PROTEIN ACTIVATOR OF CYCLIC 3':5'-NUCLEOTIDE PHOSPHODIESTERASE OF BOVINE OR RAT BRAIN ALSO ACTIVATES ITS ADENYLATE CYCLASE

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SUMMARY

Bovine or rat brain adenylate cyclase (EC 4.6.1.1) solubilized by Lubrol PX contained an activator which was separated from the enzyme by an anionic exchange resin column. Dissociation of the activator from adenylate cyclase rendered the enzyme less active, and reconstituting with an exogenous activator restored full enzyme activity. A pure protein activator of cyclic 3':5'-nucleotide phosphodiesterase (EC 3.1.4.17) isolated from bovine brain also stimulated this adenylate cyclase. Stimulation of adenylate cyclase by the activator required Ca^{++}, the effect being immediate and reversible. Although the activator was specific, it lacked tissue specificity; an activator isolated from bovine brain cross-activated effectively adenylate cyclase from rat, and vice versa. These findings indicate that brain adenylate cyclase required an activator for activity and that this activator is functionally identical to the protein activator of phosphodiesterase (J.B.C. *249*: 4943–4954, 1974).

Early workers in the field of cyclic 3':5'-nucleotide phosphodiesterase(s) (EC 3.1.4.17) experienced precipitous loss of enzyme activity during the course of purification (1–5). Cheung later found that a protein activator had dissociated from the enzyme; this resulted in apparent loss of enzyme activity. The purified enzyme regained full activity upon reconstitution with the activator (6). The activator from bovine brain has been purified to homogeneity and extensively characterized. It binds Ca^{++}, and the active form appears to be a Ca^{++}-activator complex (7,8). In the presence of Ca^{++} the activator assumed a more helical conformation (9), which may facilitate the formation of the enzyme-activator complex. The sequence of events leading to the activation of phosphodiesterase by the activator may be depicted as follows (7):

$$Ca^{++} + (\text{Activator})_{\text{inactive}} \rightleftharpoons (Ca^{++}\text{-Activator})_{\text{active}}$$

$$(\text{Enzyme})_{\text{inactive}} + (Ca^{++}\text{-Activator})_{\text{active}} \rightleftharpoons (\text{Enzyme-}Ca^{++}\text{-Activator})_{\text{active}}$$

A study on the distribution and developmental changes of phosphodiesterase and its activator in mammalian tissues and cells showed that the ratios of the two activities varied greatly from tissue to tissue as well as during ontogenetic development (10). The apparent variability of the two activities in these tissues may result from cellular heterogeneity, multiple

forms of phosphodiesterase (activator-dependent and activator-independent), or separate regulation of the two proteins (11). Another possibility is that the activator has as yet other undefined cellular function(s).

Both adenylate cyclase and phosphodiesterase from brain exhibit similar requirements for divalent cations. In the presence of molar excess of Mg^{++}, EGTA (ethylene glycol bis (β-aminoethyl ether) N,N'-tetraacetic acid) inhibited the activity of phosphodiesterase from bovine or rat brain (7,12), and Ca^{++} reversed this inhibition. Similarly, EGTA inhibited adenylate cyclase of bovine or rat brain and Ca^{++} reversed this inhibition (13,14).

Experiments described in this communication show that the effects of EGTA and Ca^{++} on brain adenylate cyclase and phosphodiesterase are interrelated and that their effects are mediated through a common Ca^{++}-binding protein, which stimulates both adenylate cyclase and phosphodiesterase *in vitro*. This finding appears similar to that of Brostrom *et al* (15), who showed that a phosphoprotein from porcine brain regulates the activity of adenylate cyclase and phosphodiesterase.

MATERIALS AND METHODS

Chemicals

[^{3}H]-ATP (S.A. 18 Ci/mmole), [^{14}C]-cAMP (S.A. 49.2 Ci/mmole) and [^{3}H]-cAMP (S.A. 27 Ci/mmole) were obtained from Schwarz/Mann or New England Nuclear. IRP-58 (200–400 mesh) was a gift of Rohm and Hass; AG 50W-X8 (200–400 mesh), and Ag 2-X8 (200–400 mesh) were obtained from Bio Rad; Lubrol-PX, phosphoenolpyruvate, and pyruvate kinase were purchased from Sigma Chemical Co.

Preparation of Adenylate Cyclase

A membranous fraction, prepared from bovine or rat brain as described previously (13), was homogenized in two volumes of 40 mM Tris-Cl (pH 7), containing 1% Lubrol PX, and 3 mM dithiothreitol. The suspension was centrifuged for 60 min at 144,000 X g; the clear supernatant fluid, 3 to 6 mg protein/ml, contained an active adenylate cyclase, which is referred to as detergent-dispersed adenylate cyclase.

