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SEPARATION OF MULTIPLE MOLECULAR FORMS OF CYCLIC ADENOSINE-3',5'-MONOPHOSPHATE PHOSPHODIESTERASE IN RAT CEREBELLUM BY POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY

The soluble supernatant fraction of rat cerebellar homogenates was subjected to electrophoresis on a preparative polyacrylamide gel column, and the eluted fractions were analyzed for phosphodiesterase activity. We found six distinct peaks of phosphodiesterase activity (designated I to VI according to the order in which they emerged from the column) and a discrete fraction containing a potent activator of phosphodiesterase. These peaks of phosphodiesterase activity had different enzymic properties. Thus the activator of phosphodiesterase increased the activity of Peak II about 4-fold and the activity of Peak VI about 2-fold, whereas the activities of Peaks I, III, IV and V were unaffected by the activator. Calcium increased the activity only of Peak II. The six peaks of phosphodiesterase also had markedly different stabilities, Peaks III IV and VI being the least stable having a half-life of about one day when stored at 4 °C. A comparison of these results with those obtained with astrocytoma and neuroblastoma cells suggests that the different molecular forms of phosphodiesterase may reside in different cell types.

INTRODUCTION

Cyclic nucleotide phosphodiesterase, the enzyme that catalyzes the hydrolysis of cyclic adenosine 3',5'-monophosphate (cyclic 3',5'-AMP)[1] and other cyclic nucleotides is not a simple molecular entity, but rather it exists in several molecular forms. This was shown by a variety of experimental approaches. As early as 1968, Brooker *et al.*[2] showed by kinetic analyses that a soluble supernatant fraction of rat brain homogenates contained two forms of phosphodiesterase activity, one with an apparent K_m of about $1 \cdot 10^{-4}$ M and one with a K_m of between $1 \cdot 10^{-5}$ M and $1 \cdot 10^{-6}$ M. Since then Thompson and Appleman[3] and several other investigators[4–6] have confirmed and extended these observations in other mammalian tissues. Thompson and Appleman[7] separated three forms of phosphodiesterase from rat brain by Agarose gel and Sephadex filtration. These enzymes had different molecular weights, Michaelis con-

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stants and substrate specificities. Kakiuchi and co-workers[8–11] isolated by Sepharose column chromatography two phosphodiesterases from rat brain. The activity of one form of this enzyme was increased by calcium and by a heat-stable, non-dialyzable factor present in tissue. This activator of phosphodiesterase has been shown earlier to be present in snake venom[12] as well as in brain[13]. Monn and Christiansen[6] demonstrated by starch-gel electrophoresis four bands of phosphodiesterase activity in a soluble supernatant fraction of rat brain homogenates. However, since they did not isolate these enzymes, they were unable to study the kinetics or other properties of these zones of activity.

Recently, Weiss and Strada[14–16] showed that the ratio of the high K_m and low K_m phosphodiesterase was markedly different in the various areas of the rat brain, with the cerebellum having a relatively high percentage of the low K_m phosphodiesterase when compared with most other areas of the brain. Moreover, they showed that the rate at which these different forms of the enzyme develop ontogenetically varied with the brain area.

It is apparent from these studies that the isolation, purification and characterization of the molecular forms of phosphodiesterase would be an important and promising avenue of research, both for understanding the physiological role of these enzymes and for finding specific pharmacological agents which could selectively alter the activity of each different form of the enzyme.

MATERIALS AND METHODS

Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethyl ethylenediamine was obtained from Eastman Organic Chemicals, Rochester, N.Y. Glycine and Tris were purchased from Sigma Chemical Co., adenylate kinase (EC 2.7.4.3) and pyruvate kinase (EC 2.7.1.40) from Boehringer, Mannheim. Firefly luciferin–luciferase from E.I. Dupont de Nemours and Co. and cyclic 3',5'-AMP from Calbiochem. The cyclic 3',5'-AMP was purified before use by the procedure described by Krishna *et al.*[17].

The molecular forms of phosphodiesterase were separated by polyacrylamide gel disc electrophoresis[18,19]. In order to study the different forms of the enzyme after isolation, we used a preparative polyacrylamide electrophoresis apparatus[20] (Shandon Sci. Co.) which allows the elution and collection of the separated material as it emerges from the bottom of the gel column.

A 7.5% acrylamide gel, 11 cm in height, was prepared with 0.37 M Tris–HCl buffer (pH 9.3). The column was washed for 2 h with the same buffer by applying a current of 25 mA.

