

GTP is not required for calmodulin stimulation of bovine brain adenylate cyclase

(calcium/guanyl nucleotides)

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ABSTRACT The importance of guanyl nucleotides for calmodulin stimulation of bovine cerebral cortex adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] was examined by using a partially purified calmodulin-sensitive adenylate cyclase that was resolved from calmodulin-insensitive forms of the enzyme. By using 5'-adenylyl imidodiphosphate as a substrate, in the absence of an ATP-regenerating system, it was determined that GTP is not required for calmodulin stimulation of the enzyme. Maximal activation by 5'-guanylyl imidodiphosphate (p[NH]ppG) was 5.3-fold, whereas the combination of p[NH]ppG and calmodulin stimulated the enzyme 27-fold. Although GDP inhibited p[NH]ppG stimulation of the calmodulin-sensitive adenylate cyclase, it did not affect calmodulin stimulation. In addition, calmodulin did not alter the kinetics for activation of the enzyme by p[NH]ppG. It is concluded that GTP is not required for calmodulin stimulation of brain adenylate cyclase and that calmodulin regulation of this enzyme is probably not due to effects of calmodulin on the affinity of the guanyl nucleotide regulatory complex for guanyl nucleotides.

In 1975 it was discovered that Ca^{2+} stimulation of brain adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] is mediated by the regulatory protein calmodulin (CaM) (1, 2). CaM forms a complex with brain adenylate cyclase, and the affinity of CaM for the enzyme is enhanced in the presence of Ca^{2+} (1-3). The mechanism for CaM regulation of brain adenylate cyclase has not been established. A CaM-insensitive form of bovine cerebral cortex adenylate cyclase has been resolved from the CaM-sensitive enzyme by using CaM-Sepharose (3). Sensitivity to CaM was restored to the former by incubation of this preparation with detergent-solubilized protein from bovine cerebral cortex, and reconstitution of CaM sensitivity required the presence of 5'-guanylyl imidodiphosphate (p[NH]ppG) (4). On the basis of these data, it was proposed that the guanyl nucleotide regulatory complex (G/F) may be required for CaM stimulation of this enzyme.

If indeed G/F is required for CaM stimulation of adenylate cyclase, then it can be hypothesized that Ca^{2+} regulation of adenylate cyclase may be similar to hormone regulation. In other words, the $\text{CaM}\cdot\text{Ca}_4^{2+}$ complex may affect the affinity of G/F for GDP, GTP, or both by analogy with the original Cassel and Selinger model for hormone stimulation (5). Because of these considerations, we have examined the influence of GDP and GTP on CaM stimulation of a partially purified adenylate cyclase from bovine brain. The data reported in this study indicate that GTP is not required for CaM stimulation and therefore it seems unlikely that CaM stimulation of brain adenylate cyclase involves energy coupling between binding of CaM and guanyl nucleotides to the enzyme system.

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MATERIALS AND METHODS

Materials. DEAE-Sepharcel and cyanogen bromide-activated Sepharose 4B were purchased from Pharmacia. Bio-Gel P-4 was obtained from Bio-Rad. p[NH]ppG, ATP, and cAMP were purchased from Sigma. [α - ^{32}P]ATP and α - ^{32}P -labeled 5'-adenylyl imidodiphosphate (p[NH]ppA) were obtained from New England Nuclear and International Chemical and Nuclear, respectively. All other reagents were of the best available grades from commercial sources.

Adenylate Cyclase Assay. Adenylate cyclase was assayed by the general method of Salomon *et al.* (6), using either [α - ^{32}P]ATP or [α - ^{32}P]p[NH]ppA as a substrate and [^3H]cAMP to monitor product recovery. Assay mixtures contained in a 250- μl final volume: 20 mM Tris-HCl at pH 7.5, 1 mM [α - ^{32}P]ATP or 1 mM [α - ^{32}P]p[NH]ppA (20 cpm/pmol), 2 mM cAMP, 5 mM MgCl_2 , 1 mM CaCl_2 , 1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM theophylline, 0.1% bovine serum albumin, and 10-20 μg of enzyme preparation. No ATP-regenerating system was used because ATPase activity was determined to be low and because commercial preparations of creatine kinase used in the regenerating system have been found to be contaminated by calmodulin. This contamination raises the basal enzyme activity levels, lowering apparent calmodulin stimulation. All results are reported as the mean of triplicate assays with standard error of less than 8%. Proteins were determined by the method of Peterson (7).

