Binding of [^{3}H]Guanylylimidodiphosphate to Membranes: Lack of Correlation with Adenylate Cyclase Activation

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Received April 5,1976

SUMMARY: The binding of tritiated guanylylimidodiphosphate ([^{3}H]GMP-P(NH)P) to turkey erythrocyte ghosts was studied in parallel with the activation by GMP-P(NH)P of adenylate cyclase. The high affinity binding capacity for GMP-P(NH)P, 50 pmoles per mg protein, exceeds the estimated quantity of adenylate cyclase of 1 pmole per mg of protein. The rate of nucleotide binding is not affected by isoproterenol. Further, in the presence of the hormone the rate of binding is much slower than the rate of activation. Although the rate of dissociation of bound [^{3}H]GMP-P(NH)P is negligible at 37°, it is increased dramatically by unlabeled GMP-P(NH)P, GTP, EDTA, ATP, AMP-P(CH_2)P, or p-aminophenylmercuric acetate. In contrast, the rate of decay of the GMP-P(NH)P-simulated state is not altered by these agents. Thus, the major fraction of GMP-P(NH)P binding to membranes is not relevant to cyclase activation.

In several tissues, including turkey erythrocyte ghosts, GTP and the synthetic analogs, guanylylimidodiphosphate [GMP-P(NH)P], guanylylmethylene diphosphonate [GMP-P(CH_2)P] and guanosine-5'-O-(3 thiotriphosphate) [GMP-PP(S)], interact at the same regulatory site of adenylate cyclase to activate the enzyme. Activation by GTP is reversible in the sense that removal of free GTP results in loss of activity. However, activation by the synthetic analogs, GMP-P(NH)P, GMP-P(CH_2)P and GMP-PP(S) persists after the free nucleotide is removed by washing (1-6) and is for practical purposes irreversible (3-5). These guanylate nucleotides bind to membranes and solubilized membrane preparations containing adenylate cyclase activity, and a class of binding sites with a dissociation constant similar to that of adenylate cyclase activation has been reported (1,7-10).

This report describes that the characteristics of binding of [^{3}H]GMP-P(NH)P to turkey erythrocyte ghosts are grossly different from

those of adenylate cyclase activation, suggesting that the major fraction of measurable GMP-P(NH)P binding is irrelevant to the process of adenylate cyclase activation.

MATERIALS AND METHODS: [^{3}H]GMP-P(NH)P (8.7 Ci per mM) and GMP-P(NH)P were purchased from ICN. Turkey erythrocyte ghosts were prepared by hyposmotic lysis (11). [α^{32}P]ATP was synthesized by the method of Symons (12,13). Adenylate cyclase activity was measured by previously described methods (13). [^{3}H]GMP-P(NH)P binding was determined under conditions similar to those used to obtain permanent enzyme activation. Turkey erythrocyte ghosts (about 200 µg protein) were incubated at 37° in 0.2 ml of 50 mM Tris-HCl, pH 7.4. Following incubation, 3 ml of ice-cold Tris-HCl buffer were added and the mixture filtered over Whatman GFB glass filters. The filters were shaken vigorously in SDS for twelve hours and counted in BBS-3.

RESULTS: Figure 1 shows the rate of GMP-P(NH)P binding to turkey erythrocyte ghosts and the rate of activation of adenylate cyclase under similar conditions. Isoproterenol has no effect on the rate of binding of GMP-P(NH)P whereas it dramatically increases the rate of activation.

In the presence of isoproterenol and 10^{-6}M GMP-P(NH)P, a near-saturating concentration, about 50 pmoles of GMP-P(NH)P are bound per mg of membrane protein at equilibrium. Only about one half of this is bound in four minutes. In contrast to binding, maximal activation of adenylate cyclase occurs within the first four minutes under the same conditions.

