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Effect of Cholera Toxin on the Activation of Adenylate Cyclase by Calmodulin in Bovine Striatum

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SUMMARY

The effect of cholera toxin on activation of adenylate cyclase by the endogenous Ca^{2+}-binding protein, calmodulin, GTP, dopamine, and forskolin was investigated in bovine striatum. Adenylate cyclase activity was measured in washed membrane fractions prepared from homogenates that had been preincubated with cholera toxin. Pretreatment of striatal membranes with cholera toxin increased the response of adenylate cyclase to GTP, calmodulin, and forskolin as compared to vehicle controls. After cholera toxin pretreatment, the maximal response of adenylate cyclase to GTP was increased 4.7-fold and the apparent K_a for GTP was reduced 3-fold. The apparent V_{max} for calmodulin was doubled after cholera toxin pretreatment. The activation of adenylate cyclase by forskolin was increased by cholera toxin, but the effect on kinetic parameters was not determined due to solubility considerations. In contrast, dopamine-stimulated adenylate cyclase activity was abolished after cholera toxin pretreatment. Examination of a concentration-response curve for cholera toxin in altering these activities revealed that calmodulin-stimulated adenylate cyclase was maximally affected at lower concentrations of cholera toxin than was activation by GTP and forskolin. Cholera toxin also affected the interaction between calmodulin and GTP. In the absence of cholera toxin, calmodulin decreased the apparent K_a for GTP nearly 10-fold. After cholera toxin pretreatment, however, calmodulin could not further decrease the apparent K_a for GTP but increased the maximal response to GTP by 30%. Calmodulin could potentiate GTP activation by stabilizing the interaction between N_s and the catalytic subunit, an action which could be negated by prior treatment with cholera toxin. ADP-ribosylation of the striatal homogenates with [^{32}P]NAD demonstrated predominant labeling of a band of M_r 45,000 which corresponds to the known molecular weight of the α-subunit of the stimulatory GTP-binding protein, N_s. These results suggest that the activational state of N_s can affect the stimulation of adenylate cyclase by calmodulin and forskolin. Calmodulin and forskolin may act at separate sites on the catalytic subunit that can allosterically interact with N_s.

Activation of adenylate cyclase (EC 4.6.1.1.) by hormones or neurotransmitters requires a hormone receptor, a heterotrimeric GTP-binding protein, termed N_s, and a catalytic subunit that produces cyclic AMP (1). In brain, adenylate cyclase activity is also activated by the endogenous Ca^{2+}-binding protein, CaM (2, 3). Several studies have suggested that there are both CaM-sensitive and CaM-insensitive components of adenylate cyclase activity in brain (4–6). CaM can directly activate the catalytic subunit in a Ca^{2+}-dependent manner (7, 8) and GTP is not required for its activation (5, 9). There is evidence, however, that CaM interacts with guanyl nucleotides in the activation of adenylate cyclase. CaM has been found to potentiate the activity of GTP and hormones in the stimulation of adenylate cyclase in rat brain (4, 10–12) and bovine retina (13). In bovine striatum, we found that CaM could increase the potency of GTP for stimulation of adenylate cyclase up to 5-fold (14). CaM could also enhance the inhibition of adenylate cyclase by the nonhydrolyzable analog of GTP, GppNHp, in rat hippocampus (15) and cerebellum (16). This suggests that CaM, N_s, and the GTP-binding protein mediating inhibition of adenylate cyclase, N_i, may act at separate but interacting sites on the catalytic subunit of adenylate cyclase.

We wished to investigate further the interaction of CaM and guanyl nucleotides by examining the effect of the activation of N_s by cholera toxin on the ability of CaM to stimulate adenylate cyclase activity. Cholera toxin activates adenylate cyclase by ADP-ribosylating N_s in a reaction that requires NAD as a substrate as well as GTP and a cytosolic factor (for reviews, see Refs. 17 and 18). Cholera toxin ADP-ribosylates the α-subunit of N_s, which binds GTP and directly activates the catalytic subunit of adenylate cyclase (1). ADP-ribosylation of N_s inhibits the hormone-stimulated GTPase activity associated with N_s which functions to inactivate the cyclase reaction (19), increases the rate of exchange of guanyl nucleotides at N_s (20),

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ABBREVIATIONS: N_s, stimulatory guanyl nucleotide-regulatory protein; CaM, calmodulin; GppNHp, guanosine-5′ (β,γ-imido)triphosphate; N_i, inhibitory guanyl nucleotide-regulatory protein; DA, dopamine (3-hydroxytyramine); DTT, dithiothreitol; PEP, phospho*enol*pyruvate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

and promotes the dissociation of the α- and β-subunits of N_s (21). These actions result in a long-lived activation of adenylate cyclase by cholera toxin (17, 18). Activation of adenylate cyclase by cholera toxin in brain has been more difficult to demonstrate than in peripheral tissues, although both enzyme activation and ADP-ribosylation of N_s by cholera toxin in rat and bovine brain have been demonstrated (22–25). The activation of brain adenylate cyclase by cholera toxin is probably not as sensitive as in other tissues because of high proteolytic activity and possible NAD glycohydrolase activity (25).

We have investigated the effect of cholera toxin pretreatment on the ability of CaM to activate adenylate cyclase activity in order to determine whether there would be a competitive, potentiative, or additive interaction between CaM and activated N_s. The activation of adenylate cyclase by CaM was potentiated after treatment of bovine striatal homogenate fractions with cholera toxin. These results further support an interaction between N_s- and CaM-binding sites at the catalytic subunit of adenylate cyclase and demonstrate that the CaM-sensitive component of adenylate cyclase is very sensitive to activation by N_s.

