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THE REGULATORY CONTROL OF β-RECEPTOR DEPENDENT ADENYLATE CYCLASE

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The characteristics of the β-receptor in turkey erythrocyte adenylate cyclase were studied using both kinetics of enzyme activation and direct binding measurement of the β-agonists and antagonists to the β-receptor. The regulatory ligands Gpp(NH)p and Ca^{2+} do not have any direct effect on the β-receptor, but modulate the enzyme activity through the interaction with specific regulatory sites.

It was found that the role of the catecholamine hormone is to facilitate the activation of the enzyme by the guanyl nucleotide. The regulatory guanyl nucleotide binds to its allosteric site in the absence of hormone, but the activation of the enzyme is slow in the absence of hormone. This role of the hormone can be described by the scheme:

$$R.E \xrightarrow[\text{fast}]{\text{Gpp(NH)p}} R.EG \xrightarrow[\text{fast}]{\text{Hormone}} HR.EG \xrightarrow[\text{limiting}]{\text{rate}} HR.E'G$$

Where R is the receptor, E the enzyme, G the guanyl nucleotide, H the hormone, and E′ the activated form of the enzyme. The binding steps are fast and reversible but the conversion of the inactive enzyme E to its active form occurs with a $k \sim 1.0\ min^{-1}$ In the absence of the β-agonist (*l*-catecholamine) at the β-receptor and at physiological free Mg^{2+} concentrations, the activation of the enzyme is insignificant. 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. At high (nonphysiological) Mg^{2+} concentration the conversion of E to E′ occurs slowly in the absence of hormone probably by another pathway.

There are two classes of Gpp(NH)p regulatory sites: tight sites and loose sites,

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Abbreviations: Gpp(NH)p, guanylyl-imidodiphosphate.

both of which can be identified kinetically. We have also identified the tight sites by direct binding studies using ^{3}H-Gpp(NH)p. It is not clear, however, whether these are two distinct classes of sites or whether their existence reflects the presence of negative cooperativity among the guanyl nucleotide regulatory sites.

Calcium was found to be a negative allosteric inhibitor of adenylate cyclase. The inhibitory effect of Ca^{2+} is exerted on the nonactivated enzyme as well as on the Gpp(NH)p preactivated enzyme.

The hormone dependent adenylate cyclases are regulatory enzymes embedded in membranes and provide one of the means of communication between the extracellular fluid and the intracellular biochemical events. The control of this enzyme by extracellular hormones and intracellular regulatory ligands is, therefore, of prime importance. It is known that occupancy of the hormone receptor is an obligatory condition for enzyme activation, but it has only recently become apparent that other regulatory ligands are involved.

Recently it was shown that GTP plays a key role as a regulatory ligand in the action of hormone activated adenylate cyclases (1–3). It has also been shown that the GTP analogs Gpp(NH)p and to a lesser extent Gpp(CH_2)p are superior to GTP in stimulating hormone activated adenylate cyclases (2 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 effects. This synergistic effect is seen with GTP analogs stable to phosphotransferase reactions such as Gpp(NH)p and Gpp(CH_2)p, probably because the hydrolysis products of GTP inhibit GTP induced activation.

Similar to many other hormone stimulated adenylate cyclases (2), it was demonstrated that *l*-epinephrine stimulated adenylate cyclase from nucleated erythrocytes is also activated by Gpp (NH)p (4–6). Guanyl nucleotides are not the only regulatory ligands that modulate adenylate cyclase activity. It was found that Ca^{2+} inhibits catecholamine stimulated adenylate cyclase by affecting only the k_{cat} of the enzyme (7). Calcium was shown to have no effect on the affinity of the β-receptor toward β-agonists and β-antagonists (7, 8). The Ca^{2+} inhibitory effect could also be demonstrated in the intact turkey erythrocyte (9).

This communication attempts to integrate our knowledge about the role of the different effector ligands on the adenylate cyclase-receptor complex.

METHODS

α-^{32}P-ATP was obtained from Radiochemical Center, England. ^{3}H-d*l* propranolol (5.3, Ci/mmole) was obtained from the Negev Nuclear Research Center, Dimona, Israel. ATP, cAMP, d-propranolol, *l*-epinephrine, d-norepinephrine, *l*-norepinephrine, d-epinephrine, ephedrine, metanephrine, phenylephrine, tyramine, and dopamine were obtained from Sigma. Gpp(NH)p was obtained from Boehringer. 2-isopropyl-ethanolamine was a gift of Dr. S. Mayani from Tel Aviv University. All other chemicals were of the highest analytical grade available. All solutions were made in Corning double distilled water.

Turkey erythrocyte plasma membranes were prepared as described elsewhere (7). Ghosts in 20% sucrose were kept at -196°C in liquid nitrogen in glass test tubes to allow fast thawing thus insuring no inactivation. Following this procedure the specific activity of the plasma membrane adenylate cyclase remains high and constant.

Adenylate cyclase activity was measured by the method of White and Zinser as described earlier (7). ^{3}H-propranolol and catecholamine binding were measured as described earlier (8, 10, 11).

