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DIFFERENTIAL SUSCEPTIBILITY TO GTP FORMED FROM ADDED GDP VIA MEMBRANE-ASSOCIATED NUCLEOSIDE DIPHOSPHATE KINASE OF GTP-SENSITIVE ADENYLATE CYCLASES ACHIEVED BY HORMONE AND CHOLERA TOXIN

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SUMMARY: GTP-sensitive adenylate cyclases in liver membranes achieved by glucagon and by cholera toxin pretreatment displayed similar responses to added GTP in assay with respect to magnitude and sensitivity. However, their susceptibility to GTP formed during incubation from added GDP catalized by membrane-associated nucleoside diphosphate kinase(mNDPK) was different. Adenylate cyclase pretreated with cholera toxin was essentially unaffected by added GDP, while further addition of glucagon produced activation. GTP-stimulated adenylate cyclase activity in toxin-treated membranes was inhibited by added GDP, whereas glucagon addition reduced the inhibitory action of GDP by two orders of magnitude. Since neither pretreatment with toxin nor glucagon addition altered GTP formation by mNDPK, these observations suggest a possible presence of a mechanism by which hormone makes adenylate cyclase susceptible to the GTP formed via mNDPK for activation.

Recent studies using resolved components of hormone sensitive adenylate cyclase systems have demonstrated that GTP, but not GDP, is an active cofactor not only for a stimulatory guanine nucleotide binding protein(Gs) but also for an inhibitory one(Gi)(1). In membrane associated adenylate cyclase systems, however, it has been observed that GDP is as effective as GTP with no ATP regenerating system in assay in, at least, hormonal activation of mammalian adenylate cyclases(2,3). Whether activation of the enzyme observed with GDP is transphosphorylation dependent has been examined under various conditions, e.g. with transphosphorylation resistant analogs of ATP and GDP(App(NH)p and

The abbreviations used are: Gs and Gi, the stimulatory and inhibitory guanine nucleotide-binding regulatory components of adenylate cyclase, respectively; mNDPK, membrane-associated nucleoside diphosphate kinase; App(NH)p, adenosine 5'-(β,γ-imino)triphosphate; GDPβS, guanosine 5'-o-(2-thiodiphosphate)

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GDPβS)(4-10) but conclusions are conflicting(11). Recently we have pointed out the problems of using these analogs(5,12,13) and provided the conclusive evidence showing that the stimulatory effect of hormone with added GDP occurs only in a transphosphorylation dependent manner(5,12,13); GDP itself was found to be a strong competitive inhibitor with an apparent Ki of about 1μM regardless of the presence of hormone(12). Through these studies from this laboratory it was observed that nucleoside diphosphate kinase, the enzyme responsible for production of GTP from added GDP, is tightly associated with membranes(12,13). Furthermore, the following observations suggesting a possible interaction of the membrane-associated nucleotide diphosphate kinase(mNDPK) with adenylate cyclase regulation by hormone were obtained. 1) A small amount of produced GTP(less than 5% of added GDP) via mNDPK is enough to cause an activation of adenylate cyclase by hormone(6,7,12,13). 2) Hormone can reduce an inhibitory action of GDP by causing a rightward shift in inhibition curve(12). This reduction, however, is not a result of reduced affinity for GDP of its binding site but is a phenomonon related to GTP formation by mNDPK since suppression of the GTP formation was followed by disappearance of the hormone-induced reduction of GDP action(12). 3) Under a certain condition, an enhanced guanine nucleotide dependent adenylate cyclase activity as a basis of an increased hormonal responsiveness is accompanied by an increased mNDPK activity(14). These observations make it likely that mNDPK could be a device to form a functional GTP pool in the close proximity of adenylate cyclase system in order that the enzyme, though surrounded by an inhibitory nucleotide(GDP), can be activated by hormone.

In order to understand the functional relationship between mNDPK and adenylate cyclase system, two GTP-sensitive adenylate cyclases achieved by different stimulants, glucagon and cholera toxin, were compared in terms of their susceptibility to GTP formed from added GDP via mNDPK as well as to added GTP in assay.

