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Activation of Adenylate Cyclase by Phosphoramidate and Phosphonate Analogs of GTP: Possible Role of Covalent Enzyme-Substrate Intermediates in the Mechanism of Hormonal Activation

(membranes/active site pyrophosphorylation/organomercurial affinity column/ solubilized adenylate cyclase/hormone receptors)

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ABSTRACT **Incubation of rat fat pad membranes with 5′-guanylyliminodiphosphonate [Gpp(NH)p] and 5′-guanylylmethylenediphosphonate [Gpp(CH_2)p], but not GTP (with or without hormones), at 24° or 30° (but not at 4°) greatly stimulates adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] measured after thoroughly washing the membranes. The rate of activation is relatively slow, even with very high (and saturating) concentrations of the analogs. Binding alone appears to be insufficient for activation. Hormones (catecholamines, glucagon) increase the rate but not the extent of activation, even when saturating analog concentrations are used. The dependence on analog concentration (apparent K_m) varies with the time of incubation. GTP and very high concentrations of ATP inhibit the activation by Gpp(NH)p, but this effect is dependent on the length of incubation and can be overcome with time. The activated state is not reversed upon incubation of the washed membranes at 30°, even in the presence of GTP, or by solubilization with nonionic detergents. Also, Gpp(NH)p can directly stimulate the control, solubilized enzyme. The activated state of the solubilized enzyme persists upon specific adsorption to and subsequent elution from an organomercurial-agarose column. It is suggested that after forming reversible Michaelis complexes of relatively low affinity, these analogs may react irreversibly with the GTP regulatory site of the enzyme, perhaps forming p-(NH)p- and p(CH_2)p-covalent enzyme intermediates which capture the activated state of the enzyme. GTP, after binding, may normally activate the enzyme by forming a "labile" pyrophosphoryl enzyme intermediate, and hormone receptors may function to increase the rate of formation (and thus concentration) of this active state of the enzyme.**

There is considerable evidence that stimulation of adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] by a variety of hormones is enhanced or facilitated by the interaction of GTP with noncatalytic, regulatory sites on the enzyme (1–11). Although GTP has only minimal or no stimulatory effects in the absence of hormones, the analog of GTP, 5′-guanylyliminodiphosphonate [Gpp(NH)p], stimulates the enzyme in the presence or absence of hormones in a variety of tissues (12–15). With this analog, enzyme activity is enhanced to levels very much greater than are observed with GTP plus hormones, or with sodium fluoride, especially if it is used in combination with hormones. The evidence indicates that Gpp(NH)p and GTP are acting at a common site which is distinct from the catalytic site and which is intimately involved in the regulation of hormonal activation (12–15). The present studies indicate that the stimulatory effects of Gpp(NH)p as well as of Gpp(CH_2)p, unlike those of GTP, are essentially irreversible and suggestive of the existence of covalent enzyme–substrate intermediates.

Abbreviations: Gpp(NH)p, 5′-guanylyliminodiphosphonate; App(NH)p, 5′-adenylyliminodiphosphonate; Gpp(CH_2)p, 5′-guanylylmethylenediphosphonate; cAMP, 3′:5′-adenosine-monophosphate.

MATERIALS AND METHODS

[α-^{32}P]ATP (10–20 Ci/mmol) was synthesized by the method of Symons (16, 26). Organomercurial-agarose was prepared by coupling (in 30% dimethylformamide) *p*-aminophenylmercuric acetate to Sepharose-4B with CNBr (17). Adenylate cyclase activity was determined (18, 26) in 0.1 ml containing [α-^{32}P]ATP (0.12–0.25 mM, 200–400 cpm/pmol), 6 mM $MgCl_2$, 5 mM aminophylline, an ATP-regenerating system (5 mM phosphoenolpyruvate and 50 μg/ml of pyruvate kinase), 50 mM Tris·HCl, pH 8.0, and 20–100 μg of protein. After 10 min at 30°, the assays were stopped by boiling (1 min) and cAMP was isolated from neutral alumina columns (19, 20). Fat pad "membranes" were prepared by homogenizing (Polytron) fat pads from 120–180 g Sprague-Dawley rats in 50 mM Tris·HCl, pH 8, or Krebs-Ringer-bicarbonate, pH 7.4, followed by centrifugation (30 min, 40,000 × *g*). Such particulate fractions (instead of fat cell membranes) greatly facilitate the recovery of membrane protein during the repeated washing procedures.

