Ca^{++}-DEPENDENT FORMATION OF BRAIN ADENYLATE CYCLASE-PROTEIN ACTIVATOR COMPLEX

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SUMMARY

In the presence of EGTA (ethyleneglycol-bis-(β-aminoethyl-ether) N,N'-tetraacetic acid), a Lubrol-PX solubilized rat brain adenylate cyclase (E.C. 4.4.1.1) and its protein activator were separated from each other in a Sephadex G-200 column. No activator was associated with the eluted enzyme, which required an exogenous activator for maximum activity. On the other hand, in the presence of Ca^{++}, some of the activator was eluted with the enzyme, which was independent of an exogenous activator for maximum activity. Because neither Ca^{++} nor EGTA affected the elution profile of the activator in the filtration column, these results suggest that the formation of the enzyme-activator complex is dependent on Ca^{++}. Separate experiments indicated that the effect of Ca^{++} on the formation of the enzyme-activator complex was immediate and reversible. Because the activator appears to be in excess of the enzyme, adenylate cyclase activity *in vivo* could be modulated by the cellular flux of Ca^{++}.

A Ca^{++}-binding protein activator originally discovered for cyclic 3':5'-nucleotide phosphodiesterase (E.C. 3.1.4.17) (1) has now been shown to activate brain adenylate cyclase (E.C. 4.6.1.1) as well (2,3). The activator, purified to homogeneity from bovine brain, has been extensively characterized with respect to its biochemical properties (4). The protein consists of a single polypeptide with a molecular weight of 18,920 and a pI of 4.3. It exhibits 4 Ca^{++}-binding sites; the dissociation constant varies from 4 to 18 μM (5). In the case of the phosphodiesterase system, the active form of the enzyme appears to be an enzyme-activator complex (5-7). The formation of the complex is dependent on Ca^{++} (4,8,9), which confers a more helical structure to the activator (10), and thus enables

it to interact with the apoenzyme to form an active holoenzyme.

In the case of adenylate cyclase, however, the mechanism of activation by the Ca^{++}-binding protein has not been elucidated. Experiments described herein suggest that Ca^{++} causes the formation of an active adenylate cyclase-activator complex, in a manner analogous to the mode of stimulation proposed for phosphodiesterase (4,8,9).

MATERIALS AND METHODS

Chemicals

[^{3}H]-ATP (S.A. 18 Ci/mmole), and [^{3}H]-cAMP (S.A. 27 Ci/mmole) were obtained from Schwarz/Mann. IRP-58 (200-400 mesh) was a gift of Rohm and Hass; AG 50W-X8 (200-400 mesh) was obtained from Bio Rad; Lubrol-PX, phosphoenolpyruvate, and pyruvate kinase were purchased from Sigma Chemical Co.

Preparation of Adenylate Cyclase

Lubrol-PX solubilized adenylate cyclase was prepared essentially as described by Bradham (11). Briefly, frozen rat brain cortices were thawed and then homogenized in 2 volumes of 50% glycerol containing 5 mM NaCl and 5 mM KCl. All procedures were carried out at 4°C. The homogenate was centrifuged for 30 min at 6000 xg. The pellet was suspended in one volume of 2 M sucrose, homogenized as before, and centrifuged for 10 min at 13,000 xg. The supernatant fluid containing membranous structures was diluted with 7 volumes of glass-distilled water and then centrifuged for 10 min at 12,000 xg. The supernatant was carefully removed and then recentrifuged at 100,000 xg for 15 min. The sediment was dissolved in 1 volume of 40 mM Tris-HCl (pH 7.0) containing 1 mM dithiothreitol and 1% Lubrol-PX; the suspension was centrifuged at 140,000 xg for 60 min to remove insoluble particles. The supernatant fluid was kept in small fractions at -90°. Adenylate cyclase activity did not decrease appreciably within 3 to 4 weeks.

Assay of Adenylate Cyclase

Crude preparations of adenylate cyclase are usually contaminated with cAMP phosphodiesterase and ATPase. The assay system, therefore, contained caffeine to inhibit phosphodiesterase, carrier cAMP to protect the newly synthesized [^{3}H]-cAMP from degradation, and an ATP regeneration system to counteract the effect of ATPase. The reaction mixture in a final volume of 0.1 ml contained 40 mM Tris-HCl (pH 7.5), 40 mM caffeine, 10 mM NaF, 5 mM $MgCl_2$, 2 mM cAMP, 1 mM [^{3}H]-ATP (sp. act. 0.9 mCi/mmole), 4 mM phosphoenolpyruvate, and 40 μg/ml of pyruvate kinase. Other additions are indicated in the legends. At the end of the incubation at 37° usually lasting 10 to 20 minutes, the reaction was terminated by adding 50 μl of 1 N HCl. The mixture was then adjusted to approximate neutrality with 1 M Tris. Cyclic AMP was purified from the reaction mixture by $Ba(OH)_2$-$ZnSO_4$ precipitation (12), followed by passage through a column of 50 W-X 8 cationic exchange resin.