The enzyme material was prepared by homogenizing rat cerebella in 3 vol. of 0.32 M sucrose, sonicating for 5 min and centrifuging at 100 000 × *g* for 1 h. About 25 mg protein in 2 ml were placed on the column beneath the electrolyte buffer (Tris–glycine, 0.08 M, pH 8.3). Current was applied with a voltage and current-regulated DC power supply (Buchler Instruments), and the eluted material was collected in 1-ml fractions with an LKB 7000 Ultrorac Fraction Collector by passing a stream of 0.43 M Tris–acetic acid buffer (pH 7.6), adjusted to a flow rate of 12 ml/h, across the lower end of the gel column. The electrophoresis was continued for 20 h. For the first h the current was 25 mA. After the protein entered the gel, we increased the current to 60 mA and kept this amperage constant throughout the remainder of the separation.

About 250 fractions in all were collected, accounting for about 25% of the initial phos phodiesterase activity. The entire procedure was conducted in a room maintained a 4 °C. At the conclusion of the separation the temperature of the gel was 6 °C.

The phosphodiesterase activity of each fraction was measured by the method o Weiss *et al.*[21], using 10 μl of sample and $2 \cdot 10^{-4}$ M cyclic 3',5'-AMP as substrate in a total volume of 100 μl. In this procedure, the 5'-AMP formed from the hydrolysis o cyclic 3',5'-AMP is converted stoichiometrically to ATP by the addition of exces myokinase and pyruvate kinase. ATP is then assayed by the firefly luciferin–luciferas technique, the generated light being detected with a Luminescence Biometer (Dupon de Nemours and Co.,). This procedure is particularly suited for this type of study a it is sensitive enough to assay the phosphodiesterase activity in samples containin ng quantities of protein, and it is simple and rapid enough to perform several hundre assays a day.

A heat-stable, non-dialyzable activator of phosphodiesterase was prepared ac cording to the procedure described by Cheung[22]. Several grams of whole rat brain wer homogenized in 3 vol. of water and the pH was adjusted to pH 5.9. The suspensio was centrifuged for 30 min at 13 000 $\times$ *g*. The supernatant fluid was placed in a boiling water bath for 5 min. After cooling, the preparation was again centrifuged fo 30 min at 13 000 $\times$ *g* and the supernatant fluid was subjected once more to heatin and centrifugation. The supernatant fluid was finally dialyzed for 24 h against 20 m glycylglycine buffer (pH 7.5) with two changes of buffer. The samples were diluted t 5 mg protein per ml and were stored frozen.

Protein content was monitored with an LKB ultraviolet absorptometer an each fraction was subsequently assayed by the method of Lowry *et al.*[23].

RESULTS AND DISCUSSION

Fig. 1 depicts the results of one of several similar experiments in which we sub jected the soluble supernatant fraction of rat cerebellar homogenates to polyacryl amide gel electrophoresis. As may be seen, there are at least 6 distinct peaks of phos phodiesterase activity, none of which coincides with either of the two major protei peaks, but which do coincide with other minor peaks of protein. Peaks I, II, III, V an VI appeared consistently in each of these experiments, but the relative phosphodi esterase activity of the peak IV varied considerably among experiments and ma represent a very labile or inducible form of the enzyme. When assayed in the presenc of optimum quantities of the heat-stable activator prepared as described by Cheung[22] the ratio of the phosphodiesterase activities among Peaks I through VI was estimate to be 1:57:91:43:16:21.

This activator, however, did not stimulate each peak of phosphodiesteras equally. For example, phosphodiesterase activity in Peaks I, III, IV and V was unaf fected by the activator, whereas the activator (5 μg protein) produced a 4-fold in crease in the activity of Peak II and about a 2-fold increase in the activity of Peak V (Fig. 2).

We next asked whether the peaks (I, III, IV and V) not responding to the acti vator were truly unresponsive or were they already activated maximally by an acti vator bound to the enzyme. This possibility was examined by assaying combination of the different peaks. In each case the total activity was equal to the sum of thei