RESULTS

The Characteristics of the β-Receptor

The affinity of a number of partial agonists and antagonists, all of which are ethylamine derivatives, was measured by competition with epinephrine in the adenylate cyclase assay (8). The value for the dissociation constants $\frac{1}{K_2}$ (or K'_D) for the various ligands was calculated from equation:

$$v_{obs} = \frac{K_2 L_2 V_2}{1 + K_2 L_2} + \frac{K_1}{1 + K_2 L_2} (V_1 - v_{obs})L^1 \quad (1)$$

where v_{obs} is measured specific activity; L_2, the concentration of the partial agonist or antagonist in the assay; K_2, the intrinsic association constant; V_2, the maximal specific activity obtained with the partial agonist; V_1, the maximal specific activity with the full agonist; L_1, the concentration of the full agonist in the assay; and K_1, its intrinsic affinity. From the study of the v_{obs} dependence on L_1 at different but constant values of L_2, one can obtain the parameters K_2 and V_2 (8). From the displacement of ^{3}H-propranolol by partial agonists and antagonists the dissociation constant of the ligand-receptor complex was computed according to equation 2:

$$K'_D = \frac{D_{0.5}}{[^3\text{H-PPL}]} K'_{PPL} \quad (2)$$

where K'_{PPL} is the propranolol-receptor dissociation constant; K'_D is the dissociation constant for the ligand; $D_{0.5}$ the ligand concentration required for 50% displacement; and [^{3}H-PPL] the concentration of ^{3}H-propranolol in the binding experiment (8, 11). The close correspondence between the values obtained by the kinetic method and the values obtained from the binding experiment is shown in Table I.

THE ACTIVATION OF THE ENZYME BY Gpp(NH)p

Preactivating of the Enzyme

It was found (4–6) that Gpp(NH)p, the nonhydrolyzable analog of GTP, potentiates the activity of β-receptor dependent adenylate cyclase, as well as the activity of other hormone-dependent adenylate cyclases (2). It was also found that if the enzyme is preactivated by incubation with *l*-epinephrine and Gpp(NH)p and then the added ligands are washed thoroughly, the enzyme activity remains stable in the absence of hormone for many hours. This finding was reported earlier for the frog erythrocyte adenylate cyclase (5, 6). The preactivation process can be inhibited by propranolol (Fig. 1). Propranolol, however, stops the activation process but does not reverse it. Thus, 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 is observed. This finding is identical whether or not the hormone and the Gpp(NH)p are removed prior to propranolol addition.

TABLE I. The Ligand Specificity of β-Receptors

	Maximal velocity induced in adenylate cyclase pmoles cAMP/mg/min	β-Receptor-ligand dissociation constant μM	
		From adenylate cyclase activation	From binding measurements
l-isoproterenol	160 ± 15	0.50 ± 0.05	0.21 ± 0.02[a]
d*l*-isoproterenol	160 ± 15	1.00 ± 0.10	0.25 ± 0.02[b]
l-epinephrine	160 ± 15	6.0 ± 1.0	2.0 ± 0.4
d-epinephrine	1 ± 1[c]	No effect[d]	No binding[d]
l-norepinephrine	160 ± 15	6.0 ± 0.5	2.6 ± 0.5
d-norepinephrine	1 ± 1[c]	No effect[d]	No binding[d]
dopamine	16 ± 1	22 ± 2	24 ± 3
l-phenylephrine	16 ± 1	61 ± 5	50 ± 5
l-ephedrine	0.0	450 ± 50	370 ± 50
tyramine	0.0	1000 ± 200	410 ± 50
d*l*-metanephrine	0.0	800 ± 150	500 ± 100
2-isopropyl-ethanolamine	0.0	No effect[d]	No binding[d]
l-propranolol	0.0	0.0012 ± 0.0002	0.0012 ± 0.0002
d-propranolol	1 ± 1	No blocking[e]	No binding[e]

[a]Data from Atlas et al. (1974)

[b]From equilibirum dialysis studies using ^{3}H-d*l*-isoproterenol in the absence of propranolol and in the presence of propranolol (Levitzki et al. (1974)).

[c]The basal adenylate cyclase activity in the absence of hormone or F^- is between zero and 1 pmoles cAMP/min/mg.

[d]Concentrations checked were up to 5×10^{-4}M.

[e]Concentrations checked were up to 25-fold higher than the concentration of *l*-propranolol giving the maximal effect.

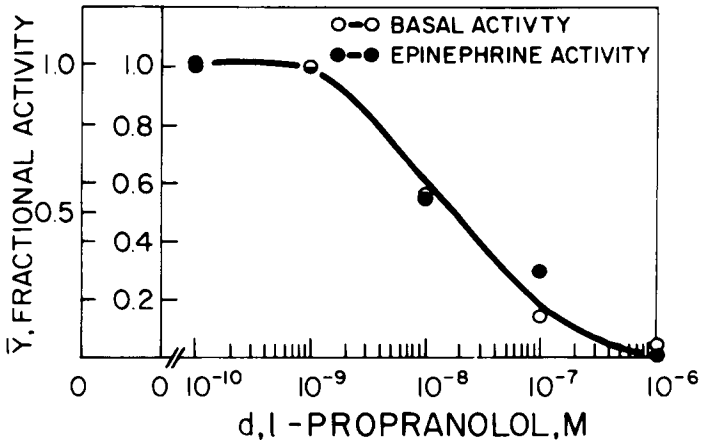

Fig. 1. Inhibition of enzymic activation by propranolol. One milligram of membrane enzyme was incubated with *l*-epinephrine (10^{-7} M) and Gpp(NH)p (10^{-4} M), 2.0 mM $MgCl_2$, 1 mM EDTA, Tris-HCl pH 7.4 (0.05 M) at 37°C, for 40 min. Different concentrations were added to the incubation mixture. After the incubation period the enzyme was thoroughly washed and assayed in the absence of added ligands. The maximal activity in the absence of propranolol was 1,250 pmoles cAMP × mg^{-1} × min^{-1} and in the presence of epinephrine, 500 pmole cAMP × mg^{-1} min^{-1}.