Assay of Adenylate Cyclase

Crude preparations of adenylate cyclase are usually contaminated with cAMP phosphodiesterase and ATPase. The assay system 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 or 0.5 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. Cyclic AMP was purified from the reaction mixture by the $Ba(OH)_2$-$ZnSO_4$ precipitation, followed by passage through a column of cationic exchange resin (16). Alternatively, cAMP was purified by a scaled-down version of a double-column procedure (13).

Determination of Protein

Protein was determined according to Lowry *et al* (17).

RESULTS AND DISCUSSION

Previous studies showed that many tissue extracts, including bovine and rat brain,

contained large quantities of the protein activator of phosphodiesterase (18), and that the activator was separated from phosphodiesterase by an anionic exchange column (5). Fig. 1 shows that bovine or rat brain adenylate cyclase solubilized by Lubrol-PX contained a similar activator and that it was separated from the enzyme by an anionic exchange column. The majority of adenylate cyclase came out with the void volume, followed by a minor peak of activity. Adenylate cyclase eluted from this column depended greatly on the composition of the reaction mixture. With the inclusion of a pure protein activator of phosphodiesterase (8), adenylate cyclase activity was increased several fold. This experiment indicates that brain adenylate cyclase, like brain phosphodiesterase, requires a protein activator for maximum activity. All the experiments described herein used the enzyme from the first, main peak. The

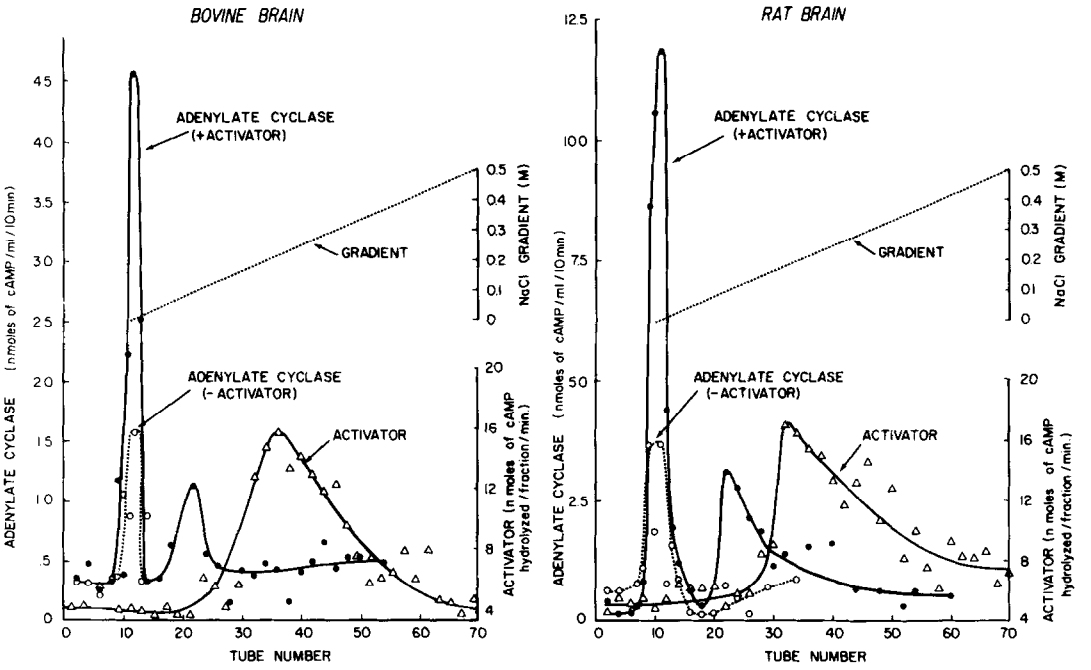

Fig. 1. Preparation of activator-deficient adenylate cyclase solubilized from bovine brain (Panel A) and rat brain (Panel B) with an anionic-exchange column. Fifteen ml (6 mg protein/ml) of solubilized brain adenylate cyclase was applied to an ECTEOLA-cellulose column (1.5 X 30 cm) which had been equilibrated with 20 mM Tris-HCl (pH 7.5), 1 mM $MgSO_4$, 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM dithiothreitol, 1% Lubrol PX, and 250 mM sucrose (Buffer A). Five ml fractions were collected at a flow rate of 40 ml/hr. After the enzyme was loaded on the column, it was washed with Buffer A until 10 fractions were collected. Then the column was eluted with a linear gradient generated by 150 ml of Buffer A containing 0.5 M NaCl, and 150 ml of Buffer A. An aliquot of these fractions was assayed for adenylate cyclase activity in the presence or absence of a pure protein activator of phosphodiesterase isolated from bovine brain (8). Another aliquot was assayed for activator by its ability to stimulate a bovine brain cAMP phosphodiesterase activity (18). Fractions pooled from the major adenylate cyclase peak are referred to as activator-deficient adenylate cyclase.

enzyme associated with the minor peak was not studied further.

