Evidence for Distinct Guanine Nucleotide Sites in the Regulation of the Glucagon Receptor and of Adenylate Cyclase Activity

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Previous studies have shown that GTP or guanyl-5'-yl-imidodiphosphate (Gpp(NH)p) stimulate the activity of hepatic adenylate cyclase and decrease the affinity of the glucagon receptor for the hormone. The studies reported here provide evidence that the effects of the nucleotides on these processes are exerted through functionally and possibly structurally distinct nucleotide sites. The evidence is as follows: (a) the concentration dependency for Gpp(NH)p action on hormone binding is at least 1 order of magnitude higher than for enzyme activation; (b) whereas Gpp(NH)p and GTP are about equipotent on enzyme activation, Gpp(NH)p is considerably less potent than GTP on the hormone binding process; (c) ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetate decreases the potency of Gpp(NH)p activation of adenylate cyclase but does not alter the affinity of hormone binding; (d) pretreatment of hepatic membranes with Gpp(NH)p leads to an activated state of adenylate cyclase which persists after washing or addition of GTP. The same pretreatment leads to a low affinity state of the receptor that is readily reversed to a high affinity state by washing the membranes; the high affinity state remains susceptible to GTP action after pretreatment with Gpp(NH)p and washing; (e) treatment of the membranes with phospholipase C (from Bacillus cereus) abolishes the action of Gpp(NH)p on glucagon binding but does not alter the ability of the nucleotide to activate adenylate cyclase.

An hypothesis is presented which serves to explain the role of the two guanine nucleotide sites in the overall regulation of adenylate cyclase activation and in the "coupling" reaction between receptor and enzyme.

It is generally recognized that hormone-sensitive adenylate cyclase systems contain, in addition to hormone receptor and catalytic units, nucleotide regulatory sites that preferentially interact with GTP (1-4). Considerable emphasis has been placed on the activating role of GTP or the synthetic analog, Gpp(NH)p, on the activity of these systems. However, there is another effect of GTP that has received relatively less attention. This effect, first described for the glucagon receptor in hepatic membranes (1, 2), is to decrease the affinity of the receptor for the hormone. In this study we have examined the possibility that the nucleotide site(s) responsible for altering receptor conformation and enzyme activity may be functionally and structurally distinct. For this purpose, several types of probes were used that may distinguish the functional and structural features of the guanine nucleotide sites affecting receptor conformation and enzyme activity. Although indirect, the results indicate that there are indeed marked differences in the properties of these sites. The implications of the findings for the overall regulation of adenylate cyclase activity by glucagon and GTP are discussed.

EXPERIMENTAL PROCEDURES

Materials — [α-32P]ATP, App(NH)p, and Gpp(NH)p were obtained from International Chemical and Nuclear Corp.; Tris base and dithiothreitol were purchased from Schwarz/Mann. ATP, GTP, GDP, and phospholipase C (Bacillus cereus, type III) was obtained from the Sigma Chemical Co. Bovine serum albumin was from Pentex. Glucagon was a gift from Eli Lilly Co. Oxoid filters were obtained from Med-Ox Chemicals (Ontario).

Isolation of Hepatic Plasma Membranes — Partially purified plasma membranes were prepared from rat liver by a modification (5) of the method of Neville (6). Membranes were stored in liquid nitrogen.

Adenylate Cyclase Assay — Adenylate cyclase was assayed according to the method of Salomon et al. (7). The assay mixture contained 30 mM Tris/HCl (pH 7.5), 10 mM MgCl2, 5 mM creatine phosphate, 3.3 units of creatine phosphokinase, 1 mM dithiothreitol, 100 μM [α-32P]ATP (100 to 500 cpm/pmol), and 20 μM cAMP. The reaction volume was 100 μl. Assays were performed for 10 min at 30° with 50 to 100 μg of protein. The reaction velocity was linear with time under all conditions used in this study.