MATERIALS AND METHODS

Materials [8-^{3}H]GDP was purchased from the Radiochemical Center, Amersham. Cholera toxin was from The Chemo-Sero-Therapeutic Research Institute(Kumamoto, Japan). Reagents for radioimminoassay of cyclic AMP, a Yamasa cyclic AMP assay kit, were generous gifts from Yamasa Shoyu Co(Choshi, Japan). ATP, GTP, and GDP were used after purification on DEAE-Sephadex column as described previously(2,12). A certain lot of Sigma grade ATP and ATP with the highest purity from Boehringer could be used for adenylate cyclase assay with no further purification since they contained no appreciable amount of GTP-like substances. Other chemicals used were reagent grade.

Preparation of liver plasma membranes Liver plasma membranes from Wistar male rats(200-250g) were prepared as previously described(3).

Enzyme assays Adenylate cyclase was assayed as described previously-(12). The standard assay mixture(100µl) contained 1mM ATP, 5mM $MgCl_2$, 10mM theophylline, 50mM Tris/HCl, pH 7.4 and appropriate amount of liver plasma membranes(1-2µg of protein/tube). Cyclic AMP formed was measured by radioimmunoassay after succinylation.

Nucleoside diphosphate kinase activity was measured essentially according to the method previously described(12).

Others Pretreatment of membranes with cholera toxin was carried out as previously mentioned(15).

Protein contents were determined by the method of Lowry et al.(16) using bovine serum albumin as a standard.

RESULTS

Susceptibility to GTP

A unique characteristic of adenylate cyclase pretreated with cholera toxin is that the enzyme can be activated and manifest its full activity solely by added GTP in assay. The activation induced by GTP was concentration-dependent; the concentration which induced half maximal stimulation was approximately 0.2µM(Fig.1 A). This activation occurred after a small lag time(about 30s)(Fig.2). Simultaneous addition of glucagon in assay abolished the lag and further increased the reaction rate. However, extent of the increase caused by hormone addition was small compared with that observed with the toxin-untreated membranes(not shown, see references 3 and 12), demonstrating that glucagon and cholera toxin share a pool of adenylate cyclase as their target. Glucagon showed no appreciable change in apparent Ka for GTP(Fig.1 A), indicating that hormone seems unlikely to affect the affinity of the enzyme to GTP. The results demonstrate that GTP-sensitive form of adenylate cyclase achieved by glucagon addition and that by cholera toxin treatment are similar in response to the added GTP with respect to magnitude and sensitivity.

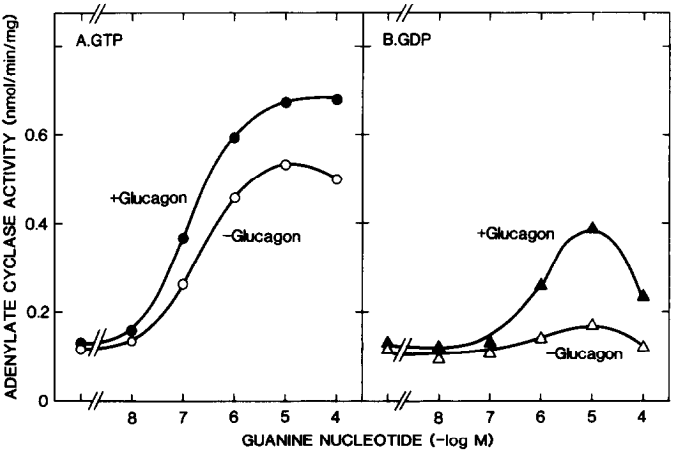

Fig.1. Effects of GTP(A) and GDP(B) on adenylate cyclase activity in chlera toxin-pretreated membranes. Pretreatment of adenylate cyclase in membranes with cholera toxin was carried out with 0.1mM GTP as described in the text. After washing adenylate cyclase activity in the toxin-pretreated membranes was determined under the standard assay condition as described in the text in the presence of indicated amount of guanine nucleotides. Glucagon, when present, was at 1μM.

