Evidence for a dissociable protein subunit required for calmodulin stimulation of brain adenylate cyclase

(calcium/guanyl nucleotides/sodium fluoride/reconstitution)

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ABSTRACT   An adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] preparation that is not stimulated by NaF, 5'-guanylyl imidodiphosphate, or $Ca^{2+} \cdot$calmodulin has been isolated from bovine cerebral cortex by Affi-Gel Blue chromatography and calmodulin-Sepharose chromatography. Sensitivity to these effectors was restored by incubation of the adenylate cyclase preparation with detergent-solubilized protein from bovine cerebral cortex. Reconstitution of $Ca^{2+} \cdot$calmodulin activation required the presence of 5'-guanylyl imidodiphosphate. The factor required for restoration of $Ca^{2+} \cdot$calmodulin stimulation was sensitive to heat, trypsin digestion, and N-ethylmaleimide. These observations suggest that this adenylate cyclase activity requires the presence of one or more guanyl nucleotide binding subunits for calmodulin sensitivity.

Calcium stimulation of brain adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] is mediated by calmodulin (CaM) (1–5) and there is considerable evidence that this regulatory protein forms a complex with adenylate cyclase in the presence of Ca^{2+} (1, 2, 6). A partially purified adenylate cyclase from bovine cerebral cortex has been resolved into two fractions based on their affinity for CaM-Sepharose in the presence of Ca^{2+} (6). One form was adsorbed to the affinity resin in the presence of Ca^{2+} and was stimulated 4-fold by CaM and Ca^{2+}. The other fraction, designated fraction I, exhibited no affinity for CaM-Sepharose and was insensitive to $Ca^{2+} \cdot$CaM stimulation. These observations supported the proposal of Brostrom et al. (5) that cerebral cortex may contain a mixture of CaM-dependent and CaM-independent adenylate cyclase activities. However, preliminary studies in this laboratory showed that fraction I was also insensitive to 5'-guanylyl imidodiphosphate (p[NH]ppG) and NaF; therefore, it was also possible that the CaM-insensitive form was generated by prior purification steps that removed one or more subunits required for CaM sensitivity. There is evidence from several laboratories that the adenylate cyclase system in eukaryotic cells is comprised of several regulatory subunits and a catalytic subunit (7–11).

In the present study, $Ca^{2+} \cdot$CaM sensitivity of fraction I was restored by reconstitution with a detergent-solubilized membrane preparation. These data suggest that one or more guanyl nucleotide binding subunits are required for CaM stimulation of adenylate cyclase.

MATERIALS AND METHODS

Materials. Radioisotopes were purchased from New England Nuclear; p[NH]ppG was obtained from PL Laboratories. Trypsin, soybean trypsin inhibitor, ATP, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), N-ethylmaleimide, and Lubrol PX were obtained from Sigma.

All other reagents were of the best available grade from commercial sources.

Solubilization of Brain Membranes. Fresh bovine cerebral cortex was suspended in 2 vol of homogenization buffer (20 mM glycylglycine, pH 7.2/1 mM $MgCl_2$/1 mM EDTA/0.25 M sucrose/3 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride). The cortex was disrupted in a blender and the homogenate was centrifuged 4000 × g for 30 min at 4°C. The supernatant was discarded and the pellet was suspended in 2 vol of homogenization buffer, Dounce homogenized, and passed through four layers of cheesecloth. The centrifugation step was repeated twice; the final pellet was suspended in a minimal amount of homogenization buffer and frozen in aliquots at −60°C. Membrane preparations were solubilized with 20 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$/1 mM EDTA/0.25 M sucrose/1% Lubrol PX at a detergent-to-protein ratio of 2.5:1. This mixture was allowed to incubate, with occasional stirring, for 1 hr at 0°C. The supernatant containing solubilized membrane proteins was recovered by centrifugation at 100,000 × g for 1 hr at 4°C.