Experimental Procedures

Materials. [α-^{32}P]ATP (specific activity 38 Ci/mmol) and [^{3}H]cAMP (41.7 Ci/mmol) were purchased from Amersham Searle, Arlington Heights, IL. [^{32}P]NAD (32 Ci/mmol) was purchased from New England Nuclear, Boston MA. cAMP, PEP, ATP, DA HCl, DTT, ADP ribose, thymidine, and Tris buffer were obtained from Sigma Chemical Co., St. Louis, MO. GTP, GppNHp, and pyruvate kinase were from Boehringer-Mannheim, Indianapolis, IN. Cholera toxin and forskolin were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Forskolin was dissolved in ethanol and diluted into buffer. Isonicotinic acid hydrazide was purchased from Aldrich Chemical Co., Milwaukee, WI. Proteins used as molecular weight standards were β-glactosidase (M_r 116,000), phosphorylase b (M_r 97,400), carbonic anhydrase (M_r 31,000), ovalbumin (M_r 45,000), and bovine serum albumin (M_r 66,000), and were purchased from Sigma Chemical Co.

Preparation of bovine striatal homogenates and particulate fractions. Bovine striatum was dissected at a local slaughterhouse and immediately placed in liquid N_2. Tissue was stored at −70°. Tissue was thawed and homogenized in 2 volumes of 0.1 M Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$, 75 mM sucrose, 1 mM DTT, 1 μg/ml of lima bean trypsin inhibitor, 1 μg/ml of soybean trypsin inhibitor, and 3 mM benzamidine (tissue buffer). Particulate fractions were prepared by homogenizing striatal tissue in 9 volumes of tissue buffer and centrifuging at 30,000 × g for 15 min. The pellet was washed twice and resuspended in tissue buffer to a concentration of 35 mg/ml.

Cholera toxin intoxication and adenylate cyclase assay. The cholera toxin was stored as a 1 mg/ml stock and was heated for 2 min at 37° before use to ensure its solubility. The cholera toxin was activated for 10 min at 37° in the presence of 2 mM DTT and 0.1% sodium dodecyl sulfate. The intoxication assay is a modification of that of Gill and Woolkalis (25). The homogenate or particulate preparation was incubated at a concentration of 3–5 mg/ml with 1 mM GTP, 10 mM PEP, 39 μg/ml of pyruvate kinase, 1 mM ATP, and 20 mM isonicotinic acid hydrazide, an inhibitor of NAD glycohydrolase (25), and cholera toxin at various concentrations, usually 80 μg/ml for 5 min at 30°. Two volumes of NAD were then added to give a final concentration of 500 μM. The incubation was continued for 20 min at 30°, which was the time required for maximum activation of the fractions by cholera toxin. The incubation was stopped by the addition of 30 volumes of tissue buffer and the samples were centrifuged at 20,000 × g for 15 min. The pellet was washed twice in a buffer containing 80 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 1.2 mM EGTA and resuspended in this buffer to a final concentration of 5 mg/ml. No activation by cholera toxin occurred when 1 mM GppNHp was substituted for GTP. The basal adenylate cyclase activity remained constant during preincubation with vehicle for at least 60 min. Activity measured after pretreatment with cholera toxin was maximal at 20 min of preincubation and declined by only 20% when preincubated 60 min.

Adenylate cyclase activity of the washed particulate fractions was determined in an assay (200-μl volume) containing 80 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 2 mM cAMP, 4 mM PEP, 20 μg of pyruvate kinase, 0.12 mM isobutylmethylxanthine, 50–100 μg of particulate membrane protein, 0.15 mM EGTA, 1 mM [^{32}P]ATP (1 μCi/assay), with or without various effectors such as GTP, Ca^{2+}, and CaM. Assays were incubated for 5 min at 37° and the reaction was stopped by heating for 1 min at 95°. A solution containing 20 mM ATP and 0.7 mM [^{3}H]cAMP (200-μl volume) was then added to the tubes. The particulate material was centrifuged, and the ^{32}P-labeled cAMP in the supernatant fraction was determined by the method of Krishna *et al.* (26). Recovery of cAMP was measured using the [^{3}H]cAMP and was usually 80–90%. Assays measuring stimulation by DA contained 1 μM GTP. DA-stimulated adenylate cyclase activity is defined as the pmol of cAMP/min/mg of protein produced above that of 1 μM GTP. Free or effective concentrations of Ca^{2+} were calculated using a dissociation constant for Ca-EGTA of 4.08×10^{-8} M according to the method of Nanninga and Kempen (27). A cytosolic protein factor that enhances cholera toxin activation was prepared from rat liver as described by Enomoto and Asakawa (23) and concentrated to 55 mg/ml. Protein was determined by the method of Lowry *et al.* (28). Kinetic constants were determined by the method of Wilkinson (29).