RESULTS

Stimulation of Gpp(NH)p Persists After Washing Membranes. In agreement with recent reports (12–15), addition of Gpp(NH)p directly into the adenylate cyclase assay stimulates enzyme activity (Table 1). However, even greater stimulation results if the membranes are incubated with Gpp(NH)p and then washed thoroughly before assay (Table 1). Such pretreated preparations show linear kinetics of cAMP production during the period of assay. In contrast, preincubation with GTP with or without hormones does not stimulate the enzyme after washing. Because of the apparently greater degree of stimulation found by preincubation, the existence of complex "lag" phases when Gpp(NH)p is added directly to the assay (12), the evidence for a slow irreversible activation process, and the possible interference by ATP, GTP, Gpp(NH)p itself or other nucleotides, all subsequent studies were performed by preincubating (and washing) before assay.

Time and Temperature Dependence of Gpp(NH)p Stimulation. Virtually no effect of Gpp(NH)p is seen at 4° (Fig. 1)

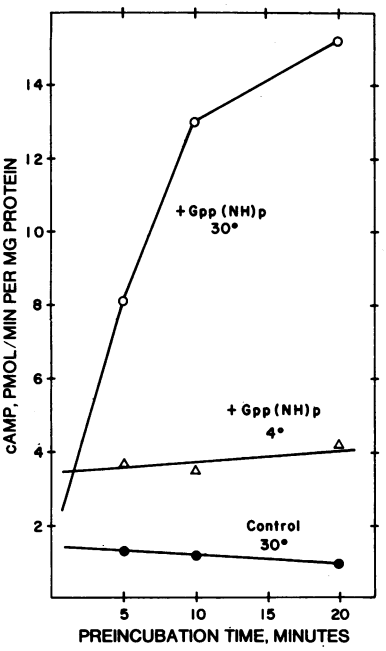

FIG. 1. Dependence of Gpp(NH)p stimulation of adenylate cyclase activity on time and temperature of preincubation with membranes. Membranes from fat pads of four 200 g rats were suspended in 4 ml of 50 mM Tris·HCl, pH 8. Portions (0.5 ml) were incubated in the presence (○, △) and absence (●) of 50 μM Gpp(NH)p for various times at 4° (△) or at 30° (○, ●), after which they were placed at 4° so that the total period of preincubation was 20 min for all the samples. The suspensions were brought to 14 ml with ice-cold 50 mM Tris·HCl, pH 8, and centrifuged for 20 min at 40,000 × g. The pellets were washed twice by suspension and centrifugation using 14 ml of the same buffer but containing 2 μg/ml of staphylococcal nuclease and 0.2 mM $CaCl_2$. The final pellets were suspended in 300 μl of 50 mM Tris·HCl, pH 8.

even with very high concentrations (e.g., 0.2 mM). Since it is very likely that simple "binding" can occur under these conditions, enzyme activation probably involves a process subsequent to binding. Further, membranes incubated at 4° and washed and incubated for 10 min at 30° before assay do not demonstrate activation. Even at 30° activation occurs relatively slowly (Fig. 1), although it can occur more rapidly with hormones or different buffer conditions.† Activation does, however, occur in a simple Tris buffer.

Effect of Hormones and Gpp(NH)p Concentration. The stimulatory effects of Gpp(NH)p are enhanced by (−)-isoproterenol (Fig. 2) or glucagon (not shown) when relatively short incubation periods are used. The effect of hormones appears to be strictly on the rate of the activation (Fig. 3). The rate of activation is relatively slow, even at concentrations of Gpp(NH)p beyond which no further effect on the rate is observed. Hormones can enhance the rate even under these conditions, suggesting that their action occurs at a step subsequent to the initial binding of the analog.†

It is difficult to describe the Gpp(NH)p concentration dependence in terms of an "apparent K_m" for the nucleotide. Since activation is a slow process, the "apparent K_m" value will vary with the time of incubation.† Similarly, although hormones may appear to change the apparent K_m for Gpp(NH)p, and Gpp(NH)p may appear to change the K_m for hormones, the kinetic significance of such effects is doubtful. Since the data are most consistent with an irreversible step following binding, K_m values for activation are not meaningful and K_m values for binding cannot be estimated simply.