Proteins. Partially purified adenylate cyclase was prepared by the method described by Westcott et al. (6). Fraction I from the CaM-Sepharose column was used throughout this study. Fraction I recovered from CaM-Sepharose affinity chromatography represented approximately 80% of applied adenylate cyclase activity. Fraction I was desalted on Bio-Gel P-4 with elution buffer (20 mM Tris·HCl, pH 7.4/0.25 M sucrose/0.1% Lubrol PX). Purified CaM was prepared from bovine brain by the method of LaPorte et al. (12).

Adenylate Cyclase Assay. Adenylate cyclase activity was measured in a final volume of 50 μl. [α-^{32}P]ATP, prepared by the method of Symons (13), was used as the substrate; [^{3}H]cyclic AMP was utilized to monitor recovery. ATP used in the assays was purified by chromatography on DEAE-Sephadex A-25 followed by Dowex AG-50 chromatography. The assay mixture contained 1 mM [α-^{32}P]ATP (150–250 cpm/pmol), 5 mM $MgCl_2$, 2 mM cyclic AMP, 5 mM theophylline, 20 mM Tris·HCl (pH 7.6), and an ATP-regenerating system consisting of 20 mM creatine phosphate and 120 units of creatine phosphokinase per ml. Assays were carried out in triplicate for 2 min at 30°C with a standard deviation of less than 5%. The reaction was stopped and the [^{32}P]cyclic AMP formed was recovered by the method of Salomon et al. (14). Protein was determined by the procedure of Peterson (15) with bovine serum albumin as a standard.

Reconstitution System. Incubation mixtures contained 10 mM $MgCl_2$, 52 μg of solubilized membrane proteins per ml, 83 μg of fraction I in 20 mM Tris·HCl, pH 7.6/0.25 M sucrose/0.1% Lubrol PX in a final volume of 0.8 ml. When present, p[NH]ppG was at 100 μM, NaF was at 10 mM, and

Abbreviations: CaM, calmodulin [formerly known as calcium-dependent regulatory protein (CDR)]; p[NH]ppG, 5'-guanylyl imidodiphosphate.

CaM was at 25 μg/ml. Preassay activation was carried out from 0 to 20 min at 30°C, with samples withdrawn at 2-min intervals and stored on ice. Twenty-microliter aliquots were assayed for adenylate cyclase activity.

Trypsin Treatment. Solubilized brain membrane protein or fraction I was exposed to 10 μg of trypsin per ml at 37°C for 15 min, followed by exposure to an equivalent quantity of soybean trypsin inhibitor. The mixture was diluted and used in reconstitution studies as described above.

N-Ethylmaleimide Treatment. Solubilized brain membrane protein was treated with 1 mM N-ethylmaleimide for 20 min at 0°C; the reaction was stopped by addition of 1 mM dithiothreitol, and the treated preparation was used in reconstitution studies as described above.

RESULTS

p[NH]ppG Activation of Adenylate Cyclase. Adenylate cyclase activity in membranes prepared from bovine cerebral cortex was sensitive to p[NH]ppG (Fig. 1A). Membranes were incubated either in the presence or absence of 100 μM p[NH]ppG for various periods of time and assayed for adenylate cyclase activity. In the presence of p[NH]ppG, adenylate cyclase activity increased progressively with time and reached maximal stimulation after 12 min. At early stages of the incubation with p[NH]ppG, the nucleotide actually caused a slight inhibition of adenylate cyclase activity. The reason for this initial inhibition has not been adequately explained. However, it has been proposed that guanyl nucleotide inhibition may be due to a regulatory protein distinct from the regulatory components responsible for GTP activation (16). The maximal stimulation observed in the presence of p[NH]ppG was 3-fold greater than the activity observed with untreated membranes.

Adenylate cyclase solubilized from membranes with Lubrol PX was also stimulated by p[NH]ppG (Fig. 1B) in a time-dependent manner. Stimulation was observed after a lag of 2 min. Whereas basal activity in intact membranes was stable for over 20 min, solubilized basal activity decreased approximately 35% during the same time period.