ADP-ribosylation of bovine striatal homogenates and particulate fraction. ADP-ribosylation of bovine striatal homogenates or particulate fractions was conducted under the identical conditions described above for cholera toxin except that the intoxication assay contained 20 μM [^{32}P]NAD as opposed to 500 μM unlabeled NAD. Some of the assays contained 10 mM ADP-ribose and 10 mM thymidine. The reaction was stopped for dilution as described above and centrifuged at 20,000 × g for 15 min. The pellets were resuspended in tissue buffer and 1% sodium dodecyl sulfate to a concentration of 2 mg/ml and subjected to slab gel electrophoresis using 10% polyacrylamide as described by Laemmli (30). After electrophoresis, the gels were dried and autoradiographed using 8 × 10 XAR2 X-OMAT ^{3}H-sensitive film for 24 hr. The apparent molecular weight of the labeled bands was determined by comparison with protein standards of known molecular weight.

Preparation of CaM. CaM was purified from bovine testes by the method of Dedman *et al.* (31) and demonstrated a single band on disc gel electrophoresis containing 10% polyacrylamide. CaM was prepared in the presence of mM concentrations of EGTA, dialyzed against 0.05 M $(NH_4)HCO_3$, and lyophilized. The CaM was redissolved in 10 mM Tris-maleate buffer, pH 7.5. The protein concentration was determined by ultraviolet absorption (31) and the method of Lowry *et al.* (28). The M_r determined by slab gel electrophoresis using standards of known molecular weight was 16,700. This mass was used to calculate the concentrations of CaM reported in this study.

Results

Activation of adenylate cyclase by CaM after pretreatment of bovine striatal homogenate fractions with cholera toxin. Pretreatment of bovine striatal homogenate fractions with cholera toxin increased the activation of adenylate cyclase by Ca^{2+} and CaM. As shown in Fig. 1, the activation of adenylate cyclase by CaM was increased at least 2-fold in membranes that had been pretreated with cholera toxin as compared to vehicle controls. The CaM-stimulated adenylate cyclase activity is illustrated in the *inset* of Fig. 1. CaM-stimulated adenylate cyclase activity in both cholera toxin-treated and -nontreated membranes is consistent with one kinetically activated species. The apparent V_{max} was increased

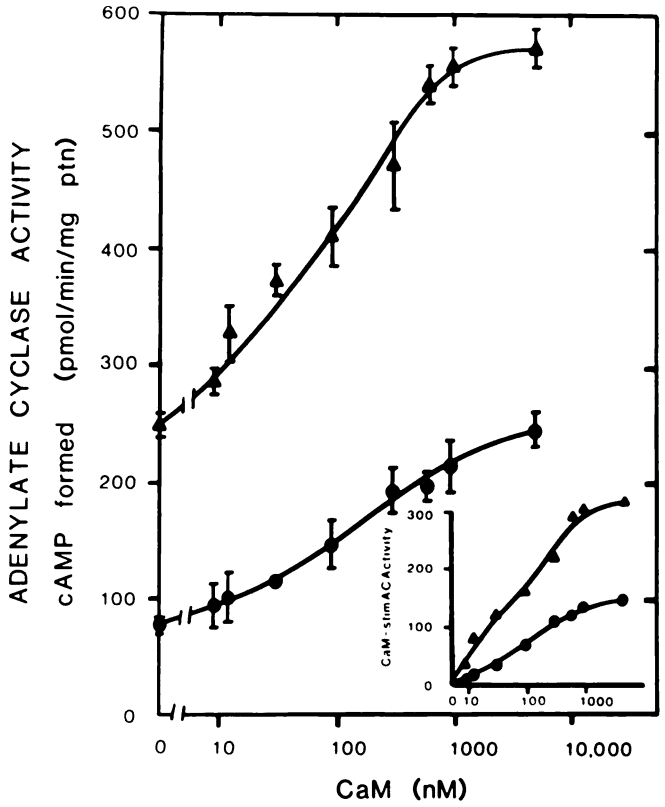

Fig. 1. Effect of cholera toxin on activation of adenylate cyclase by Ca^{2+} and CaM in bovine striatal membranes. The concentration-dependent activation of adenylate cyclase by CaM was measured in washed membranes prepared from bovine striatal homogenates that had been incubated in the absence (●) and presence (▲) of 80 μg/ml of cholera toxin as described in Experimental Procedures. All assays contained 125 μM $CaCl_2$ (0.12 μM free Ca^{2+}). $CaCl_2$ did not affect adenylate cyclase activity in the absence of CaM. The results are the average of three experiments done in duplicate ± the standard error. *Inset:* Stimulation of adenylate cyclase activity above basal levels by Ca^{2+} and CaM calculated from the data presented in Fig. 1. The *ordinate* is given in pmol/min/mg of protein and the abcissa is given in nM.

from 142 ± 18 to 309 ± 11 pmol/min/mg of protein ($p \leq 0.005$, $N = 3$) in vehicle- and cholera toxin-treated fractions, respectively. The apparent affinity for CaM was only slightly affected by cholera toxin pretreatment, being 87 ± 14 nM and 62 ± 7 nM after pretreatment in the absence and presence of cholera toxin, respectively ($p \leq 0.02$ in paired t analysis).

Effect of cholera toxin on activation of adenylate cyclase by DA, forskolin, and Mn^{2+}. We next determined the effect of cholera toxin pretreatment on activation of adenylate cyclase by the neurotransmitter, DA, forskolin, and Mn^{2+}. Since GTP is required for activation of DA, DA-stimulated adenylate cyclase activity is considered to be the pmol of cAMP formed/min/mg of protein above that of GTP itself. As shown in Table 1, the activation of adenylate cyclase by DA was completely inhibited after pretreatment with high concentrations of cholera toxin. ADP-ribosylation was most likely complete at the pool of N_s subunits accessible to the DA receptor. We also examined the effect of cholera toxin pretreatment on the activation of adenylate cyclase by 5 mM $MnCl_2$. The amount of cAMP produced in pmol/min/mg of protein by Mn^{2+} was slightly reduced after cholera toxin pretreatment.

