Effects of Nucleoside Triphosphates on Choleragen-Activated Brain Adenylate Cyclase†

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ABSTRACT: To investigate the effects of nucleoside triphosphates on the activation of adenylate cyclase by choleragen and on the stability and catalytic function of the choleragen-activated enzyme, we treated samples of particulate preparation from bovine brain successively in three separate incubations with extensive washing between each step. In incubation I, choleragen and NAD were present to activate the adenylate cyclase. In incubation II, conditions were varied to assess enzyme stability. Finally, adenylate cyclase activity was assayed with ATP or adenylyl imidodiphosphate [App(NH)p] as the substrate. Even when assays contained an optimal concentration of GTP, nucleoside triphosphate (plus a regenerating system) was required in incubation I for maximal choleragen activation; in order of effectiveness, GTP > ITP ≫ ATP ≥ CTP = UTP. During incubation II (at 30 °C), activity of the choleragen-treated fractions was essentially completely stable when 100 μM GTP (plus a regenerating system) was present. ITP and ATP were less effective. Activation produced by guanylyl imidodiphosphate was more stable than that resulting from choleragen, GTP, and NAD. After activation of membranes with choleragen, NAD, and GTP, nucleoside triphosphate plus a regenerating system (but not NAD or additional choleragen) was essential for expression of maximal activity. In order of effectiveness, GTP > ITP ≫ ATP ≥ CTP = UTP. It appears that GTP, which was effective in micromolar concentrations, plays an important role not only in the activation of adenylate cyclase by choleragen but also in the stabilization and expression of the catalytic function of the activated enzyme.

Choleragen, an enterotoxin secreted by *Vibrio cholerae*, exerts its effects on cells through activation of adenylate cyclase (Finkelstein, 1973; Gill, 1977; Moss & Vaughan, 1979; van Heyningen, 1977a). Activation of adenylate cyclase catalyzed by the A_1 peptide of choleragen requires NAD and is believed to result from the ADP ribosylation of a protein that influences adenylate cyclase activity (Cassel & Pfeuffer, 1978; Gill, 1975, 1976; Gill & King, 1975; Gill & Meren, 1978; Johnson et al., 1978; Moss & Vaughan, 1977a; Moss et al., 1976a,b; van Heyningen, 1977b). In addition to NAD, several other factors, including nucleoside triphosphates and cytosolic protein(s), contribute to the activation by choleragen, stabilization, and/or catalytic activity of adenylate cyclase (Enomoto & Gill, 1979; Gill, 1975, 1976; Moss & Vaughan, 1977b; Wheeler et al., 1976), but it has been difficult to ascertain at which step each is involved. Enomoto & Gill (1979) and Lin et al. (1978) observed effects of GTP present during the activation by choleragen and NAD or added in the assay, respectively. It was unclear, however, whether the presence of GTP in either activation or assay alone would suffice to produce a choleragen-activated enzyme. By separating the activation and assay steps, carefully washing the cyclase preparation between, and using optimal conditions in one step when attempting to define the effects of nucleotides in the other, we have now shown effects of GTP at both steps. In addition, as reported here, the choleragen-activated adenylate cyclase is extremely labile at 30 °C and can be stabilized by GTP.

Experimental Procedures

Frozen bovine brain was homogenized in 50 mM glycine, pH 8.0 (5 mL/g of tissue), with nine strokes of a Dounce homogenizer. The homogenate was filtered through gauze and centrifuged (20000*g*; 20 min). The sedimented particulate fraction was washed twice by homogenization in 50 volumes of 50 mM glycine, pH 8.0, containing 0.5 mM EGTA, collected by centrifugation each time, and finally suspended in 50 mM glycine, pH 8.0 (5 mL/g of tissue).

