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Identification of a Calcium-Binding Protein as a Calcium-Dependent Regulator of Brain Adenylate Cyclase

(cAMP/cGMP/cyclic nucleotide phosphodiesterase)

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ABSTRACT **An activating factor of adenylate cyclase (EC 4.6.1.1) has been obtained from detergent-dispersed preparations of porcine cerebral cortex by column chromatography on ECTEOLA-cellulose. The factor was identified by acrylamide gel electrophoresis and by enzyme activation studies as the Ca^{2+}-binding protein that regulates the activity of a brain cyclic nucleotide phosphodiesterase. This Ca^{2+}-binding protein confers a Ca^{2+}-dependent activation upon the adenylate cyclase, which is reversed by the subsequent addition of EGTA in excess of the free Ca^{2+}. It is proposed that this Ca^{2+}-dependent regulator controls enzymatic activities responsible for the synthesis of adenosine 3′:5′-monophosphate and for the hydrolysis of guanosine 3′:5′-monophosphate.**

Adenylate cyclase activity is a major determinant of the intracellular concentration of adenosine 3′:5′-monophosphate (cAMP). Rapid activation of the enzyme by humoral factors is believed to involve constituents of the plasmalemma which serve as receptors linked to a catalytic unit in an indeterminant manner (1). Less rapid alterations of adenylate cyclase activity occur in cultured mammalian cells during various stages of growth, differentiation, and transformation by oncogenic viruses (2, 3). Among the agents that have been implicated in adenylate cyclase modulation is Ca^{2+}, but the nature of its involvement remains ill-defined. Generally, Ca^{2+} has been regarded as an inhibitor of adenylate cyclase. However, low concentrations (micromolar range) have been reported to increase the accumulation of cAMP in intact cells and to stimulate adenylate cyclase activity (4–9).

This report demonstrates that a specific protein confers a reversible Ca^{2+}-dependent regulation on a detergent-dispersed preparation of adenylate cyclase from brain. Previously this protein has been purified, physically characterized as a Ca^{2+}-binding protein (10, 11), and identified as a Ca^{2+}-dependent regulator (CDR) of a cyclic nucleotide phosphodiesterase (12, 13).

EXPERIMENTAL PROCEDURE

Materials. [8-^{3}H]Guanosine 3′:5′-monophosphate ([^{3}H]-cGMP) (4 Ci/mmol), [8-^{3}H]GTP (5.6 Ci/mmol), and [2,8-^{3}H]-ATP (33.2 Ci/mmol) were purchased as the ammonium salts from New England Nuclear Corp. ECTEOLA-cellulose and AG 50 X 8 (100–200 mesh) were obtained from Bio Rad Laboratories. Pyruvate kinase from rabbit muscle was purchased from the Boehringer Mannheim Corporation, and Lubrol-PX from the Sigma Chemical Co. Homogeneous CDR from porcine brain was prepared by the procedure of Wolff and Siegel (10). CDR-dependent cyclic nucleotide phosphodiesterase was prepared from brain extracts by removing endogenous CDR through ECTEOLA-cellulose column chromatography as described (12).

Preparation of Brain Adenylate Cyclase. The procedure developed by Johnson and Sutherland (14) for preparing rat brain, detergent-dispersed adenylate cyclase [EC 4.6.1.1; ATP pyrophosphate-lyase (cyclizing)] was adapted with minor modifications to the preparation of porcine brain adenylate cyclase. Cerebral cortex was removed from whole brains purchased from a local supplier. Cortex (100 g) was suspended in 9 volumes (relative to tissue weight) of ice-cold 0.25 M sucrose, 2 mM $MgCl_2$, 1 mM ethylenediamine tetraacetic acid (EDTA), 3 mM dithiothreitol, and 0.1 M Tris·HCl, pH 7.5, and homogenized for 30 sec in a Waring Blendor at a setting of "medium." The homogenate was centrifuged at 3000 × *g* for 10 min, and the pellet was washed by resuspension and homogenization in 9 volumes of the homogenizing fluid. The homogenization and centrifugation procedure was performed a total of three times. The final pellet was resuspended and rehomogenized in 9 volumes of homogenizing fluid containing 1% Lubrol-PX and centrifuged at 27,000 × *g* for 20 min. The supernatant fraction, containing the detergent-dispersed adenylate cyclase activity, was retained.