Cholera toxin pretreatment of bovine striatal homogenates increased the subsequent activation of adenylate cyclase by

TABLE 1

Activation of bovine striatal adenylate cyclase by DA and Mn^{2+} after pretreatment with Cholera toxin

Addition to assay[a]	Cholera toxin (μg/ml)[b]			
	0	40	80	160
None	122	350	383	533
GTP	193	520	604	741
GTP + DA	282	570	645	740
Mn^{2+}	903		1071	

[a] Adenylate cyclase activity was determined in washed membrane fractions after cholera toxin intoxication as described in Experimental Procedures. Concentrations of reactants in the assay are: GTP, 1 μM; DA, 100 μM; and $MnCl_2$, 5 mM.

[b] Bovine striatal homogenate fractions were preincubated with various concentrations of cholera toxin as described in Experimental Procedures. The results are the average of two separate experiments performed in triplicate that did not vary by more than 5%.

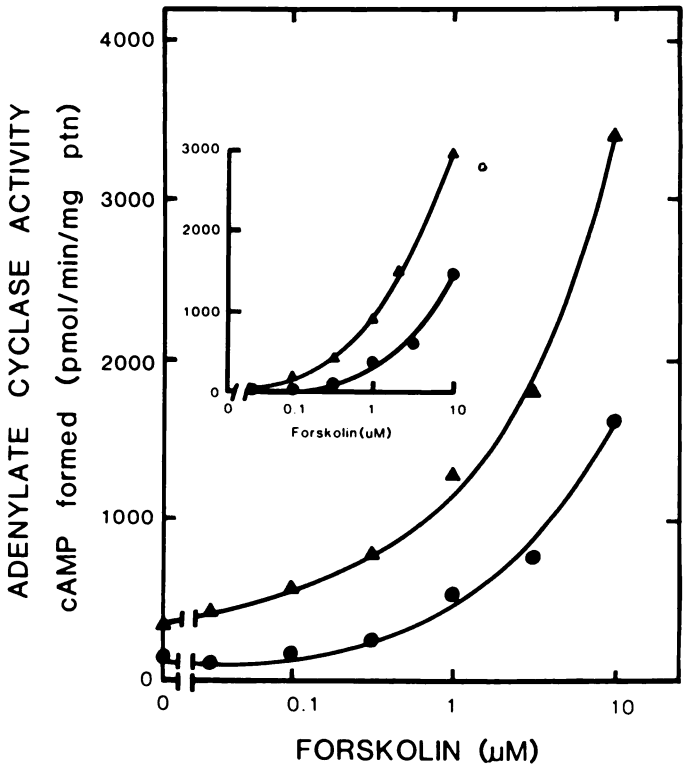

Fig. 2. Effect of cholera toxin on the activation of adenylate cyclase by forskolin in bovine striatal membranes. The concentration-dependent activation of adenylate cyclase by forskolin was measured in bovine striatal membranes prepared from homogenates that had been incubated in the absence (●) and presence (▲) of cholera toxin as described in Experimental Procedures. *Inset:* Stimulation of adenylate cyclase activity above basal levels by forskolin calculated from the data presented in Fig. 2. The *ordinate* is given in pmol/min/mg protein and the abcissa in μM.

forskolin, as shown in Fig. 2. The results clearly demonstrate that cholera toxin pretreatment increased activation by all concentrations of forskolin. We were unable to reach maximal activation by forskolin due to the limitations in solubility of the drug, so that we could not determine whether the apparent V_{max} or K_a for forskolin was affected by cholera toxin.

Concentration dependence of cholera toxin on activation of adenylate cyclase by CaM, GTP, and forskolin. The activation of adenylate cyclase by CaM, GTP, and forskolin after treatment of bovine striatal homogenate fractions with various doses of cholera toxin was examined. Examination of

Fig. 3 reveals that activation of adenylate cyclase by these ligands was differentially affected by cholera toxin. Basal adenylate cyclase activity increased with a steep rise to 40 μg/ml of cholera toxin followed by a linear increase in activity to at least 200 μg/ml. Activation of adenylate cyclase by low concentrations of Ca^{2+} did not significantly differ from basal activity. Activation by CaM, however, was increased after preincubation with varying amounts of cholera toxin to a maximum at 120 μg/ml of cholera toxin. The CaM-stimulated adenylate cyclase activity is shown in the *inset* to Fig. 3. In contrast, activation by GTP continued to be increased after pretreatment with up to 200 μg/ml of cholera toxin (Fig. 3 and *inset*). Activation of adenylate cyclase by forskolin was also linear to at least 200 μg/ml of cholera toxin.

