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ISOLATION OF AN ACTIVATOR OF MULTIPLE FORMS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE OF RAT CEREBRUM BY ISOELECTRIC FOCUSING

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Summary

An isoelectric focusing technique was used to isolate multiple forms of cyclic nucleotide phosphodiesterase from a 105 000 × *g* soluble supernatant fraction of sonicated rat cerebrum. These separated peaks of activity had isoelectric points of 5.1, 5.6, 6.0, 6.6, 8.0, and 9.0. The activities were not stimulated by an endogenous activator of the enzyme but were inhibited by EGTA treatment. However, activator-sensitive forms of the enzyme could be separated from brain if the preparation of rat cerebrum was dialyzed against an EGTA containing buffer prior to electrofocusing. The procedure was also used to isolate a column fraction that stimulated maximum velocities of cyclic AMP and cyclic GMP hydrolysis. This fraction was itself devoid of phosphodiesterase activity and had an isoelectric point of 4.7.

Introduction

Brain and other tissues contain a protein factor that is capable of activating partially purified cyclic nucleotide phosphodiesterase (3′,5′-cyclic nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) [1—5]. Only certain forms of phosphodiesterase can be activated by the protein activator. For example, activatable forms have been separated by subjecting crude homogenate activities to DEAE-cellulose chromatography [3], gel filtration [6], or polyacrylamide gel electrophoresis [4,7,8]. Moreover, an activator protein, dependent on Ca^{2+} for its activity [6,9,10] and generally incapable of activating crude enzyme homogenates, has been purified to homogeneity [11—14].

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We have recently reported separation of six forms of cyclic nucleotide phosphodiesterase from rat cerebellum using the technique of isoelectrofocusing [15]. Each of the separated activities hydrolyzed cyclic AMP at high (200 μM) and low (0.25 μM) substrate concentrations but only three of the forms hydrolyzed cyclic GMP significantly. Five of the six separated forms displayed multiple cyclic AMP phosphodiesterase kinetic profiles, while none were activated by a crude exogenous activator preparation [3,7]. In this paper we show that (1) phosphodiesterase activity isolated from rat cerebrum by isoelectrofocusing is inhibited by EGTA, (2) that dialysis of a crude brain homogenate against EGTA yields activatable phosphodiesterase activities after separation by isoelectric focusing, and (3) that isoelectrofocusing can separate the activator from all enzyme activity.

Materials and methods

Crude rat cerebral homogenates were prepared in 10 vol. of 40 mM Tris (pH 8.0). The supernatant from a 105 000 $\times$ *g* centrifugation was dialyzed against 1 l of homogenizing buffer containing 1 mM EGTA (2 changes) for 24 h and then 1 l of buffer alone (2 changes) for 24 h. Following dialysis, a sample containing 12 mg of protein was applied to the isoelectrofocusing column as described previously [15].

Cyclic nucleotide phosphodiesterase was measured by the methods of Thompson and Appleman [16] with 0.25 μM cyclic AMP as substrate, Weiss et al. [17] with 200 μM cyclic AMP substrate, and Thompson and Appleman [18] with 20 μM cyclic GMP as substrate.

A crude protein activator was prepared by a modified method of Cheung [3] as previously described [7,8,15]. This preparation was used to study the activation of the separated forms of phosphodiesterase and for isoelectric focusing.

Results

A preparation of rat cerebrum was applied to the isoelectrofocusing column and individual fractions were analyzed for total cyclic AMP (200 μM) phosphodiesterase activity. Fig. 1 shows the rat cerebral enzyme separation profile whn enzyme activities were measured in the absence and in the presence of 0.2 mM or 2.0 mM EGTA. All of the six cyclic AMP phosphodiesterase activity peaks were reduced by the EGTA treatment.