Kinetics of Gpp(NH)p Activation as a Function of Hormone Concentration

When the adenylate cyclase preparation is allowed to preincubate with hormone and Gpp(NH)p, prior to its assay, it is found that the enzyme is irreversibly converted to its stimulated form. Thus, when the enzyme is preincubated in the presence of 10^{-4} M Gpp(NH)p and increasing *l*-catecholamine concentrations the specific activity of the enzyme increases as a function of time (Fig. 2). Each time point represents the time at which the activation process was stopped by the addition of propranolol and then the enzyme thoroughly washed (see experimental details, and the legends to Figs. 2 and 3). At each time point the enzyme is assayed in the absence of added stimulating ligands and thus represents the true state of activity to which the enzyme was stimulated.

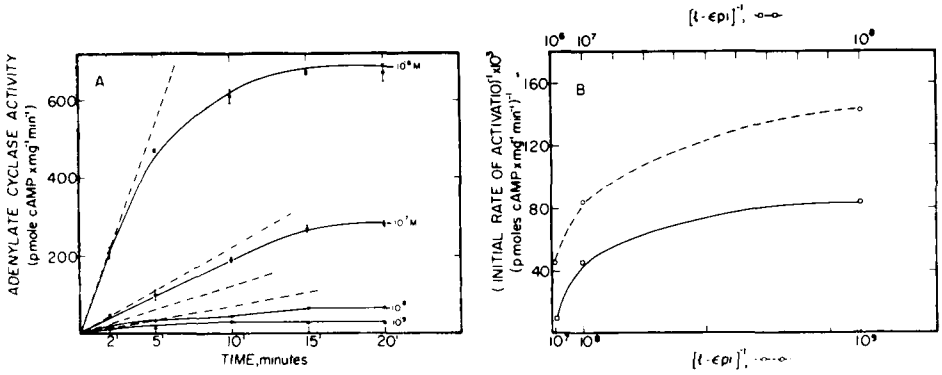

Fig. 2. Rate of adenylate cyclase activation by Gpp(NH)p as a function of *l*-epinephrine concentration. Plasma membranes were incubated with Gpp(NH)p 10^{-4} M, $MgCl_2$ 0.8 mM and varying concentrations of *l*-epinephrine at 37°C. Propranolol was added to stop the activation process at different times and then samples were taken, washed thoroughly and assayed for adenylate cyclase "basal" activity.

Let us assume that the activation rate of Gpp(NH)p saturated enzyme (GE) by hormone follows the scheme:

$$GE + H \underset{}{\overset{K_H}{\rightleftharpoons}} GEH \xrightarrow{k_3} GE'H \qquad (3)$$

where GE is the Gpp(NH)p-enzyme complex, K_H the receptor-hormone dissociation constant and k_3 is the rate constant at which E is converted to E′, then the rate of conversion of the enzyme to its active state, GE′, is given by:

$$v - v_o = \frac{k_3 E_o [H]}{K_H + [H]} \qquad (4)$$

where v_o in equation 4 represents the rate of enzyme activation by Gpp(NH)p alone. The data in Fig. 2A are analyzed in Fig. 2B by plotting the double reciprocal plot $\frac{1}{v}$ vs $\frac{1}{[H]}$. The extrapolated value on the double reciprocal plot (Fig. 2B) yields the maximal rate of enzyme activation $V_{max} = k_3 E_o$. Assuming that 1 mg of membrane protein contains about one picomole of enzyme (8), one can calculate a value of 1.0 min^{-1} for k_3.

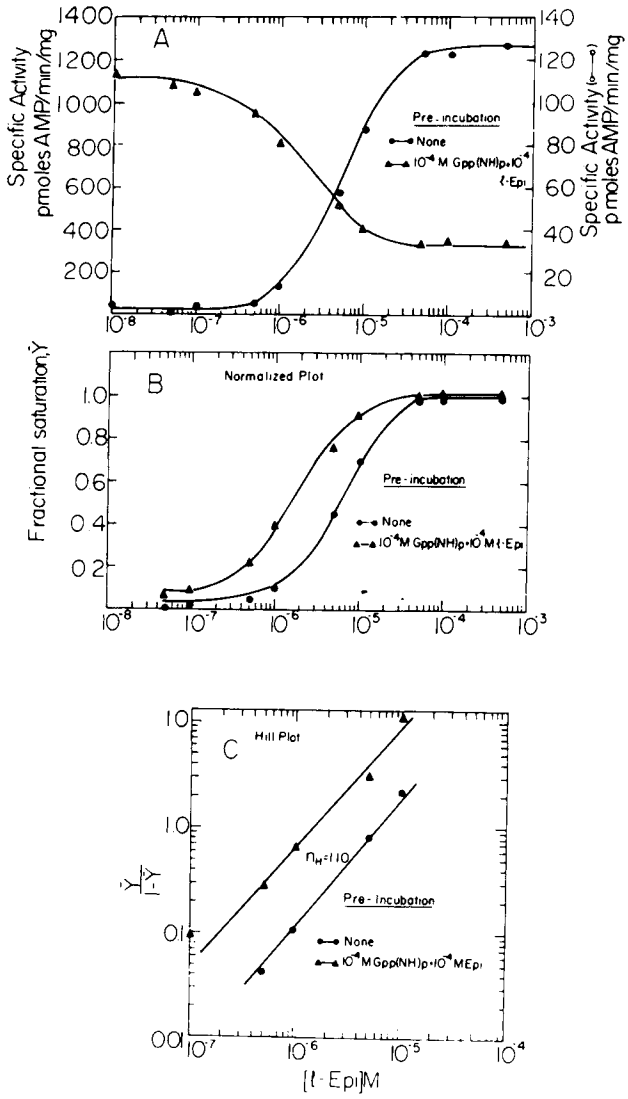

Fig. 3. The affinity of the β-receptor-cyclase complex toward *l*-epinephrine in preactivated membranes. Plasma membranes were incubated for 40 min at 37°C with Gpp(NH)p 10^{-4} M, or Gpp(NH)p 10^{-4} M + *l*-epinephrine 10^{-4}, 0.8 mM $MgCl_2$, washed thoroughly (dilution factor $1:10^8$) at the end of preincubation and assayed for adenylate cyclase activity as a function of *l*-epinephrine concentration in assay. Absolute values given (A), fractional activity (B), Hill plot of the above results (C).