Effect of cholera toxin pretreatment on the interaction of CaM and GTP in activation of bovine striatal adenylate cyclase. The effect of cholera toxin on the activation of adenylate cyclase by CaM and GTP was examined. In the presence of CaM, the apparent affinity for GTP in the activation of adenylate cyclase was decreased but the apparent V_{max} was unchanged. As shown in Fig. 4, A and B, activation of bovine striatal adenylate cyclase by GTP was maximal at 100 μM. When CaM was present, however, only 1 μM GTP was required to reach maximal activation. The apparent K_a for

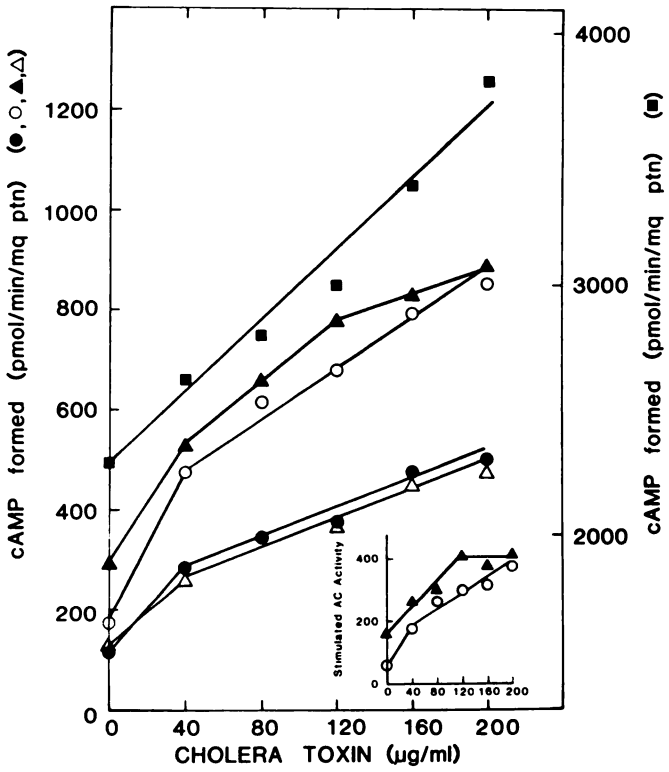

Fig. 3. Concentration dependence of cholera toxin on the activation of adenylate cyclase by GTP, CaM, and forskolin in bovine striatal membranes. Adenylate cyclase activation was measured in the presence of 125 μM $CaCl_2$ (△), 125 μM $CaCl_2$, and 0.9 μM CaM (▲), 1 μM GTP (○), and 1 μM forskolin (■) and in the absence of added ligands (●) in membranes that had been prepared from homogenates incubated in the absence or presence of various concentrations of cholera toxin. The results are the average of three experiments performed in duplicate that did not differ by more than 5%. *Inset:* GTP-stimulated (○) and CaM-stimulated (▲) adenylate cyclase activities in membranes pretreated with various concentrations of cholera toxin. Values are calculated from the data presented in Fig. 3.

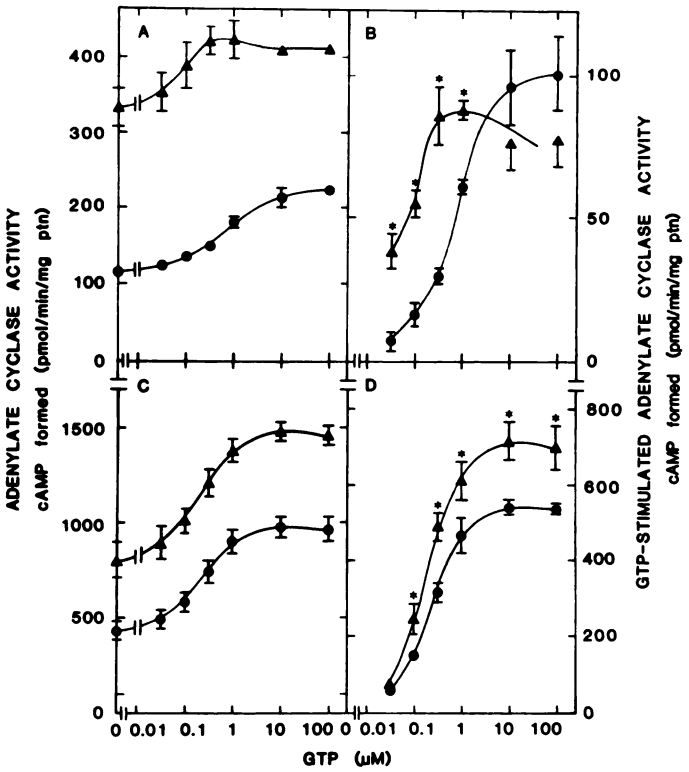

Fig. 4. Effect of CaM on the activation of adenylate cyclase by GTP in the absence (A and B) and presence (C and D) of cholera toxin. A. Concentration-dependent activation of adenylate cyclase by GTP in bovine striatal membranes in the absence (●) and presence (▲) of 125 μM $CaCl_2$ and 0.9 μM CaM. Membranes were prepared from homogenate fractions incubated in the absence of cholera toxin as described in Experimental Procedures. B. GTP-stimulated adenylate cyclase activity calculated from A as that amount of cAMP in pmol/min/mg of protein stimulated by GTP above that produced in the absence (●) and presence (▲) of $CaCl_2$ and CaM. C. Concentration-dependent activation of adenylate cyclase by GTP in bovine striatal membranes that had been preincubated in the presence of 80 μg/ml of cholera toxin in the absence (●) and presence (▲) of 125 μM $CaCl_2$ and 0.9 μM CaM. Membranes were prepared from homogenate fractions incubated in the absence or presence of cholera toxin as described in Experimental Procedures. D. GTP-stimulated adenylate cyclase activity calculated from C as that amount of cAMP in pmol/min/mg of protein stimulated by GTP above that produced in the absence (●) and presence (▲) of $CaCl_2$ and CaM. The results are the average of three separate experiments performed in duplicate ± the standard error.