Choleragen activation of adenylate cyclase (incubation I) was carried out in a total volume of 0.9 mL containing unless otherwise stated ~5.4 mg of particulate protein, 45 μg of choleragen [previously activated by incubation for 10 min at 30 °C with 20 mM dithiothreitol as described in Moss & Vaughan (1977b)], 1 mM NAD, 10 mM $MgCl_2$, 200 mM NaCl, 10 mM Tris-HCl (pH 8.0), 33 mM glycine buffer (pH 8.0), and other additions as indicated. (When choleragen was omitted, an equivalent amount of the choleragen-activation buffer was added.) After 20 min at 30 °C, the particulate was sedimented by centrifugation, washed with 9 mL of 50 mM glycine, pH 8.0, and suspended in 400 μL of the same buffer. Samples (20 μL) were then taken for assay of adenylate cyclase activity. Essentially maximal activation of adenylate cyclase was produced by incubation with 25 μg of choleragen for 20 min at 30 or 37 °C in the presence of GTP and a regenerating system (data not shown).[1]

For evaluation of the stability of the choleragen-activated enzyme following incubation I, the washed particulate fraction was incubated again (incubation II) before assay of adenylate cyclase activity. The adenylate cyclase assay was carried out in a total volume of 100 μL containing unless otherwise stated 25 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM cAMP, 3 mM theophylline, 2 mM dithiothreitol, 300 mM NaCl, 0.1 mg of bovine serum albumin, and either 100 μM [α-^{32}P]-App(NH)p[2] with 10 μM GTP, 0.2 mM phosphoenolpyruvate, and pyruvate kinase (12 units/mL) or 1 mM [α-^{32}P]ATP with 100 μM GTP, 2 mM phosphoenolpyruvate, and pyruvate kinase (12 units/mL). Assays were initiated with the addition of the washed particulate fraction (~98 μg of protein) and

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[1] The regenerating system consisted of phosphoenolpyruvate–pyruvate kinase unless otherwise indicated.

[2] Abbreviations used: G_{M1}, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; Gpp(NH)p, 5′-guanyl β,γ-imidodiphosphate; App(NH)p, adenylyl β,γ-imidodiphosphate.

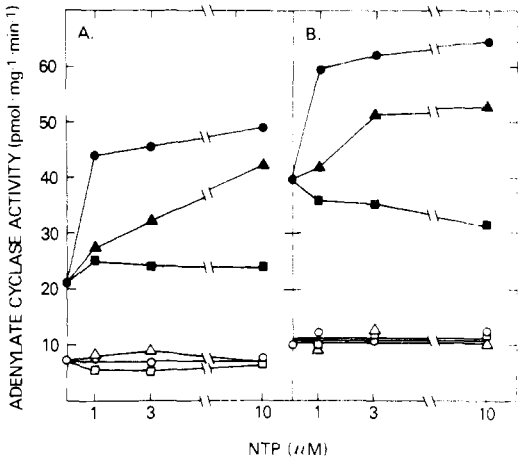

FIGURE 1: Effect of nucleoside triphosphates in incubation I (A) and assay (B). Samples of the particulate fraction were incubated without (○, △, and □) or with (●, ▲, and ■) choleragen, 10 mM phosphoenolpyruvate, and pyruvate kinase (100 units/mL), washed, and assayed with App(NH)p, 0.2 mM phosphoenolpyruvate, and pyruvate kinase (12 units/mL). (A) Incubation I contained GTP (○ and ●), ITP (△ and ▲), or ATP (□ and ■) at the indicated concentration, and assays contained 10 μM GTP. (B) Incubation I contained 10 μM GTP and assays contained GTP (○ and ●), ITP (△ and ▲), or ATP (□ and ■) as indicated. NTP = nucleoside triphosphate.

incubated for 10 min at 30 °C. Then, 0.1 mM cAMP with ~6000 cpm of [^{3}H]cAMP and 2% sodium dodecyl sulfate were added. [^{32}P]cAMP and [^{3}H]cAMP were isolated by the procedure of Salomon et al. (1974) for radioassay. Protein was determined by the method of Lowry et al. (1951).