Restoration of p[NH]ppG Sensitivity of Fraction I. Fraction I obtained by Affi-Gel Blue and CaM-Sepharose chromatography was not stimulated by p[NH]ppG (Fig. 2). Because solubilized adenylate cyclase activity was stimulated by p[NH]ppG, it seemed likely that the p[NH]ppG-insensitive form was generated by prior purification steps. Therefore, reconstitution experiments were designed to test for the existence of a dissociable subunit present in detergent-solubilized fractions that would reconstitute p[NH]ppG sensitivity to fraction I. When fraction I was preincubated with diluted solubilized brain membrane protein in the presence of p[NH]ppG, sensitivity to this guanyl nucleotide was restored to the level of that observed with solubilized adenylate cyclase (Fig. 2). A lag of approximately 2 min was observed. Activation by p[NH]ppG after a 20-min exposure to the nucleotide in the reconstitution system was 2.5-fold. It should be emphasized that the solubilized adenylate cyclase activity in the reconstituted system contributed <10% to total activity. Reconstitution experiments with diluted solubilized preparations as a source of guanyl nucleotide regulatory subunits are feasible because the GTP binding subunit is in large excess over adenylate cyclase (17). GTP did not stimulate either native fraction I or the reconstituted system. However, the lack of GTP stimulation may have been due to an active GTPase in the reconstituted system. (Specific activity = 1.15 mmol/mg per hr.)

Restoration of Fluoride Sensitivity. Fraction I was inhibited approximately 25% by 10 mM NaF (Fig. 3). However, the reconstituted adenylate cyclase system was stimulated 1.6-fold by NaF over a period of 20 min. Addition of p[NH]ppG to the reconstitution system containing NaF did not appreciably affect stimulation of adenylate cyclase activity by fluoride ion.

Reconstitution of CaM Sensitivity. As previously reported,
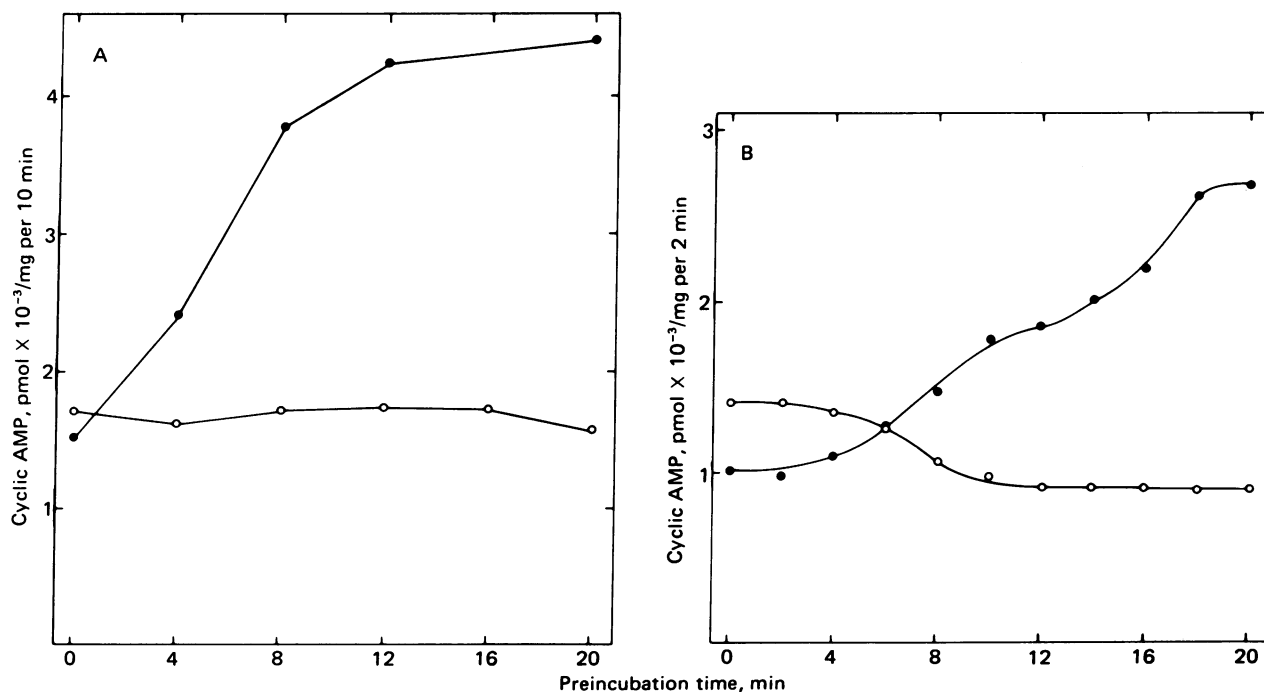