† Manuscript in preparation.

TABLE 1. *Stimulation of adenylate cyclase activity by Gpp(NH)p when added directly in the assay and when preincubated (and washed) with membranes*

Experiment	Adenylate cyclase activity[a]
In assay	
No Gpp(NH)p	9.1 ± 0.8
Gpp(NH)p, 10 μM	19.2 ± 0.7
100 μM	25.0 ± 1.2
In preincubation	
No Gpp(NH)p	5.1 ± 0.6
Gpp(NH)p, 50 μM	62.5 ± 2.8
100 μM	108.2 ± 8.8

Fat pad membranes were either assayed directly in the presence and absence of Gpp(NH)p, or first incubated with Gpp(NH)p for 10 min at 30° in 50 mM Tris·HCl, pH 7.8. In the latter procedure, the membranes were washed two times with large volumes of ice-cold Tris·HCl buffer (Fig. 1) before assay.

[a] Picomoles of cAMP produced/min per mg of protein, mean value ± SEM.

Stimulation of Adenylate Cyclase Activity by 5'-Guanylylmethylenediphosphonate [Gpp(CH₂)p]. Preincubation of membranes with Gpp(CH₂)p also leads to stimulation (Table 2). However, the concentration of nucleotide required is greater and the maximal extent of activation achieved is lower than with Gpp(NH)p.† Hormones also increase the rate of activation by Gpp(CH₂)p.†

Inhibition of Gpp(NH)p Stimulation by GTP. In agreement with a common site of action of GTP and Gpp(NH)p (12–15), GTP can inhibit the stimulatory effects of Gpp(NH)p (Table 3). However, this inhibition is demonstrable only when low concentrations of Gpp(NH)p or short incubation periods are used. The inhibition by GTP can be reversed with long peri-

TABLE 2. *Effect of preincubating (and washing) fat pad membranes with Gpp(NH)p, Gpp(CH₂)p, App(NH)p, App(CH₂)p, and GTP*

Nucleotides in preincubation	Adenylate cyclase activity[a]
None	0.28 ± 0.06
Gpp(NH)p, 2 μM	1.24 ± 0.03
50 μM	6.16 ± 0.06
Gpp(CH₂)p, 50 μM	1.75 ± 0.03
200 μM	2.61 ± 0.08
App(NH)p, 50 μM	0.54 ± 0.04
200 μM	0.60 ± 0.05
App(CH₂)p, 200 μM	0.50 ± 0.06
GTP[b], 200 μM	0.41 ± 0.04

Fat pad membranes were incubated at 30° for 20 min in 50 mM Tris·HCl, pH 8, in the presence of various nucleotide analogs. The membranes were washed (4°) three times (Fig. 1) before adenylate cyclase activity was determined. The effects of the last three nucleotides in the Table do not represent true stimulation but rather stabilization of spontaneous inactivation.†

[a] Picomoles of cAMP/min per mg of protein, mean value ± SEM.

[b] These effects are unaltered if the preincubation is performed in the presence of (−)-isoproterenol or glucagon.

TABLE 3. *Inhibition by GTP of Gpp(NH)p stimulation of adenylate cyclase activity*

Additions during preincubation	Adenylate cyclase activity[a]
None	2.7 ± 0.1
Gpp(NH)p	23.0 ± 0.8
Gpp(NH)p +	
GTP, 1 mM	2.6 ± 0.1
ATP, 1 mM	22.7 ± 0.7
5 mM	18.1 ± 0.4
App(CH_2)p, 1 mM	21.1 ± 0.6
3 mM	15.5 ± 0.3

Fat pad membranes were incubated at 24° for 10 min in 50 mM Tris·HCl, pH 8.0, in the presence or absence of 30 μM Gpp(NH)p and other nucleotides, as indicated. Adenylate cyclase activity was determined after washing the membranes twice with the same buffer at 4°.

