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SEPARATION OF MULTIPLE FORMS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASES FROM RAT BRAIN BY ISOELECTROFOCUSING*

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SUMMARY

Multiple forms of cyclic nucleotide phosphodiesterase(s) (EC 3.1.4.–) have been investigated using isoelectric focusing techniques. Six distinct peaks of cyclic AMP phosphodiesterase activity are apparent in a 105 000 × *g* soluble supernatant fraction of sonicated rat cerebellum. These peaks, designated A–F, have isoelectric points (p*I* values) of 4.4, 4.8, 5.0, 6.1, 8.3 and 9.0, respectively, varying affinities for cyclic AMP and cyclic GMP, different kinetic behavior, and divergent subcellular localization. Peaks B, C and D contain appreciable cyclic GMP phosphodiesterase activities, while Peaks A, E and F hydrolyze little or no cyclic GMP. Kinetic analysis of five of the focused peaks showed non-linear Lineweaver–Burk plots closely approximating those of the original cerebellar homogenate. Discontinuous sucrose gradient fractionation before isoelectric focusing indicates that Peaks B, D and E are cytosolic forms and the others appear particulate in nature. In contrast to multiple forms separated by preparative polyacrylamide gel electrophoresis, activity peaks separated by isoelectric focusing do not respond to an endogenous activator of the cyclic AMP phosphodiesterase activity. Since the isoelectric focusing technique has preparative and analytical advantages over other electrophoretic methods, it provides a good procedure to investigate components of the complex cyclic nucleotide phosphodiesterase enzyme system.

INTRODUCTION

Kinetic analysis of cyclic nucleotide phosphodiesterase (EC 3.1.4.–) in homogenates of brain and other tissues shows non-linear kinetic patterns which are suggestive of multiple enzyme forms [1]. However, such data are also explicable in terms of one enzyme exhibiting negative cooperativity [2]. More definitive evidence for the existence of multiple forms of phosphodiesterase in brain and other tissues has come from physical separation of enzyme forms by gel filtrations [3–7], starch gel electrophoresis [8, 9], and polyacrylamide gel electrophoresis [10–13]. These enzyme

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activities, separable by chromatographic and electrophoretic techniques, have been shown to possess different molecular weights, substrate affinities, kinetic properties, stabilities, and sensitivities to endogenous and pharmacological activators and inhibitors.

Isoelectrofocusing is an electrophoretic procedure often used to separate heterogeneous proteins [14, 15]. We have investigated this method as a technique to study multiple phosphodiesterase activities. The data thus obtained support further the existence of multiple molecular forms of cyclic nucleotide phosphodiesterase in mammalian tissues and demonstrate the preparative, analytical, and comparative utility of the isoelectrofocusing technique.

METHODS AND MATERIALS

Cerebella of 250–300 g male Sprague–Dawley rats were homogenized in 3 vol. of 0.32 M sucrose (4 °C). In sonication experiments, a Labline ultra tip sonicator was used at a setting of 50 W (30 s/ml). Both the sonicated and non-sonicated cerebellar preparations were centrifuged at 105 000 × *g* for 1 h. The 105 000 × *g* supernatant fractions referred to as "soluble" cyclic nucleotide phosphodiesterase activities were then electrofocused. Electrofocusing was performed using an LKB 8101 apparatus. Linear sucrose gradients (10–40%, w/v) containing 1% ampholine carrier ampholytes (pH 3–10) in 100 ml volume were employed. The anodal solution was H_2SO_4 and the cathode solution was NaOH. Enzyme preparations (15–20 mg protein in 2–3 ml) were applied to the column. A Brinkman Lauda K-2/R circulating, cooling bath maintained the column at 2–4 °C.

Electrofocusing was performed for 48 h using an LKB 3371E D.C. power supply. A constant voltage of 560 V was applied with an initial amperage of 12–14 mA which dropped within 24 h to less than 1 mA. About 100 fractions of 1 ml each were routinely collected.

Cyclic AMP phosphodiesterase activity (EC 3.1.4.17) was measured by the method of Weiss et al. [16] when 200 μM cyclic AMP substrate concentration was employed, or by the method of Thompson and Appleman [17] with 0.25 μM cyclic AMP substrate. Cyclic GMP phosphodiesterase activity was measured according to Thompson and Appleman [3] using 20 μM cyclic GMP substrate concentrations. Linearity was established for incubation time and appropriate dilutions.