Preparation and Assay of Protein Activator

Protein activator from bovine brain was purified to the stage of DEAE-cellulose column chromatography (5). The activator was measured by its ability to stimulate an activator-deficient phosphodiesterase (13) that was partially purified from bovine brain (14).

Determination of Protein

Protein was determined according to Lowry *et al.* (15) with bovine serum albumin as standard or according to Warburg and Christian (16).

RESULTS AND DISCUSSION

We have shown that adenylate cyclase solubilized by a detergent, Lubrol-PX, from a bovine or rat brain membrane fraction contains a Ca^{++}-binding protein activator and that the activator was separated from adenylate cyclase on an anionic exchange column (3). The activator-deficient adenylate cyclase was partially inactive; full enzyme activity

TABLE I

Differential Effect of EGTA on Adenylate Cyclase Activity in a Rat Brain Extract and in an Eluent from an Anionic Exchange Resin Column

Tube	Additions	Adenylate Cyclase Activity (pmoles/mg/min)
1	Extract	1443
2	Extract + Activator	1663
3	Extract + EGTA	190
4	Eluent from column	480
5	Eluent from column + Activator	1184
6	Eluent from column + EGTA	183

Adenylate cyclase was assayed as described under Materials and Methods. The extract used in Tubes 1 to 3 refers to a Lubrol-PX solubilized adenylate cyclase; the eluent in Tubes 4 to 6 refers to the enzyme after an anionic exchange resin column (3). When EGTA (final conc. 0.25 mM) was present no Ca^{++} was added to the reaction mixture (Tube 3 and Tube 6). When activator was present, 0.1 mM Ca^{++} was present (Tube 2 and Tube 5). Tube 1 and Tube 4 also contained 0.1 mM Ca^{++}.

was reconstituted by an exogenous activator (Table I). The adenylate cyclase preparation prior to treatment on the anionic exchange column appears to contain sufficient endogenous activator to give full enzymic activity, as addition of an exogenous activator gave essentially the same activity. Another way to demonstrate that dissociation of the activator from adenylate cyclase occurred in the column was the use of EGTA. EGTA inhibited adenylate cyclase by chelating the Ca^{++} that is required by the activator for activity. In the presence of EGTA, the activity of adenylate cyclase in the extract and in the eluent from the anionic column decreased. The decrease, however, was more pronounced in the extract because it contained relatively more of the activator. These results are summarized in Table I.