Glucagon-binding Assay — In most cases, [3H]glucagon binding was performed at 30° for 5 min in a 250-μl reaction mixture containing the components described in the table and figure legends. Thus, hormone binding was measured under equilibrium conditions where the rates of association and dissociation are rapid compared to the

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1 The abbreviations used are: Gpp(NH)p, guanyl-5'-yl imidodiphosphate; App(NH)p, adenylyl-5'-yl imidodiphosphate; EGTA, ethyleneglycol bis(β-aminoethyl ether)N,N'-tetraacetate; BSA, bovine serum albumin.
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Dose-Response Effects of GTP and Gpp(NH)p on 125I-Glucagon Binding and Adenylate Cyclase Activity - Figure 1 illustrates the concentration dependency for Gpp(NH)p activation of adenylate cyclase and for the effects of this nucleotide on the binding of \( 125^{\text{I}} \)-glucagon to its receptor. Half-maximal concentration for activation \( \beta \) was 0.4 \( \mu \text{M} \), whereas the half-maximal concentration required for effects on glucagon binding was about 20-fold higher (7 \( \mu \text{M} \)). By contrast potency for the effects of GTP on these processes was essentially identical (0.2 \( \mu \text{M} \)) as seen in Fig. 2. These differences in the potency of Gpp(NH)p and GTP on hormonal binding and enzyme activation gave the first indication that the guanine nucleotide sites responsible for these effects on the receptor and enzyme may be functionally distinct.

Effects of Chelators on Actions of Gpp(NH)p - Spiegel et al. (11) have recently shown with the turkey erythrocyte adenylate cyclase system that EDTA inhibits the ability of Gpp(NH)p to activate adenylate cyclase during pretreatment. We have found with the hepatic adenylate cyclase system that this apparent inhibitory effect of chelators (EGTA or EDTA) results from a shift in the concentration of Gpp(NH)p required for activation during pretreatment. This is illustrated with EGTA in Fig. 2A where an approximately 7-fold shift to the right was observed in the presence of chelator. In contrast, when the effects of Gpp(NH)p on \( 125^{\text{I}} \)-glucagon binding to its receptor were assayed in the presence of chelators, essentially no change was observed for the potency of Gpp(NH)p action on hormone binding to the receptor. It should be emphasized that the effects of Gpp(NH)p on activity and binding were assayed in the presence of 30 \( \mu \text{M} \) App(NH)p to minimize the hydrolysis of Gpp(NH)p by nucleotide pyrophosphohydrolases present in the hepatic plasma membrane (12). If the effects of the chelators were also simply to protect against enzymatic degradation of the nucleotide, one would have expected a marked leftward shift.

In these studies we were concerned only with the concentration of Gpp(NH)p needed for one-half maximal response. The relation of the true \( K_a \) to this value may be quite complex due to considerations such as the irreversible nature of the activation by this nucleotide.

RESULTS

FIG. 1. Effect of varying concentrations of Gpp(NH)p on activation of adenylate cyclase and alteration of \( 125^{\text{I}} \)-glucagon binding. Liver plasma membranes were thawed, suspended in 20 mm Tris/HCl, pH 7.0, containing 1 mm dithiothreitol, centrifuged at 27,000 \( \times \) g for 15 min and resuspended in the above buffer for assays. Adenylate cyclase was assayed as described under "Experimental Procedures" in the presence of 10\( \times 10^{-8} \) \( \text{M} \) glucagon and the indicated concentrations of Gpp(NH)p. \( 125^{\text{I}} \)-glucagon binding was assayed in a mixture containing 30 \( \mu \text{M} \) App(NH)p, 1 mm dithiothreitol, 0.2% BSA, 20 mm Tris/HCl (pH 7.0), 1 \( \times 10^{-9} \) \( \text{M} \) \( 125^{\text{I}} \)-glucagon (240,000 cpm/pmol), and the indicated concentrations of Gpp(NH)p. Under these conditions, App(NH)p had no effect on the binding.