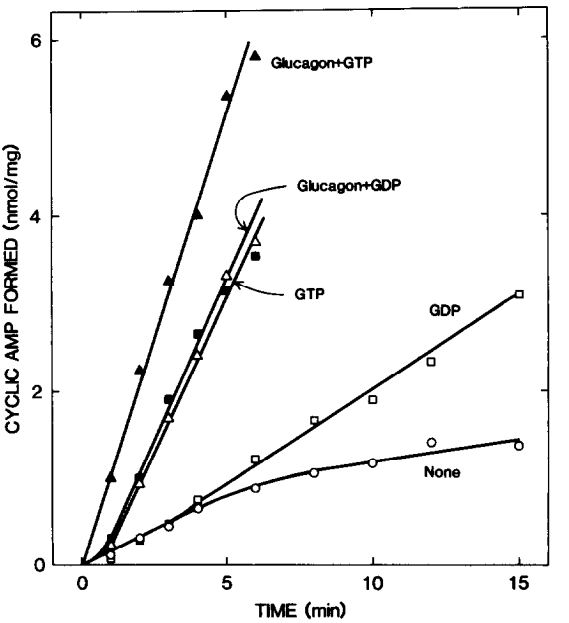

Fig.2. Time course of cyclic AMP accumulation in cholera toxin-treated membranes. Cholera toxin-treated membranes were obtained as described under the legend to Fig.1. Adenylate cyclase assay was carried out under the standard assay condition. Reactions were started by adding toxin-treated, washed membranes(14μg of protein) into pooled assay mixture(total volume of 1.4ml) prewarmed for 2 min at 37°C. 100μl aliquots were withdrawn from each tube at indicated times and accumulated cyclic AMP was measured. Additions were: glucagon, 1μM; GTP, 10μM; GDP, 10μM.

Susceptibility to GTP formed from added GDP via mNDPK

Next, we compared these two GTP-sensitive adenylate cyclases in terms of their susceptibility to GTP formed via mNDPK. For this purpose two types of experiments were carried out. First, since we know that hormone can activate adenylate cyclase with added GDP under a standard assay condition(3,12,13), we have done a similar experiment as shown in Fig.1 B and examined whether the cholera toxin-pretreated, GTP-sensitive enzyme can be activated by added GDP in assay. In contrast with added GTP, GDP, at concentrations ranging from 0.01 to 100μM, caused essentially no stimulation on the enzyme activity, while further addition of glucagon produced activation of the toxin-treated enzyme under the conditions. Time course study demonstrated that the absence of the effect of added GDP lasted for 4 min of incubation(Fig.2). Thereafter, however, GDP sustained the initial basal reaction rate. Upon addition of glucagon along with GDP, the reaction rate increased and became similar to that with added GTP. It is surprising that glucagon action with added GDP is rapid in onset and is as large as that with added GTP with respect to steady state rate because this implies that these glucagon actions can be elicited under the condition where GTP formation proceeds less than 1% of the added GDP(not shown, see reference 12). Prolonged incubation undoubtedly allows GTP to form in substantial amount in assay medium, which explains a delayed activation of adenylate cyclase observed with added GDP alone in the toxin-treated membranes(Fig.2).

A recent study(12) from this laboratory shows that hormone is capable of reducing an inhibitory action of GDP against GTP- or its analog-stimulated adenylate cyclase activity as shown by causing a rightward-shift in inhibition curve. In Fig.3, potency of the added GDP to inhibit GTP-stimulated cyclase activity in cholera toxin-pretreated membranes was studied in the absence and presence of glucagon. The GTP-stimulated cyclase activity was suppressed to unstimulated levels by

