GDP does not support activation of adenylate cyclase nor ADP-ribosylation of a guanine nucleotide binding protein by cholera toxin

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The actions of cholera toxin (i.e., activation of adenylate cyclase and ADP-ribosylation of a guanine nucleotide binding protein in purified membranes from rat liver) were GTP dependent. Neither of these actions of cholera toxin was reproduced with GDP. Simultaneous addition of ATP and $MgCl_2$ along with GDP allowed cholera toxin to exert these actions. The role of GDP in adenylate cyclase regulation was discussed.

Cholera toxin *Adenylate cyclase* *GDP* *ADP-ribosylation* *Liver plasma membrane*

1. INTRODUCTION

Recent studies have revealed that the regulation of adenylate cyclase from eukaryotic cells requires the presence of guanine nucleotides as an essential regulatory factor in addition to hormones [1–3]. Both activation of adenylate cyclase [4–7] and ADP-ribosylation of the guanine nucleotide binding protein [8,9] induced by cholera toxin treatment also require a guanine nucleotide GTP as well as NAD as an essential factor. Cholera toxin treatment decreases low K_m GTPase activity stimulated by a hormone with concomitant increase in adenylate cyclase activity [10]. Based on these studies, GTP regulatory cycle hypothesis has been proposed to explain adenylate cyclase regulation [11]. According to this hypothesis, GTP is an active nucleotide and GDP, a hydrolytic product of GTP, is inactive for adenylate cyclase. An argument exists that hormone is capable of mediating its signal to adenylate cyclase with GDP as well as with GTP [12–14]. Thus, a question has been raised as to whether the GTP regulatory cycle hypothesis is a general feature of eukaryotic adenylate cyclase system [15,16]. However, we have obtained the conclusive evidence that GDP, in contrast with GTP, cannot mediate hormonal signal to adenylate cyclase [17,18]. This observation not only supports the GTP regulatory cycle hypothesis as a general model but also clearly demonstrates that the purity of materials used (e.g., membranes and nucleotides) and metabolism of guanine nucleotides in membranes should be carefully considered in analyses of adenylate cyclase regulation. To extend this notion, using a purified system we have studied whether cholera toxin can activate adenylate cyclase and transfer an ADP-ribose moiety into the guanine nucleotide binding protein in the presence of GDP.

2. MATERIALS AND METHODS

[^{32}P]NAD was purchased from New England Nuclear (Boston MA); cholera toxin from Schwartz-Mann; reagents for radioimmunoassay

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of cyclic AMP, a Yamasa cyclic AMP assay kit, were kindly donated from Yamasa Shoyu Co. (Choshi, Chiba). Other chemicals used were described in [18].

Purified rat liver plasma membranes were obtained as in [1].

Adenylate cyclase activity was determined as in [18] by radioimmunoassay. Protein contents were determined as in [19] using bovine serum albumin as a standard.

Activation of cholera toxin and preincubation of plasma membranes with cholera toxin were performed as in [20]. For labeling experiments, [^{32}P]NAD (1–10 μCi/mmol) was added at 10 μM instead of 2 mM NAD with other constituents.

Membranes labeled with [^{32}P]NAD were dissolved in Laemmli's final sample buffer [21] and subsequent SDS–polyacrylamide gel electrophoresis was done as in [20,21]. Autoradiography was performed with Kodak X-ray film (X-Omat AR) with intensifying screens.

3. RESULTS AND DISCUSSION

Cholera toxin treatment resulted in an activation of adenylate cyclase in membranes from rat liver in a time- and dose-dependent manner; the effect of cholera toxin was maximal at >50 μg/ml after 5 min preincubation at 37°C (not shown). Activation of adenylate cyclase by cholera toxin treatment required NAD and GTP as essential factors [4–7] in addition to cholera toxin itself in preincubation period (table 1). ATP had no such an effect as GTP. Addition of GTP in adenylate cyclase assay medium further enhanced the enzyme activity (table 1). Under this condition we examined whether GDP can replace GTP in activating adenylate cyclase. In contrast with GTP, GDP could not support the action of cholera toxin (table 2). Addition of ATP and $MgCl_2$ along with GDP, however, allowed cholera toxin to activate adenylate cyclase, indicating that GTP formed by the catalytic reaction of nucleoside diphosphate kinase is essential for the effect of cholera toxin to be expressed in the presence of GDP.

In accordance with activation experiments, ADP-ribosylation of the guanine nucleotide binding proteins of M_r 47000, 43000 and 38000 occurred entirely dependently on cholera toxin, the M_r

Table 1

Requirements for activation of adenylate cyclase by cholera toxin (pmol.min^{-1}.mg protein^{-1})

Conditions in preincubation	Adenylate cyclase activity	
	– GTP	+ GTP
Complete	55.7	128.9
– cholera toxin	6.3	11.0
– GTP	8.1	65.3
– ATP	51.7	112.8
– thymidine	57.4	123.1
– NAD	6.8	12.8
– GTP, – ATP	5.4	53.6
– GTP, – NAD	3.8	5.3

