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Choleragen activation of solubilized adenylate cyclase: Requirement for GTP and protein activator for demonstration of enzymatic activity

(phosphodiesterase activator/guanylyl imidodiphosphate/cholera toxin)

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ABSTRACT **The requirements for choleragen activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] were investigated by using an enzyme preparation solubilized with Triton X-100 from an extensively washed brain particulate fraction and partially purified with DEAE-cellulose. Unlike the particulate enzyme, this preparation was not activated after incubation with choleragen plus dithiothreitol, ATP, and NAD. Addition of the purified protein activator of cyclic nucleotide phosphodiesterase and calcium to the partially purified enzyme increased basal activity somewhat, but choleragen activation was minimal. When cyclase was incubated with GTP plus the protein activator (and calcium), choleragen markedly increased the activity 3- to 6-fold. When GppNHp and protein activator were incubated with the cyclase prior to assay, activity was elevated but no effect of choleragen was observed. GTP and GppNHp had relatively small effects on cyclase activity in the absence of protein activator or if they were added directly to the assay. Boiled brain supernatant was consistently more effective than protein activator (plus calcium) and GTP, suggesting that other factors are required for maximal cyclase activity after choleragen treatment.**

It appears that the cyclase system is dissociable into several components, all of which may be necessary for optimal regulation of activity. It is probable that one of these is the heat-stable calcium-dependent protein activator of cyclic nucleotide phosphodiesterase and adenylate cyclase that we have found is required along with GTP for demonstration of choleragen activation of partially purified brain adenylate cyclase.

Choleragen (cholera toxin) exerts its effects on vertebrate cells through activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] (1). Activation of the cyclase in cell-free systems requires the A protomer of the toxin but not the B or binding subunit (2–4), is dependent on NAD (5–7), and may involve ADP-ribosylation of a cellular protein (8–10). Investigations by Gill and King (2) and Lai and coworkers (11, 12) provided evidence that a macromolecule from the cell supernatant and a low-molecular-weight compound were essential for maximal *in vitro* activation of adenylate cyclase by choleragen.

In the studies reported here, the requirement for supernatant factors for maximal expression of enzymatic activity after incubation of adenylate cyclase with choleragen was confirmed. Using a partially purified enzyme preparation solubilized with Triton X-100, we have found that GTP and the protein activator of cyclic nucleotide phosphodiesterase, previously shown to activate cyclase (13–16), can, at least in part, replace the heat-stable supernatant factors and permit demonstration of adenylate cyclase activation by choleragen.

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EXPERIMENTAL PROCEDURES

Purification of Adenylate Cyclase from Rat Brain. Male Osborne–Mendel rats were decapitated; the brains were removed, minced, and homogenized with 50 mM glycine·HCl, pH 8.0 (5 ml/g of tissue), by 20 strokes of a Dounce homogenizer. The homogenate was centrifuged at 15,000 × *g* for 20 min, and the particulate fraction was suspended in 50 volumes of 50 mM glycine·HCl, pH 8.0/0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) in a Dounce homogenizer. The resulting suspension was centrifuged as before and the pellet was washed two more times with 50 mM glycine·HCl, pH 8.0/0.5 mM EGTA. This preparation constituted the particulate enzyme.

For solubilization of adenylate cyclase, the pellet, after the final wash, was suspended in an equal volume of 50 mM glycine, pH 8.0/1% Triton X-100. After 20 min at 4°, the enzyme was centrifuged at 100,000 × *g* for 30 min. This supernatant was used for the experiments shown in Table 2. For all other experiments, 2 M NaCl was added to the enzyme at this stage to a final concentration of 0.2 M. The mixture was stirred for 20 min with an equal volume of DEAE-cellulose (DE 23) previously equilibrated with 50 mM glycine·HCl, pH 8.0/0.2 M NaCl/1% Triton X-100/0.2 mM EGTA. The supernatant obtained after centrifugation at 20,000 × *g* for 30 min is designated as the partially purified adenylate cyclase in the text and table legends.

The supernatant from the first centrifugation of brain homogenate was boiled for 10 min and centrifuged at 50,000 × *g* for 20 min. The resulting supernatant is referred to as boiled supernatant. For all experiments, enzyme and boiled supernatant were prepared on the day of use.