The assumption that 1 mg of membrane protein contains 1 pmole of enzyme is based on the supposition that the receptor to enzyme stoichiometry is 1 : 1 (8, 10). The possible meaning of the deviation of the double reciprocal plot from linearity is discussed below (see Discussion).

The Hormone-Induced Antagonism of Preactivated Enzyme

When the enzyme is activated by Gpp(NH)p and *l*-epinephrine to the highest

activity and assayed in the absence of any regulatory ligand, the specific activity is the highest. When the assay is conducted in the presence of *l*-epinephrine, the activity is reduced (Fig 3A). The extent of decrease in activity depends on the hormone concentration and follows a saturation curve. This response curve can be linearized according to equation 5:

$$v_{obs} - V^{(1)}_{max} = K_H(V^{(2)}_{max} - v_{obs})\,[Epi] \tag{5}$$

where v_{obs} is the observed specific activity; $V^{(1)}_{max}$, the maximal specific activity in the presence of epinephrine; $V^{(2)}_{max}$, the maximal specific activity in the absence of epinephrine; and K_H, the epinephrine-receptor association constant. It can be seen (Figs. 3 and 4) that

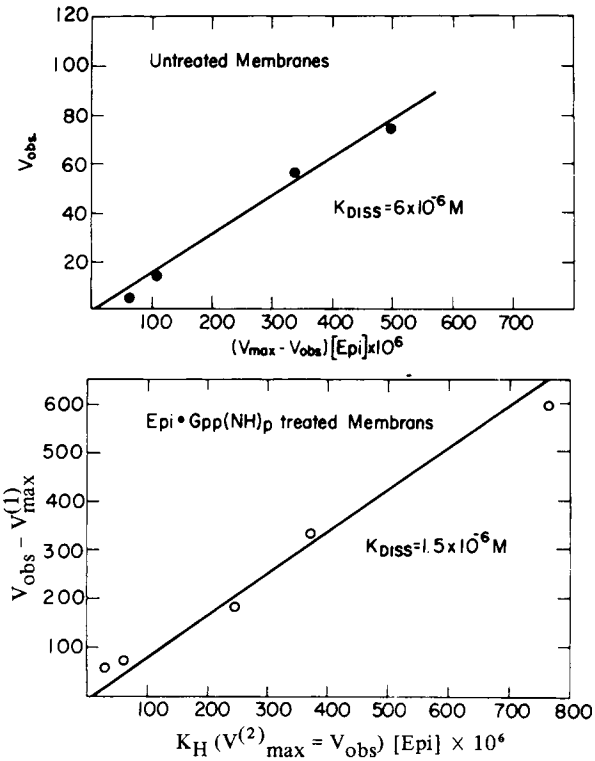

Fig. 4. The affinity of the β-receptor toward *l*-epinephrine in the preactivated enzyme and the non-activated enzyme. Analysis of results from Fig. 3 according to equation 5.

the affinity for the hormone is increased in the activated cyclase as compared to the enzyme not pretreated with Gpp(NH)p. The specific activity is always maximal when the enzyme is assayed in the presence of 10^{-4} M *l*-epinephrine and excess Gpp(NH)p (10^{-4} M) or when the preactivated enzyme is assayed in the absence of hormone or any other added ligand (basal activity) (Table II).

Reversibility of the Hormone Antagonism

When the fully activated enzyme is washed with *l*-epinephrine and assayed in the

TABLE II. Reversible Nature of the Partial Inhibition by Hormone of the Gpp(NH)p Activated Enzyme

		Assay		
Preincubation	Washings	Basal	10^{-4} M *l*-epinephrine	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*	538 ± 35	–
Gpp(NH)p + *l*-epinephrine	10^{-4} M *l*-epinephrine then buffer	1130 ± 50	649 ± 65	–

*Assay in the presence of 10^{-4} M *l*-epinephrine.