Polyacrylamide disc gel electrophoretic separations of cyclic nucleotide phosphodiesterase were performed by modifications of the procedure described by Uzunov and Weiss [10]. A sonicated 105 000 × *g* soluble supernatant fraction of rat cerebellum was applied to a 7% acrylamide column (60 mm; Poly-Prep 200, Buchler Instruments); the column had been pre-electrophoresised for 2 h at 25 mA. Buffer concentrations were as follows: upper buffer, 0.5 M Tris and 0.05 M glycine (pH 8.9); lower buffer, 0.1 M Tris–HCl (pH 8.1); elution membrane buffer, 0.43 Tris–acetate (pH 7.6) containing 3.75 mM 2-mercaptoethanol; gel buffer, 0.75 M Tris–HCl (pH 9.3). Following application of the sample to the gel, current was maintained (− to +) at 25 mA until protein entered the gel; the current was then increased to 60 mA. Approx. 200 fractions in 1-ml volumes were collected at a flow rate of 12 ml/h at 4 °C.

A heat-stable non-dialyzable activator of cyclic AMP phosphodiesterase

activity was prepared by the procedure of Cheung [18] as modified by Uzunov and Weiss [10]. Potency of the activator preparation was tested by adding varying quantities to phosphodiesterase fractions. Activity changes with the activator added were compared to basal enzyme activities measured with equivalent amounts of bovine serum albumin.

Protein concentration was assayed by the method of Lowry et al. [19] using bovine serum albumin (Fraction V) as standard.

Acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate, and *N,N, N',N'*-tertramethylethylene diamine were purchased from Eastman Organic Chemicals and ampholytes (pH 3–10) from LKB Instruments.

RESULTS AND DISCUSSION

Six peaks of cyclic AMP phosphodiesterase activity were observed when sonicated cerebellar homogenates reached isoelectric neutrality on an electrofocusing column (Fig. 1a, b). The six peaks designated A, B, C, D, E and F had p*I* values of 4.4, 4.8, 5.0, 6.1, 8.3 and 9.0, respectively. Fig. 1 (Panels a and b) compares a representative activity profile when cyclic AMP phosphodiesterase activity was measured with 200 or 0.25 μM cyclic AMP substrate concentrations. Closely similar activity profiles are apparent irrespective of the substrate concentration of cyclic nucleotide employed; the ratios in activities of the individual peaks were 1:2:2:3:1:1 with either 200 or 0.25 μM cyclic AMP.

When cyclic GMP phosphodiesterase activity was measured in these fractions (Fig. 1, Panel c) only three major peaks and one minor peak of activity were found. The major fractions of cyclic GMP phosphodiesterase activities appeared in Peaks B, C and D. The ratios of the activities of these peaks were 1:8:12:19.

Approx. 40% of the cyclic AMP phosphodiesterase activity (measured with 200 or 0.25 μM substrate concentration in the original 105 000 $\times$ *g* sonicated preparation) was recovered with isoelectrofocusing.

Six peaks of soluble cyclic AMP phosphodiesterase activity have been previously demonstrated in sonicated cerebellar preparations by preparative polyacrylamide gel electrophoretic separation [10, 20]. With this technique an endogenous factor that stimulates the activity of two of the six peaks was found in fractions other than those containing phosphodiesterase [10]. In these experiments, a similar activator preparation produced a 6-fold activation of an electrophoretically isolated Peak II cerebellar phosphodiesterase but did not stimulate any isoelectrofocused peak of cyclic AMP phosphodiesterase. We have been unable to obtain activation of a responsive Peak II phosphodiesterase activity using material present in the six activity peaks separated by electrofocusing.

Kinetic analysis of the focused peaks (Fig. 2) revealed non-linear Lineweaver–Burk kinetic plots which paralleled closely those observed in the original homogenate preparation [3, 21]. Thus the activator studies and kinetic analyses would suggest that, in contrast to polyacrylamide gel electrophoresis, isoelectric focusing technique separates cyclic nucleotide enzyme forms while preserving the characteristics of homogenate enzyme activity.

Experiments were conducted to test the effects of sonication on soluble and particulate forms of enzyme activities. When a 105 000 $\times$ *g* supernatant fraction of