FIG. 2. Effect of varying concentrations of GTP on the activation of adenylate cyclase and alteration of \( 125^{\text{I}} \)-glucagon binding. See Fig. 1 for details.

As discussed by others, the effects of chelators on the adenylate cyclase system are complex (5, 13). For example, we have observed that pretreatment with chelators can cause an increase in the maximal velocity attained in the presence of Gpp(NH)p; however, under a variety of conditions both EGTA as well as EDTA cause the rightward shift in the concentration dependency of Gpp(NH)p activation. Thus despite such
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Fig. 3. The effect of EGTA on the ability of Gpp(NH)p to alter 125I-glucagon binding (Panel A) and activate adenylate cyclase (Panel B). Prior to assays, liver plasma membranes were thawed, resuspended in 20 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol, and centrifuged at 27,000 × g for 15 min. For 125I-glucagon binding assays, the membranes were then resuspended in the same buffer. Binding was carried out as described under "Experimental Procedures" in an assay mixture containing 30 μM App(NH)p, 1 mM dithiothreitol, 0.2% BSA, 20 mM Tris/HCl (pH 7.0), 1 × 10^{-5} M 125I-glucagon, varying concentrations of Gpp(NH)p, and 1 mM EGTA, where indicated. Activation of adenylate cyclase was assessed by incubating membranes (0.3 mg/ml) at 30°C for 15 min in the presence of 30 μM App(NH)p, 1 mM dithiothreitol, 20 mM Tris/HCl (pH 7.5), varying concentrations of Gpp(NH)p and 1 mM EGTA, where indicated. These "pretreated" membranes were then resuspended at 27,000 × g for 15 min and resuspended in 20 mM Tris/ HCl, pH 7.5, containing 1 mM dithiothreitol for assay of adenylate cyclase activity as described under "Experimental Procedures."

Rat liver plasma membranes were thawed, resuspended in 20 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol, and centrifuged at 27,000 × g for 15 min. These membranes were then either resuspended in 20 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol and incubated on ice (control) or resuspended in 30 μM App(NH)p, 1 mM dithiothreitol, 5 mM MgCl₂, 20 mM Tris/HCl, pH 7.5, and 10^{-7} M Gpp(NH)p to a protein concentration of 0.4 mg/ml and incubated at 30°C for 15 min (pretreated membranes). The "pretreated" membranes were centrifuged at 27,000 × g for 15 min and then resuspended in 20 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol and assayed for glucagon binding. The binding assay was performed in a medium containing 20 mM Tris/HCl, 0.2% BSA, the indicated amounts of 125I-glucagon, varying concentrations of Gpp(NH)p, and 1 mM EGTA, where indicated. Activation of adenylate cyclase was assessed by incubating membranes (0.3 mg/ml) at 30°C for 15 min in the presence of 30 μM App(NH)p, 1 mM dithiothreitol, 20 mM Tris/HCl (pH 7.5), varying concentrations of Gpp(NH)p and 1 mM EGTA, where indicated. These "pretreated" membranes were then resuspended at 27,000 × g for 15 min and resuspended in 20 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol for assay of adenylate cyclase activity as described under "Experimental Procedures."

| Membranes | GTP 5 × 10^{-10} M | 1.4 × 10^{-8} M | 5 × 10^{-9} M |
|-----------|-----------------|----------------|----------------|
| Control   | 646             | 1882           | 3033           |
| Gpp(NH)p  | 348             | 1007           | 3901           |
| Pretreated| 762             | 2028           | 3888           |