FIG. 1.   Stimulation of adenylate cyclase from bovine cerebral cortex by p[NH]ppG. (A) Membranes were preincubated in the presence (●) and absence (○) of 100 μM p[NH]ppG for the times indicated at 30°C. The samples were withdrawn and stored on ice, and adenylate cyclase activity was measured at 30°C for 10 min. (B) Membranes from bovine cerebral cortex were solubilized with Lubrol PX and preincubated at 30°C in the presence (●) and absence (○) of 100 μM p[NH]ppG. The samples were withdrawn, stored on ice, and assayed for adenylate cyclase activity at 30°C for 2 min.
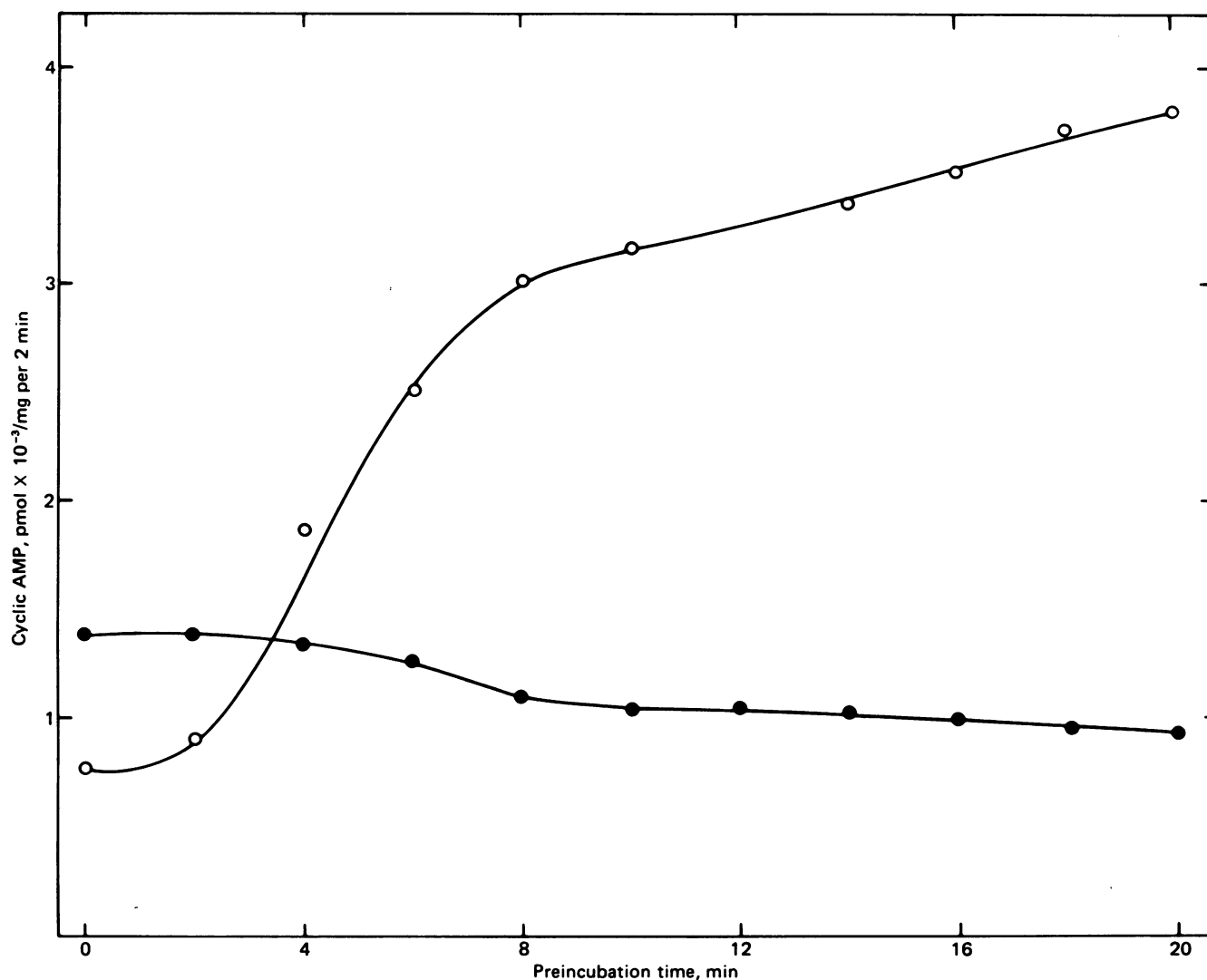
FIG. 2.   Restoration of p[NH]ppG sensitivity. Fraction I was passed through a Bio-Gel P-4 column and desalted into 50 mM Tris·HCl, pH 7.4/0.25 M sucrose/0.1% Lubrol PX and mixed with 100 μM p[NH]ppG in the absence (●) or presence (O) of solubilized bovine cerebral cortex membranes that had been diluted 1:10 in desalting buffer. The mixture was preincubated at 30°C and samples were withdrawn at the times indicated, stored on ice, and assayed for adenylate cyclase activity for 2 min. The data plotted represent net adenylate cyclase activity with the contribution by the solubilized membrane extract subtracted from gross activity.