Assay of Adenylate Cyclase. Adenylate cyclase activity was determined by a modification of the method of Krishna *et al.* (15). Unless otherwise specified, the standard reaction mixture of 150 μl contained 5 mM $MgCl_2$, 10 mM theophylline, 2 mM cAMP, 1 mM dithiothreitol, 1 mg/ml of bovine serum albumin, 0.2 mM (0.13 Ci/mmol) [^{3}H]ATP, 50 mM glycylglycine buffer (pH 7.5), 5 mM phospho*enol*pyruvate, 20 mM KCl, and 10 μg of rabbit muscle pyruvate kinase. Reaction mixtures were preincubated for 2 min at 37°, and the reaction was initiated by the addition of enzyme. After 10 min of incubation, the reactions were terminated with 50 μl of 1 M HCl. The samples were neutralized with 75 μl of 1 M Tris·hydroxide and treated with 100 μl of 0.3 M $Ba(OH)_2$ followed by 100 μl of 0.3 M $ZnSO_4$. The resulting precipitate was collected by centrifugation at 8000 × *g* for 15 min. The Ba-Zn precipitation procedure was repeated, and an aliquot of the supernatant liquid was applied to an AG 50 X 8 (0.5 × 6.5-cm) column equilibrated with 1 mM HCl. After

Abbreviations: CDR, calcium-dependent regulator; EGTA, ethylene glycol-bis-(β-aminoethyl ether)*N,N*′-tetraacetic acid; cGMP, guanosine 3′:5′-monophosphate.

TABLE 1. *Ca^{2+} requirement of porcine brain adenylate cyclase*

Additions	pmol of cAMP/mg × min
None	54 ± 3
EGTA	1 ± 2
Ca^{2+} (30 μM)	83 ± 7
Ca^{2+} (150 μM)	47 ± 5
EGTA + CDR	4 ± 1
Ca^{2+} (30 μM) + CDR	75 ± 14
Ca^{2+} (150 μM) + CDR	52 ± 3

Detergent-dispersed adenylate cyclase was assayed under standard conditions with the exception that [³H]ATP was adjusted to 0.5 mM. EGTA (50 μM) was present in all incubations. Ca^{2+} concentrations, where indicated, represent $CaCl_2$ added in excess of the EGTA. One microgram of homogeneous CDR was added to the indicated incubations. Values given are the mean ± SEM of three separate determinations.

a 7-ml wash with 1 mM HCl, which was discarded, [³H]cAMP was eluted with 3 ml of H_2O and collected in scintillation vials. The samples were evaporated at 80°, redissolved in 0.5 ml of H_2O, and analyzed for radioactivity by scintillation counting. Samples were normally conducted in triplicate and the results were averaged. The values were corrected for losses of [³H]-cAMP by determining spectrophotometrically (A_{259}) the recoveries of the added, unlabeled cAMP. Controls without enzyme were found to contain less than 0.02% of the initial radioactivity contained in the incubation. Samples with enzyme normally ranged from 1 to 20 times the value of these controls.

Assay of Guanylate Cyclase. Guanylate cyclase [EC 4.6.1.2; GTP pyrophosphate-lyase (cyclizing)] was assayed in incubations conducted identically to those described above for adenylate cyclase, with the exception that 0.2 mM [³H]GTP was substituted for [³H]ATP, and 2 mM cGMP for cAMP. Reactions were terminated with 50 μl of 1 M HCl, neutralized with 1 M Tris·hydroxide, diluted with 1 ml of H_2O, and applied to an AG 1 X 8 column (0.5 × 5 cm, formate form). The column was washed with 9 ml of 1 M formic acid. The cGMP was selectively eluted with 9 ml of 3 M formic acid. The sample was lyophilized and dissolved in 1 ml of H_2O and applied to a QAE-A25 column (0.5 × 5 cm, chloride form). The column was washed with 5 ml of H_2O, followed by 5 ml of 0.1 M NH_4HCO_3, pH 9.0, and then eluted with 5 ml of 0.1 M NH_4CO_3, pH 7.0, to collect cGMP. The samples were evaporated and assayed for radioactivity by scintillation counting.