Preparation of Protein Activator. The protein activator of phosphodiesterase was purified from rat brain by a modification of the methods of Cheung and coworkers (17), through the DEAE-cellulose fractionation. This preparation exhibited one major protein band corresponding to the activator after electrophoresis on polyacrylamide gel with sodium dodecyl sulfate. Purified protein activator from porcine brain was kindly provided by Claude Klee (18). Both activators were tested with the cyclic GMP phosphodiesterase isolated from rat liver and were effective (19).

Adenylate Cyclase Assay. Activation and assay of adenylate cyclase was carried out with sequential incubations and additions of several solutions as noted in the tables. The choleragen solution was prepared by incubation of 0.4 mg of choleragen

Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; GppNHp, guanylyl imidodiphosphate.

Table 1. Activation of particulate adenylate cyclase by choleragen (toxin)

Exp.	Additions	Adenylate cyclase activity, pmol/assay: No toxin	Plus toxin
1	None	28	22
	NAD, ATP	40	100
	NAD, ATP, and supernatant	8.0	14.4
2	NAD, ATP	32	70
	NAD, ATP, and boiled supernatant	56	158

Samples of the particulate preparation (0.3 mg of protein in Exp. 1 and 0.11 mg in Exp. 2) were incubated with 10 μl of NAD/ATP and 10 μl of choleragen (or control) solutions and 10 μl of supernatant as indicated in a total volume of 50 μl for 10 min at 30°. Cyclase activity was then determined after the addition of 25 μl of assay solution. The protein concentration of the fresh supernatant used in Exp. 1 was 17 mg/ml and of the boiled supernatant in Exp. 2, 12 mg/ml.

and 0.4 mg of ovalbumin in 0.4 ml of 50 mM glycine·HCl, pH 8.0/20 mM dithiothreitol for 10 min at 30°; 10 μl of this (or of the same mixture minus choleragen) was added to each assay. The NAD/ATP solution contained 12.5 mM ATP, 20 mM NAD, 50 mM $MgCl_2$, 185 mM phosphoenol pyruvate, and pyruvate kinase at 1160 units/ml; 10 μl of this (or of the mixture minus NAD or ATP) was added to each assay.

The enzyme preparation was first incubated for 6 min at 30° (incubation I) with additions as indicated. The NAD/ATP and choleragen solutions were then added and incubation was continued for 10 min at 30° (incubation II), following which adenylate cyclase assay was carried out in a total volume of 75 μl after the addition of 25 μl (unless otherwise specified) of a solution containing [^{3}H]ATP (1.7×10^6 cpm), 100 mM glycine·HCl (pH 8.0), 1 mM dithiothreitol, and bovine serum albumin, 9 mg/ml. Assays were incubated for 10 min at 30°. Sodium dodecyl sulfate was then added and cyclic [^{3}H]AMP

Table 2. Effect of boiled supernatant (S_B) on activation of solubilized adenylate cyclase by choleragen (toxin)

Exp.	Additions to: Incubation I	Incubation II	Assay	Adenylate cyclase activity, pmol/assay: No toxin	Plus toxin
1	—*	—	—	14.6	16
	S_B	—	—	20	66
	—	S_B	—	22	84
	—	—	S_B	14.0	22
2	—	—	—	9.4	16
	S_B	—	—	14.6	62
	S_B, EGTA	—	—	5.0	20
	S_B	—	EGTA	8.2	23

During incubation I (6 min at 30°), 10 μl of boiled supernatant (S_B) and 1.25 mM EGTA were present as indicated with the solubilized cyclase preparation (8.5 μg of protein in Exp. 1 and 6.9 μg in Exp. 2). The NAD/ATP solution (10 μl) and 10 μl of choleragen (or control) solution were added for incubation II (10 min at 30°). Cyclase activity was then determined after addition of 25 μl of assay solution. The concentration of EGTA in the cyclase assay was 0.5 mM. The protein content of the boiled supernatant was 14.2 mg/ml in Exp. 1 and 12.5 mg/ml in Exp. 2.

* No S_B or EGTA added.