Choleragen, dithiothreitol, and Tris base were purchased from Schwarz/Mann; phosphoenolpyruvate sodium salt, cyclic AMP sodium salt, ATP, GTP, ITP, UTP, ovalbumin, pyruvate kinase [865 units/mg in 2.2 M $(NH_4)_2SO_4$], alumina WN-3, NaF, imidazole, and theophylline were from Sigma Chemical Co.; NAD, Gpp(NH)p, App(NH)P, and CTP were from Boehringer Mannheim; glycine and AG 50-X8 were from Bio-Rad; $MgCl_2$ was from Baker; albumin (fraction V from bovine serum) was from Armour Pharmaceutical; [α-^{32}P]-App(NH)p (20 Ci/mmol) was from ICN; [α-^{32}P]ATP (22 Ci/mmol), [^{3}H]cAMP (42.5 Ci/mmol), and Aquasol were from New England Nuclear.

Results

Activation of the brain particulate adenylate cyclase by choleragen was markedly increased by the addition of GTP, which was more effective than ITP; ATP (also CTP or UTP in other experiments) at similar concentrations had little effect (Figure 1A). In this experiment, all samples were assayed with 10 μM GTP. In the assay, GTP was more effective than ITP, and ATP was ineffective in increasing catalytic activity of the enzyme activated by choleragen with 10 μM GTP present in incubation I (Figure 1B). The nucleoside triphosphates whether present in incubation I or in the assay had no effect on activity of the fractions not exposed to choleragen.

The concentration of GTP that produced a half-maximal effect in incubation I and in the assay was significantly decreased by the addition of pyruvate kinase and phosphoenolpyruvate (Figures 2 and 3). In the presence of the regenerating system, maximal effects were observed with <5 μM GTP. A nucleoside triphosphate regenerating system consisting of creatine phosphate and creatine phosphokinase was similarly effective (data not shown).

When the particulate fractions following incubation I were incubated at 30 °C before assay (incubation II), the activity

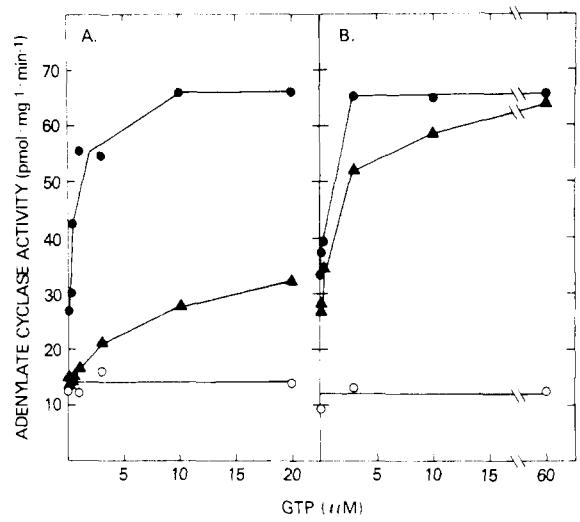

FIGURE 2: Effect of regenerating system on the GTP requirement in incubation I (A) and assay (B). Samples of the particulate fraction were incubated without (○) or with (● and ▲) choleragen, washed, and assayed with App(NH)p. (A) Incubation I contained the indicated concentration of GTP without (▲) or with (○ and ●) 10 mM phosphoenolpyruvate and pyruvate kinase (100 units/mL). Assays contained 10 μM GTP, 0.2 mM phosphoenolpyruvate, and pyruvate kinase (12 units/mL). (B) Incubation I contained 10 μM phosphoenolpyruvate and pyruvate kinase (100 units/mL). Assays contained GTP at the indicated concentration without (▲) or with (○ and ●) 0.2 mM phosphoenolpyruvate and pyruvate kinase (12 units/mL).