[a] Picomoles of cAMP/min per mg of protein, mean ± SEM.

ods of incubation,† in harmony with the irreversible nature of the Gpp(NH)p effect. K_i values for GTP therefore cannot be determined simply. Very high concentrations of ATP can also cause some inhibition. Since App(CH_2)p can also inhibit, it is likely that ATP itself, rather than contaminating GTP, can bind to the GTP site (albeit with low affinity).

Irreversibility of the Stimulated Enzyme. The activity stimulated by Gpp(NH)p (Table 4) or Gpp(CH_2)p is not reversed upon incubation (at 30°) of the thoroughly washed membranes. High concentrations of GTP or ATP, the presence (Table 4) or absence of divalent cations, or EDTA† does not reverse the stimulation. Further, after such incubations Gpp(NH)p does not stimulate further. The stabilization of enzyme activity by ATP and GTP against spontaneous inactivation (Table 4) will be discussed elsewhere.†

Detergent-Solubilized Adenylate Cyclase. The Gpp(NH)p-stimulated state is completely retained after solubilizing (18) the enzyme with Lubrol PX (Table 5). Furthermore, a time- and temperature-dependent, but hormone-independent, activation occurs if Gpp(NH)p is added directly to the solubilized enzyme.† The Gpp(NH)p-stimulated, solubilized enzyme can adsorb selectively to an organomercurial-agarose column (Fig. 4). After washing (24°) of the column with detergent, the enzyme, still in the activated state, can be eluted in good yield with dithiothreitol.

TABLE 4. *Absence of reversibility of adenylate cyclase activity previously stimulated with Gpp(NH)p*

Incubation			Adenylate cyclase activity[a]
First (30°, 20 min)	Second (30°, 15 min)	Second (30°, 30 min)	
No additions	No additions		1.5
No additions	GTP		4.1[b]
No additions	ATP		5.9[b]
Gpp(NH)p	No additions		55.4
Gpp(NH)p	GTP		50.6
Gpp(NH)p	ATP		66.2
Gpp(NH)p		No additions	27.5
Gpp(NH)p		GTP	35.6
Gpp(NH)p		ATP	48.8

Fat pad membranes were incubated for 20 min at 30° in Krebs-Ringer-bicarbonate buffer, 1 mM dithiothreitol, without additions or with 0.1 mM Gpp(NH)p. The membranes were washed (4°), resuspended in the same buffer, incubated at 30° for 15 or 30 min in the absence or presence of 2 mM GTP or ATP, and washed again before assay.

[a] Picomoles cAMP/min per mg protein.

[b] This "increase" represents protection from inactivation.†

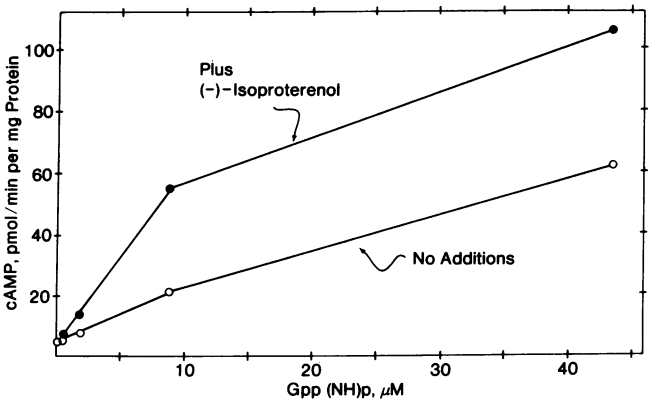

FIG. 2. Stimulation of adenylate cyclase activity by prior incubation (and washing) of fat pad membranes with various Gpp(NH)p concentrations in the presence (○) and absence (●) of 0.5 μM (−)-isoproterenol. Fat pad membranes were incubated for 10 min at 30° in 50 mM Tris·HCl buffer, pH 8.0, containing Gpp(NH)p.

DISCUSSION

The data presented here and elsewhere (12–15) indicate that Gpp(NH)p and Gpp(CH_2)p interact at the same site as GTP, although the properties of these analogs are very different from those of GTP. Nearly maximal enzyme activation by these analogs can occur in the absence of hormones, although the *rate* of this activation is clearly enhanced by hormones. Unlike the effects of GTP, the stimulatory effects of Gpp(NH)p and Gpp(CH_2)p persist unabated after thorough washing of the membranes.