The activator, which was eluted after adenylate cyclase, was measured by its ability to stimulate a purified phosphodiesterase from bovine brain. As shown later in Table II, this activator also stimulated adenylate cyclase and is therefore functionally identical to the pure protein activator used to assay adenylate cyclase in Fig. 1 (A and B).

The requirement of the activator for Ca^{++} in the adenylate cyclase system is illustrated in Table I. Neither Ca^{++} nor the activator affected adenylate cyclase activity. EGTA diminished the enzyme activity because the preparation probably contained Ca^{++} (19) and trace of activator (see Fig. 1). In the presence of both, however, the activity was markedly increased.

TABLE I
Requirement of Activator for Ca^{++} in Stimulating the Activities of Brain Adenylate Cyclase or Phosphodiesterase

Additions	Adenylate Cyclase Activity	Phosphodiesterase Activity
	(pmoles/mg/min)	(nmoles/mg/min)
None	65	73
Ca^{++}	77	65
EGTA	37	77
Activator	69	247
EGTA + Activator	80	77
Activator + Ca^{++}	166	298

Activator-deficient adenylate cyclase was prepared from rat brain as described in Fig. 1B. The activator, prepared from bovine brain to homogenity (8) was the same sample used in Fig. 2. Ca^{++} was 100 μM and EGTA was 200 μM. Phosphodiesterase was determined by a two-stage procedure with the batch use of an anionic exchange resin, IRP-58 (23). The reaction mixture in a final volume of 0.1 ml contained 40 mM Tris-Cl (pH 8.0), 3.3 mM $MgSO_4$, 0.1 mM $CaCl_2$ 2 mM [^{3}H]-cAMP (sp. act. 0.9 mCi/mmole) and an appropriate amount of enzyme. Where indicated, EGTA was 0.2 mM and the activator was 2 μg. The incubation was at 30° for 10 min.

Table I also shows the effects of Ca^{++} and the activator on bovine brain phosphodiesterase activity. As shown elsewhere, Ca^{++} or EGTA did not affect the activity of this activator-deficient phosphodiesterase (7). The elevated activity in the presence of the activator observed in Table I probably resulted from trace amounts of Ca^{++} in the reaction system, because chelation of this Ca^{++} by EGTA reduced the activity to basal level (also see Ref. 7). In the presence of Ca^{++} and the activator, however, the activity of phosphodiesterase was increased 4-fold.

The results presented in Table I suggest that the active form of the activator for adenylate cyclase is a Ca^{++}-activator complex, as has been shown for phosphodiesterase (7,8).

Table II documents the lack of specificity of the activator with respect to brain adenylate cyclase. The activator, whether prepared from bovine or rat brain, stimulated adenylate cyclase from these tissues to the same extent. Although the activator lacks tissues

TABLE II

Lack of Species Specificity of Activator on Adenylate Cyclase

Tube	Additions	Activity (pmoles/mg protein/min)
1	Bovine adenylate cyclase	28
2	Bovine activator	0
3	Bovine adenylate cyclase + bovine activator	98
4	Rat adenylate cyclase	36
5	Rat activator	0
6	Rat adenylate cyclase + rat activator	196
7	Bovine adenylate cyclase + rat activator	98
8	Rat adenylate cyclase + bovine activator	182

The reaction mixture contained the standard components and activator-deficient cyclase, or activator or both. Adenylate cyclase and the activator were taken from active fractions of Fig. 1 (A and B).

specificity, its action on brain adenylate cyclase is specific. None of several proteins tested mimicked its stimulation of adenylate cyclase (Table III). Similar findings have been made on phosphodiesterase (18).

Figure 2 shows the time course of stimulation of adenylate cyclase by the activator. When activator was added to the reaction system containing Ca^{++}, adenylate cyclase activity was augmented with no apparent time lag. The enhanced activity was maintained until EGTA was added; thereafter the activity diminished to the level before the addition of the activator. This experiment indicated that the stimulation of adenylate cyclase by the activator was immediate and readily reversible, in a manner similar to that of phosphodiesterase (18,20,21).

The results presented in this communication indicate strongly that the protein activator of brain phosphodiesterase also serves as an activator for brain adenylate cyclase. Several common features of the activator are notable: (1) separation from either enzyme by an

TABLE III

Specificity of Protein Activator on Rat Brain Adenylate Cyclase Activity

Additions	Activity
	(pmoles/mg protein/min)
None	155
Cytochrome c (mol. wt. 12,400; 5 μg)	161
RNase (mol. wt. 12,700; 5 μg)	151
Trypsin inhibitor (mol. wt. 21,500; 5 μg)	149
Ovalbumin (mol. wt. 45,000; 5 μg)	148
DNase (mol. wt. 63,000; 5 μg)	135
Bovine serum albumin (mol. wt. 67,000; 5 μg)	138
Bovine brain activator (mol. wt. 18,900; 5 μg)	709
Rat Brain activator (mol. wt. not determined; 4 μg)	684

The reaction mixture contained the standard components, rat brain activator-deficient adenylate cyclase, and the test protein as indicated in the Table. The bovine brain activator was purified through the stage of DEAE-cellulose chroma tography (8); the rat brain activator was taken from the active fractions of Fig. 1B.