Preparation of CaM. CaM was prepared by a modification of the procedure of Dedman *et al.* (8) as previously reported (9). CaM-Sepharose was prepared from purified CaM and cyanogen bromide-activated Sepharose 4B by following the procedure of Westcott *et al.* (3).

Preparation of CaM-Sensitive Adenylate Cyclase. Adenylate cyclase was partially purified by using a published procedure (10). Briefly, membranes prepared from bovine cerebral cortex were solubilized in 20 mM Tris-HCl pH 7.4/1 mM MgCl_2 /1 mM EDTA/250 mM sucrose/1% Lubrol PX, at a detergent-to-protein ratio of 2.5:1 (wt/wt). The detergent/membrane mixture was stirred for 90 min at 4°C and centrifuged for 2 hr at 10,000 $\times g$. The supernatant enzyme solution was decanted and the pellet was discarded. DEAE-Sepharcel was equilibrated in 50 mM Tris-HCl, pH 7.4/250 mM sucrose/5 mM MgCl_2 /1 mM EDTA/0.1% Lubrol PX/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride (buffer A). Approximately 5 liters of detergent extract was incubated with 2 liters of DEAE-Sepharcel and stirred at 4°C for 90 min. The gel was washed on a sintered glass funnel with 4 liters of buffer A containing 50 mM KCl and poured into a 9 \times 30 cm column, and the column was eluted with a linear 150-600 mM KCl gradient in buffer A.

Abbreviations: CaM, calmodulin; p[NH]ppG, 5'-guanylyl imidodiphosphate; p[NH]ppA, 5'-adenylyl imidodiphosphate; G/F, the guanyl nucleotide regulatory complex of adenylate cyclase.

Adenylate cyclase was pooled on the basis of enzyme activity, avoiding fractions containing more than 250 mM KCl to ensure separation of the enzyme from endogenous CaM. The pooled enzyme solution was desalted on a Bio-Gel P-4 column into buffer containing 50 mM Tris·HCl, 250 mM sucrose, 5 mM MgCl₂, 1 mM EDTA, 1.1 mM CaCl₂, 0.1% Lubrol PX, and 1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride (buffer B). The desalted solution was applied to a CaM-Sepharose column equilibrated in buffer B, and the column was washed with 4 column volumes of buffer B and eluted with buffer A. Adenylate cyclase flowing through the column without binding in the presence of Ca²⁺ had no sensitivity to calmodulin. Adenylate cyclase specifically eluted from the CaM-Sepharose column with EDTA was stimulated up to 20-fold by CaM and is referred to as CaM-sensitive adenylate cyclase. This enzyme preparation was purified over 85-fold and was used in all the experiments reported. The specific activities of these preparations varied between 10 and 20 nmol of cAMP formed per mg of protein per min in the presence of 2.5 μM CaM.

RESULTS

Effect of GTP on CaM Stimulation. In order to determine whether or not GTP is required for CaM stimulation, it was necessary to eliminate contaminating GTP present in commercial ATP preparations (11) and to prevent the formation of GTP by phosphorylation of endogenous GDP (12). The use of GTP-free p[NH]ppA, which is a poor phosphate donor, minimizes GTP contamination in adenylate cyclase assays (13). In addition, the assays were carried out in the absence of an ATP-regenerating system in order to ensure no formation of GTP by phosphorylation of endogenous GDP. Assays of adenylate cyclase activity carried out in the presence and absence of an ATP-regenerating system gave identical activities. This system was earlier used by Rodbell *et al.* (14) to demonstrate that guanyl nucleotides are required for hormone stimulation of adenylate cyclase.