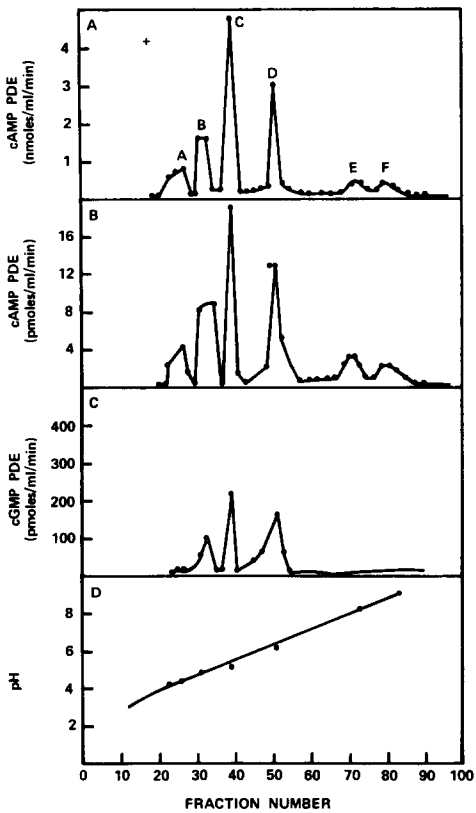

Fig. 1. Rat cerebellum was homogenized in 3 vol. of 0.32 M sucrose, sonicated, and centrifuged at 105 000 × *g* for 1 h. 3 ml of the 105 000 × *g* "soluble" supernatant was applied to an isoelectrofocusing column. The sample was isoelectrofocused for 48 h in a 10–40% (w/v) sucrose gradient containing 1% carrier ampholytes (pH 3–10). 100 fractions were collected and cyclic nucleotide phosphodiesterase activities measured in the individual fractions. (A) Cyclic AMP phosphodiesterase activities measured in 20-μl aliquots at high substrate concentrations (200 μM) by the method of Weiss et al. [16]. (B) Cyclic AMP phosphodiesterase activities measured in 0.1-ml aliquots at low substrate concentrations (0.25 μM) of cyclic AMP according to Thompson and Appleman [3]. (C) Cyclic GMP phosphodiesterases activities measured in 0.1-ml aliquots at 20 μM substrate concentrations as described in Methods. (D) pH profile of the fractionation.

cerebellum was sonicated, the activity profiles (Fig. 3) differed markedly from those observed when the homogenate was sonicated prior to ultracentrifugation. Only Peaks B, D and E of cyclic AMP phosphodiesterase activity with p*I* values of 4.8, 6.1 and 8.2, respectively, as compared to the six peaks observed previously, were apparent. Kinetic studies revealed that these three peaks of activity have high and low affinities for cyclic AMP and that only Peaks B and D hydrolyzed cyclic GMP. Three peaks of cyclic AMP phosphodiesterase activity have also been seen when a soluble cerebellar preparation is sonicated and subjected to polyacrylamide disc gel electrophoresis (unpublished data). It appears, therefore, that the B, D and E forms of the enzyme are cytosolic and that A, C and F may be particulate enzyme forms. This is also supported by the recovery of three peaks (B, D and E) in 105 000 × *g*

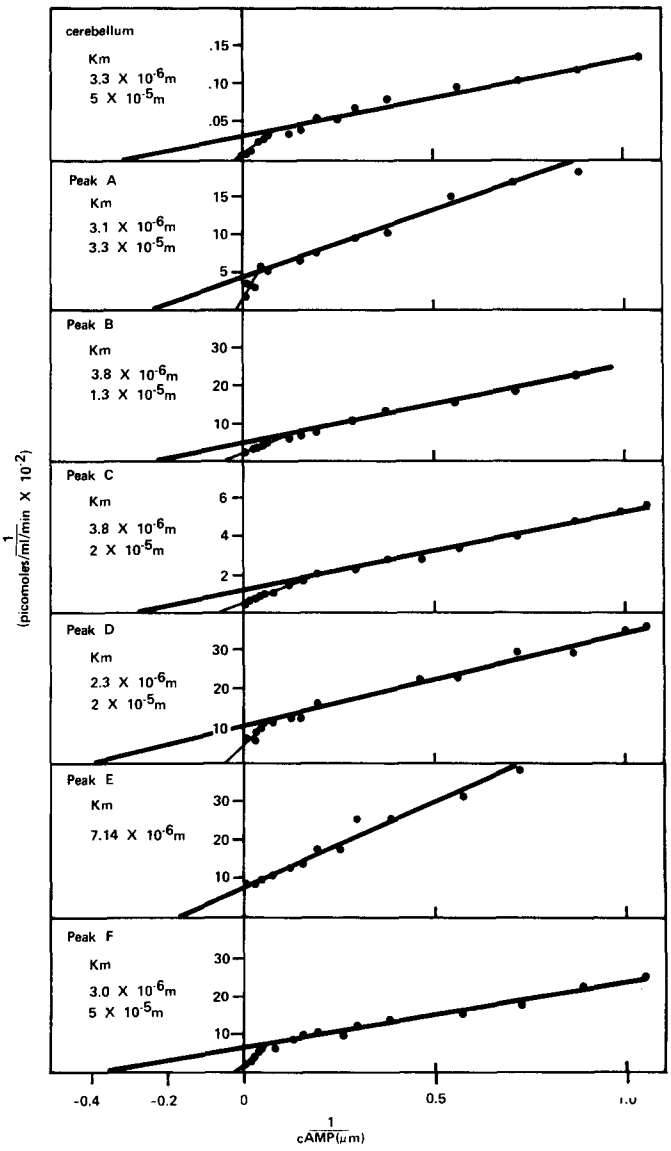

Fig. 2. The original cerebellar homogenate applied to the electrofocusing column and peak fractions of cyclic AMP phosphodiesterase activity indicated in Fig. 1 were analyzed kinetically. The apparent K_m values listed in above panels were extrapolated from double reciprocal plots as described previously [3].