Published procedures were used for the assay of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase activity (13), for analytical electrophoresis on acrylamide gels (16), and for the preparation of ECTEOLA-cellulose for chromatography (10). CDR was standardized by light absorbance at 280 nm (10), and protein was measured by the procedure of Lowry *et al.* (17).

RESULTS

Activation of Adenylate Cyclase by Ca^{2+} and CDR. Preparations of adenylate cyclase dispersed with 1% Lubrol-PX were examined for Ca^{2+} sensitivity. The porcine enzyme was similar to brain adenylate cyclase from other species (7, 8, 14)

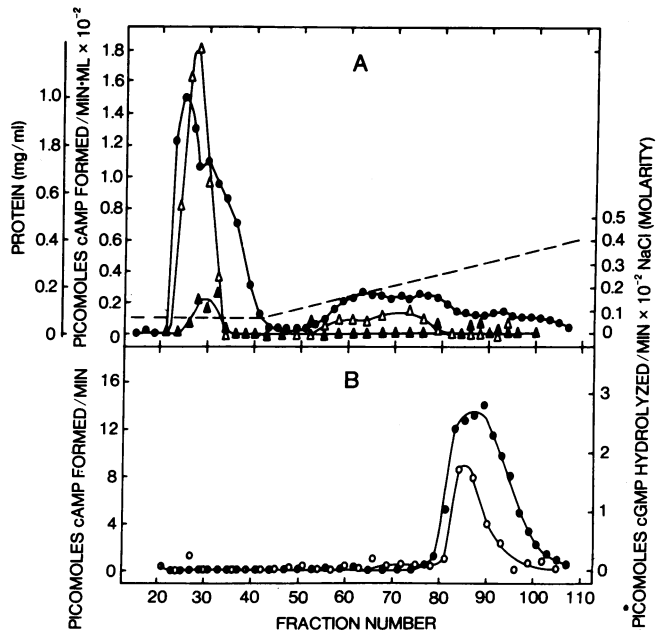

FIG. 1. Separation of an activator fraction from brain adenylate cyclase by ECTEOLA-cellulose chromotography. Detergent-dispersed adenylate cyclase (30 ml) was applied to a 2.5 × 60-cm column of ECTEOLA-cellulose equilibrated with 0.25 M sucrose, 75 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 2.5 mM dithiothreitol, 1% Lubrol-PX, and 10 mM Tris·HCl, pH 7.4. Twenty-two fractions (10 ml) were collected, and an 800-ml linear gradient to 0.5 M NaCl was initiated. (A) Aliquots (25 μl) of the column fractions were assayed for adenylate cyclase activity by the standard assay procedure at 50 μM free $CaCl_2$ with (△) and without (▲) the addition of CDR (1 μg). Protein (●). (B) Portions of the column fractions were heated to 90° for 1 min. An aliquot of the heated material (25 μl) was tested for the presence of an activating factor of the adenylate cyclase described in A. Adenylate cyclase activity (○) was measured by the standard assay conducted with 50 μM free $CaCl_2$. A second aliquot (10 μl) of heated material was tested for the presence of CDR by recombination with and activation of CDR-dependent phosphodiesterase from porcine brain (●) with 25 μM cGMP as substrate.

in that it was inhibited by ethylene glycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), and the inhibition was reversed by micromolar concentrations of Ca^{2+} in excess of the EGTA (Table 1). Concentrations of Ca^{2+} above 0.1 mM were inhibitory. CDR, added at concentrations previously established as sufficient to fully activate preparations of CDR-dependent cyclic nucleotide phosphodiesterase from brain (12, 13), did not influence the adenylate cyclase activity. Samples of the adenylate cyclase preparation, however, when heated to 90° to denature endogenous phosphodiesterase activity, were found to contain large amounts of CDR when tested for the ability to activate CDR-dependent phosphodiesterase from brain (12, 13) (data not illustrated).