The specific activity of CaM-sensitive adenylate cyclase was significantly lower when p[NH]ppA was used as a substrate compared to ATP. With p[NH]ppA and ATP as substrates the specific activities were 1 and 10 nmol cAMP/mg·min, respectively. In the absence of added GTP, 2.5 μM CaM stimulated the enzyme approximately 10-fold (Fig. 1). GTP concentrations as high as 100 μM had no effect on CaM stimulation. Higher concentrations of GTP inhibited both basal and CaM-stimulated activities to the same extent.

Effect of CaM on the Activity of p[NH]ppG-Activated Adenylate Cyclase. The CaM-sensitive enzyme was stimulated by p[NH]ppG when either Mg²⁺ or Mn²⁺ was present as the divalent cation (Table 1). Maximal stimulation was obtained with 4 hr of treatment using 100 μM p[NH]ppG at 22°C. Longer periods of incubation or increases in p[NH]ppG concentration

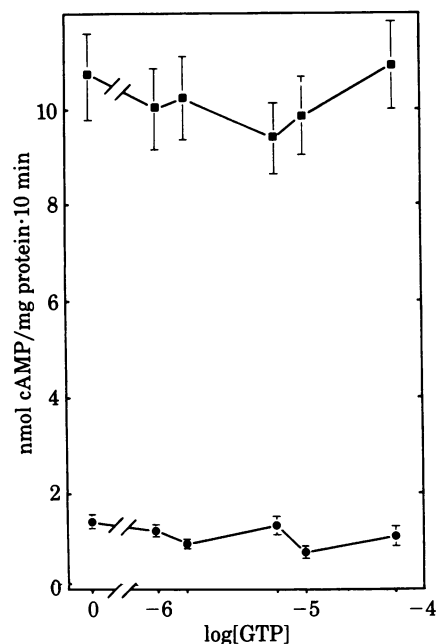


FIG. 1. Response of the CaM-sensitive adenylate cyclase to GTP. CaM-sensitive adenylate cyclase activity was assayed as described in the text, using p[NH]ppA as a substrate in the presence (■) or absence (●) of 2.5 μM CaM at the indicated GTP concentrations. Error bars indicate SEM.

did not cause any further activation of adenylate cyclase activity. p[NH]ppG stimulated adenylate cyclase activity 5.3-fold and 2.2-fold in the presence of Mg²⁺ and Mn²⁺, respectively. Under these conditions, in which it was reasonable to assume that the guanyl nucleotide binding sites were saturated, CaM caused further stimulation of activity. For example, addition of CaM increased the activity of the p[NH]ppG-activated enzyme from 17.9 to 90.8 nmol cAMP/mg·10 min when Mg²⁺ was present. In the presence of Mn²⁺, the effect of CaM was less; however, CaM still stimulated the enzyme maximally activated by p[NH]ppG. Although it is possible that CaM may have caused an increase in the number of p[NH]ppG binding sites, it seems more likely in the light of other data presented in this manuscript that CaM stimulation is independent of guanyl nucleotide binding.

Effect of CaM on the Kinetics for p[NH]ppG Activation of Adenylate Cyclase Activity. The addition of hormones to hormonally sensitive adenylate cyclase has been shown to accelerate the kinetics for activation of the enzyme by p[NH]ppG (15, 16). In addition, guanyl nucleotides are known to affect the affinity of hormone receptors for hormones. These general observations strongly suggest energy coupling for the binding of

Table 1. p[NH]ppG and CaM stimulation of adenylate cyclase activity

Addition	Mg ²⁺		Mn ²⁺	
	Enzyme activity, nmol cAMP/mg·10 min	Fold stimulation over basal	Enzyme activity, nmol cAMP/mg·10 min	Fold stimulation over basal
None	3.4	1	30.7	1
p[NH]ppG	17.9	5.3	66.6	2.2
p[NH]ppG + CaM	90.8	27.0	170.0	5.6

Adenylate cyclase was assayed as described in *Materials and Methods* except that where indicated 10 mM MnCl₂ was substituted for MgCl₂. Adenylate cyclase was activated with p[NH]ppG by incubating the enzyme in buffer A with 100 μM p[NH]ppG at 22°C for 4 hr. When present, CaM was at 2.5 μM. CaM by itself stimulated the enzyme approximately 10-fold.