Complete system in preincubation mixture contained 2 mM NAD, 1 mM GTP, 1 mM ATP, 10 mM thymidine, 100 μg/ml of activated cholera toxin in a total volume of 100 μl of 200 mM potassium phosphate buffer (pH 7.0). Activation of cholera toxin was done with 20 mM dithiothreitol as in [20]. Adenylate cyclase activity was determined with or without GTP in assay mixture after membranes were centrifuged

Table 2

Comparison of the effects of GTP and GDP in activation of adenylate cyclase by cholera toxin (pmol.min^{-1}.mg protein^{-1})

Additions in preincubation				Adenylate cyclase
ATP	$MgCl_2$	GTP	GDP	
–	–	–	–	19.2
–	–	–	+	20.7
–	–	+	–	65.5
+	–	–	–	17.1
+	–	–	+	25.5
+	–	+	–	78.6
+	+	–	–	13.0
+	+	–	+	56.5
+	+	+	–	124.6

Preincubation was performed with 2 mM NAD, 10 mM thymidine, 100 μg/ml of activated cholera toxin in the absence or presence of other additions as indicated. Additives were: ATP, 1 mM; $MgCl_2$, 5 mM; GTP, 1 mM; GDP, 1 mM. Adenylate cyclase activity was determined without additions after the membranes were washed as in table 1

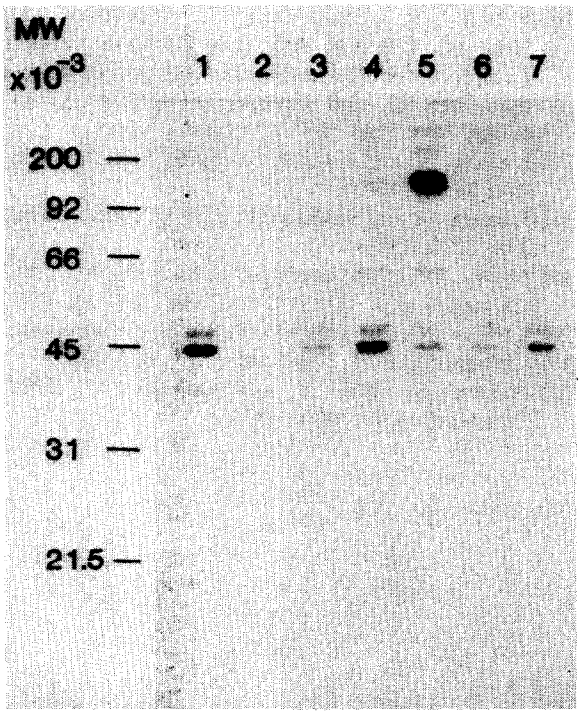

Fig.1. Requirements for [^{32}P]ADP-ribosylation of membrane proteins by cholera toxin treatment: (1) complete system containing 1 mM GTP, 1 mM ATP, 10 mM thymidine and 100 μg/ml activated cholera toxin along with 10 μM [^{32}P]NAD; (2) complete minus cholera toxin; (3) complete minus GTP; (4) complete minus ATP; (5) complete minus GTP and ATP; (6) complete minus GTP plus GDP (1 mM); (7) complete minus GTP plus GDP (1 mM) and $MgCl_2$ (5 mM).

43000 protein being the major substrate (fig.1, lanes 1,2). Removal of GTP from the incubation mixture resulted in a marked decrease in ADP-ribose transfer into these proteins (lane 3). Removal of ATP had essentially no effect on ADP-ribosylation (lane 4). Under these conditions, GDP in place of GTP had no stimulatory effect on this reaction (lane 6). Further addition of $MgCl_2$ along with GDP and ATP slightly increased the ADP-ribosylation reaction (lane 7). Although materials used in our system were essentially GTP-free [1,17,18] a small amount of ADP-ribose was transferred into these 3 proteins without added GTP (lanes 3,5,6 in fig.1). Whether the ADP-ribosylation observed without GTP added was due to contaminating a small amount of GTP in membrane preparation or other reagents, or due to a reaction independent of the presence of GTP is not known. It was of interest that proteins of M_r 120000 and 60000 were labeled under these conditions without ATP and GTP (lane 5), and without ATP (lanes 4 and 5), respectively. The significance of the phenomena and the roles of these proteins are not understood.

It is generally accepted that GTP facilitates covalent modification of the guanine nucleotide binding protein [8,9], stabilizes the ADP-ribosylated regulatory protein [22] and activates adenylate cyclase [4–7]. The present results confirm this notion. Furthermore, none of these functions elicited by GTP was reproduced by GDP. This study and [17,18] clearly show that, regardless of stimulators, hormones or cholera toxin, in the guanine nucleotide-dependent regulation of adenylate cyclase, especially in a positively regulated system, GTP is an essential active factor, and GDP is entirely inactive; GDP rather acts inhibitorily. This observation supports the recent GTP regulatory cycle hypothesis [11] as a general mechanism of adenylate cyclase regulation; GTP can make the guanine nucleotide binding protein active, resulting in an enhanced adenylate cyclase activity, and hydrolysis of GTP to GDP in turn renders the system inactive. In this cycle, the guanine nucleotide binding protein which binds GTP can serve as a substrate of an ADP-ribosylation reaction catalyzed by cholera toxin.

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