The spontaneous rate of dissociation of membrane-bound [^{3}H]GMP-P(NH)P is negligible at 37°. However, in the presence of added excess, unlabeled GMP-P(NH)P more than 60% dissociates in 10 minutes (Fig. 2a). Similar results were reported by Spiegel and Aurbach (8). GTP and EDTA also cause the rapid dissociation of bound [^{3}H]GMP-P(NH)P from erythrocyte

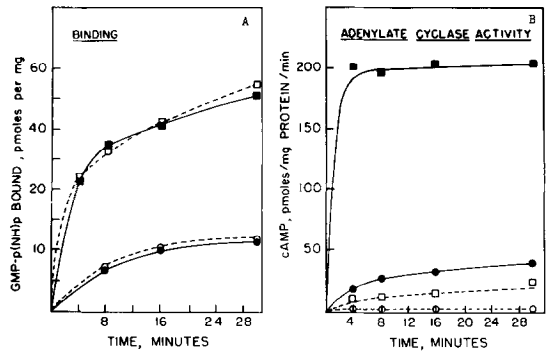

Figure 1. Time course of binding of [^{3}H]GMP-P(NH)P and of activation of adenylate cyclase by GMP-P(NH)P. A) Ghosts were incubated at 37° with 10^{-7} M [^{3}H]GMP-P(NH)P with (●) or without (○) 10^{-5} M (−)-isoproterenol, or 10^{-6} M [^{3}H]GMP-P(NH)P with (■) or without (□) 10^{-5} M isoproterenol in 50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol. At various times binding was measured as described in Methods. Nondisplaceable binding was determined by carrying out the above incubations in the presence of 5 x 10^{-4} M unlabeled GMP-P(NH)P. Displaceable binding was calculated by subtracting nondisplaceable binding from total binding. B) Ghosts were incubated at 37° with 10^{-7} M GMP-P(NH)P with (●) or without (○) 10^{-5} M isoproterenol or 10^{-6} M GMP-P(NH)P with (■) or without (□) 10^{-5} M isoproterenol with the same buffer as A. At various times the ghosts were diluted 50-fold with ice-cold Krebs-Ringer-bicarbonate, 10^{-5} M propranolol. The ghosts were centrifuged at 40,000 x g and the pellet resuspended and assayed for adenylate cyclase activity for 10 minutes at 37°.

ghosts (Fig. 2a). The rate of dissociation is not affected by the presence of isoproterenol either during incubation with GMP-P(NH)P or during dissociation (data not shown).

In contrast to these binding properties, incubation of ghosts previously activated by GMP-P(NH)P with GTP or EDTA does not reverse the activated state of adenylate cyclase (Fig. 2b). The persistence of the activated state in the presence of EDTA and GTP has been reported previously (2-4,7). In fact, when ghosts previously activated by GMP-P(NH)P are incubated at 37° the loss of activity is actually slower in the presence of EDTA and GTP (4). This is probably because these agents decrease the lability of adenylate cyclase. It is also important that ATP and AMP-P(CH_2)P also increase the rate of dissociation of GMP-P(NH)P bound to turkey erythrocyte ghosts without reversing the activated state of adenylate cyclase induced by prior exposure to GMP-P(NH)P (Table 1).