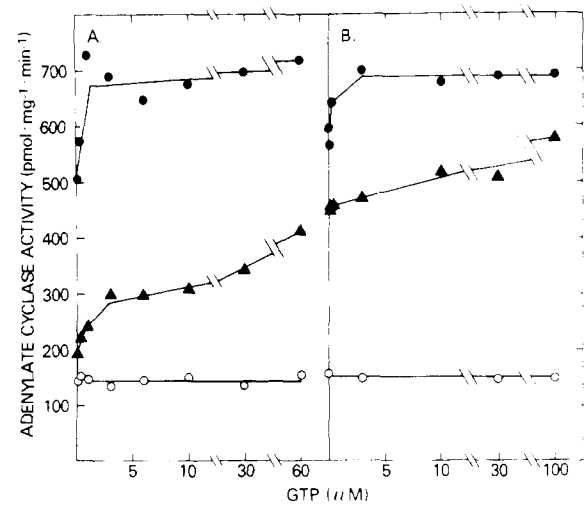

FIGURE 3: Effect of the regenerating system on the GTP requirement in incubation I (A) and assay (B). Experiment and symbols are as described in the legend for Figure 2, except that assays were with ATP. (A) Incubation I contained the indicated concentration of GTP. Assays contained 100 μM GTP, 2 mM phosphoenolpyruvate, and pyruvate kinase (12 units/mL). (B) Incubation I contained 100 μM GTP, 10 mM phosphoenolpyruvate, and pyruvate kinase (100 units/mL). Assays contained GTP at the indicated concentration.

of the choleragen-activated samples declined dramatically; activity decreased ~50% in the first 20 min and thereafter more slowly at a rate similar to that of the samples not exposed to choleragen (Figure 4). At 4 °C, activity was relatively stable (Figure 4). As shown in Figure 5, after incubation at 30 °C, a second incubation with choleragen resulted in only a small increase in adenylate cyclase activity which at all times was less than the activation observed with samples that had not been previously exposed to choleragen.

Activity of the choleragen-treated (and control) fractions was essentially completely stable for 2 h at 30 °C when GTP (and the regenerating system) were present in incubation II

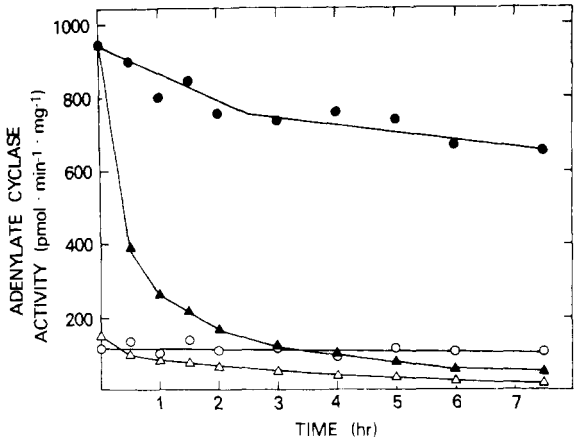

FIGURE 4: Stability of choleragen-activated adenylate cyclase at 4 °C and at 30 °C. Samples of the particulate fraction were incubated without (○ and △) or with (● and ▲) choleragen with 100 μM GTP, 35 mM phosphoenolpyruvate, and pyruvate kinase (232 units/mL), washed, suspended in 400 μL of 50 mM glycine (pH 8.0), and incubated (incubation II) at 30 °C (△ and ▲) or at 4 °C (○ and ●). At the indicated times, samples were removed for assay of adenylate cyclase activity with ATP as described under Experimental Procedures.

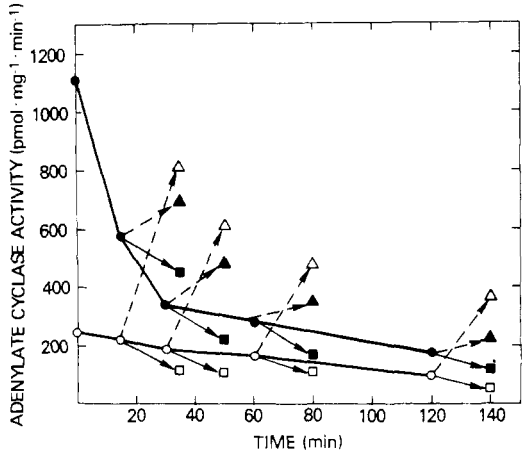

FIGURE 5: Choleragen activation after incubation of particulate fractions at 30 °C. Samples of the particulate fraction were incubated (incubation I) without (○, △, and □) or with (●, ▲, and ■) choleragen and other additions as described in the legend for Figure 4, washed, suspended in 400 μL of 50 mM glycine (pH 8.0), and incubated at 30 °C (Incubation II). At the indicated times, samples (20 μL) were assayed for adenylate cyclase (○ and ●) with ATP as described under Experimental Procedures. Other samples were incubated with choleragen and a regenerating system as in incubation I without (□ and ■) or with (△ and ▲) GTP and NAD, washed, and assayed with ATP.