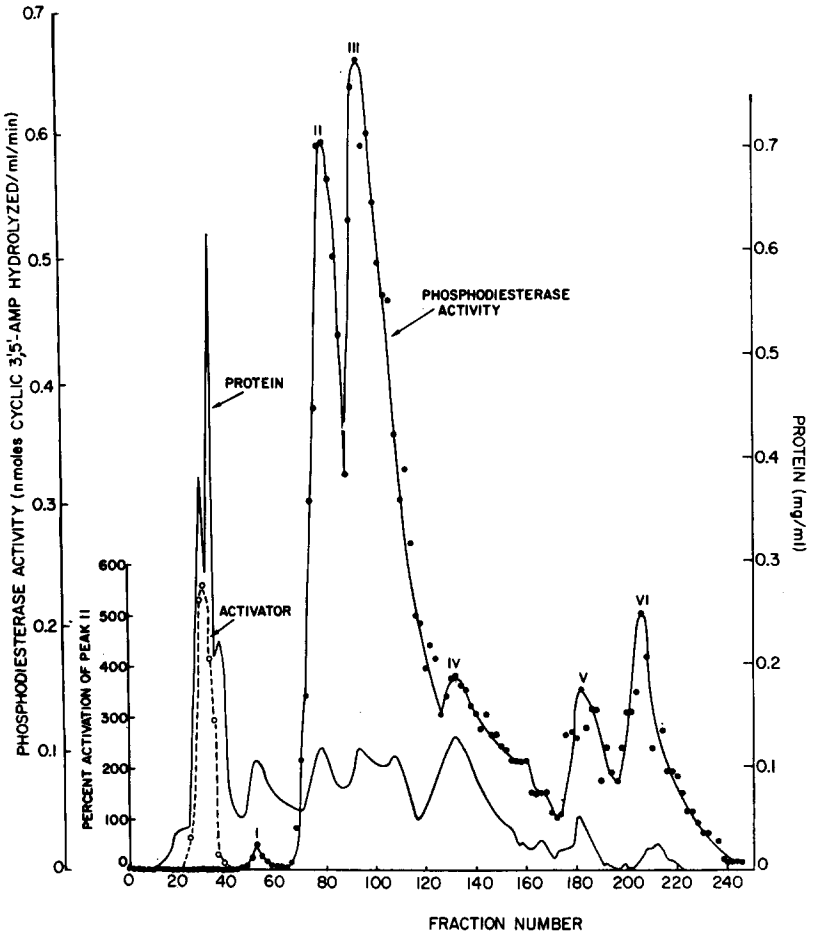

Fig. 1. Separation of multiple forms of phosphodiesterase and isolation of an activator of phosphodiesterase in rat cerebellum by polyacrylamide gel electrophoresis. A soluble 100 000 × *g* supernatant fraction of rat cerebellum was prepared and subjected to polyacrylamide gel electrophoresis as described in Materials and Methods. Each fraction was analyzed for phosphodiesterase activity and protein content as described in the text (solid lines). The presence of an endogenous activator of phosphodiesterase (interrupted line) was determined as follows: 10-μl portions of the phosphodiesterase of Peak II (which can be activated by Cheung's activator) was incubated with 10 μl of each of the 250 fractions eluted from the column and the phosphodiesterase activity was measured. Only in Fractions 26–38 was the phosphodiesterase activity greater than the sum of the individual activities. These fractions (26–38) had no phosphodiesterase activity themselves but markedly increased the activity of Peak II. In the figure we have plotted the percent increase in the phosphodiesterase activity of Peak II induced by each 10-μl portion of the column eluate.

individual activities. Moreover, taking advantage of the fact that phosphodiesterase is inactivated by heating but the activator is not, we added a heated preparation of the various peaks to the phosphodiesterase of Peak II; no increase in the activity of Peak II was noted, again indicating that the unresponsive peaks of activity did not possess an activator bound to the enzyme. These results are different from those reported by Cheung[22] who showed that a boiled purified preparation of phosphodiesterase increased the activity of another unboiled preparation separated on DEAE-cellu-

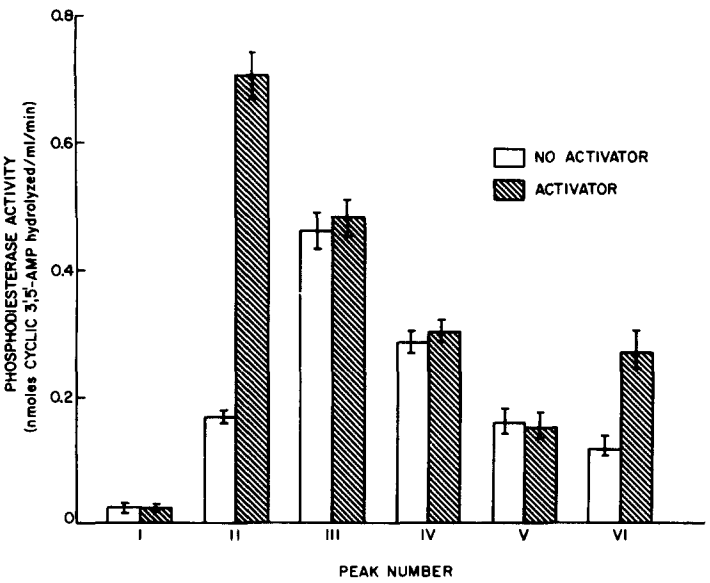

Fig. 2. Effect of a phosphodiesterase activator on the multiple forms of phosphodiesterase of rat cerebellum. An activator of phosphodiesterase was prepared as described by Cheung[22]. Phosphodiesterase activity was determined in each of the 6 peaks of phosphodiesterase of rat cerebellum (shown in Fig. 1) in the presence of this activator (equivalent to 5 μg protein) and in the presence of 5 μg bovine serum albumin (control samples). Each value represents the mean of 10 experiments. Vertical brackets indicate the standard error.

lose columns. The difference may be in the relative purities of the samples. Perhaps polyacrylamide gel electrophoresis separates the activator from the enzyme more completely than does DEAE-cellulose chromatography.