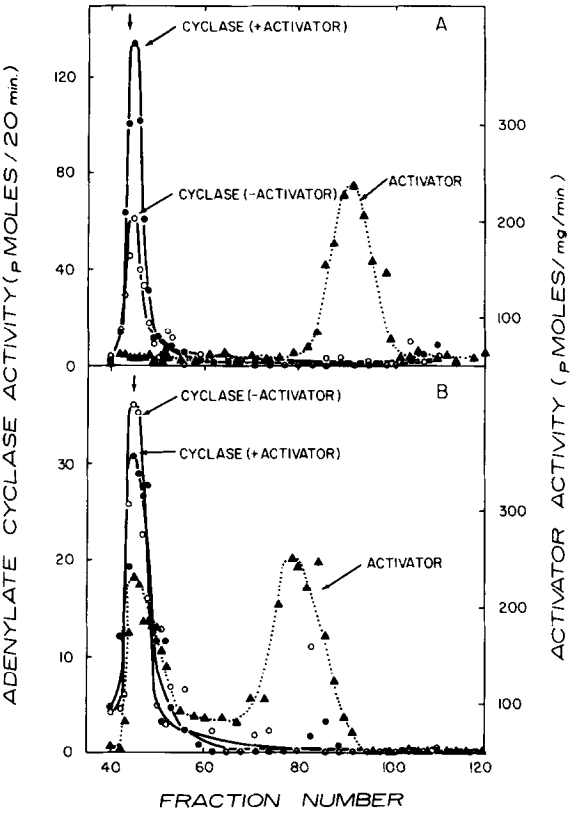

Fig. 1. Ca^{++}-dependent association of adenylate cyclase and its protein activator in a Sephadex G-200 column (99 x 2.5 cm). Panel A: The column was equilibrated with a buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM sucrose, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM $MgSO_4$, 0.5 mM DTT and 1% Lubrol-PX (Buffer A) at 4°C. Eight ml of a Lubrol-PX solubilized rat brain adenylate cyclase containing 26.4 mg protein was applied to the column. The column was eluted with Buffer A; fractions of 4 ml were collected at a flow rate of 19 ml/hr. Aliquots were assayed for the activator and for adenylate cyclase in the presence or absence of an exogenous activator highly purified from bovine brain (5). The reaction mixture for adenylate cyclase assays was the same as described in the Materials and Methods except 50 μM [^{3}H]-ATP was used. Panel B: The column was equilibrated with a buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM sucrose, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 mM DTT and 1% Lubrol-PX (Buffer B). Eight ml of a Lubrol-PX solubilized adenylate cyclase containing 26.4 mg protein was loaded on the column, which was eluted with Buffer B. Fractions were collected and assayed as in Panel A. The arrows indicated the void volume of the column.

The protein activator was dissociated from adenylate cyclase by a salt gradient on an anionic exchange resin column (3). The activator, being an acidic protein (5), was retarded in the column by charge. Dissociation of the activator reduced adenylate cyclase to its basal

activity. On the other hand, the inclusion of EGTA in the reaction mixture also diminished adenylate cyclase to its basal activity, even though the enzyme contained sufficient activator (see Table I). The possibility that EGTA inhibits adenylate cyclase by dissociating the activator from the enzyme and hence reduced its activity was examined. An extract containing both adenylate cyclase and the protein activator was passed through a Sephadex G-200 column that had been equilibrated with EGTA. As shown in Fig. 1A, the activity of adenylate cyclase was considerably higher when assayed in the presence of an exogenous protein activator than when assayed in its absence. The differential activity was due to the fact that its endogenous protein activator was eluted after the enzyme and none was detected with the enzyme. This experiment shows that chelation of Ca^{++} by EGTA dissociates the activator from adenylate cyclase, making it less active.

In a separate experiment (Fig. 1B), a similar extract was passed through the column that had been equilibrated with Ca^{++} instead of EGTA. In contrast to the results seen in Fig. 1A, some of the activator was eluted with the enzyme and adenylate cyclase activity was virtually the same in the presence or absence of an exogenous activator. Further, the activator not associated with the enzyme was eluted ahead of its position in Fig. 1A. This probably resulted from association-dissociation of the enzyme and the activator during the course of filtration through the column. It has been shown previously that neither Ca^{++} nor EGTA affected the elution profile of the activator in a Sephadex G-200 column (9). These results suggest that in the presence of Ca^{++} the enzyme and the activator form a complex, whereas in its absence, the two proteins remain separated.

Note that in the presence of Ca^{++} (Fig. 1B) a considerable amount of activator was separated from adenylate cyclase even though the enzyme retained sufficient activator for full activity. This suggested that in the extract the activator was present in excess over adenylate cyclase. This point is more clearly shown in a mixed experiment (Table II), which

TABLE II

Excess of Activator over Adenylate Cyclase

Tube	Additions	Adenylate Cyclase Activity (pmoles/min)
1	Extract	104
2	Activator-deficient enzyme	55
3	(1) + (2)	244

The extract refers to a Lubrol-PX solubilized adenylate cyclase; the activator-deficient enzyme refers to the enzyme eluted from an anionic exchange resin column (3). Adenylate cyclase was assayed as described under Materials and Methods.

compares the activity of an extract and an activator-deficient adenylate cyclase assayed individually to that of a mixture of the two enzymes assayed together. The activity of the mixture was more than the summed activities of the individual preparations, indicating therefore that the activator in the extract was present in excess over adenylate cyclase.

Although the experiments shown in Fig. 1 clearly demonstrate that the formation of the enzyme activator complex is Ca^{++}-dependent, they did not reveal the time for the process to take place. The time for the formation or the dissociation of the enzyme-activator complex was next examined. Because the enzyme-activator complex appears to be the active species, the rate of increase of adenylate cyclase activity in the presence of Ca^{++} would be a measure of the rate of complex formation. Similarly, the rate of decrease of enzymic activity by EGTA would be a measure of the rate of complex dissociation. Fig. 2 shows the rapid change of adenylate cyclase activity as a result of the addition and chelation of Ca^{++}. The initial portion of the curve represents the basal adenylate cyclase activity. Upon addition of Ca^{++} to the reaction mixture, the rate of cAMP formation was increased immediately. The elevated enzyme activity was maintained until EGTA was added, which reduced the activity to its prestimulated level.

