the membranes were washed thoroughly and assayed for adenylate cyclase activity in the presence of increasing Ca^{2+} concentrations as depicted in the figure. The assay was conducted in the absence (○ — ○), or in the presence (● — ●) of l-epinephrine (5×10^{-5} M). The maximal activity of the preactivated enzyme was 1100 pmol of cAMP mg^{-1} min^{-1} in the absence of epinephrine and 410 pmol of cAMP mg^{-1} min^{-1} in the presence of epinephrine. The Ca^{2+} inhibition of the hormone-stimulated activity in the nonactivated state (specific activity = 100 pmol of cAMP mg^{-1} min^{-1}) was measured in parallel (□ — □).

fourfold activation by GTP can be demonstrated (Cassel and Selinger, unpublished).

The Allosteric Inhibition of Adenylate Cyclase by Ca^{2+}. It was demonstrated earlier (Steer and Levitzki, 1975a,b) that the hormone-activated enzyme is inhibited by Ca^{2+} both in the isolated plasma membranes and in whole turkey red blood cells in the presence of a specific Ca^{2+} ionophore (Steer and Levitzki, 1975a,b). It was therefore of interest to examine if the permanently-activated enzyme also responds to calcium inhibition. It was found that calcium inhibits the fully stimulated enzyme whether it is assayed in the absence of any added hormone, or when assayed in the presence of epinephrine, conditions under which its specific activity is about one-third of the maximal specific activity (Figure 6). Thus, the responses of the enzyme to Ca^{2+} when stimulated by hormone alone or by hormone + Gpp(NH)p are identical. In both cases the $S_{0.5}$ for Ca^{2+} is identical and both inhibition curves are positively cooperative with a Hill coefficient of $n_H = 1.5$. Thus, although the two enzyme species differ in their k_{cat} values by an order of magnitude, they respond to Ca^{2+} in an identical pattern. Calcium, on the other hand, does not interfere with the process of enzyme activation by hormone and Gpp(NH)p. When Ca^{2+} is included in the activating mixture of epinephrine and Gpp(NH)p, no inhibition of enzyme activation is observed.

Discussion

Recently, it has been shown that GTP and even more so its nonhydrolyzable analogue, Gpp(NH)p, stimulate cyclase from turkey erythrocytes synergistically with the catecholamine hormone (Pfeuffer and Helmreich, 1975; Schramm and Rodbell, 1975). The apparent increase in the affinity of adenylate cyclase toward hormone (Figure 1) is due to the fact that the enzyme is activated by Gpp(NH)p to a stable active enzyme at a rate depending on hormone concentration. The maximal rate at which the enzyme is converted to its active form is 0.7 min^{-1} at 0.8 mM free Mg^{2+}. In the absence of hormone and at low free Mg^{2+} concentration (Figure 3A) the rate of enzyme activation is negligible in the absence of hormone, even at high concentrations of Gpp(NH)p. One can, therefore, conclude that that hormone functions as an allosteric activator enhancing the adenylate cyclase activation by the regulatory nucleotide Gpp(NH)p.

This effect of the hormone can be blocked by propranolol (Figure 2), a potent β blocker. Propranolol, however, cannot reverse the process as was already shown by Schramm and Rodbell (1975), although it binds to the β receptor of the preactivated enzyme, with an affinity identical with that reported for the untreated membranes (Levitzki et al., 1975).

It seems, therefore, that occupancy of the β receptors by the agonist is obligatory but not sufficient for achieving adenylate cyclase activation. The guanyl nucleotide must occupy simultaneously the regulatory site in order for the adenylate cyclase to become active. It seems, therefore, that the two ligands when present simultaneously induce the fast transformation of the enzyme to its active form.

The binding of Gpp(NH)p to the membrane-containing enzyme is hormone independent. Subsequent to the nucleotide binding step and removal of the excess free Gpp(NH)p, l-epinephrine is capable of catalyzing the formation of the high-activity form of the enzyme (Table III). It should be noted that the binding of the antagonist [^{3}H]propranolol is not affected by treating the membrane by Gpp(NH)p (Levitzki et al., 1975). This observation does not eliminate the possibility that agonist binding may be altered. So far, evidence as to the latter possibility has not been found. It seems that the binding of the regulatory ligands hormone and Gpp(NH)p is not the limiting factor in determining the rate of enzyme activation. It is likely that the process of enzyme activation can be viewed according to the following scheme:

$$R \cdot E + G \overset{K_G}{\rightleftharpoons} R \cdot EG \quad (3)$$

$$RE \cdot G + H \overset{K_H}{\rightleftharpoons} HR \cdot EG \quad (4)$$

$$HR \cdot EG \overset{k}{\longrightarrow} HR \cdot E''G \quad (5)$$

where R·E is the receptor enzyme complex, G the guanyl nucleotide, K_G its dissociation constant with the enzyme, H the hormone, K_H the hormone dissociation constant, and k the first-order rate constant characterizing the conversion of the low-activity form of the enzyme, E, to its high activity form E″. In the presence of high Gpp(NH)p concentrations eq 2 may adequately describe the dependence of the rate of enzyme activation on hormone concentration. The deviation of the double-reciprocal plot in Figure 3B may be indicative of negative cooperativity (Levitzki and Koshland, 1969) but preexistent heterogeneity in enzyme molecules cannot be excluded at this point.