Table 3. Effect of activator protein and GTP on activity of partially purified adenylate cyclase

Additions to incubation I: Activator	Ca^{2+}	GTP	Adenylate cyclase activity, pmol/assay: No toxin	Plus toxin
0	0	0	0	2.2
0	+	0	1.4	0.8
Rat	+	0	11.6	11.8
Porcine	+	0	7.6	12.4
0	0	+	2.0	7.0
0	+	+	0.6	6.0
Rat	+	+	11.0	26
Porcine	+	+	12.4	28
*	0	0	11.0	40

During incubation I (6 min at 30°), protein activator, 107 μM Ca^{2+}, and 322 μM GTP were present as indicated with partially purified cyclase (41 μg of protein) in a total volume of 35 μl. The NAD/ATP solution (10 μl) and 10 μl of choleragen (or control) solution were added for incubation II (10 min at 30°). Cyclase activity was then determined after addition of 20 μl of assay solution. (Final concentrations of assay components were the same as in other experiments.) Concentrations of GTP and Ca^{2+} during the assay period were 150 and 50 μM, respectively; 14 μg of rat brain or 4 μg of porcine activator was used per assay.

* Boiled supernatant (10 μl; 38 μg of protein).

was isolated as described earlier (20). Data from representative experiments with assays in duplicate are reported.

Materials. Choleragen and dithiothreitol were purchased from Schwarz/Mann; phosphoenolpyruvate (sodium salt), cyclic AMP (sodium salt), ATP, GTP, ovalbumin, and pyruvate kinase [465 units/mg in 2.2 M $(NH_4)_2SO_4$] from Sigma; guanylyl imidodiphosphate (GppNHp) from ICN; AG 1-X2 (200–400 mesh) and AG 50W-X8 (100–200 mesh) from Bio-Rad; sodium fluoride and glycine from Fisher Scientific; Triton X-100 from Packard; bovine serum albumin from Armour Pharmaceutical; EGTA from Eastman; [8-^{3}H]ATP (29.3 Ci/mmol) from New England Nuclear.

DEAE-cellulose (DE 23, Whatman) was prepared for use by the procedure of Gregolin *et al.* (21). Protein was determined by the method of Lowry *et al.* (22).

RESULTS

Gill (6) and Martin *et al.* (23) reported that both NAD and ATP were required for choleragen activation of adenylate cyclase. As shown in Table 1, activation of the particulate brain cyclase was demonstrated when ATP and NAD were present with choleragen during incubation before assay. The presence of boiled supernatant along with NAD and ATP increased by about 100% the activities of preparations incubated with or without choleragen—i.e., the apparent magnitude (in absolute or percentage terms) of choleragen activation was enhanced by the boiled supernatant. Fresh (unheated) supernatant decreased cyclase activity under the same conditions.

With the solubilized cyclase, addition of boiled supernatant during incubation I or incubation II somewhat increased basal cyclase activity and markedly increased choleragen activation (Table 2, Exp. 1). The effects of the supernatant were minimal when it was present only during the assay period. Addition of EGTA to incubation I or during the assay period markedly decreased the effect of the boiled supernatant (Table 2, Exp. 2).

The partially purified cyclase preparation that was solubi-

Table 4. Effect of Ca^{2+} and EGTA in the presence of activator protein and GTP on activity of partially purified adenylate cyclase

Incubation I		Adenylate cyclase activity, pmol/assay	
Ca^{2+}	EGTA	No toxin	Plus toxin
0	0	10.8	38
+	0	14.0	54
0	+	6.4	28
+	+	17.6	54

During incubation I, rat activator (14 μg) and 321 μM GTP, with 214 μM EGTA and 107 μM Ca^{2+}, were present as indicated with partially purified cyclase (43 μg of protein) in a total volume of 35 μl. The NAD/ATP and choleragen (or control) solutions were added for incubation II and cyclase activity was determined with addition of 20 μl of assay solution as in Table 3. Cyclase assays contained 150 μM GTP with 100 μM EGTA and/or 50 μM Ca^{2+} as indicated.

Table 5. Effect of NAD on activity of partially purified adenylate cyclase

Incubation I Activator	Incubation II		Adenylate cyclase activity, pmol/assay	
+ Ca^{2+} + GTP	ATP	NAD	No toxin	Plus toxin
0	+	0	6.2	3.4
0	+	+	0.6	4.6
+	+	0	17.4	15.8
+	+	+	12.2	60

During incubation I, rat activator protein (14 μg), 125 μM Ca^{2+}, and 385 μM GTP were present as indicated with partially purified cyclase (42 μg of protein) in a total volume of 30 μl. The NAD/ATP or ATP solution followed by choleragen (or control) solution was then added to initiate incubation II. Concentrations of GTP and Ca^{2+} when present in the cyclase assay were 150 and 50 μM, respectively.

lized in the presence of EGTA exhibited essentially no basal activity and was activated little if at all by choleragen (plus NAD and ATP), as shown in Table 3. Addition of cyclic nucleotide phosphodiesterase activator protein (from rat or porcine brain) plus calcium during incubation I increased basal cyclase activity but choleragen activation was still minimal. The presence of GTP during incubation I had only a small effect on basal activity or choleragen activation. When GTP, protein activator, and Ca^{2+} were added together during incubation I a clear effect of choleragen was demonstrable. However, the magnitude of choleragen activation with GTP, activator, and Ca^{2+} was not as great as it was when boiled supernatant was used during incubation I. Increasing the amounts of activator above those used in the experiment shown in Table 3 had no further effect.