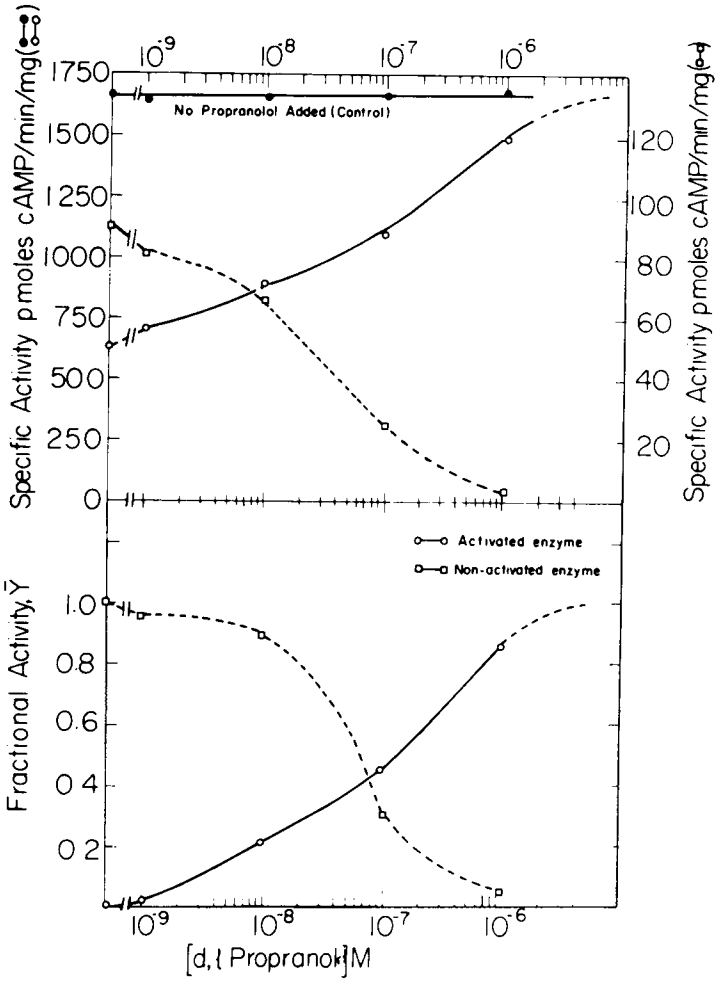

Fig. 5. The inhibition of the partial hormone Gpp(NH)p antagonism by propranolol. Plasma membranes were pretreated with Gpp(NH)p 10^{-4} M + *l*-epinephrine 10^{-4} M for 40 min at 37°C, then thoroughly washed and assayed for adenylate cyclase as a function of propranolol in assay in the absence or in the presence of 5×10^{-5} M *l*-epinephrine.

presence of *l*-epinephrine, the enzyme possesses 35–50% of the maximal activity (Figs. 1 and 5, Table II). When the hormone is removed, the specific activity returns to its maximal value (Table II). The hormone inhibition can also be reversed by excess Gpp(NH)p (10^{-4}M Gpp(NH)p, Table II).

The Inhibition of the Hormone Antagonism by Propranolol

When the fully activated enzyme is assayed in the presence of *l*-epinephrine and increasing concentrations of d*l*-propranolol, the antagonistic effect of the hormone (inhibition) is reversed (Fig. 5). From the mid-point ($S_{0.5}$) of the propranolol effect and the known hormone concentration in the assay (10^{-4}M), one can calculate (8, 11) the dissociation constant for propranolol K'_{ppL} from the formula:

$$K'_{ppL} = \frac{K'_H}{[H]} S_{0.5}^{ppL} \tag{6}$$

where $K'_H = 1.5 \times 10^{-6}$M (Figs. 3 and 4). The value calculated is 2.1×10^{-9}M, which is in very close agreement with the known dissociation constant of d*l*-propranolol in this system (8, 10, 11). This result may mean that although the affinity of the β-receptor toward *l*-epinephrine is increased, its affinity toward the blocker propranolol remains essentially unchanged (see Discussion). Indeed, direct binding measurements of ^{3}H-propranolol show that the affinity of the β-receptor toward the blocker is unchanged in the presence of Gpp(NH)p (Fig. 6).

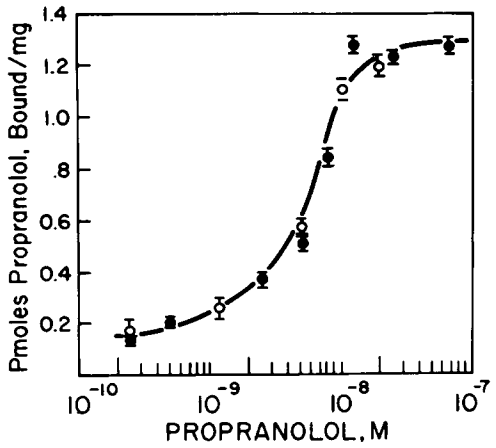

Fig. 6. The binding of ^{3}H-propranolol in the absence and in the presence of Gpp(NH)p. The binding experiments were conducted as described earlier (Levitzki et al., 1974); membranes (1. 5 mg protein) were suspended in 0.05 M Tris-HCl pH 7.6-1 mM EDTA-2 mM $MgCl_2$.

The Activity of Adenylate Cyclase by Gpp(NH)p Alone

It was consistently found that the activation of adenylate cyclase by hormone and Gpp(NH)p requires low concentrations of Mg^{2+} (12). It was also found that under the same conditions the enzyme can also be activated by Gpp(NH)p alone but extremely slowly. However, at increased Mg^{2+} concentrations the extent of enzyme activation by Gpp(NH)p alone can reach 70% of the maximal activation within 24 hr and with 5 mM free Mg^{2+}.

In the presence of magnesium concentrations higher than this value, close to 100% activity is achieved.

The Allosteric Inhibition of Adenylate Cyclase by Ca^{2+}

It was demonstrated earlier (7, 9) that the hormone activated enzyme is inhibited by Ca^{2+} both in the isolated plasma membranes (7) and in whole turkey red cells in the presence of Ca^{2+} ionophore (9). It was, therefore, of interest to examine if the Gpp(NH)p + hormone activated enzyme still 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 *l*-epinephrine, when its specific activity is about one-half of the maximal activity (Fig. 7). The responses to Ca^{2+} of the enzyme when stimulated by hormone alone and by hormone + Gpp(NH)p are identical. In both cases $S_{0.5}$ 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 species of the enzyme differ in their k_{cat} values by an order of magnitude, their response to Ca^{2+} is identical. Calcium on the other hand does not interfere with the process of enzyme activation by hormone and Gpp(NH)p (8).