When a cerebral preparation was dialyzed against 1 mM EGTA before its application to the isoelectrofocusing column only four peaks of activity were evident. EGTA treatment reduced recovery of the total phosphodiesterase activity measured in the original homogenate (Table I). Phosphodiesterase activity was assayed in individual focusing fractions using 200 μM cyclic AMP, 0.25 μM cyclic AMP, and 20 μM cyclic GMP substrate concentrations (Fig. 2). Control activities were compared to those in the presence of 1 mM calcium and 10 μg of exogenous brain activator. The original total activity could no be restored by the addition of excess quantities of Ca^{2+} and activator protein. The forms of cyclic nucleotide phosphodiesterase separated on the isoelectric focusing

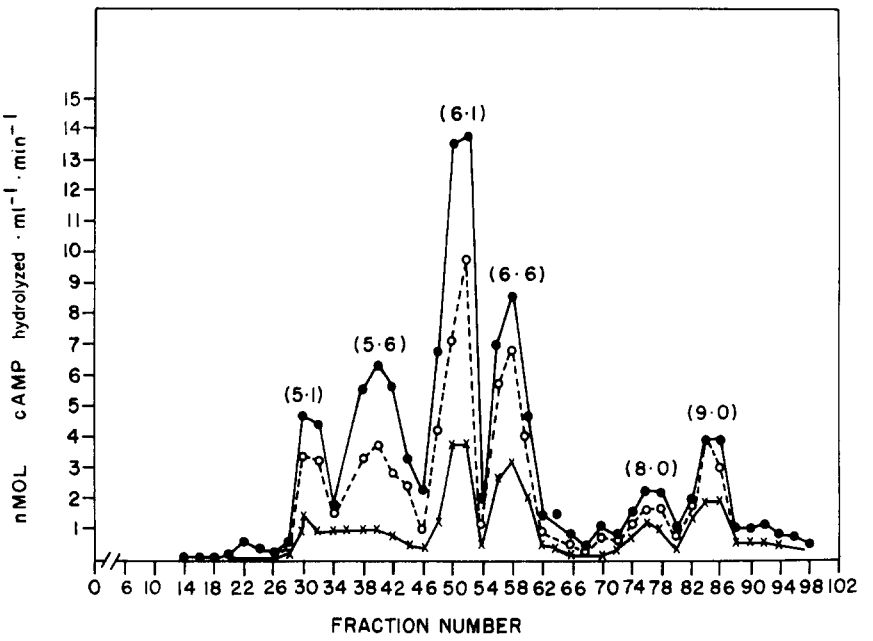

Fig. 1. Rat cerebral preparations were prepared and applied to an isoelectric focusing column as described by Pledger et al. [15]. Column fractions of 25 drops were collected from a nondialyzed preparation. The figure shows activity of cyclic AMP phosphodiesterase measured by the method of Weiss et al. [17] with no EGTA (●———●), 0.2 mM EGTA (○———○), and (X———X) 2.0 mM EGTA.

column after being dialyzed against EGTA had isoelectric points of 5.3, 6.0, 8.0, 9.2. Of the four remaining peaks of activity, the 6.0 form, a peak which hydrolyzed both cyclic AMP and cyclic GMP, showed the most significant degree of activation.

Fig. 3 shows the isolation of an activator fraction from cerebrum by the isoelectrofocusing procedure. Potency of the activation was studied on an activatable form of phosphodiesterase that had been separated by isoelectric focusing (p*I* of 6.0 seen in Fig. 2) or on a form isolated by polyacrylamide gel

TABLE I

RECOVERY OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITIES AFTER ISOELECTRIC FOCUSING SEPARATION

Cyclic nucleotide phosphodiesterase activities of a 105 000 × *g* soluble supernatant fraction of sonicated rat cerebrum were measured at the substrate concentration indicated in parenthesis and recoveries determined after isoelectrofocusing of a nondialyzed preparation (A). A second preparation (B) was dialyzed in EGTA containing buffer as described in Methods. Following application of this preparation to the isoelectric focusing column, individual fractions were analyzed in the absence (B) and presence of 10 μg of activator and 1 mM Ca^{2+} (C).

Treatment	% Recovery of original homogenate activity		
	200 μM cAMP	0.25 μM cAMP	20 μM cGMP
A	29	10	12
B	3	2	1
C	6	3	2

electrophoresis (peak II, data not shown). Identical results were seen with either source of phosphodiesterase.