The Reversibility of the Gpp(NH)p Activation. l-Epinephrine, in the presence of ATP and regenerating system, reverts the permanently activated state to a lower activity form. The extent of reversion is hormone-dependent and increases with hormone concentration (Figure 4). High concentrations of Gpp(NH)p in the presence of the hormone will shift the enzyme back to its high activity form. Propranolol, a potent β blocker, is capable of blocking *both* hormone-dependent processes, thus suggesting that both the activation process and the deactivation process are hormone dependent. These results can be summarized in the following scheme:

$$R \cdot E \underset{\text{hormone}}{\overset{\text{hormone, Gpp(NH)p}}{\rightleftharpoons}} E' \underset{\text{hormone}}{\overset{\text{hormone, Gpp(NH)p}}{\rightleftharpoons}} E'' \qquad (5)$$

where E is an enzyme form possessing a k_{cat} of about 120 min^{-1} typical for the nonactivated state and E″ represents the most active form of the enzyme possessing a turnover number of k_{cat} = 900–1200 min^{-1}. The form E″ is stable in the absence of hormone or in the presence of hormone provided that excess free Gpp(NH)p is present. In the presence of hormone and in the absence of excess Gpp(NH)p, E″ is converted to a less active form, E′, which possesses an intermediate turnover number of around k_{cat} = 530 min^{-1}. An alternative explanation would be that E″ cannot in fact revert all the way back to E but the process is inefficient due to partial desensitization of E″ toward the hormone. Under these circumstances, the effect of hormone on E″ will be incomplete. Therefore, the intermediate activity designated as E′ in fact represents a mixture of E and E″.

The Nature of Gpp(NH)p Activation. The nature of the process that is responsible for the Gpp(NH)p activation is still not known. It was suggested (Pfeuffer and Helmreich, 1975) that the activation of the turkey erythrocyte adenylate cyclase by Gpp(NH)p results from a physical separation between the regulatory subunit and the catalytic subunit, a process similar to the activation of protein kinase by cAMP. Another possibility was suggested by Cuatrecasas et al. (1975). They considered the possibility that enzyme activation involves a covalent pyrophosphorylation by Gpp(NH)p. So far, no experimental clue is available as to the chemical event occurring upon Gpp(NH)p activation. Whether a two- or a three-state model should be adopted is unclear. In the adenylate cyclase from fat cells three states were identified kinetically, in the presence of glucagon and Gpp(NH)p, by Rodbell and his colleagues (Rendell et al., 1975). It thus seems that the basic mechanisms of adenylate cyclase activation in a number of independent systems are very similar.

GTP and Gpp(NH)p. The fact that Gpp(NH)p has a dramatic effect, whereas GTP does not, indicates that although GTP is probably the physiological effector, it is devoid of any effect in this system. It should, however, be noted that the hormone activity measured in the absence of additional guanyl nucleotide may reflect the effect of saturating concentrations of GTP that could be provided as a contamination of the ATP added in the assay. Since GTP binds with affinities close to or below 2×10^{-7} M (Spiegel and Aurbach, 1974) to the regulatory sites, this possibility can indeed be real.

The fact that GTP activates the broken-membrane preparation only slightly is probably due to the formation of the nonactivating species, GDP, by phosphohydrolases and its competition with GTP for the regulatory site. As was pointed out by Pfeuffer and Helmreich (1975), the presence of a GTP regenerating system in the assay mixture does not eliminate the possibility that GDP is formed rapidly close to the regulatory site but dissociates slowly from that site. Thus, the replacement of GDP by the activator GTP is largely independent of external GTP. The fact that the hormone stimulated activity in the absence of Gpp(NH)p is a fraction of that observed in the presence of Gpp(NH)p may therefore reflect a difference in the steady state concentration of the enzyme in the E″ state.

It may be that the hydrolysis of GTP is fast enough to account for its limited availability. Furthermore, GTP breakdown may be a controlling element in the activity of β-receptor-dependent adenylate cyclase. Indeed, it has been shown recently by Cassel and Selinger (1976) that turkey erythrocyte membranes possess a β-receptor-dependent specific GTPase. In summary, we would like to suggest that the phenomena analyzed for Gpp(NH)p are fully applicable to GTP with one basic difference: the activation effect induced by GTP is readily reversible because of its rapid hydrolysis.