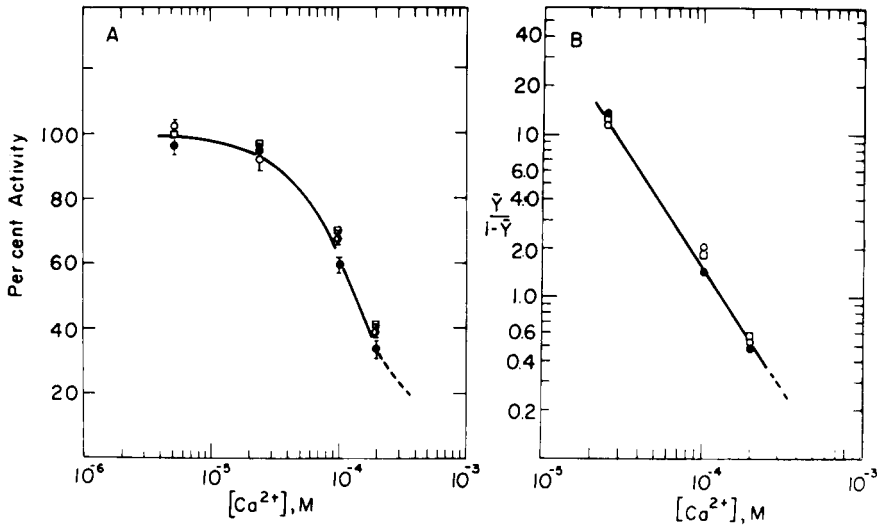

Fig. 7. Inhibition of adenylate cyclase activity by Ca^{2+} treated, or pretreated with Gpp(NH)p + *l*-epinephrine. Ghost membranes were preincubated in the presence of Gpp(NH)p 10^{-4} M + *l*-epinephrine 10^{-4} M + 0.9 mM$MgCl_2$ for 40 min at 37°C. After this period the membranes were washed thoroughly and assayed for adenylate cyclase activity as a function of Ca^{2+} concentration in the absence of (○ – ○), or in the presence (● – ●) of *l*-epinephrine 5.10^{-5} M. The maximal activity of the preactivated enzyme was 1,100 pmole/mg/min in the absence of epinephrine and 410 in the presence of epinephrine. The inhibition of hormone stimulated activity of the nonactivated enzyme (specific activity 100 ± 10 pmole/min/mg) is also shown (□–□).

DISCUSSION

The level of enzyme activity of the β-receptor dependent adenylate cyclase is not only controlled by the hormone but is modulated by a variety of ligands. These ligands include GTP and Mg^{2+} and regulatory Ca^{2+}. The binding of the β-agonist to the receptor

is an obligatory step in the activation of the enzyme but is not sufficient to switch the cyclase activity on. The process of receptor enzyme coupling involves GTP and free Mg^{2+} functioning as positive allosteric regulators. The enzyme is also controlled by a negative allosteric effector, Ca^{2+}.

The Role of GTP and Mg^{2+}

GTP and to a greater extent its nonhydrolyzable analog Gpp(NH)p stimulate adenylate cyclase synergistically with the *l*-catecholamines. It has also been demonstrated that Gpp(NH)p, which is resistant to phosphohydrolases, activates adenylate cyclase in the presence of hormone to a higher level of activity than GTP. It was suggested by Salomon and Rodbell (13) that Gpp(NH)p is more effective than GTP because GDP formed from GTP can inhibit the action of GTP by competing for the regulatory site. The maximal rate at which the enzyme is converted by Gpp(NH)p and hormone to its active form is 1.0 min^{-1} at 0.8 mM free Mg^{2+}. In the absence of hormone and at physiological free Mg^{2+} concentration (about 2 mM) the rate of enzyme activation in the absence of hormone is negligible, even at high concentrations of Gpp(NH)p. One can, therefore, conclude that the role of the hormone is as that of an allosteric activator enhancing the adenylate cyclase activation by the regulatory nucleotide Gpp(NH)p. Binding studies using ^{3}H-Gpp(NH)p (12) have indeed demonstrated that the binding process of the regulatory ligand is hormone independent. It is a post-binding conformational transition which is controlled by the *l*-catecholamine, Gpp(NH)p and free Mg^{2+}. Mg^{2+} is required for adenylate cyclase activity both in the form of the substrate MgATP and as free Mg^{2+} which binds to a regulatory site. We have also shown (14) that the process of enzyme activation by Gpp(NH)p and catecholamines requires the presence of free Mg^{2+}.

The Inhibition of Gpp(NH)p-Epinephrine Synergism by Propranolol

Propranolol, a potent β-blocker, inhibits the activation of the enzyme when added to the preincubation mixture (Fig. 1), but when added to the already activated enzyme, no inhibition occurs. It seems, therefore, that the hormone catalyzed activation of the enzyme-Gpp(NH)p complex occurs through conformational transitions induced at the β-receptor by the agonist. As in the inhibition of catecholamine stimulated adenylate cyclase, the β-blocker binds to the β-receptor, but unlike the agonist it cannot induce the proper conformational change in the enzyme.

Partial Antagonism between Epinephrine and Gpp(NH)p

When the preactivated enzyme is washed free of epinephrine and hormone and assayed (basal activity) it exhibits the highest specific activity (1,100 to 1,300 pmole/min/mg). However, when the enzyme is assayed in the presence of *l*-epinephrine this activity is lowered to 35–50% of the maximal value (Fig. 3, Table II). This inhibition is reversible since upon subsequent washing the epinephrine away, the activity returns to its maximal value (Table II). The partial inhibition of the cyclase activity of the preactivated enzyme is a function of the *l*-epinephrine concentration in the assay (Fig. 3). This concentration dependence follows a simple saturation curve, the linearized form of which (eq 5) is shown in Fig. 4. From this plot it can be seen that the affinity of the receptor toward *l*-epinephrine calculated from that effect is found to be four times higher than that of the β-receptor in the nontreated enzyme.