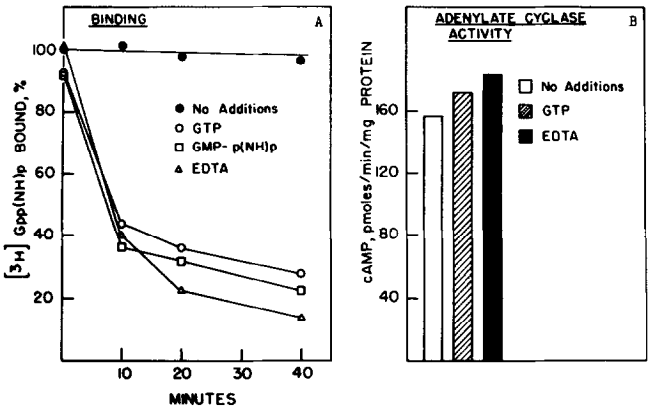

Figure 2. Effect of GTP, GMP-P(NH)P and EDTA on the rate of dissociation of bound [^{3}H]GMP-P(NH)P (left) and on the rate of decay of the active state of adenylate cyclase previously stimulated by GMP-P(NH)P (right). A) Turkey erythrocyte ghosts (5 mg of protein/ml) in 50 mM Tris HCl pH 7.4, 5 mM $MgCl_2$, were incubated for 10 minutes at 37° with 8.4 x 10^{-8}M (720,000 CPM per ml) [^{3}H]GMP-P(NH)P. The suspension was diluted with buffer at 4°, washed twice by centrifugation at 4°, and resuspended (1.8 mg of protein per ml) in 50 mM Tris HCl pH 7.4 with no additions (●), 0.5 mM GTP (○), 0.1 mM GMP-P(NH)P (□), or 10 mM EDTA (▲). The samples were incubated at 37° and at varying times binding was measured as described in methods. B) Ghosts suspended in 50 mM Tris HCl pH 7.4, 5 mM $MgCl_2$ were incubated for 10 minutes at 37° with 50 μM GMP-P(NH)P and 20 μM isoproterenol. The suspension was diluted with cold buffer, washed twice by centrifugation, resuspended in 50 mM Tris HCl. pH 7.4, containing no addition (□), 0.5 mM GTP (▨), or 10 mM EDTA (■), and incubated for 10 minutes at 37°. The membranes were then diluted with cold buffer, washed by centrifugation, resuspended in 50 mM Tris HCl pH 7.4 and assayed for adenylate cyclase activity.

If turkey erythrocyte ghosts are activated by GMP-P(NH)P, washed, and then incubated with p-aminophenylmercuric acetate, measurable membrane-bound GMP-P(NH)P and adenylate cyclase activity decrease rapidly (Table II). However, the active state induced by GMP-P(NH)P can be restored by further incubation of these membranes with dithiothreitol, indicating that despite the loss of bound nucleotide the active state induced by GMP-P(NH)P is retained after treatment with organic mercurials.

DISCUSSION: Several factors complicate measurements of binding of GTP and synthetic analogs of GTP to the regulatory site of adenylate cyclase. The enzyme is present in small quantities. The specific activity of

TABLE I

Effect of Adenylate Nucleotides on the Dissociation of Membrane-Bound [^{3}H]GMP-P(NH)P and on Stimulated Adenylate Cyclase Activity

For binding studies, turkey erythrocyte ghosts in 50 mM Tris·HCl, pH 7.4, were incubated with 4 x 10^{-8} M [^{3}H]GMP-P(NH)P for 10 minutes at 37°. The ghosts were then washed by centrifugation and resuspended in the same buffer with no additions, 5 x 10^{-4} M ATP or 10^{-4} M AMP-P(CH_2)P and incubated at 37° for 10 minutes. Binding of [^{3}H]GMP-P(NH)P was then determined by filtration as described in Methods. For adenylate cyclase studies, turkey erythrocyte ghosts in 50 mM Tris·HCl, pH 7.4, 1 mM DTT and 5 mM $MgCl_2$ were incubated with 50 μM GMP-P(NH)P and 25 μM (−)-isoproterenol for 10 minutes at 37°. The ghosts were washed and resuspended in 50 mM Tris·HCl, pH 7.4, containing either no additions, ATP or AMP-P(CH_2)P using the same concentrations as above. The membranes were diluted with cold buffer, centrifuged, resuspended and assayed for adenylate cyclase activity.

	No Additions	ATP	AMP-P(CH_2)P
[^{3}H]GMP-P(NH)P Bound (cpm)	7,785	6,222	4,476
Adenylate cyclase activity[1]	210	230	200

[1] pmoles cyclic AMP per minute per mg protein

available ligands is relatively low (8.7 Ci per mM for [^{3}H]GMP-P(NH)P vs 2,000 Ci per mmole for [^{125}I]insulin). Most important is the likely presence in adenylate cyclase preparations of other membrane components which bind GTP and GTP analogs. For example, tubulin, which has been isolated from membranes (14), is known to bind GTP and GMP-P(NH)P (15). Such components may be present in concentrations which greatly exceed that of adenylate cyclase. Consistent with this, GTP binding activity from solubilized myocardial membranes elutes from DEAE cellulose columns in several peaks under conditions where adenylate cyclase activity elutes as a single peak (9). If binding of GTP to these components is to reflect a physiological, regulatory function it may be anticipated that the K_d for binding would be in the range of the local concentration of GTP. Therefore, it would not be surprising if these structures had similar K_d's for GMP-P(NH)P as adenylate cyclase. The results presented

TABLE II

Effect of Organomercurial on Dissociation of Membrane-Bound [^{3}H]GMP-P(NH)P and on Reversal of GMP-P(NH)P Stimulated Adenylate Cyclase Activity