GTP was decreased from 0.61 ± 0.1 μM to 0.05 ± 0.018 μM ($N = 3$, $p \leq 0.003$) when measured in the absence and presence, respectively, of 0.9 μM CaM. The apparent V_{max} for GTP was 101 ± 14 pmol/min/mg of protein when measured in the absence of CaM and 95 ± 9 pmol/min/mg of protein when determined in the presence of 0.9 μM CaM. In the presence of CaM, higher concentrations of GTP slightly inhibited adenylate cyclase activity.

Cholera toxin pretreatment also decreased the apparent K_a for GTP as well as increasing the maximal response to GTP. As shown in Fig. 4, C and D, cholera toxin increased the apparent V_{max} for GTP 5- to 6-fold from 101 ± 14 to 552 ± 22 pmol/min/mg of protein in the absence and presence of cholera toxin, respectively. The apparent K_a was decreased by cholera toxin pretreatment from 0.61 ± 0.1 μM to 0.24 ± 0.017 μM ($N = 3$, $p \leq 0.01$). In cholera toxin-pretreated membranes, however, CaM was not able to further increase the sensitivity of the enzyme for GTP. The apparent K_a for GTP determined in the

presence of 0.9 μM CaM was 0.20 ± 0.02 μM. On the contrary, after cholera toxin pretreatment, 0.9 μM CaM significantly increased the apparent V_{max} for GTP from 552 ± 22 to 721 ± 47 pmol/min/mg of protein ($N = 3$, $p \leq 0.01$).

Effect of Ca^{2+} on the activation of adenylate cyclase by cholera toxin. Since pretreatment with cholera toxin increased the response of adenylate cyclase to Ca^{2+} and CaM, we investigated whether Ca^{2+} and CaM had any effect on the ability of cholera toxin to activate adenylate cyclase. This effect was tested in homogenates by conducting the intoxication assay in the presence of 1 mM EGTA. We also examined the ability of cholera toxin to activate adenylate cyclase in particulate fractions that had been prepared in the presence and absence of 1.2 mM EGTA. Washing the particulate fraction with EGTA will remove substantial amounts of the endogenous Ca^{2+} and CaM (10). As shown in Table 2, the presence of 1 mM EGTA in bovine striatal homogenates had no effect on the ability of cholera toxin to activate the enzyme regardless of whether the activity was measured in the presence or absence of 10 μM GTP. Similarly, the adenylate cyclase activity in EGTA-washed particulate fractions was similar to control. The adenylate cyclase activity measured in the absence of cholera toxin was slightly higher in EGTA-washed membranes, but the activity in the presence of cholera toxin was the same as in membranes prepared without EGTA. In another experiment, readdition of 3 μg of CaM and 125 μM $CaCl_2$ to EGTA-washed membranes had no effect on cholera toxin activation (data not shown). Therefore, it appears that Ca^{2+} and CaM have no effect on the ability of cholera toxin to activate adenylate cyclase in bovine striatum.

ADP-ribosylation of homogenate fractions. In order to ensure that our results were due to ADP-ribosylation of N_s, we examined the ADP-ribosylation of proteins stimulated by cholera toxin in bovine striatal homogenates and particulate fractions. The results shown in Fig. 5 demonstrate that the [^{32}P] ADP-ribose was predominantly incorporated into a band of M_r 45,000 which corresponds to the known molecular weight of $N_s\alpha$ (1). A second band of 49,000 Da was also ADP-ribosylated in the presence of cholera toxin. This was found in both homogenate and particulate fractions from bovine striatum. ADP-ribose and thymidine, which are often included in cholera toxin intoxication assays, slightly reduced the background on the gels but did not alter the adenylate cyclase activity. There was no ADP-ribosylation of proteins in the absence of cholera toxin. Cholera toxin itself was not ADP-ribosylated in the absence of membranes. These results support the conclusion that the changes in activation of adenylate cyclase by CaM, forskolin, and guanyl nucleotides were due to ADP-ribosylation of $N_s\alpha$ by cholera toxin.

TABLE 2

Effect of EGTA on the activation of bovine striatal adenylate cyclase by cholera toxin

Preincubation conditions[a]	Adenylate cyclase in the presence of:			
	No addition		10 μM GTP	
	−CT	+CT[b]	−CT	+CT[b]
	pmol/min/mg protein			
Homogenate	53	156	86	343
Homogenate + EGTA	63	154	89	359
Membranes	52	114	85	218
EGTA-washed	88	127	116	233

[a] Homogenate or 30,000 × *g* membrane fractions were prepared and incubated with cholera toxin or vehicle as described in Experimental Procedures. In one set of intoxication assays using homogenate fractions, 1 mM EGTA was present. EGTA-washed membranes were prepared by homogenizing striatum in tissue buffer, as described in Experimental Procedures, containing 1.2 mM EGTA. A 30,000 × *g* membrane fraction was prepared, washed twice, and resuspended in this buffer. This fraction was then used in the intoxication assay which was conducted as described in Experimental Procedures.

[b] Cholera toxin (CT) was present at a concentration of 80 μg/ml.