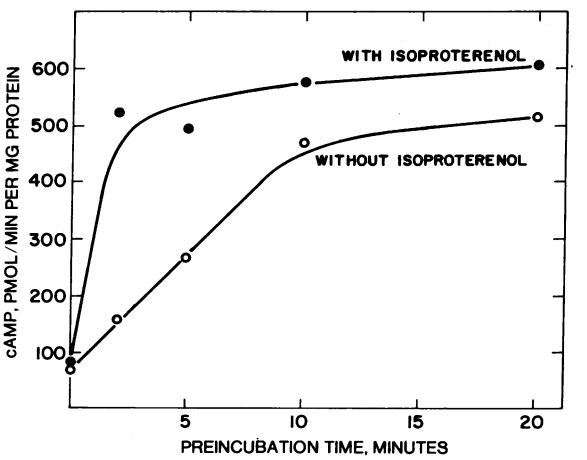

FIG. 3. Isoproterenol increases the rate of activation of adenylate cyclase by Gpp(NH)p. Membranes from isolated fat cells were incubated for various times (30°) in 50 mM Tris·HCl, pH 8, containing the ATP-regenerating system, ATP (0.25 mM), 5 mM $MgCl_2$, dithiothreitol (1 mM), and 0.1 mM Gpp(NH)p in the presence (●) or absence (○) of (−)-isoproterenol (20 μM). The reaction was stopped by diluting with 10 ml of ice-cold Krebs-Ringer bicarbonate, 10 μM propranolol. The membranes were washed twice with the same buffer and assayed in the presence of 10 μM propranolol.

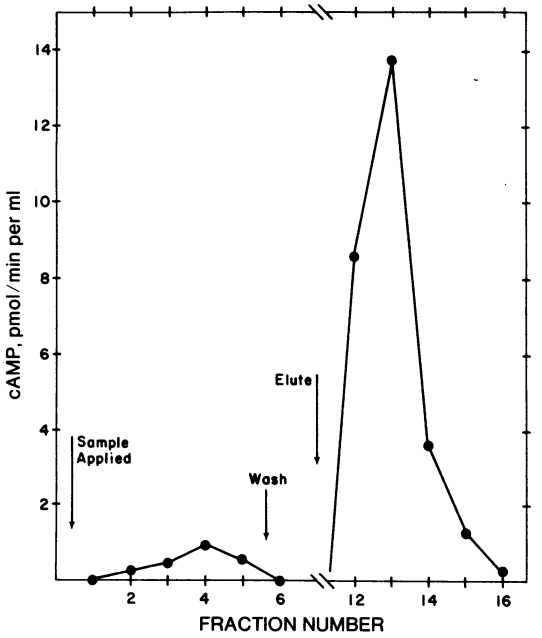

FIG. 4. Organomercurial-agarose chromatography of adenylate cyclase solubilized with Lubrol PX from fat pad membranes previously stimulated by incubation with Gpp(NH)p. The membranes from the fat pads of four rats were suspended in 1 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, incubated for 20 min at 30° with 0.2 mM Gpp(NH)p, and washed. The pellet was suspended (4°) in 2 ml of Krebs-Ringer-bicarbonate and solubilized with 1% Lubrol PX (Table 5). The solution (2.2 mg of protein per ml), after adjustment with ATP to 1 mM, was applied at 24° to a 1.5 ml column containing *p*-aminophenylmercuric acetate-agarose diluted 1:3 with unsubstituted agarose. The effluent fractions (nos. 1–6) were adjusted with dithiothreitol to 20 mM and placed at 4°. The column was washed (nos. 6–11) with Krebs-Ringer-bicarbonate, 0.5% Lubrol PX, 0.1% albumin, and eluted with the same buffer containing 20 mM dithiothreitol. Fractions of 20 μl were assayed. The total activity applied was 54 pmol of cAMP per min, while that recovered on elution was 41 pmol/min.