The preactivated and washed enzyme possesses tightly bound Gpp(NH)p which is not washed away. We can therefore tentatively conclude that an enzyme species possess-

ing tightly bound Gpp(NH)P has the maximal activity. However, the enzyme species in which both its tight regulatory sites for Gpp(NH)p are filled and its β-receptor occupied by an agonist exhibits lower activity (Fig. 3) and thus represents another conformation. The hormone induced antagonism is not due to Gpp(NH)p dissociation since the amount of ^{3}H-Gpp(NH)p bound is hormone independent (12). Also, upon removal of the hormone the enzyme reverts to its high activity form.

Propranolol reverses the partial inhibition of the preactivated enzyme by epinephrine (Fig. 5). The dissociation constant calculated for d*l*-propranolol for this competition is

$$K'_{ppL} = \frac{K'_H}{[H]} D_{0.5} = \frac{1.5 \times 10^{-6}}{5 \times 10^{-5}} \times 1.1 \times 10^{-7} = 3.2 \times 10^{-9} \quad (7)$$

The value 3.2 nM is close to the previously found values (2.5 nM to 3.2 nM) for d*l*-propranolol-β-receptor dissociation constant by either direct binding measurements or by the inhibition of hormone activated adenylate cyclase (8, 10, 11).

The preactivation of the enzyme by Gpp(NH)p and epinephrine causes a significant change (four-fold increase) in the apparent affinity toward the β-agonist epinephrine but no change in the receptor affinity toward the potent blocker propranolol. The absence of a change in β-receptor affinity toward the β-antagonist propranolol can also be demonstrated by binding studies (Fig. 6).

Propranolol does not alter the activity of the preactivated enzyme as does the agonist epinephrine. This finding is in accord with the capacity of propranolol to inhibit both the Gpp(NH)p-hormone antagonism as well as the hormone stimulated adenylate cyclase activity. Remembering that propranolol inhibits the process of hormone catalysis of enzyme activation by Gpp(NH)p (Fig. 1), it becomes clear that although propranolol binds to the β-receptor, it cannot induce the specific structural changes induced by the β-agonists.

Tight and Loose Gpp(NH)p Sites

The partial inhibition of the preactivated adenylate cyclase by epinephrine can be overcome by excess Gpp(NH)p (Table II). Adding Gpp(NH)p to the preactivated enzyme does not alter its maximal specific activity. Thus we can conclude that the enzyme species possessing Gpp(NH)p in the tight sites only, as well as in both the tight sites and loose sites possesses identical catalytic activity in the absence of a β-agonist. However, an enzyme species possessing Gpp(NH)p only in the tight sites and in the presence of a β-agonist in its receptor possesses only partial activity, close to half-maximal reactivity. This decrease in enzyme activity is not due to partial Gpp(NH)p dissociation from its regulatory site as was verified using ^{3}H-Gpp(NH)p (12). When both classes of Gpp(NH)p sites are filled and the β-receptor saturated with a β-agonist, full activity is also observed. The overall scheme describing the control of adenylate cyclase by hormone and Gpp(NH)p is shown in Fig. 8.

The hormone catalyzed activation of adenylate cyclase by Gpp(NH)p responds to *l*-epinephrine with apparent negative cooperativity (Fig. 2B) since the double reciprocal is concave downwards (15). At this stage, however, it is premature to establish whether this is true negative cooperativity with respect to hormone or whether it reflects the interaction between two classes of Gpp(NH)p sites.