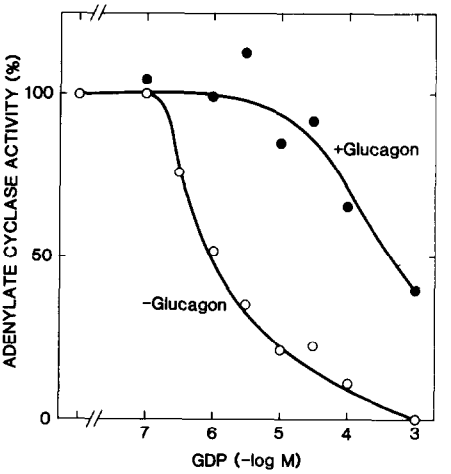

Fig.3. Inhibitory action of added GDP on GTP-stimulated adenylate cyclase activity in cholera toxin treated membranes in the absence and presence of glucagon. Cholera toxin pretreated membranes were obtained as described under the legend to Fig.1. Adenylate cyclase activity was determened with 0.3μM GTP along with indicated amounts of added GDP in the absence and presence of 1μM glucagon. For calculation, unstimulated cyclase activity(with no addition) was subtracted from these activities, and data are expressed as per cent of control values(with no GDP).

increasing amount of added GDP. Addition of glucagon, however, caused two orders of magnitude rightward shift in the inhibition curve(Fig.3). The extent of the hormone-induced shift was almost the same as that obtained with the toxin-untreated membranes(12), indicating that cholera toxin pretreatment did not affect the potency of an inhibitory action of GDP. Differential effects of these stimulants observed with added GDP were not due to altered amount of formed GTP in assay since neither glucagon addition nor toxin pretreatment changed GTP formation catalyzed by mNDPK(Table 1). Therefore, the results demonstrate that the two GTP-sensitive adenylate cyclases achieved by these stimulants are different in susceptibility to the GTP formed from added GDP via mNDPK.

DISCUSSION

The present study demonstrates that, at least in membrane-bound adenylate cyclase system, there are two different GTP-sensitive states of the enzymes; one is achieved by hormone addition and susceptible to GTP formed via mNDPK, and the other is obtained by cholera toxin

Table 1

Effects of glucagon and cholera toxin on membrane-associated nucleoside diphosphate kinase activity

Cholera toxin in preincubation	Glucagon in assay	Membrane-associated nucleoside diphosphate kinase activity: GDP concentration (μM) 1	100
		nmol / min / mg	
-	-	3.4 ± 0.1	44 ± 1
-	+	3.6 ± 0.1	50 ± 2
+	-	3.8 ± 0.3	50 ± 1
+	+	3.7 ± 0.1	48 ± 1

Pretreatment of membranes with cholera toxin was carried out with 0.1 mM GTP as described in the text. Membrane-associated nucleoside diphosphate kinase activity in those untreated and cholera toxin-treated membranes was determened in the absence and presence of 1 μM glucagon at two different substrate concentrations. Values are the means ± S.E. of triplicate samples.

treatment but insusceptible to the GTP although they display similar responses to added GTP in assay. The present observation provides an additional support for the notion(5,12) that mNDPK may play a role as a GTP supply system in hormone-dependent regulation of adenylate cyclase. The studies including this one on mNDPK and adenylate cyclase raise a possibility that the interaction between the two may become a rate-limiting step in adenylate cyclase regulation by hormone under conditions, for example, where adenylate cyclase is surrounded in large part by GDP although we do not know guanine nucleotide levels in situ adjacent to the enzyme.

The data shown in Fig.1 and 2, and Fig.3 are phenomenologically different but underlying mechanism seems to be identical since in both cases the GTP produced via mNDPK is likily to be responsible for the hormone-dependent phenomena(12). Possibilities that hormone alters GTP formation catalized by mNDPK and that it reduces an affinity for GDP of

the binding site(Gs) seem unlikely(Table 1, see also reference 12). The mechanism by which hormone makes adenylate cyclase susceptible to GTP formed via mNDPK is unclear but it is tempting to speculate that hormone, through activation of its receptor, may cause a functional(or physical) coupling between mNDPK and adenylate cyclase, rendering the cyclase(Gs) accessible to GTP formed via the mNDPK. Further studies are necessary to elucidate physiological role of mNDPK in hormonal regulation of adenylate cyclase.

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