Calcium Inhibition of Adenylate Cyclase. We have previously shown that Ca^{2+} inhibits cooperatively the catecholamine-stimulated adenylate cyclase activity in the membrane preparation as well as in the intact cell (Steer and Levitzki, 1975a,b; Steer et al., 1975). It was therefore of interest to examine whether the Gpp(NH)p fully activated enzyme is also inhibited by Ca^{2+} as well. It was, indeed, found (Figure 6) that Ca^{2+} inhibits the fully activated enzyme and the enzyme not exposed to Gpp(NH)p in an identical fashion. These observations indicate that the two regulatory ligands Gpp(NH)p and Ca^{2+} interact with the adenylate cyclase system by two independent mechanisms.

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Affinity Labeling of Bovine Colostrum Galactosyltransferase with a Uridine 5′-Diphosphate Derivative†

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ABSTRACT: The dialdehyde produced by the periodate cleavage of the ribose moiety of uridine 5′-diphosphate (UDP) has been used as an affinity label for the UDP-galactose/UDP binding site of galactosyltransferase from bovine colostrum. This derivative causes progressive inactivation of galactosyltransferase at a rate dependent on its concentration, and under certain conditions is a competitive inhibitor with respect to UDP-galactose. The substrate UDP-galactose protects the enzyme from inactivation. The inactivation is also dependent on Mn^{2+} concentration, in a range that implies that the binding of Mn^{2+} at site I is a prerequisite for the binding of the UDP derivative. The inactivation can be progressively reversed by nitrogenous bases, or stabilized by KBH_4 reduction, which is consistent with the hypothesis that a Schiff base has formed with a lysine residue. Galactosyltransferase was inactivated with a [^{3}H]UDP derivative and the predominant labeled peptide, from thermolysin digestion, isolated and characterized as: Ser-Gly-Lys-UDP

UDP-D-galactose–*N*-acetylglucosamine β-4-galactosyltransferase (EC 2.4.1.38), catalyzes two reactions of distinct physiological significance: (1) a step in the serial addition of monosaccharides during the biosynthesis of plasma-type glycoproteins: the transfer of galactose from UDP-galactose to glycoprotein bound GlcNAc[1] to form an *N*-acetyllactosamine moiety. Free GlcNAc is also an acceptor in this reaction. (2) The transfer of galactose from UDP-galactose to glucose to form lactose, a reaction for which, at physiological glucose levels, the regulatory protein α-lactalbumin is also required. Galactosyltransferase and α-lactalbumin together form the lactose synthase enzyme system (EC 2.4.1.22) that catalyzes the terminal, rate-limiting step in the biosynthesis of lactose in the lactating mammary gland. The various aspects of this complex enzyme have been reviewed recently (Brew and Hill, 1975; Hill and Brew, 1975).

Galactosyltransferase can be isolated as a soluble homogeneous glycoprotein, molecular weight 50 000, from bovine colostrum, (Powell and Brew, 1974), while an enzyme form isolated from bovine milk contains variable amounts of partial proteolytic degradation products (Barker et al., 1972; Magee et al., 1974; Powell and Brew, 1974). Galactosyltransferase in these secretions appears to originate from a membrane-bound enzyme form present in the golgi apparatus of the mammary gland, and it is used as a marker enzyme for the golgi membranes from the mammary gland and other tissues (Fleischer et al., 1969; Schachter et al., 1970). Divalent metal ions are essential for the activity of galactosyltransferase. While the enzyme was previously supposed to possess a single Mn^{2+} specific binding site (see Ebner, 1973, Brew and Hill, 1975; Hill and Brew, 1975) more recent studies have revealed the presence of two activating metal-binding sites. The first, designated site I can accept Mn^{2+} (dissociation constant of 2.3×10^{-6} M), and must be occupied before binding of UDP-galactose, regulatory protein or acceptor substrate can occur. The second site, designated site II, is a lower affinity site that can accept either Mn^{2+} or Ca^{2+} (dissociation constant of $1-2 \times 10^{-3}$ M) and has a specific kinetic interconnection with UDP-galactose, indicative of ordered equilibrium binding, but

† From the Department of Biochemistry, University of Miami School of Medicine, Miami, Florida 33152. *Received February 11, 1976.* This work was supported by a grant from the National Institutes of Health (GM 21363). A preliminary account of some of these studies has been previously reported (Powell and Brew, 1975b).

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[1] Abbreviations used are: GlcNAc, *N*-acetylglucosamine; dial-UDP, the dialdehyde formed by periodate cleavage of the ribose moiety of UDP, as described in reaction 1; DNS, dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; UDP, CDP, ADP, uridine, cytidine, and adenosine 5′-triphosphates; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; BSA, bovine serum albumin.