Kinetic properties were determined for each of the separated peaks of

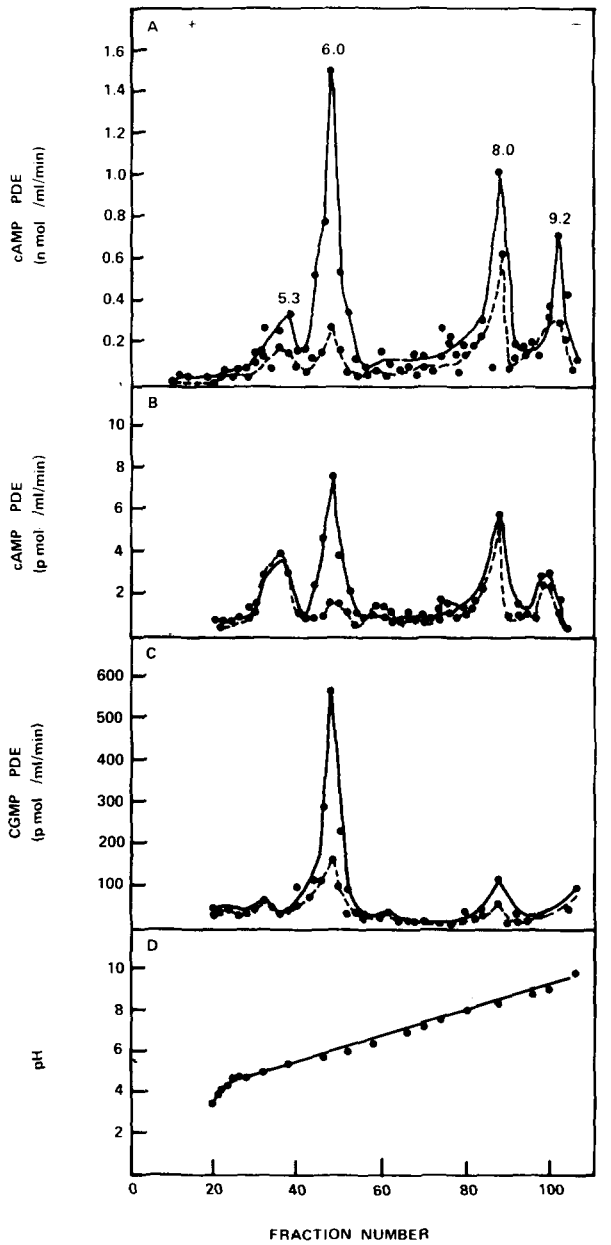

Fig. 2. A preparation of rat cerebrum was applied to a column after it had been dialyzed in 1.0 mM EGTA. Cyclic AMP phosphodiesterase activity with 200 μM cyclic AMP substrate is shown in Panel A. Panel B shows activity measured at 0.25 μM cyclic AMP. Panel C shows the cyclic GMP phosphodiesterase activity using 20 μM substrate. Panel D shows the pH profile of the experiment. In each panel the dashed lines (●- - - - -●) indicate activity in the absence of activator and solid lines (●———●) indicate activity with 10 μg activator and 1 mM Ca^{2+} added.

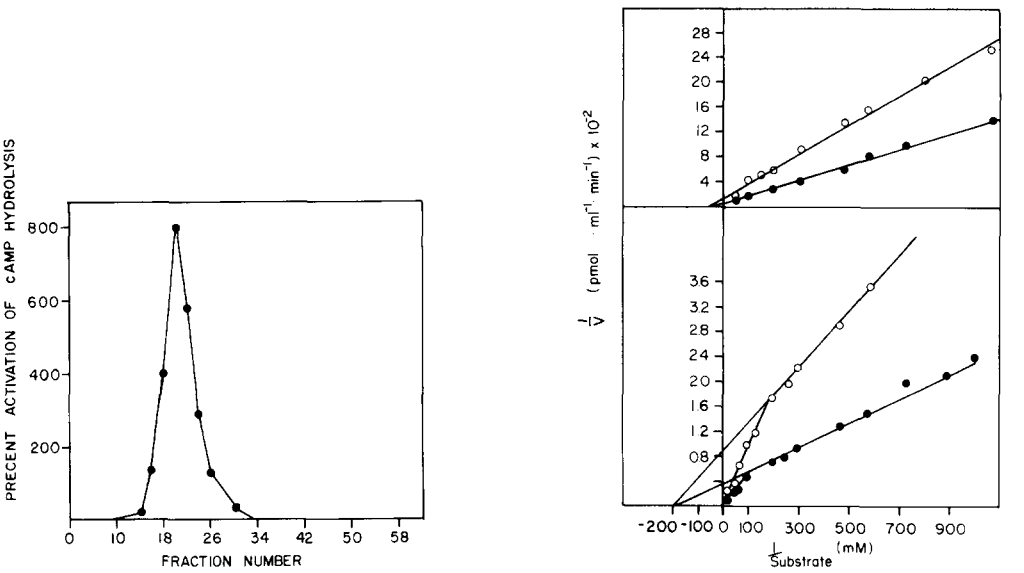

Fig. 3. A preparation of rat brain activator prepared as described by Uzunov and Weiss [7] was applied to the isoelectric focusing column. Aliquots of each fraction were tested for their ability to stimulate an activator-sensitive (p*I* 6.0) peak shown in Fig. 2. Results are expressed as a percent of the phosphodiesterase activity in the absence of added activator when measured at 200 μM cyclic AMP substrate.