Table I: Stabilization of Choleragen-Activated Adenylate Cyclase with Nucleoside Triphosphates[a]

additions (3 μM)	adenylate cyclase act. (pmol min⁻¹ mg⁻¹)
none	82
ATP	120
ITP	140
GTP	180
GTP plus ATP	240

[a] Samples of particulate fraction were incubated with choleragen (incubation I), washed, and incubated for 2 h at 30 °C (incubation II) with the indicated nucleotide before assay of adenylate cyclase activity. This experiment was carried out as described in the legend for Figure 6 except that the concentrations of nucleotides present during incubation II were only 3 μM. In the last sample, GTP and ATP were each 3 μM.

(Figure 6). At a concentration of 100 μM, ITP also prevented inactivation but ATP was much less effective (Figure 6).

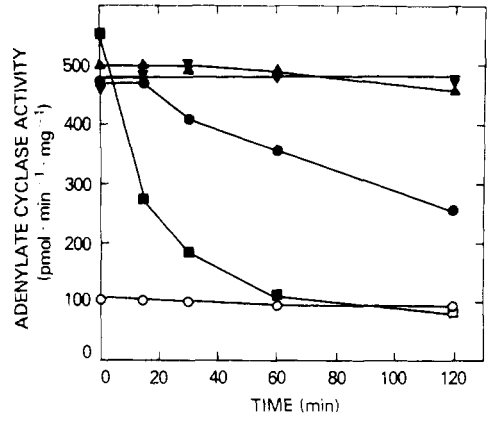

FIGURE 6: Stabilization of choleragen-activated adenylate cyclase activity with nucleoside triphosphates. Samples of the particulate fraction were incubated without (○) or with (●, ▼, ■, and ▲) choleragen (incubation I), washed, and suspended as described in the legend for Figure 4. They were then incubated (Incubation II) at 30 °C in 400 μL of 50 mM glycine (pH 8.0), containing 200 mM NaCl, 10 mM $MgCl_2$, 3.5 mM phosphoenolpyruvate, and pyruvate kinase (112 units/mL) with 100 μM GTP (○ and ▼), 100 μM ITP (▲), 100 μM ATP (●), or no nucleotide (■). At the indicated times, samples (20 μL) were removed for assay of adenylate cyclase activity with ATP as described under Experimental Procedures.

Table II: Effects of GTP and Gpp(NH)p on Adenylate Cyclase Activity[a]

addition to incubation			adenylate cyclase act. (pmol min⁻¹ mg⁻¹)	
I	II	assay	no CT	plus CT
0	0	0	10	17
0	0	Gpp(NH)p	41	31
0	GTP	Gpp(NH)p	35	43
0	Gpp(NH)p	0	64	61
0	Gpp(NH)p	GTP	63	62
0	Gpp(NH)p	Gpp(NH)p	63	65
Gpp(NH)p	0	0	93	90
Gpp(NH)p	Gpp(NH)p	0	110	107
Gpp(NH)p	0	Gpp(NH)p	89	88
Gpp(NH)p	Gpp(NH)p	Gpp(NH)p	112	95
0	GTP	0	14	21
0	0	GTP	15	24
0	GTP	GTP	15	32
GTP	0	0	14	25
GTP	GTP	0	15	29
GTP	0	GTP	19	41
GTP	GTP	GTP	19	70

[a] Samples of particulate fraction were incubated (incubation I) with or without choleragen (CT) as described under Experimental Procedures, washed, suspended in 50 mM glycine, pH 8.0, and incubated 20 min at 30 °C (incubation II) as described in the legend for Figure 4, after which samples (20 μL) were taken for assay of adenylate cyclase with App(NH)p. Where indicated, 10 μM Gpp(NH)p or 10 μM GTP plus 10 mM phospho(enol)pyruvate and pyruvate kinase (100 units/mL) was present in incubation I and/or incubation II. Assays contained the regenerating system and, where indicated, 10 μM GTP or Gpp(NH)p.