Ca^{2+} added alone or with GTP during incubation I had little effect on basal cyclase activity or on choleragen activation (Table 3). In the presence of GTP and protein activator, however, Ca^{2+} clearly increased the effect of choleragen, and the addition of EGTA (2 mol/mol of added Ca^{2+}) under these conditions had no effect (Table 4).* The same amount of EGTA in the absence of added Ca^{2+} decreased choleragen activation. The presence of protein activator, Ca^{2+}, and GTP did not relieve the requirement for NAD during incubation with choleragen (Table 5).

As shown in Table 6, the addition of GppNHp during incubation I produced a small increase in cyclase activity and this was not altered by the presence of Ca^{2+}. When protein activator was added with Ca^{2+} and GppNp during incubation I, activity was markedly increased and no further effect of choleragen was evident. Although the significance is unclear, it is notable that, in the presence of Ca^{2+} and the protein activator, the extent of cyclase activation achieved with GppNHp was similar to that observed with the same concentration of GTP plus choleragen (Table 7).

As shown in Table 8, the effects of GTP or GppNHp added to the assay were not nearly as great as those observed when the nucleotide was present during incubation I. On the other hand, when GTP was present during incubation I, addition of protein activator (plus Ca^{2+}) only during the assay period markedly increased the activity of the choleragen-activated enzyme, albeit not to the level observed when the activator was added to incubation I.

DISCUSSION

It is apparent that choleragen activation of adenylate cyclase in cell-free systems is a complex process that requires multiple factors from supernatant and particulate fractions (2, 6, 11, 12). In addition to thiol, which is necessary for the release of the A_1 subunit of the toxin (2), and NAD (5–7), which may be a substrate in a choleragen-catalyzed ADP-ribosylation reaction (8–10), Gill and King (2) and Lai and coworkers (11, 12) found supernatant factors of high and low molecular weight that increased choleragen activation of pigeon erythrocyte adenylate cyclase. To begin to define, isolate, and identify the components necessary for cyclase activity and choleragen activation, we used a solubilized enzyme preparation from rat brain partially purified by fractionation with DEAE-cellulose. Cheung and coworkers (14, 15) and Brostrom *et al.* (13, 16), using similar procedures, obtained cyclase preparations that required addition of the protein activator of cyclic nucleotide phosphodiesterase for catalytic activity. We found also that protein activator from rat or porcine brain increased the activity of the partially purified cyclase preparation, which in the absence of activator was essentially undetectable. Activation of the cyclase by choleragen was minimal, however, whether or not the protein activator (plus Ca^{2+}) was present. Thus, it was apparent that the purified activator did not in itself account for the ef-

Table 6. Effect of GppNHp and activator protein on activity of partially purified adenylate cyclase

Additions to incubation I			Adenylate cyclase activity, pmol/assay	
Activator	Ca^{2+}	GppNHp	No toxin	Plus toxin
0	0	0	1.0	1.6
+	+	0	16.2	19.6
0	0	+	5.8	7.0
0	+	+	5.6	7.6
+	+	+	94	96

During incubation I, rat activator protein (14 μg), 125 μM Ca^{2+}, and 375 μM GppNHp were present as indicated with partially purified cyclase (39 μg of protein) in a total volume of 30 μl. The NAD/ATP and choleragen (or control) solutions were added for incubation II. Concentrations of Ca^{2+} and GppNHp when present in the cyclase assay were 50 and 150 μM, respectively.

* Although we did not demonstrate that the effect of the protein activator in the adenylate cyclase system was absolutely dependent on added Ca^{2+}, it is most probable that Ca^{2+} is required and that the optimal concentration is relatively low. Under the conditions of our experiments (with ca. 25 μM EGTA present in the assay system), the addition of >50 μM Ca^{2+} inhibited adenylate cyclase activity.