Fig. 4. Lineweaver-Burk kinetic analysis of cyclic AMP phosphodiesterase activity was performed on the major isoelectric focused peak (p*I* 6.0) described in Fig. 2 in the absence and presence of 10 μg of activator and 1 mM Ca^{2+} (lower panel). The upper panel shows a similar analysis of kinetic activity using cyclic GMP as substrate. Samples with activator are shown in closed circles and open circles are without added activator.

cyclic nucleotide phosphodiesterase activities shown in Fig. 2 in the absence and presence of exogenous activator. The apparent Michaelis-Menten constants of the cyclic nucleotide phosphodiesterase (p*I* 6.0) were not affected by the addition of excess quantities of Ca^{2+} and activator, whereas the maximum velocities were increased with Ca^{2+} and activator (Fig. 4). Similar effects were noted with other peaks. Addition of 1 mM EGTA (in the assay medium) prevented the subsequent increase in maximum velocities in response to Ca^{2+} and activator.

Discussion

Polyacrylamide gel electrophoresis has been used to isolate a form of cyclic AMP phosphodiesterase activity that is activated by an endogenous protein factor [4,7,8]. The results reported here show that forms of cyclic nucleotide phosphodiesterase that are also sensitive to an endogenous protein activator can be isolated by isoelectric focusing techniques. This procedure was shown to separate the activator from all enzyme activity and the isoelectric point of the activator substance was found to be 4.7. The activator did not significantly affect the apparent K_m of an activatable form of phosphodiester-

ase, but did increase the V of the sensitive phosphodiesterase activity with both cyclic AMP or cyclic GMP as substrates. Our results are in agreement with studies of Weiss et al. [19] which showed that the activator can increase the V of a peak II phosphodiesterase without changing the K_m while others have proposed [3,4] that the activator increases the V and decreases the K_m for the phosphodiesterase. Thus, the mechanism by which the activator increases phosphodiesterase activity is not yet resolved but it seems likely that differences in the types of enzyme and activator preparations used in the various studies contribute to the apparent discrepancies. In this regard, the studies of Teo et al. [11,12] are pertinent since they reported that low concentrations of activator increase the V of phosphodiesterase without altering the K_m whereas higher concentrations of activator reduce the K_m as well.

The isoelectric focusing method offers advantages over procedures such as gel electrophoresis in studying the multiple forms of cyclic nucleotide phosphodiesterase since it can be used to separate both an activator - enzyme complex [15] or an activator-free enzyme (this report).

Four activity forms were evident when the activator was separated from rat cerebral activity by EGTA dialysis before electrofocusing. However, when the preparation was not dialyzed against EGTA prior to focusing six activity peaks of cyclic nucleotide phosphodiesterase were found. These profile differences could be due to differential stabilities of certain peaks in the presence and absence of activator. Since EGTA dialysis can apparently dissociate activator from the enzyme it is possible that the presence of activator is required for the stability of some forms of phosphodiesterase activity. Full recovery of the total homogenate phosphodiesterase activities after physical separation of the enzyme forms was not obtained but recoveries with the isoelectrofocusing technique was slightly better than those found previously with preparative gel electrophoresis [7,8,15]. Treatment of homogenates with EGTA reduced the recovery further, presumably because this treatment dissociates the activator from the enzyme, and it was not possible to add back sufficient quantities of the activator preparation to restore phosphodiesterase activities to pretreatment levels. Preliminary data showed that an apparent low K_m from of cyclic AMP phosphodiesterase was less stable then was a higher K_m form when an EGTA dialyzed cerebral preparation was heated to 45°C (unpublished data). The kinetic data showing a change in maximum enzyme velocity but not in apparent affinity in the presence of activator also supports this contention. Our results suggest the utility of isoelectrofocusing as a tool to elucidate mechanisms involved in the interaction between the activator and multiple molecular forms of cyclic nucleotide phosphodiesterase.

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