When 3 μM nucleotide was used, GTP did not completely prevent loss of activity but was more effective than ITP or ATP (Table I). Even when GTP was present during incubation II, addition of GTP in the assay markedly increased the activity of the choleragen-activated cyclase (Table II). This activity was distinctly lower when GTP was absent from incubations I and/or the assay than when it was omitted from incubation II.

In fractions incubated with Gpp(NH)p at any stage, there was no demonstrable effect of choleragen on adenylate cyclase activity (Table II). Exposure to Gpp(NH)p during incubation

I produced the highest activities; the further presence of Gpp(NH)p during incubation II or in the assay had little effect (Table II). Activation was less in samples first exposed to Gpp(NH)p during incubation II and was still lower when Gpp(NH)p was added only in the assay, whether or not GTP had been present during incubation II (Table II).

Discussion

The studies reported here clearly establish a role for GTP not only in the activation of adenylate cyclase by choleragen but also in the expression of catalytic function of the enzyme activated in the presence of an optimal concentration of the nucleotide. Activation has been reported to require GTP or ATP in millimolar concentrations (Enomoto & Gill, 1979; Gill, 1976; Wheeler et al., 1976). In our experiments with the brain particulate fraction, however, addition of a nucleoside triphosphate regenerating system markedly decreased the requirement for GTP present during choleragen activation to concentrations similar to those necessary for hormonal activation of adenylate cyclase (Rodbell et al., 1971). In the presence of a regenerating system, similarly low concentrations of GTP were sufficient to provide maximal effects on catalytic function of the choleragen-activated enzyme. For activation and catalysis, GTP was more effective than ITP, and ATP was inactive.

No choleragen activation of adenylate cyclase was observed with preparations that were exposed to Gpp(NH)p at any stage. This effect of a GTP analogue that is not hydrolyzed by GTPase is consistent with the hypothesis of Cassel & Selinger (1977) that choleragen acts by inhibiting the degradation of GTP bound to adenylate cyclase and thereby prolongs the lifetime of the catalytically active cyclase–GTP complex.

Incubation of the choleragen-activated particulate fraction at 30 °C in glycine buffer resulted in rapid loss of activity (>50% in 30 min). A second incubation of these fractions with choleragen plus NAD, GTP, and a regenerating system produced only a small increase in adenylate cyclase activity. The activity of the control enzyme also decreased with incubation at 30 °C but much more slowly than that of the choleragen-activated preparations; at all times its capacity to be activated by choleragen was greater than that of the enzyme previously exposed to the toxin. Thus, it seems that activation by choleragen enhanced the intrinsic lability of the adenylate cyclase or perhaps that the reversal of choleragen activation results in a form of the enzyme that is refractory to activation by the toxin. When the choleragen-treated enzyme was incubated at 30 °C in the presence of 100 μM GTP (with a regenerating system), activity was virtually unchanged in 2 h. Stabilization of the activated enzyme by GTP did not obviate the need for addition of GTP in the assay to permit maximal catalytic activity.

In intact cells exposed to choleragen, adenylate cyclase activation appears to be relatively irreversible (O'Keefe & Cuatrecasas, 1974). The persistence of the activated form of adenylate cyclase after the removal of choleragen from the medium might, of course, result from continuing activation by toxin that remains bound to cells even after extensive washing (Moss et al., 1980). Polyacrylamide gel electrophoresis of sodium dodecyl sulfate solubilized proteins from fibroblasts that had been exposed to [^{125}I]choleragen and then washed and incubated revealed the presence of ^{125}I-labeled peptides with mobilities identical with those of the ^{125}I-labeled A_1 and B peptides of the original toxin (Moss et al., 1980). It would appear that intact choleragen peptides may have a relatively long half-life in these cells. If so, the A_1 peptide could continue to catalyze the ADP ribosylation (or re ADP ribosylation, if this process is reversible) of its protein substrate. Alternatively, our demonstration that the choleragen-activated adenylate cyclase from brain is stabilized by GTP in micromolar concentrations is consistent with the possibility that the prolonged activation of adenylate cyclase in intact cells does not require the continuing ADP–ribosyltransferase activity of choleragen but rather results from stabilization of the ADP-ribosylated enzyme by GTP.

Acknowledgments

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