Conditions that had previously been established for the chromatographic separation of CDR from brain Ca^{2+}-dependent phosphodiesterase on ECTEOLA-cellulose (12, 13) were utilized in removing CDR from the adenylate cyclase preparation. Detergent-dispersed adenylate cyclase, adjusted to contain 0.1 mM EGTA, was applied to an ECTEOLA-cellulose column and eluted with a linear NaCl gradient (Fig. 1A). A large quantity of protein was eluted before ap-

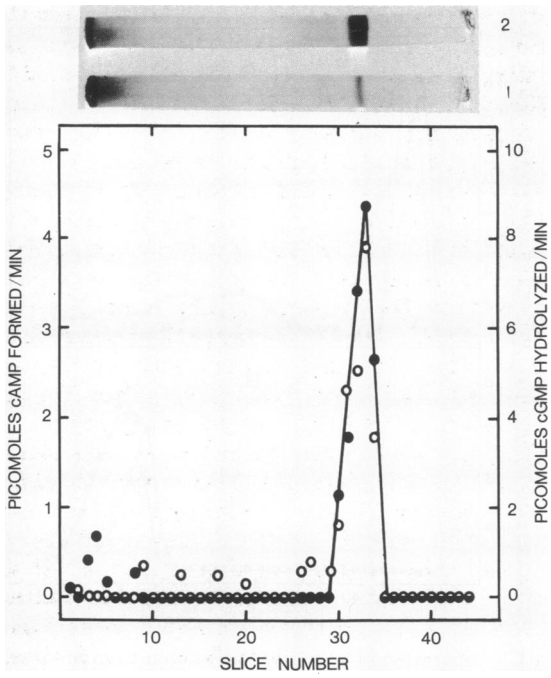

FIG. 2. Electrophoretic comparison of CDR with the crude adenylate cyclase activating factor from ECTEOLA-cellulose chromatography. Fractions 81–97 from Fig. 1 were pooled, dialyzed against two 4-liter changes of 5 mM Tris buffer, pH 7.4, concentrated to a volume of 5 ml by ultrafiltration, and heated to 90° for 1 min. Samples containing 50 μg of protein with (gel 2) and without (gel 1) 20 μg of added homogeneous, brain CDR were subjected to electrophoresis in 15% acrylamide, pH 8.3. Electrophoresis was conducted for 2.5 hr at 1.5 mA per gel. The electrophoretic fractionation of sample 1 was conducted in duplicate. After electrophoresis, one gel was stained, the second was frozen. The frozen gel, 45 cm in length, was sliced into 1-mm sections. Each section was incubated at 4° for 24 hr in 125 μl of 50 mM imidazole, pH 7.4, and aliquots (1 μl) were tested for CDR activity by recombination with and activation of CDR-dependent phosphodiesterase (●), with 25 μM cGMP as substrate. Aliquots (25 μl) were also tested for activation of the CDR-dependent adenylate cyclase (○) derived from ECTEOLA-cellulose chromatography. A peak of CDR activity was observed (slice 32) that corresponded in migration to authentic CDR.

plication of the gradient, followed by several small, broad peaks of protein as the gradient was developed. Assays of the column fractions for adenylate cyclase activity at micromolar free Ca^{2+} concentrations indicated that a dramatic loss of enzyme activity had occurred in the preparation. Reassay of the column fractions with the addition of homogeneous CDR (1 μg) resulted in a 9-fold increase in the adenylate cyclase activity of fractions 22–35 and in fractions 55–75. Calculated recoveries of adenylate cyclase activity, expressed as percentages of the total activity applied to the column, provided values of 66% for fractions 22–35 and 19% for fractions 55–75, as assayed with CDR and Ca^{2+} immediately after chromatography.