This finding that the endogenous activator of phosphodiesterase, prepared as described by Cheung[22], produced different effects on the various phosphodiesterase peaks was intriguing and stimulated us to study whether an activator of phosphodiesterase was eluted from the polyacrylamide column. Accordingly, we assayed each fraction eluted from the column for the presence of an activator of phosphodiesterase by incubating a portion of each of these fractions with the phosphodiesterase of Peak II. As may be seen from the interupted line in Fig. 1, the activator emerges from the column as a sharp peak coinciding exactly with the first major protein peak and preceding all the bands of phosphodiesterase activity. This activator does not possess phosphodiesterase activity, and it is similar to that described by Cheung[22] in that it also is heat-stable and non-dialyzable, and both activators increase the V of the phosphodiesterase.

Besides having different responses to the activator, the isolated peaks of phosphodiesterase showed several other distinguishing characteristics. Thus Ca^{2+} (5 μM) induced about a 2-fold increase in the phosphodiesterase activity of Peak II but did not significantly alter the activity of any of the other peaks. The stability of the different peaks of phosphodiesterase activity and the degree to which albumin stabilized these preparations were also markedly different. The 6 peaks of enzyme activity were collected and stored at 4 °C in the absence or presence of 1% bovine serum albumin.

Enzyme activity was determined periodically during a two-week period. In the absence of albumin, the half-life for the phosphodiesterase activity of Peaks III, IV, V and VI was approx. 1 day, whereas that of Peak II was about 1 week and that of Peak I was more than 2 weeks. When the preparations were stored in the presence of albumin the following results were obtained: Phosphodiesterase activity of Peak I was unaffected by albumin. This was not surprising since even in the absence of albumin the activity of Peak I did not decrease appreciably. Albumin extended the half-life of phosphodiesterase activity of Peak II by 2-fold and that of Peaks IV and VI about 10-fold. Moreover, there was practically no reduction of phosphodiesterase activity of Peaks III and V over a 2-week period when 1% albumin was included in the samples, whereas during this same period, in the absence of albumin, the phosphodiesterase activity of Peaks III and V declined to only about 5% of their initial activities.

Another point which must be considered is the Michaelis constant of the various forms of phosphodiesterase. Although the supernatant fraction of rat cerebellar homogenates shows multiple phosphodiesterase activities (one with an apparent K_m of about $1 \cdot 10^{-4}$ M and one with a K_m of between $1 \cdot 10^{-5}$ and $1 \cdot 10^{-6}$ M)[14] we have not as yet been able to demonstrate a purified peak of phosphodiesterase containing solely the low K_m enzyme. Moreover, we were unable to demonstrate the low K_m enzyme in these purified preparations even when we conducted our assays in the presence of the activator of phosphodiesterase which has been reported to reduce the K_m of phosphodiesterase[22]. One explanation is that this high affinity form of the enzyme may become very unstable when purified and separated from its activator. Alternatively, after separation a transformation may occur from the low K_m phosphodiesterase to the high K_m enzyme as has been shown for the phosphodiesterase of slime mold[25].

Thus our studies on the separation and characterization of the cyclic adenosine-3′,5′-monophosphate phosphodiesterases from rat cerebellum showed clearly, in agreement with several previous investigations, that this enzyme exists in several different molecular forms. Unfortunately, available data are not sufficient to enable us to conclude with certainty whether these phosphodiesterases are distinctly different molecules or are subunits of the same enzyme. Further studies including molecular weight analyses by sodium dodecyl sulfate polyacrylamide gel electrophoresis may help resolve this question. Nor have we as yet determined the substrate specificities of each of these phosphodiesterases. Nevertheless, the techniques described in this report provide for the first time the possibility of studying the properties of these multiple forms of phosphodiesterase on a routine basis. For now we can search for specific chemicals which will selectively inhibit the activity of each form of the enzyme, and since the relative activities of these phosphodiesterases are different, not only in different areas of the brain but also in different cell types[25], in the future, one might be able to manipulate the concentration of cyclic 3′,5′-AMP at discrete sites.

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