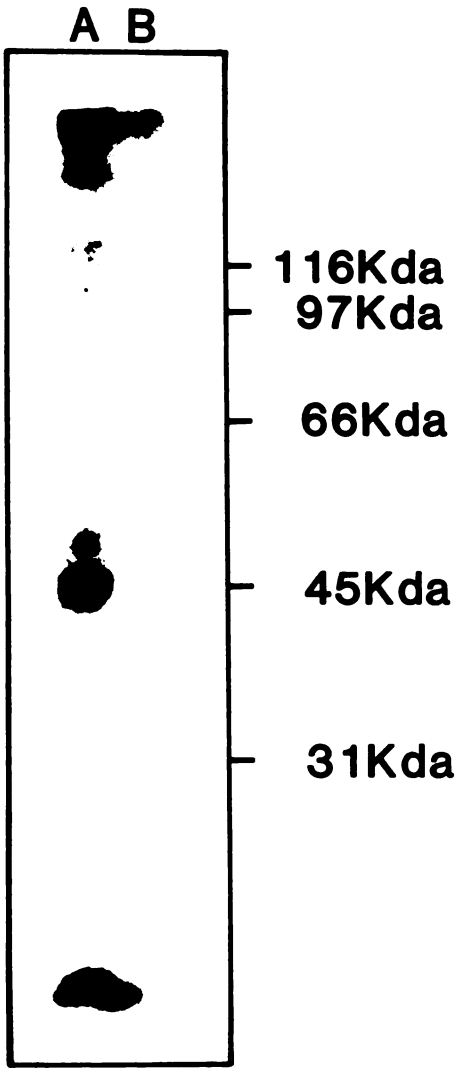

Fig. 5. Autoradiography of bovine striatal particulate proteins incubated in an ADP-ribosylation medium separated by SDS gel electrophoresis in 10% polyacrylamide gels. Membrane proteins were incubated in the presence (*lane A*) and absence (*lane B*) of cholera toxin and 20 μM [^{32}P] NAD as described in Experimental Procedures. Protein samples (100 μg) were subjected to electrophoresis in each lane as described in Experimental Procedures. *Numbers* are molecular weight × 10^{-3}. Markers were β-glactosidase (116,000), phosphorylase b(97,400), carbonic anhydrase (31,000), ovalbumin (45,000), and bovine serum albumin (66,000).

Discussion

Studies using partially purified catalytic subunit of adenylate cyclase (7) and CaM-Sepharose affinity chromatography (5) have demonstrated that CaM directly binds to the catalytic subunit and does not require GTP-binding proteins for stimulation of adenylate cyclase activity (9). Evidence from several laboratories, however, has shown that CaM can potentiate the activation of adenylate cyclase by guanyl nucleotides (4, 11, 13

14) and neurotransmitters (10–13). In this study we investigated further the interaction between CaM and guanyl nucleotides by examining the effect of persistent activation of N_s on the ability of CaM to activate adenylate cyclase activity.

Treatment of cells or tissue fragments with cholera toxin has been shown to persistently activate adenylate cyclase (17, 18). Cholera toxin ADP-ribosylates $N_s\alpha$ which inhibits several activities occurring at N_s: hormone-stimulated GTPase activity (19), guanyl nucleotide exchange (20), and the recombination of the β-subunit with the α-subunit of N_s (21). GTP-occupied $N_s\alpha$ then persistently activates adenylate cyclase activity at the catalytic subunit (1). In our study, pretreatment of bovine striatal homogenates with cholera toxin increased the stimulation of GTP 5-fold and increased the sensitivity to GTP at least 3-fold. The increased activation of adenylate cyclase by $N_s\alpha$ resulted in a potentiation of the activation of the enzyme by Ca^{2+} and CaM. If the two activities were totally independent, it might be expected that activation by CaM would be additive with that of cholera toxin. Instead, pretreatment of striatal homogenate fractions with cholera toxin increased the apparent V_{max} of CaM-stimulated adenylate cyclase activity. Our results with cholera toxin pretreatment are not unique to striatum; we found essentially the same increased activation by CaM when homogenates of bovine cerebral cortex were pretreated with cholera toxin. A similar result was reported by Moss and Vaughan (32) who found that activation of a partially purified adenylate cyclase preparation of adenylate cyclase from rat brain by Ca^{2+} and CaM was increased 3-fold.

The affinity for CaM was not altered to an appreciable extent by cholera toxin pretreatment. One explanation for this result is that cholera toxin pretreatment is increasing the availability of catalytic subunits for activation by CaM. It is also possible that cholera toxin-activated N_s could produce an increased activational state of existing CaM-sensitive catalytic subunits making them more responsive to activation by CaM. The studies of Malnoe and Cox (16) in rat cerebellum support the latter hypothesis. Their study suggested that CaM and N_s act at two allosterically linked regulatory domains at the catalytic subunit that can serve as a gain control for each other. In contrast, Smigel (33) noted that a purified catalytic subunit of adenylate cyclase prepared from bovine brain could not be activated by CaM after pretreatment with GTPγS-activated N_s. It is possible that another factor required for the interaction is missing in the purified state or that the detergent milieu has altered the nature of the interaction between the ligands from that of the membrane state.