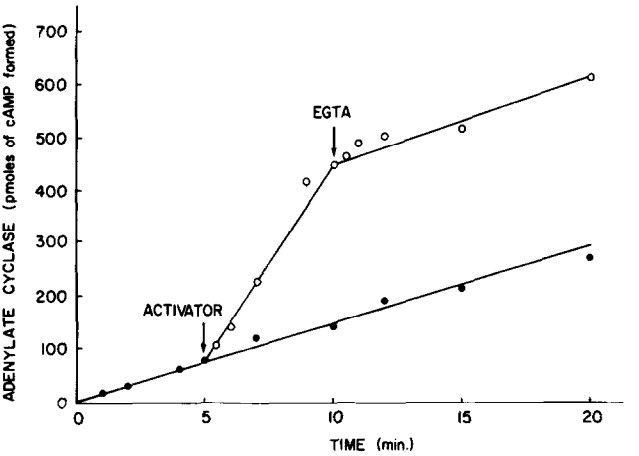

Fig. 2. Reversible stimulation of rat brain activator-deficient adenylate cyclase by the activator. The reaction mixture in a final volume of 3.4 ml contained 40 mM Tris-HCl (pH 7.5) 40 mM caffeine, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM F^-, 1 mM [^{3}H]-ATP, 2 mM cAMP, 4 mM phosphoenolpyruvate, 136 μg of pyruvate kinase and 4 mg of rat brain activator-deficient adenylate cyclase, which was added last to initiate the reaction. Incubation was maintained at 37°. At appropriate times 100 μl aliquots were withdrawn from the reaction system and were transferred to small tubes containing 50 μl of 1 N HCl. At 5 minutes, 1 ml of the reaction mixture was removed and transferred to another tube, which served as a control, and then 50 μg of a pure bovine brain activator (7) was added. At 10 minutes, EGTA was added to a final concentration of 0.4 mM. [^{3}H]-cAMP was purified from the reaction mixture as described under Materials and Methods.

anionic exchange column; (2) requirement for Ca^{++} (3) the activator is specific although it lacks tissue specificity; and (4) the stimulation is immediate and reversible.

Brostrom *et al* (15) reported that a heat-stable, Ca^{++}-binding phosphoprotein stimulated both phosphodiesterase and adenylate cyclase of porcine brain. The pure protein activator from bovine brain contains no phosphate (9). However, in view of the many common physical and chemical properties of the two Ca^{++}-binding proteins (7,8,21), it appears likely that the Ca^{++}-binding protein from porcine brain is identical to the protein activator described here and that the phosphate detected in the porcine brain protein probably comes from contaminants.

In analogy to the scheme depicted for phosphodiesterase (see Introduction), the activity of adenylate cyclase possibly may be viewed as being regulated by the cellular flux of Ca^{++}. According to this scheme, the influx of Ca^{++} through the plasma membrane or the release of membrane-bound Ca^{++} in response to stimuli activates adenylate cyclase, resulting in an increase of intracellular cAMP. Ca^{++} thus made available in the cytoplasmic space stimulates the soluble cytoplasmic phosphodiesterase, which then returns the elevated intracellular levels of cAMP to its prestimulated level. The sequential stimulation of adenylate cyclase and phosphodiesterase could allow momentary elevation of cellular cAMP level in response to a hormone. On the other hand, the cytoplasmic phosphodiesterase also catalyzes the hydrolysis of cGMP; in fact, at micromolar concentration of substrates the rate of cGMP hydrolysis is greater than that of cAMP (7,22). The influx of Ca^{++}, therefore, could result in an increase of cAMP and a concomitant decrease of cGMP (15).

Although membranous fraction from brain exhibits phosphodiesterase activity, this activity is not responsive to Ca^{++} or activator (24). The membrane enzyme is thought to maintain cAMP levels under steady state conditions whereas the cytoplasmic enzyme becomes critical when the cell is confronted with an upsurge of cAMP following perturbation (25). It is believed that Ca^{++} regulates the activity of the cytoplasmic rather than the membrane enzyme.

Finally, a word of caution may be added. The Lubrol-solubilized adenylate cyclase may have properties quite different from those of the enzyme associated with the plasma membrane. The thesis that the activator plays a physiologic role in regulating adenylate cyclase activity would appear more creditable if stimulation of membrane-associated adenylate cyclase by the activator could be demonstrated.

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