The Activation of Adenylate Cyclase by Gpp(NH)p Alone

The maximum stimulation of adenylate cyclase by hormone and Gpp(NH)p re-

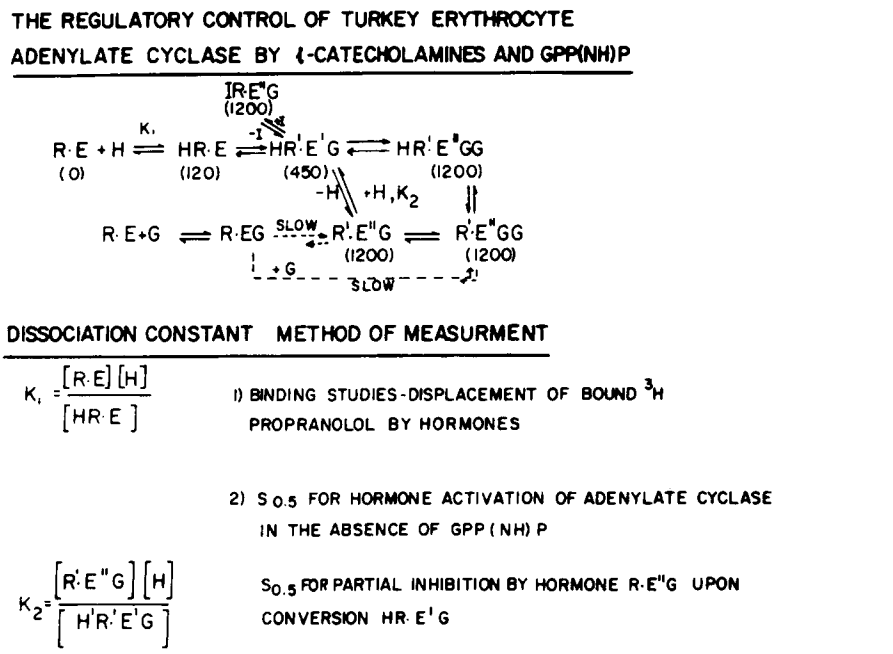

Fig. 8. The regulatory control of adenylate cyclase. The receptor enzyme complex. R · E can combine reversibly with the *l*-catecholamine to form HR · E which possesses the turnover number of 120 min^{-1} (Levitzki et al., 1974). This value may reflect the k_{cat} of the system in which the regulatory site is occupied by GTP or partly by GTP and partly by GDP (see text). In the presence of Gpp(NH)p G the enzyme has a k_{cat} value of 450 min^{-1} if it occupies only the tight regulatory sites and at the same time the β-receptor is bound with a β-agonist (H) such as *l*-epinephrine. In the absence of β-agonist or in the presence of the β-blocker propranolol (I) the enzyme is fully active (k_{cat} = 1,400 min^{-1}) when only the tight sites are filled. In the presence of hormone the enzyme will be fully active (k_{cat} = 1,400 min^{-1}), only when Gpp(NH)p is present in excess thus probably saturating a low affinity site, thus counteracting the partial Gpp(NH)p hormone antagonism. The receptor affinity toward the β-agonist H is increased as is revealed by the close response curve of hormone inhibition of preactivated enzyme binding Gpp(NH)p only at its tight site (Figs. 5 and 6); therefore is depicted as R! The enzyme can be activated slowly by Gpp(NH)p alone in the absence of hormone by a different pathway (dotted lines) provided, for example, by excess free Mg^{2+} in the preincubation medium.

quires low concentrations of free Mg^{2+} (12). The activation of the enzyme by Gpp(NH)p in the absence of a β-agonist and at low Mg^{2+} concentration is extremely small (Fig. 2). At higher Mg^{2+} (5 mM) concentrations and long incubations the enzyme can be slowly activated by Gpp(NH)p alone to at least 70% of the maximal activation obtained by the synergistic actions of hormone and Gpp(NH)p. This observation indicates that the enzyme can, in principle, be activated by Gpp(NH)p alone and further supports the supposition that the function of the hormone is to catalyze the process of enzyme activation by Gpp(NH)p.

Absence of a GTP Effect

Under the assay conditions which apply ATP and ATP regenerating system, we have found no stimulation of the hormone activation by added GTP or MgGTP (14). As pointed out by Pfeuffer and Helmreich (6), the presence of a GTP regenerating system does

not eliminate the possibility that GDP is formed rapidly close to the regulatory site. If the inhibitory GDP dissociates slowly from that site, its replacement by the activator GTP would not depend on the external GTP concentration but rather on the dissociation rate of GDP from the regulatory site. Since Gpp(NH)p is not hydrolyzed, its full effect is continuously expressed. In the in vivo situation GTP probably functions as Gpp(NH)p in vitro, since the GTP splitting activity is separated topographically from the adenylate cyclase complex.

The Role of Calcium

Calcium inhibits the catecholamine stimulated adenylate cyclase activity in the membrane preparation (7) as well as in the intact cell (9). The Ca^{2+} functions by interacting with specific allosteric sites in a positively cooperative fashion (Fig. 7). Ca^{2+} inhibits the fully activated enzyme and the enzyme not exposed to Gpp(NH)p in an identical fashion (Fig. 7). Furthermore, Ca^{2+} inhibits the activity of the enzyme-Gpp (NH)p-hormone complex where the nucleotide occupies only the tight site (species HR · E′G in Fig. 8) also in the same fashion. All three species of the enzyme are inhibited by Ca^{2+} where the effect of Ca^{2+} is positively cooperative with a Hill number $n_H = 1.5$, a value close to the one reported by us for the nonactivated enzyme (7). These observations indicate that the control of k_{cat} of the adenylate cyclase is independent of the other regulatory ligands controlling the enzyme activity. It was indeed shown that Ca^{2+} has no effect on receptor affinity toward β-agonists or toward the antagonist propranolol (7, 8). It can, however, be demonstrated that Ca^{2+} has no effect on the synergistic activation of the enzyme by Gpp(NH)p and epinephrine (12). Thus Ca^{2+} is a true negative V-effector of the adenylate cyclase system.

REFERENCES

1. Rodbell, M, Birnbaumer, L., Pohl, S.L., and Krans, H. M. J. J. Biol. Chem. 246:1877 (1971).
2. Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J., and Rodbell, M. Proc. Nat. Acad. Sci. U.S.A. 71:3087 (1974).
3. Rodbell, M., Krans, H. M. J., Pohl, S. L., and Brinbaumer, L. J. Biol. Chem. 246:1872 (1971).
4. Spiegel, A. M., and Aurbach, G. D. J. Biol. Chem. 249:7630 (1974).
5. Schramm, M., and Rodbell, M. J. Biol. Chem. 250:2232, (1975).
6. Pfeuffer, T., and Helmreich, E. J. M. J. Biol. Chem. 250:867 (1975).
7. Steer, M. L., and Levitzki, A. J. Biol. Chem. 250:2080 (1975).
8. Levitzki, A., Sevilla, N., Atlas, D., and Steer, M. L. J. Mol. Biol. in press (1975).
9. Steer, M. L., and Levitzki, A. Arch. Biochem. Biophys. 167:371 (1975).
10. Levitzki, A., Steer, M. L., and Atlas, D. Proc. Nat. Acad. Sci. U.S.A. 71:2773 (1974).
11. Atlas, D., Steer, M. L., and Levitzki, A. Proc. Nat. Acad. Sci. U.S.A. 71:4246 (1974).
12. Sevilla, N., and Levitzki, A. (manuscript in preparation).
13. Salomon, Y., and Rodbell, M. J. Biol. Chem. in press (1975).
14. Sevilla, N., Steer, M. L., and Levitzki, A., submitted to Biochemistry.
15. Levitzki, A., and Koshland, D. E. Jr. Proc. Natl. Acad. Sci. U.S.A. 62:1121 (1969).