The evidence suggests that Gpp(NH)p and Gpp(CH_2)p are acting by a mechanism which involves a specific, irreversible chemical reaction. The stimulation is time-dependent and very sensitive to temperature. Virtually no activation occurs at 4°, even with very high analog concentrations, and whether or not the membranes are subsequently washed and incubated at higher temperatures before assay. Thus, "binding" alone would seem to be insufficient for activation. More importantly, the stimulated state persists after removal of the free compounds by thorough washing and incubation (at 30°) of the membranes, even in the presence of high concentrations of GTP, which presumably "binds" to the same site. If activation were to depend simply on binding of the analog, the complex would have to display an extraordinarily slow rate of "dissociation," and hence an equally extraordinarily high "apparent affinity." Unless an irreversible process occurs after initial binding, it is most difficult to reconcile such a high "affinity" for the overall process with the rather high analog concentrations required to achieve a maximal *rate* of activation and with the slow nature of this rate.† Furthermore, the analog apparent "affinity" varies with time, and the inhibition by GTP is readily overcome with time, observations most consistent with an irreversible process occurring after binding.

TABLE 5. *Retention of stimulated adenylate cyclase activity upon solubilizing membranes previously incubated (and washed) with Gpp(NH)p*

Solubilized enzyme preparation	Adenylate cyclase activity[a]
Control	2.4 ± 0.1
Control, + NaF (10 mM)	5.1 ± 0.2
Gpp(NH)p-treated	57.9 ± 1.2
Gpp(NH)p-treated, + NaF (10 mM)	49.9 ± 2.2

Membranes from eight fat pads were suspended in 50 mM Tris·HCl, pH 8, and incubated for 20 min at 30° with no additions (control) or with 0.1 mM Gpp(NH)p. After the membranes were washed two times (Fig. 1) they were suspended in 0.5 ml of Tris·HCl, pH 8, adjusted to 1% Lubrol PX, and sonicated three times for 1 sec. The suspension was then adjusted with $MgCl_2$ to 6 mM and albumin to 0.015% and centrifuged for 40 min at 40,000 × *g* (18). The supernatant was assayed in the presence and absence of NaF.

[a] Picomoles of cAMP/min per mg of protein, mean ± SEM.

In addition, the stimulated state is maintained upon solubilizing with detergents. Activation persists when the solubilized enzyme is selectively adsorbed to, washed extensively with detergent, and eluted from an organomercurial affinity column run at 24°.

The irreversible nature of Gpp(NH)p and Gpp(CH_2)p activation of adenylate cyclase calls for caution in interpreting values for the "K_m" of these analogs, of competitive substances (e.g., GTP), and of hormones that affect the reaction. When Gpp(NH)p is added directly to the adenylate cyclase assay mixture, the effects, although dramatic, are understandably complex (12). Since the rate of activation is slow and temperature- and hormone-dependent, it is clear why a "lag" should occur, and why this lag is diminished by hormones or higher temperatures. Also, in the assay inhibition by other nucleotides may occur, and it is difficult to discern which process is affected by certain agents (e.g., hormones, Mg^{++}). These complexities are simplified by incubating (and washing) prior to assay under standard conditions.

What may the chemical nature of the reaction of adenylate cyclase with Gpp(NN)p and Gpp(CH_2)p be? The specificity, persistent nature, and other properties of the activation suggest that, after the initial binding (probably of low affinity), these analogs are covalently reacting with the GTP regulatory site by a reaction that can be uniquely distinguished from the the GTP reaction by the greater stability of the bonds formed. Although the terminal, gamma phosphate moiety of Gpp(NH)p is chemically at least as labile as that of GTP (21, 22), and it can serve as a substrate for enzymes such as alkaline phosphatase (21), it is unlikely that simple phosphorylation is involved, since the analogous reaction with Gpp(CH_2)p would not be expected chemically and since the analogous enzyme intermediate formed from GTP would not be different. Formation of an enzyme-(NH)p intermediate is very unlikely because formation of the analogous enzyme-(CH_2)p complex from Gpp(NH)p would be chemically most improbable. Since the α–β phosphodiester linkage of the analogs is susceptible to enzymatic attack, as evidenced by the fact that App(NH)p is a substrate for enzymes such as snake venom phosphodiesterase (23) and adenylate cyclase (1, 24), the possibility of an enzyme-guanosine or enzyme-GMP inter-