hormones and guanyl nucleotides to adenylate cyclase systems. If CaM stimulates adenylate cyclase by a mechanism analogous to that of hormones, then one might expect that CaM would affect the rate of p[NH]ppG stimulation. At 30°C the half-life for activation of the enzyme by 100 μ M p[NH]ppG was approximately 20 min. Fig. 2 illustrates that CaM did not affect the rate of activation by p[NH]ppG. Data is presented giving net accumulation of cAMP as a function of time (Fig. 2A) and the specific activity of the enzyme at various times after p[NH]ppG treatment (Fig. 2B). Although CaM clearly stimulates the p[NH]ppG-treated enzyme, the first-order rate constants for the kinetics of p[NH]ppG activation are identical in the presence and absence of CaM.

Effects of GDP on p[NH]ppG and CaM Stimulation. GDP has been shown to block the activation of brain adenylate cyclase by GTP and p[NH]ppG (17). Presumably, GDP competes

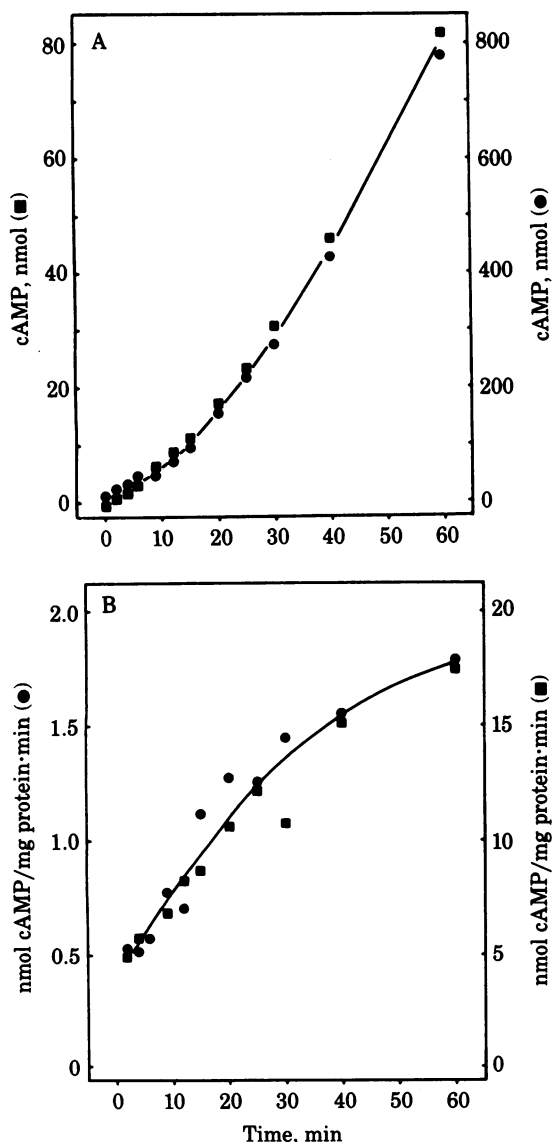


FIG. 2. Effect of CaM on the kinetics for p[NH]ppG stimulation of adenylate cyclase. The accumulation of cAMP in the presence (■) or absence (●) of 2.5 mM CaM (A) and the specific activity of adenylate cyclase at each time point (B) are reported. In all cases, p[NH]ppG was present at 100 μ M. Adenylate cyclase activity was monitored as described in *Materials and Methods* except that the volume of assays was increased to allow withdrawal of triplicate 250- μ l aliquots at the times indicated. In addition, the cAMP in the assay was increased to 4 mM. p[NH]ppG activation was carried out at 30°C.