It should be noted that the lack of effect of GTP at higher concentrations of the hormone is consistent with the fact that GTP alters the affinity of the receptor for glucagon but not the concentration of receptors that bind the hormone (2, 10).
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Fig. 4. The effect of phospholipase C (Bacillus cereus) digestion on the ability of Gpp(NH)p to displace 'H-glucagon from hepatic plasma membranes. The procedure for phospholipase C treatment of membranes was described in the legend to Table II. After this treatment, the membranes were suspended to a protein concentration of 0.2 mg/ml in 20 mM Tris/HCl, pH 7.5, containing 0.2% BSA, and incubated in 7-ml aliquots with 1.5, 4.5, or 9 x 10^-16 M 'H-glucagon (240,000 cpm/pmol) at 30°. After 10 and 12 min, 1-ml aliquots were withdrawn and the amount bound was determined by filtration as described under "Experimental Procedures." After 14 min, 10^-6 M glucagon (O-O) or 10^-4 M glucagon and 10^-4 M Gpp(NH)p (closed circle) were added to the incubation mixture (arrows) and the displacement of 'H-glucagon from liver plasma membranes were assessed by filtering 1-ml aliquots from the incubation mixture at 16, 18, 20, and 22 min. Panel A is for control membranes while Panel B is for phospholipase C-treated membranes.

Table II

Effect of Phospholipase C (Bacillus cereus) digestion on adenylate cyclase activities in hepatic plasma membrane

Liver membranes were thawed, resuspended to 2 mg/ml in 10 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol, and centrifuged at 27,000 x g for 15 min. The pelleted membranes were then suspended in 10 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol and 1 mM CaCl2 to a protein concentration of 0.5 mg/ml, incubated at 25° for 5 min with phospholipase C (12 pg/ml of membrane protein), and then centrifuged at 27,000 x g for 15 min. The pelleted membranes were resuspended in 10 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol, and recentrifuged at 27,000 x g for 15 min prior to resuspension in the same buffer to assay enzymatic activity. The concentration of Gpp(NH)p used in each assay was 10^-5 M, while the concentration of glucagon was 10^-6 M and that of GTP was 10^-5 M. All enzymatic activities are expressed as picomoles per 10 min per mg of protein. The phospholipase C had a specific activity of 120 pmol/min/mg. Under the conditions described above 76% of the phospholipid phosphate was hydrolyzed by the phospholipase.  

| Treatment of membranes | Adenylate cyclase activities | GTP Glucagon + GTP Gpp(NH)p pmol CAMP/mg protein |
|------------------------|-----------------------------|------------------------------------------------|
| Control                | Basal Glucagon GTP Glucagon | 32 82 115 211 205 |
| Treated                | 12 15 43 41 90 |

GTP-, and Gpp(NH)p-stimulated activities and in complete loss of hormone response. These changes required hydrolysis in excess of 40% of the membrane lipids (data not shown). It can be seen that under those conditions in which a loss of guanine nucleotide action on the receptor took place the fold stimulation of adenylate cyclase activity by either GTP or Gpp(NH)p remained the same as that seen in control membranes. The differential effect of phospholipase digestion on receptor regulation and enzymatic activation by Gpp(NH)p again suggests that functionally distinct sites were involved in these two processes, although we cannot eliminate the possibility that those differential effects may be due, in part, to an alteration in the hormone receptor site.

Discussion

This study provides substantial evidence that guanine nucleotides modify the states of the receptor and adenylate cyclase through functionally distinct sites. In addition to the results presented here, there is other evidence which supports this view and which suggests, furthermore, that these sites reside on structurally different binding components. Such evidence includes previous findings (1, 2) that GDP is equipotent with GTP in glucagon receptor regulation but is a potent competitive inhibitor of the activation process (4). These findings support different structural requirements for guanine nucleotides at the two sites. More recently, we have shown that after Lubrol solubilization of the adenylate cyclase complex, the nucleotide-sensitive receptor can be resolved from the nucleotide-activatable catalytic unit.