fraction I was insensitive to CaM-mediated stimulation by Ca^{2+} (Fig. 4). Ca^{2+} inhibited fraction I adenylate cyclase activity at all Ca^{2+} concentrations examined. Because the previous data demonstrated that p[NH]ppG and NaF sensitivity was restored to fraction I by incubation with diluted solubilized extracts, the Ca^{2+} sensitivity of the reconstituted system was examined in the presence and absence of p[NH]ppG. Adenylate cyclase activity in the reconstituted system was not stimulated by Ca^{2+} in the absence of p[NH]ppG (Fig. 4). In the presence of p[NH]ppG, CaM stimulated adenylate cyclase activity of fraction I 3-fold over the reconstituted p[NH]ppG system and 6-fold over the fraction I reconstitution system in the absence of p[NH]ppG. Sodium fluoride did not substitute for p[NH]ppG in effecting reconstitution of Ca^{2+} stimulation. Ca^{2+}·CaM sensitivity in the presence of p[NH]ppG was stable for 20 min at 30°C. Fig. 4 also reports the absolute activities contributed by solubilized adenylate cyclase to the reconstitution system. These data illustrated that restoration of Ca^{2+}·CaM sensitivity requires fraction I, the solubilized fraction, and the presence of p[NH]ppG.

Effects of Temperature, Trypsin, and *N*-Ethylmaleimide

on Reconstitution. The above data indicated that p[NH]ppG was required for restoration of Ca^{2+}·CaM sensitivity, suggesting that a guanine nucleotide binding subunit was involved. The solubilized extract was heated to 50°C for 1 min and treated with *N*-ethylmaleimide or trypsin to determine if the obligatory factor(s) was a protein. As indicated from the data in Table 1, reconstitution of p[NH]ppG, NaF, and Ca^{2+}·CaM activation was sensitive to these treatments, suggesting that the factor(s) required for reconstitution is a protein. The protein required for reconstitution was not CaM present in soluble extracts because it has been established that CaM is heat stable and contains no sulfhydryl groups (18, 19). In addition, previous studies have shown that purified CaM does not restore Ca^{2+} sensitivity to fraction I (6). Analogous experiments were done in which fraction I was heated or treated with trypsin and reconstituted with the solubilized fraction. Under these conditions, the observed activities were identical to those exhibited by the solubilized fraction alone. These experiments suggest, but do not prove, that the enhanced activity in the reconstituted system was not due to stimulation of the solubilized activity by a factor present in fraction I.