For binding studies, ghosts (5 mg/ml protein) were incubated for 20 minutes at 37° in 50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 8.7×10^{-8} M [^{3}H]GMP-P(NH)P (8.7 Ci/mM). The ghosts were washed twice and suspended in the same buffer containing 1% DMF or para-aminophenylmercuric acetate (0.5 mM), 1% DMF. The membranes were incubated at 4° for 10 minutes. Binding was determined as described in methods. For cyclase studies, ghosts were incubated for 10 minutes at 37° in 60 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 5×10^{-5} M GMP-P(NH)P, and 2.5 μM (−)-isoproterenol. The control membranes were washed, suspended in 50 mM Tris·HCl, pH 7.4, 1% DMF. The mercurial treated membranes were suspended in the same buffer plus p-aminophenylmercuric acetate (0.5 mM). The membranes were incubated at 30° for 10 minutes, and washed. The control membranes were resuspended in Tris·HCl, 20 mM dithiothreitol. The mercury-treated membranes were resuspended in the same buffer or Tris·HCl without dithiothreitol. The membranes were incubated for 10 minutes at 37°, washed and assayed for adenylate cyclase activity.

	[^{3}H]GMP-P(NH)P Bound[1]	Adenylate Cyclase Activity[2]
Control	71,000	210
Plus mercury	20,000	
without DTT		7
with DTT		150

[1] cpm

[2] pmoles of cyclic AMP produced per minute per mg of protein

in this report suggest that the major fraction of [^{3}H]GMP-P(NH)P bound to turkey erythrocyte ghosts is irrelevant to the process of adenylate cyclase activation.

The quantity of high affinity GMP-P(NH)P-binding sites on turkey erythrocyte ghosts appears to be very much greater than the quantity of adenylate cyclase. Although there are about 50 pmoles of GMP-P(NH)P-binding sites per mg of membrane protein (Fig. 1 and Ref. 7), the quantity of β-adrenergic receptors is only about 1 pmole per mg of protein (16). Assuming that adenylate cyclase is present in a one to one molar ratio with the β-adrenergic receptor, this may be taken as an estimate of the

concentration of adenylate cyclase. Levitski et al. (17) also estimated the concentration of adenylate cyclase of turkey erythrocyte ghosts to be about 1 pmole per mg of membrane protein by assuming that maximally activated adenylate cyclase has a turnover number similar to that of bacterial adenylate cyclase (18). If these assumptions are correct, and if it is further assumed that GMP-P(NH)P interacts with adenylate cyclase in a one-to-one molar ratio, then a maximum of only 2% of the measured [^{3}H]GMP-P(NH)P-binding would be relevant to adenylate cyclase activation. In pigeon erythrocytes 140 pmoles GMP-P(CH_2)P are bound per mg of membrane protein (1), a value which also seems excessive when compared with reasonably expected adenylate cyclase concentrations.

The binding of GMP-P(NH)P can be clearly dissociated from adenylate cyclase activation in several other important respects. In the presence of isoproterenol, the rate of GMP-P(NH)P binding is much slower than the rate of adenylate cyclase activation. Agents which affect binding of GMP-P(NH)P do not alter activation of adenylate cyclase. EDTA, GTP (Fig. 2), ATP, AMP-P(CH_2)P (Table 1) and organic mercurials (Table 2) all greatly increase the rate of dissociation of measurable GMP-P(NH)P binding without reversing the active state of adenylate cyclase induced by GMP-P(NH)P. The failure of these agents to reverse the GMP-P(NH)P induced active state has been observed previously (2-7).

A major argument against the proposal that activation of adenylate cyclase by GMP-P(NH)P may involve a covalent reaction (3-5) is the fact that GMP-P(NH)P bound to adenylate cyclase preparations can be extracted in an unaltered state (1,19). Since the major fraction of GMP-P(NH)P bound to such preparations is irrelevant to adenylate cyclase activation, this argument must be questioned. If the activation of adenylate cyclase by GMP-P(NH)P involves the covalent formation of an enzyme-P(NH)P complex, as previously suggested (3-5), GMP-P(NH)P analogs labeled on the base or the alpha phosphate would not be suitable ligands to measure the pertinent binding.

In view of the above considerations, direct measurement of the reaction of adenylate cyclase with GMP-P(NH)P may require highly purified preparations of adenylate cyclase and the synthesis of highly radioactive ligands labeled on the β- or γ-phosphate.

Acknowledgment: We would like to acknowledge the excellent technical assistance of L. Hernaez.

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