Table 7. Effects of GTP and GppNHp in the presence of protein activator and Ca^{2+} on partially purified adenylate cyclase

Incubation I nucleotide, μM	Adenylate cyclase activity, pmol/assay	
	No toxin	Plus toxin
GTP:		
25	24	52
125	26	72
375	26	96
750	26	104
GppNHp:		
25	62	58
125	78	78
375	94	96
750	102	102

During incubation I, rat activator protein (14 μg) and 125 μM Ca^{2+} with GTP or GppNHp as indicated were present with partially purified cyclase (39 μg of protein) in a total volume of 30 μl. The NAD/ATP and choleragen (or control) solutions were then added for incubation II. In the cyclase assay the Ca^{2+} concentration was 50 μM and the nucleotides were present at 40% of the concentration indicated during incubation I.

Table 8. Effects of GTP, GppNHp, protein activator, and Ca^{2+} on partially purified adenylate cyclase

Additions to incubation I		Additions to assay		Adenylate cyclase activity, pmol/assay	
Activator + Ca^{2+}	GTP	Activator + Ca^{2+}	GTP	No toxin	Plus toxin
0	0	0	0	0	2.4
+	0	0	0	11.6	11.8
0	0	+	0	5.6	9.2
0	0	+	+	4.8	7.8
+	0	0	+	6.8	13
0	+	+	0	11.2	38
+	+	0	0	10.2	66
	GppNHp		GppNHp		
0	0	+	+	2.8	6.4
+	0	0	+	13.4	13.2
+	+	0	0	60	66

Rat activator protein (14 μg), Ca^{2+}, and GTP or GppNHp were added at the beginning of incubation I or the assay period, as indicated. The NAD/ATP and choleragen (or control) solutions were added for incubation II. Each assay contained 42 μg of partially purified cyclase. In the cyclase assay the concentration of Ca^{2+} was 50 μM and of nucleotide, 150 μM. Concentrations of these components during incubation I were 125 μM and 375 μM, respectively.

fectiveness of boiled brain supernatant which markedly enhanced choleragen activation of the cyclase.

In view of reports that the activity of adenylate cyclase activated by choleragen in intact cells or in homogenates was increased by guanine nucleotides (23, 24), we investigated the effects of GTP and GppNHp. When the partially purified brain cyclase was incubated with GTP, only a slight increase in basal activity and choleragen activation was observed. Addition of GTP with protein activator and Ca^{2+}, however, resulted in a clear enhancement of cyclase activity after choleragen activation with relatively little effect on basal enzyme activity. Martin *et al.* (23) found that the choleragen-activated cyclase in rat liver membranes responded to GTP. It now appears likely that these effects were dependent on the presence of endogenous protein activator in the membrane preparation. As has been found in many other cyclase systems, the effects of GppNHp were clearly different from those of GTP. When the protein activator (and Ca^{2+}) were present, GppNHp dramatically increased the activity of the partially purified adenylate cyclase but, in the presence of GppNHp (even with concentrations that were less than maximally effective), no choleragen activation was demonstrable.

Although the presence of GTP and protein activator during incubation I clearly enhanced basal activity and permitted demonstration of severalfold activation by choleragen, the activity of the choleragen-activated cyclase was consistently lower under these conditions than it was when boiled supernatant was used in incubation I. This would suggest that, in addition to NAD, GTP, and protein activator, other factors are necessary for choleragen activation or for expression of catalytic activity of the choleragen-activated enzyme. Indeed, the protein activator and GTP may be distinct from the supernatant factors described by Gill and King (2) and Lai and coworkers (11). Pfeuffer and Helmreich (25) found that a guanyl nucleotide binding protein, separable from the cyclase and of much higher apparent molecular weight than the protein activator of phosphodiesterase, may be necessary for activation of pigeon erythrocyte adenylate cyclase. In addition, Pecker and Hanoune (26) recently reported that a cytosolic protein may be involved in the effect of GTP on hepatic cyclase. Although the molecular mechanisms through which adenylate cyclase activity is controlled remain to be elucidated, it appears that the cyclase system is dissociable into several components (stimulatory and inhibitory), all of which are necessary for optimal regulation of activity. It is probable that one of these is the heat-stable protein activator of cyclic nucleotide phosphodiesterase (17) and adenylate cyclase (13–16) that we found to be required for demonstration of activation of the partially purified brain adenylate cyclase by GppNHp or by choleragen. The role of protein activator in relation to the ADP-ribosyl transferase activity of choleragen (9) is at present unclear.

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