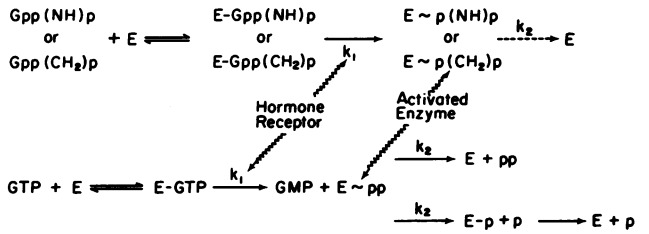

FIG. 5. Proposed mechanisms for the activation of adenylate cyclase by Gpp(NH)p, Gpp(CH_2)p, GTP, and hormones. The enzyme-nucleotide Michaelis complex is probably of relatively low affinity, at least for the analogs. Hormone receptor complexes increase k_1 (after initial binding). The *rate* but not the extent of formation of the active covalent-enzyme intermediate is affected by hormones when Gpp(NH)p or Gpp(CH_2)p is used because k_2 in this case is negligibly small compared to k_1. With GTP alone no activation occurs because k_2 is as great as or greater than k_1. k_1 is thus the rate limiting step in enzyme activation, i.e., in the formation of significant steady state concentrations of the activated intermediate. By analogy, the net effect of hormone-receptor complexes is to increase k_1 so that it exceeds k_2. GTP could activate in the absence of hormone if the conditions are such (e.g., certain tissues or incubation conditions) that $k_1 > k_2$. An essential feature of enzyme activation is the reaction with GTP, which is facilitated by hormones. High concentrations of ATP may substitute for GTP, and in certain tissues other nucleotides besides GTP (4, 25) may be involved.

mediate must be considered. However, these are unlikely, since the same intermediates would be formed from GTP. The most likely possibility, also involving α–β bond cleavage, is formation of enzyme-p(NH)p and enzyme-p(CH_2)p intermediates.

Extension of the above rationale leads to the postulate that with GTP the analogous, activated enzyme intermediate may normally be an adenylate cyclase–pp complex which breaks down very rapidly. The comparable enzyme-p(NH)p or -p-(CH_2)p intermediates would be much more stable, not necessarily because these are weaker leaving groups but because they are unfavorable enzyme-substrate intermediates for hydrolysis by the enzyme or by other pyrophosphatases or phosphatases.

On the basis of the available evidence, a possible mechanism for activation of adenylate cyclase by GTP, Gpp(NH)p, Gpp(CH_2)p and hormones is suggested in Fig. 5. Activation would normally involve formation at the regulatory site of a GTP-derived enzyme-pyrophosphoryl intermediate, and hormone receptor complexes would act principally to enhance the *rate* of formation, and thus to increase the steady state concentration, of such an intermediate. GTP necessarily plays an obligatory role, which is consistent with the absolute dependence of glucagon (and probably other hormones, refs. 2–11) action on GTP (1). Hormonal activation may normally occur in the absence of added GTP, but this usually requires high concentrations of the substrate, ATP, which, as suggested here (Table 3) and elsewhere (1) may act like (and thus replace) GTP at the regulatory site. In certain circumstances it is possible that nucleotides other than GTP (4, 25) may act preferentially, but by similar mechanisms. The scheme depicted in Fig. 5 predicts very complex kinetics of enzyme activation by hormones, nucleotides, divalent cations and temperature.

These studies suggest direct methods for identifying the postulated enzyme intermediates, since the analogs under study may in effect be active site-directed reagents. For example, the proposed mechanisms predict that specific labeling would occur with β- or γ-labeled [^{32}P]GTP, but not with α-labeled [^{32}P]GTP or [^{3}H]guanosine-labeled derivatives. Such studies are complicated by the scarcity and impure state of adenylate cyclase and the unavailability of the proper radioactive analogs. Extensive binding studies of ^{3}H-labeled Gpp(NH)p (8 Ci/mmol) in fat membranes and turkey erythrocyte ghosts indicate that the overall "binding" observed is not related to the process of activation described in this report.†

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