The mechanism by which forskolin activates adenylate cyclase is not totally clear. Forskolin can interact directly with the catalytic subunit to activate adenylate cyclase (34, 35). Several studies have shown, however, that N_s has a role in the activation of adenylate cyclase by forskolin (35–37). We found that pretreatment of bovine striatal homogenates with cholera toxin potentiated the activation by forskolin. We were unable to attain maximal activation by forskolin due to solubility considerations; thus, we could not determine whether the apparent K_a or V_{max} would have been changed by cholera toxin pretreatment. Barovsky and Brooker (38) reported that forskolin potentiated the ability of cholera toxin to stimulate cyclic AMP accumulation in intact C6-2B cells and, similarly, that cholera toxin treatment augmented forskolin-stimulated cyclic AMP accumulation and shifted the forskolin concentration-response curve to the left. Our results demonstrate that the activation by both forskolin and CaM are modulated by the activity state of N_s. On the contrary, activation of adenylate cyclase by Mn^{2+} was not potentiated and was even slightly decreased after pretreatment with cholera toxin. Mn^{2+} also acts directly at the catalytic subunit and its activation is not affected by pretreatment with activated N_s (35).

The increase in activation of adenylate cyclase by CaM was maximal after pretreatment with 120 μg/ml of cholera toxin as opposed to the activation by GTP and forskolin. Activation by these ligands, as well as the basal adenylate cyclase activity, increased linearly to at least 200 μg/ml of cholera toxin. This suggests either that the CaM-sensitive component of adenylate cyclase has a greater affinity for activated $N_s\alpha$ or is present in a more limiting quantity than $N_s\alpha$ or the CaM-insensitive component. The stoichiometric amounts of CaM-sensitive and CaM-insensitive adenylate cyclase in brain membranes have not yet been determined. There was a marked increase in adenylate cyclase activity from 0 to 40 μg/ml of cholera toxin which then rose linearly to at least 200 μg/ml. It is possible that higher doses of cholera toxin could lead to some destabilization of the enzyme which could then occur at a constant rate with increasing activation, resulting in linearity. Nakaya *et al.* (22) found that activation by cholera toxin enhanced the intrinsic lability of the adenylate cyclase. We found, however, that adenylate cyclase measured in the presence or absence of cholera toxin was stable over the duration of the intoxication assay and decreased less than 20% in the presence of cholera toxin when the intoxication assay proceeded longer than 20 min.

CaM potentiated the activation by GTP in bovine striatum by decreasing the apparent K_a for GTP as much as 10-fold without increasing the apparent V_{max}. In rat striatum the effect of CaM in potentiating GTP activation was only apparent in the presence of DA (11). Since cholera toxin can also decrease the apparent activation constant for GTP, it was of interest to determine whether the action of CaM and cholera toxin were additive in this respect. We found that after pretreatment with cholera toxin, CaM could no longer decrease the apparent K_a for GTP in activating adenylate cyclase. CaM inhibited a low K_m GTPase activity in rat striatum (11), but it is uncertain whether inhibition of the N_s-associated GTPase activity is the primary mechanism of adenylate cyclase activation by cholera toxin (21). These results suggest that CaM and cholera toxin may have a common mechanism in causing a potentiation of GTP-stimulated activity. CaM could potentiate the activation by GTP by stabilizing the interaction between N_s and the catalytic subunit. Since this sort of stabilization may be similarly effected by cholera toxin, this action of CaM could then be negated. In contrast to the results in vehicle controls, CaM elicited a 30% increase in maximal activation by GTP after cholera toxin treatment. It is possible that CaM would increase the maximal stimulation by GTP in the absence of cholera toxin, but this action is obscured by the inhibition occurring at high concentrations of GTP. N_i can inhibit CaM-stimulated adenylate cyclase activity (15, 16). In preliminary experiments, however, we have found that treatment of bovine striatal membranes with pertussis toxin blocks the slight decrease in activity by 10 and 100 μM GTP in the presence of CaM, but CaM cannot increase the maximal stimulation by GTP.

Although cholera toxin pretreatment could increase the ac-

tivation by Ca^{2+} and CaM, we could find no evidence that Ca^{2+} and CaM could alter the ability of cholera toxin to activate adenylate cyclase. Neither addition of EGTA to striatal homogenates nor washing membranes with EGTA affected the -fold stimulation of activity of cholera toxin whether adenylate cyclase was measured in the presence or absence of GTP. Brostrom *et al.* (39) showed that Ca^{2+} enhanced the rate of accumulation of cyclic AMP in response to cholera toxin in C6 glioma cells. Once maximal cell cycle cyclic AMP accumulation was achieved, however, Ca^{2+} had no effect. It is possible that, at very short times of intoxication with cholera toxin, we could have detected an effect of Ca^{2+}, but it is more likely that in this system Ca^{2+} does not affect activation by cholera toxin. Moss and Vaughan (32) reported that CaM was required for activation by cholera toxin in rat brain, but this was in a partially purified preparation of adenylate cyclase.

In conclusion, we have demonstrated that pretreatment of bovine striatal homogenates with cholera toxin increases the activation of adenylate cyclase by CaM and forskolin as well as GTP. In this system, cholera toxin predominantly ADP-ribosylated a protein that corresponds in molecular size to the α-subunit of N_s. The ability of CaM to decrease the apparent K_a for GTP was blocked by pretreatment with cholera toxin, suggesting at least one shared mechanism of action between these agents in activation of adenylate cyclase. Although cholera toxin increased the activation of adenylate cyclase by Ca^{2+} and CaM, these agents had no effect on the ability of cholera toxin to activate adenylate cyclase. This study further demonstrates a potentiative interaction between CaM and N_s and suggests that the activity state of N_s can modulate the activation of adenylate cyclase by CaM.

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