Identification of an Endogenous Adenylate Cyclase Activating Factor as CDR. Aliquots of the ECTEOLA-cellulose column fractions were heated to inactivate endogenous adenylate cyclase and cyclic nucleotide phosphodiesterase activities and examined for the ability to activate the deactivated (CDR-dependent) adenylate cyclase and the CDR-dependent

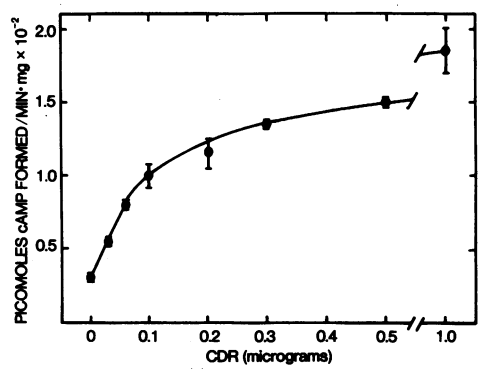

FIG. 3. Variation of adenylate cyclase activity with concentration of CDR. CDR-dependent adenylate cyclase was assayed under standard conditions with 50 μM free Ca^{2+}.

phosphodiesterase. Coincident peaks of activating factor were found (fractions 81–97) for the two enzymes (Fig. 1B). Fractions 81–97 were pooled, concentrated, dialyzed against water, heated to 90°, and examined by analytical acrylamide gel electrophoresis (Fig. 2). A protein from the concentrate was observed to migrate on 15% gels to a position identical to that of homogeneous CDR. A duplicate gel of the heated concentrate (gel 1) was frozen, sliced, and assayed for the presence of factors activating the CDR-dependent adenylate cyclase and the CDR-dependent phosphodiesterase. The presence of a single activating factor in the gel slices was found for each enzyme, corresponding to the migration position of brain CDR.

Regulation of Adenylate Cyclase by CDR. Activity of various preparations of the CDR-dependent adenylate cyclase declined several-fold in activity during 2 days at 4°. Enzyme preparations generally exhibited linear rates of product formation for as long as 30 min. Although the CDR-dependent enzyme was largely inactive when incubated with 100 μM EGTA, the addition of micromolar concentrations of Ca^{2+} in excess of the EGTA increased the enzymatic activity of some preparations to as much as 25% of the values obtained with the addition of 1 μg of CDR. This was taken to indicate that traces of CDR still remained.

Detergent-dispersed preparations of adenylate cyclase before and after exposure to ECTEOLA-cellulose chromatography were analyzed for the presence of guanylate cyclase activity. At conditions of assay that were similar to those used in determining adenylate cyclase activity, the guanylate cyclase activity of the preparations was less than 1% that of the adenylate cyclase activity as measured with and without the addition of 50 μM free Ca^{2+} and 1 μg of CDR.

Degrees of activation of the CDR-dependent adenylate cyclase were examined as a function of increasing amounts of CDR (Fig. 3). Half-maximal activation of the enzyme occurred at about 0.7 μg of CDR per ml of incubation contents. Similar CDR requirements have been reported for the CDR-dependent phosphodiesterase (12).

The Ca^{2+} requirement of the CDR-dependent adenylate cyclase was examined to determine whether the enzyme was activated in a reversible or irreversible fashion (Fig. 4). A sample of CDR-dependent adenylate cyclase incubated with EGTA and CDR exhibited only traces of activity. Addition of Ca^{2+} in excess of the EGTA (at 5.5 min) produced more than a 10-fold increase in activity. Addition of EGTA (at 10.5