Biochemistry: Toscano *et al.*

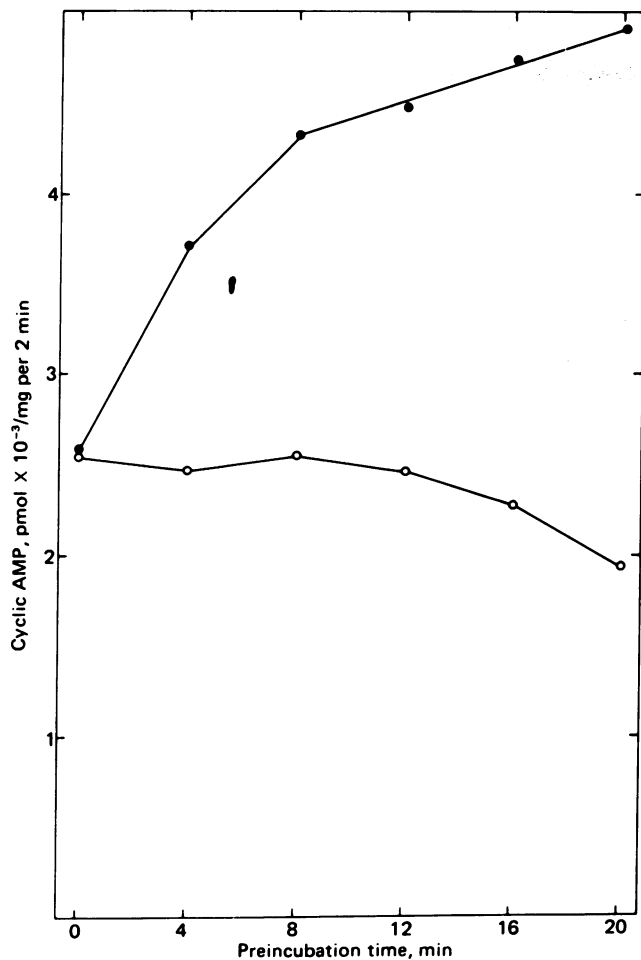*Proc. Natl. Acad. Sci. USA 76 (1979)* 5585

FIG. 3. Restoration of NaF sensitivity to fraction I. Preincubation conditions were the same as described in the legend to Fig. 2 except 10 mM NaF was added in place of p[NH]ppG. ●, Reconstituted fraction I; ○, fraction I in presence of 10 mM NaF.

FIG. 4. Restoration of Ca^{2+}·CaM sensitivity to fraction I. Fraction I was desalted and mixed with solubilized bovine cerebral membranes as described in the legend of Fig. 2, except the incubation mixtures contained 25 μg of CaM per ml, 250 μM EGTA, and Ca^{2+} in the concentrations labeled on the abscissa. ■, Detergent-solubilized fraction; □, fraction I; ○, fraction I plus solubilized fraction with no p[NH]ppG; ●, fraction I plus solubilized fraction in the presence of 100 μM p[NH]ppG. The samples were incubated for 12 min and assayed for adenylate cyclase activity.

DISCUSSION

The role of guanyl nucleotides in regulation of hormone activation of adenylate cyclase has been extensively characterized (20–22). In most eukaryotic cells studied to date, guanyl nucleotides activate adenylate cyclase systems in the absence of hormones. Recent studies on the reconstitution of guanylnucleotide and fluoride activation of rat brain adenylate cyclase by Cuatrecasas (23, 24) and genetic studies reported by the Bourne (25) and Gilman (11, 26–28) laboratories have demonstrated that a dissociable protein factor is required for guanyl nucleotide stimulation of adenylate cyclase. The elegant work of Pfeuffer and Helmreich (7, 17) indicates that even though many GTP binding proteins are present in eukaryotic cells, at least one specific GTP binding protein is required for restoration of guanyl nucleotide stimulation of adenylate cyclase.