supernatants from non-sonicated cerebellar homogenates applied to the isoelectric focusing column.

Although these experiments provide additional information on the subcellular distribution of individual enzyme forms, the actual origins of each of the six peaks seen in the sonicated preparations subjected to isoelectric focusing or gel electrophoresis requires further delineation. Nevertheless, differential centrifugation, soni-

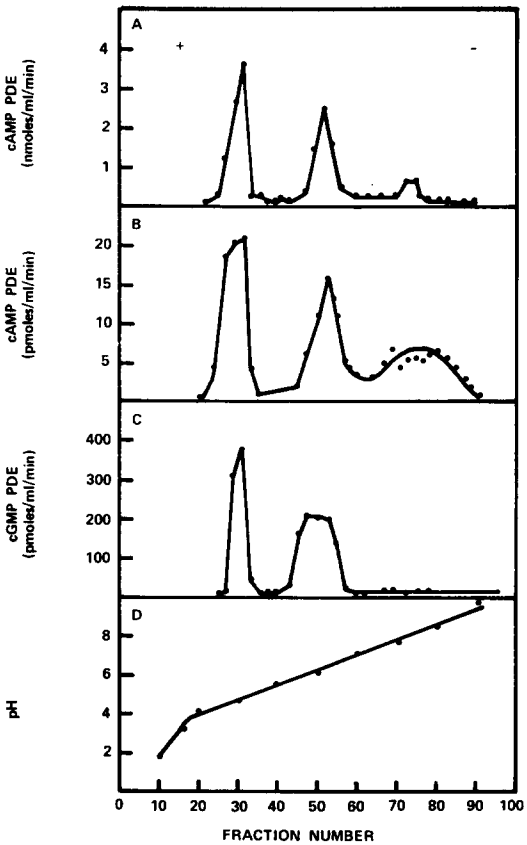

Fig. 3. Rat cerebellum was homogenized in 0.32 M sucrose, centrifuged at 105 000 × *g* to obtain a non-sonicated 105 000 × *g* soluble supernatant fraction. This fraction was then sonicated at a 50-W setting (30 s/ml) prior to its application to an isoelectrofocusing column. Isoelectrofocusing procedures were identical to those described in Fig. 1 legend. (A) Cyclic AMP phosphodiesterase activities measured in 20-μl aliquots at 200 μM substrate concentrations. (B) Cyclic AMP phosphodiesterase activities measured in 100-μl aliquots at 0.25 μM substrate concentrations. (C) Cyclic GMP phosphodiesterase activities measured in 100-μl aliquots at 20 μM substrate concentrations. (D) pH profile of the fractions.

cation, and electrophoretic analysis have indicated the presence of soluble and particulate enzyme forms.

Whereas sonicated or non-sonicated homogenate or soluble supernatant fractions of rat cerebellum display anomalous kinetic patterns [21] with Michaelis constants approximating 10^{-4} and 10^{-6} M cyclic AMP [17], electrophoretically separated peaks exhibit only high K_m forms of the enzyme. In contrast, most of the electrofocused peaks display multiple kinetic plots (Fig. 2). Since an endogenous cyclic AMP phosphodiesterase activator has been reported to affect the affinity as well as the velocity of semi-purified phosphodiesterase preparations [11, 18, 22], it is tempting to speculate that differences in activator responsiveness between electrophoretic and focused forms accounts in part for the different kinetic properties. These studies provide additional evidence that cyclic nucleotide phosphodiesterase(s) exist in several

different molecular forms. In agreement with previous investigations [10, 20], six forms were found in a 105 000 × *g* soluble fraction of sonicated rat cerebellum. Differences in the properties of the forms separated by isoelectrofocusing and those separated by gel electrophoresis were the lack of activator separation by focusing techniques and the different kinetic properties of these peaks.

Isoelectrofocusing thus appears to be a useful tool to be be used singly or in convert with other fractionation techniques towards the isolation and characterization of multiple molecular forms of cyclic nucleotide phosphodiesterase.

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