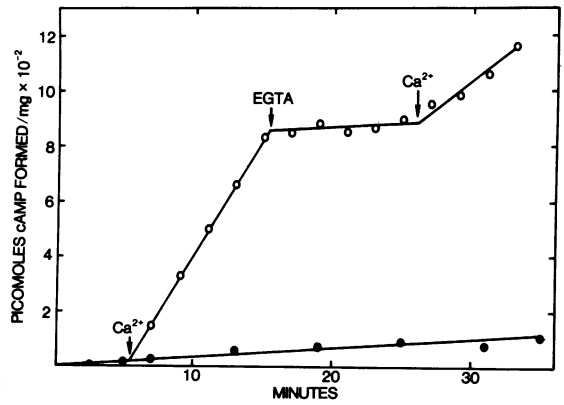

FIG. 4. Regulation of brain adenylate cyclase by Ca^{2+}. Two incubation mixtures were constructed containing (in 1.7 ml) the components and concentrations of the standard reaction, CDR (7 μg/ml), EGTA (0.15 mM), and CDR-dependent adenylate cyclase (2.0 mg of protein). The reaction was initiated by the addition of the enzyme. Aliquots (100 μl) were removed at indicated times, denatured with 1 M HCl (50 μl), and assayed for [^{3}H]cAMP. One incubation sample was retained throughout the course of the experiment as a control (●), to which no further additions were made. The other sample (○) was adjusted to 0.2 mM $CaCl_2$ at 5.5 min with 20 mM $CaCl_2$, to 1.5 mM EGTA at 15.5 min with 100 mM EGTA, and to 1.6 mM $CaCl_2$ at 26 min with 20 mM $CaCl_2$.

min) in excess of the Ca^{2+} returned the reaction rate to that of the control. Subsequent addition of Ca^{2+} in excess of the EGTA (at 26 min) reestablished an increased rate of product formation.

DISCUSSION

The present results demonstrate that Ca^{2+}-dependent regulation is conferred upon detergent-dispersed adenylate cyclase activity by the Ca^{2+}-binding protein, CDR, previously identified as a regulator of brain cyclic nucleotide phosphodiesterase activity (12). The CDR appears to be functionally identical to the heat-stable phosphodiesterase-activating factor(s) described by Cheung (18), Kakiuchi *et al.* (19), and Teo *et al.* (20). However, the preparation of homogeneous CDR, unlike the procedures used to obtain these phosphodiesterase activating factor(s), does not involve a heating step. Therefore, CDR is not generated in the heating process. Because heated and nonheated samples of CDR are comparable in activating the phosphodiesterase (12), the use of heated aliquots of fractions from ECTEOLA–cellulose columns in the present study for measurements of CDR content appears to be valid.

The low concentrations of Ca^{2+} required for the activation of the adenylate cyclase and the cyclic nucleotide phosphodiesterase, combined with the rapid reversal of their activation by EGTA, provide a basis for suggesting that their activities *in vivo* are regulated by variations of intracellular Ca^{2+} concentration. It is well known that Ca^{2+} influx is one component of the flow of ions which comprises the normal propagated impulse of depolarization in nerve tissue (21), and it has been noted that extracellular Ca^{2+} is required in order to obtain increased concentrations of cAMP in cerebral cortex slices in response to depolarizing agents (6, 9). Kakiuchi *et al.* (19) have proposed, on the basis of kinetic evidence, that the Ca^{2+}-dependent cyclic nucleotide phosphodiesterase preferentially hydrolyzes cGMP rather than cAMP at substrate concentrations in the micromolar range. An attractive hypothesis consistent with this proposal and the results reported here is that Ca^{2+} influx results in the formation of a Ca^{2+}·CDR complex. The ensuing activation of adenylate cyclase would increase intracellular cAMP, and the concomitant activation of the phosphodiesterase would decrease cGMP. Clearly, information in addition to that presently available is needed before concluding that the Ca^{2+}·CDR complex mediates the numerous instances of reciprocal changes of cAMP and cGMP that have been observed (22). For example, the cellular distribution of CDR and of the two CDR-dependent enzymes remains to be established. CDR-dependency may not be characteristic of all forms of adenylate cyclase. The possible relationship of CDR to Ca^{2+}-dependent guanylate cyclase activity (23) requires systematic exploration. In spite of these limitations, the potential applicability of the present findings to the bidirectional cyclic nucleotide control of cell proliferation (22, 24, 25) should be noted. Anderson and Pastan (26) have evidence for the existence of an adenylate cyclase activating factor that is present in fibroblasts grown in culture. The factor is contained in normal chick embryo fibroblasts, but lacking in cells transformed by a Rous sarcoma virus. As a basis for future studies on cell proliferation, it is suggested that interactions between Ca^{2+}, Ca^{2+}-binding proteins such as CDR, and the enzymes of cyclic nucleotide metabolism may underlie important changes in cAMP and cGMP concentrations.

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