Given evidence for functionally and probably structurally distinct nucleotide sites involved in the regulation of receptor and enzyme activity, what role do these sites play in the overall regulation of enzyme activity and, most importantly, in the so-called "coupling" reaction between receptor and enzyme? De Haen (17) and Cuatrecasas et al. (18) have summarized evidence suggesting that the hormone receptors and adenylate cyclase are separate molecular entities and have proposed that the receptors and enzyme need not be present in stoichiometric amounts in the membrane or even normally juxtaposed within the plane of the membrane. Orly and Schramm (19) have shown recently that catecholamine β receptors can be transferred separate from adenylate cyclase and in a functional state between membranes of different cell types. Their findings provide strong evidence for the separateness of catalytic and receptor units. What factors are involved
that promote interaction between these units? It has been proposed (20) that binding of the hormone to a receptor state having a favored coupling conformation is a prime factor in the coupling reaction, and a role for GTP was suggested in this process. We have shown in previous studies (21) that GTP regulates the conformation of the glucagon receptor by changing the receptor state from one having a high affinity \( K_a = 2 \) nm and which displays essentially irreversible binding characteristics to at least two other states of the receptor. Approximately 90% of the receptor sites are converted to low affinity \( (K_a = 10 \) to 20 nm), whereas the remaining sites have considerably higher affinity.

Since GTP regulates, through independent sites, both the glucagon receptor and adenylate cyclase, we suggest that the following hypothesis, presented schematically below, may explain the formation of high and low affinity states of the receptor, provides the basis of the coupling reaction, and places in focus the role of GTP and glucagon in the overall regulation of enzyme activity:

\[
\begin{align*}
GTP + R & \rightleftharpoons R_{c} \cdot GTP \\
GTP + E_{i} & \rightleftharpoons E_{c} \cdot GTP \\
R_{c} \cdot GTP + E_{i} \cdot GTP & \rightleftharpoons \left( R_{c} \cdot GTP \right) \left( E_{c} \cdot GTP \right) \\
\left( R_{c} \cdot GTP \right) \left( E_{c} \cdot GTP \right) + H & \rightleftharpoons \left( H_{c} \cdot GTP \right) \left( E_{c} \cdot GTP \right)
\end{align*}
\]

In the "uncoupled" Equilibrium (I) the hormone receptor \( (R) \) exists in two states. The \( R_{c} \) ("desensitized") form has a high affinity for hormone \( (H) \) and low affinity for nucleotide. The converse holds for the \( R_{c} \) ("sensitized") form. Similarly, the catalytic unit \( (E) \) exists in two forms in the uncoupled Equilibrium (II). \( E_{i} \) is inactive and has low affinity for guanine nucleotides, whereas \( E_{c} \) is active and has higher affinity for the nucleotides. In the simplest case, only the \( E_{c} \) and \( R_{c} \) forms are conformationally compatible for coupling. Such linkage gives rise to the third "coupled" state (III) of the receptor \( (K_{c}) \) and catalytic units and is accompanied by an increase in the affinity for the hormone (IV). According to this hypothesis, the bulk of the low affinity receptor states induced by GTP are relevant to the coupling process and can be considered as the favored precursor states in the process.

The advantage of a scheme of uncoupled equilibria is the fact that not only the effective concentrations of hormone and nucleotide but also the effective stoichiometry and configuration of the macromolecular components are important in the overall regulation. Such an hypothesis may explain two of our earlier observations. First, activation of adenylate cyclase by glucagon displays a hyperbolic function of glucagon receptor occupation (4). Second, GTP causes a rightward shift in the glucagon binding curve (2), but a leftward shift in the dose-response curve for hormonal activation of the enzyme (4). The latter can be explained readily by an excess of receptors over the catalytic unit. The hypothesis also predicts that, in the absence of GTP, increasing hormone concentrations would shift the equilibrium to the "uncoupled" high affinity form of the receptor and concomitant shifts toward the \( E_{i} \) or inactive state of the enzyme. This may be the basis of the "desensitization" phenomenon that has been reported recently for the catecholamine-sensitive adenylate cyclase system in frog erythrocytes (22).

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