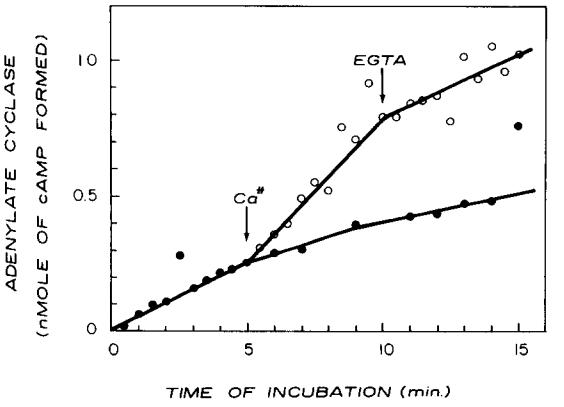

Fig. 2. Effect of Ca^{++} or EGTA on the rate of cAMP synthesis catalyzed by rat brain adenylate cyclase solubilized by Lubrol-PX. A reaction mixture of 5 ml contained 40 mM Tris-HCl (pH 7.5), 40 mM caffeine, 10 mM NaF, 5 mM $MgSO_4$, 0.1 mM EGTA, 4 mM cAMP, 1 mM [^{3}H]-ATP, 4 mM phosphoenolpyruvate, 40 µg/ml of pyruvate kinase and 4.1 mg Lubrol-PX solubilized rat brain adenylate cyclase, which was added last to initiate the reaction. At appropriate times, 0.1 ml aliquots were withdrawn from the reaction system and were transferred to small tubes containing 50 µl of 1 N HCl. At 5 min, 1 ml of the reaction mixture was removed and transferred to another tube, which served as a control, and then Ca^{++} was added to a final concentration of 0.1 mM. At 10 min, EGTA was added to a final concentration of 0.1 mM. [^{3}H]-cAMP was purified from the reaction mixture as described under Materials and Methods.

This experiment demonstrates that both the formation and dissociation of the enzyme-activator complex were rapid and that the process was readily reversible. Because the protein activator binds Ca^{++} and the active species appears to be a Ca^{++}-activator complex (9), stimulation of adenylate cyclase may be depicted as follows:

$$Ca^{++} + \underset{\text{inactive}}{(\text{Activator})} \rightleftharpoons \underset{\text{active}}{(Ca^{++}\text{-Activator*})} \quad ..(1)$$

$$\underset{\text{inactive}}{(\text{Enzyme})} + \underset{\text{active}}{(Ca^{++}\text{-Activator*})} \rightleftharpoons \underset{\text{active}}{(\text{Enzyme-}Ca^{++}\text{-Activator*})} \quad ..(2)$$

where Ca^{++}-activator* depicts a more helical conformation than the Ca^{++}-free activator (10). The stoichiometry of the components in the above equations has not been established; the equations simply depict the necessary interaction of the two proteins.

On the basis of these experiments, it appears that the mechanism of

stimulation of adenylate cyclase by the protein activator is analogous to that of phosphodiesterase (5-7). Because the activator is present in excess in the tissue (16), the activity of both adenylate cyclase and phosphodiesterase could be regulated by the cellular flux of Ca^{++}.

The stimulation of adenylate cyclase and phosphodiesterase by the same protein activator raises a question of its utility in terms of physiological function. The protein activator increases the activity of the cytoplasmic but not the membrane phosphodiesterase. It is believed that the influx of Ca^{++} through the plasma membrane or the release of membrane-bound Ca^{++} in response to stimuli activates adenylate cyclase, leading to an increase of intracellular cAMP. Ca^{++} subsequently arriving at the cytosol then activates the soluble phosphodiesterase, thus returning the elevated level of cAMP to its prestimulated level. The sequential stimulation of the two enzymes would allow transient elevation of cAMP as seen in many tissues. Alternatively, the cytoplasmic phosphodiesterase also catalyzes the hydrolysis of cGMP; in fact, at micromolar concentration of substrates the rate of cGMP hydrolysis exceeds that of cAMP (5). The influx of Ca^{++}, therefore, could increase cAMP and concommitantly decrease cGMP (2,3).

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