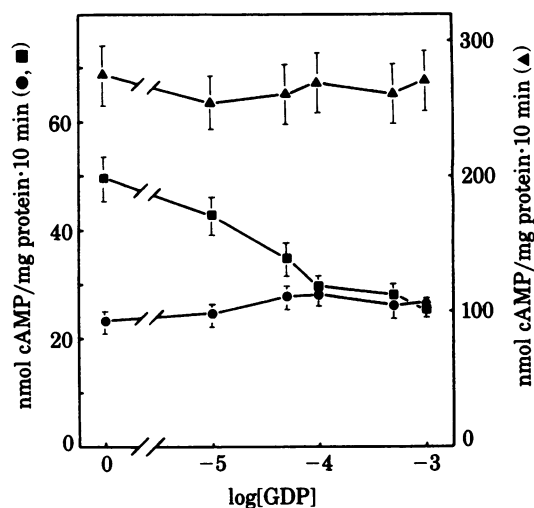


FIG. 3. Effects of GDP on CaM and p[NH]ppG stimulation of adenylate cyclase. Adenylate cyclase was assayed in the presence of 2.5 μ M CaM (\blacktriangle), 100 μ M p[NH]ppG (\blacksquare), or no additions (\bullet) and the indicated concentrations of GDP as described in *Materials and Methods* except that the assay mixtures were incubated at 30°C for 30 min to allow p[NH]ppG activation to occur.

with p[NH]ppG for binding to the guanyl nucleotide binding site and GDP does not activate the enzyme. If CaM·Ca₄²⁺ affects the binding of guanyl nucleotide to G/F by a mechanism analogous to hormone stimulation, then one might expect GDP to inhibit CaM stimulation. The addition of GDP at 100 μ M completely inhibited p[NH]ppG stimulation of the CaM-sensitive adenylate cyclase (Fig. 3). In contrast, CaM stimulation was unaffected by concentrations of GDP as high as 1 mM.

DISCUSSION

Several prior observations have indirectly implicated G/F as playing a role for CaM stimulation of brain adenylate cyclase. The partially purified CaM-sensitive enzyme is sensitive to p[NH]ppG, NaF, and cholera toxin. We have reported that CaM sensitivity can be restored to a CaM-insensitive enzyme by using a reconstitution procedure that also restored p[NH]ppG and NaF sensitivity (4). More recently, we have discovered by using ¹²⁵I-labeled azido-CaM that CaM forms a Ca²⁺-dependent complex with purified G/F (data not shown). The purpose of this study was to determine whether or not CaM stimulation of brain adenylate cyclase requires GTP.

Taken collectively, the evidence reported in this manuscript suggests that GTP is not required for CaM stimulation of brain adenylate cyclase. Using an assay system that minimizes any contribution from contaminating GTP, we found significant CaM stimulation even in the absence of added GTP. The enzyme maximally activated by p[NH]ppG was stimulated 5-fold by CaM. CaM had no effect on the kinetics for p[NH]ppG activation, and GDP inhibited p[NH]ppG stimulation but not CaM stimulation. We conclude that the mechanism for CaM stimulation may be distinct from that underlying hormone stimulation of adenylate cyclase.

If G/F or one of the G/F polypeptides is required for CaM stimulation, it appears unlikely that CaM functions by affecting the affinity of G/F for guanyl nucleotides. Salter *et al.* (18) have recently reported that a brain adenylate cyclase preparation lacking p[NH]ppG sensitivity was activated by CaM. These investigators concluded that CaM activates the catalytic subunit directly but does not seem to affect the function of the G/F complex. However, Salter *et al.* acknowledged that their prep-

aration may have contained low amounts of G/F not detectable by p[NH]ppG sensitivity. The studies by Toscano *et al.* (4) and by Salter *et al.* (18) are both compromised by the uncertainties associated with reconstitution studies using heterogenous undefined preparations. The fact that CaM stimulates adenylate cyclase from only a limited number of tissues (1, 19, 20) suggests that these enzymes are distinct from adenylate cyclases that are not regulated by CaM. By analogy with other CaM-regulated enzymes, it seems likely that direct interactions between CaM and the adenylate cyclase catalytic subunit contribute, at least in part, to CaM stimulation of the enzyme. Interactions between CaM and G/F cannot be solely responsible for CaM stimulation of brain adenylate cyclase because a variety of adenylate cyclases from other tissues contain functional G/F complexes but are not stimulated by CaM. In addition, *Bordetella pertussis* adenylate cyclase is activated by CaM and this enzyme is insensitive to guanyl nucleotides and does not contain G/F (21). Although the functional significance of CaM-G/F interactions is still open to question and requires further investigation, the data reported in this study clearly indicate that GTP is not required for CaM stimulation of the partially purified enzyme.

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