Bovine cerebral cortex adenylate cyclase is sensitive to stimulation by the nonhydrolyzable GTP analog, p[NH]ppG, both in the membrane and in extracts solubilized with Lubrol PX. A CaM-insensitive form of adenylate cyclase, designated fraction I, was also insensitive to p[NH]ppG and inhibited by NaF. Restoration of p[NH]ppG, NaF, and CaM stimulation to fraction I requires one or more protein factors present in solubilized bovine brain membranes. The factor(s) is inactivated by heat, trypsin, or N-ethylmaleimide treatment. The factor(s) appears to be functionally similar to the N (25) or G/F (27) proteins of S49 lymphoma cells and to the GTP binding protein from avian erythrocytes (17).
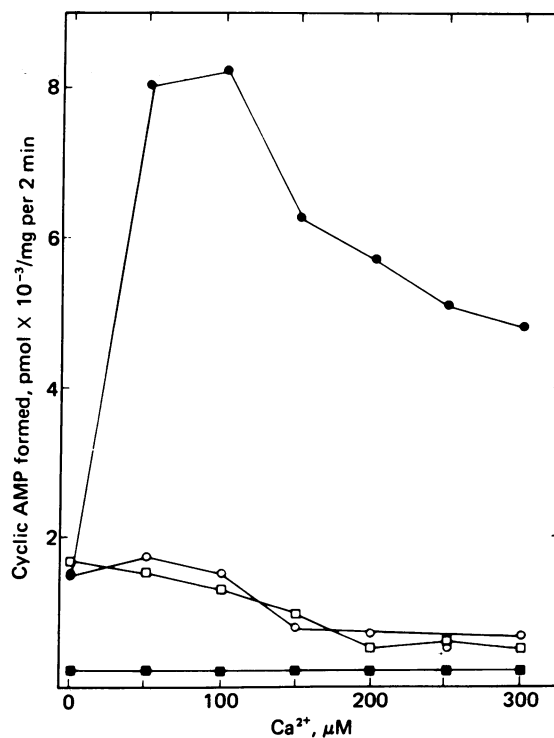
The data reported in the present study illustrate that CaM-dependent Ca^{2+} stimulation can be restored to a previously insensitive form of adenylate cyclase by addition of dissociable factors. This process required p[NH]ppG, indicating that res-

Table 1. Characterization of the factor required for reconstitution of p[NH]ppG, NaF, and Ca^{2+}·CaM stimulation of fraction I

Treatment of solubilized membranes	Adenylate cyclase, (pmol/2 min/mg)			
	None	p[NH]ppG	NaF	p[NH]ppG + CaM·Ca^{2+}
None*	3395	5216	5338	10,093
N-Ethylmaleimide†	3395	3119	3253	3,679
Trypsin (10 μg/ml)‡	3395	3568	3588	3,678
Heat§	3395	3277	3449	3,705

* Fraction I was desalted as described in the legend of Fig. 2, and reconstitution was carried out with a preincubation time of 12 min. Adenylate cyclase activity was measured for 2 min. When present, p[NH]ppG was at 100 μM; NaF, 10 mM; Ca^{2+}, 200 μM.
† Solubilized brain membrane was treated with 1 mM N-ethylmaleimide at 0°C for 20 min; the reaction was stopped by the addition of an equivalent quantity of dithiothreitol, and the mixture was desalted on Bio-Gel P-4 into 20 mM Tris·HCl, pH 7.4/0.25 M sucrose/0.1% Lubrol PX prior to use in reconstitution.
‡ Solubilized brain membranes were treated with 10 μg of trypsin per ml at 37°C for 15 min. The reaction was stopped with an equivalent quantity of soybean trypsin inhibitor before use in reconstitution.
§ Solubilized brain membrane was heated at 50°C for 1 min prior to use in reconstitution.

toration of CaM-dependent Ca^{2+} stimulation is coupled to guanyl nucleotide sensitivity. Identification of the coupling factor as the guanyl nucleotide binding subunit remains to be unambiguously established. It is interesting that restoration of CaM responsiveness, similar to hormone activation of adenylate cyclase, is guanyl nucleotide dependent. The generality of this observation remains to be established for other CaM-sensitive adenylate cyclase systems. More definitive characterization of adenylate cyclase requires further purification of the adenylate cyclase system.

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