A BRAIN PROTEIN AND ITS EFFECT ON THE CA^{2+}-AND PROTEIN MODULATOR-ACTIVATED CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

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Summary

Bovine brain cyclic nucleotide phosphodiesterase was found to require higher concentrations of the Ca^{2+}-regulated protein modulator for activation than the heart enzyme. This was due to the presence of an inhibitory factor in brain preparations. The factor could be separated from the enzyme by gel filtration on a G-200 Sephadex column. Rechtromatography of the factor on a G-200 Sephadex column showed a single activity peak with an estimated molecular weight of 110,000. The factor appears to be a protein since it was susceptible to trypsin or chymotrypsin but not to RNase, DNase or amylase. The protein factor specifically counteracts the activation of phosphodiesterase by the protein modulator. This protein factor may be either a new regulator for phosphodiesterase or an additional modulator-regulated enzyme.

Introduction

Cheung (1) and Kakiuchi and Yamazaki (2) independently discovered the existence in mammalian brains of a protein that modulates the activation of a Ca^{2+}-activated cyclic nucleotide phosphodiesterase (3). This protein modulator is also present in other mammalian tissues and animal species examined (4-6). Using a homogeneous preparation from bovine heart we have shown that the modulator is a Ca^{2+} binding protein (7). Similar observations have been made with modulators from many other sources (8-10). The mechanism of phosphodiesterase activation by Ca^{2+} and the protein modulator has been intensively studied (9-13) and a reaction mechanism has been proposed as described in the following scheme:

$$Ca^{2+} + \underset{\text{(Inactive)}}{\text{Modulator}} \rightleftharpoons \text{Modulator-Ca}^{2+} \rightleftharpoons \underset{\text{(Activated)}}{\text{Modulator}^{*}\text{-Ca}^{2+}} \quad (1)$$

$$\underset{\text{(Less Active)}}{\text{Phospho-diesterase}} + \text{Modulator}^{*}\text{-Ca}^{2+} \rightleftharpoons \underset{\text{Phosphodiesterase}}{\text{Modulator}^{*}\text{-Ca}^{2+}} \rightleftharpoons \underset{\text{(Activated)}}{\underset{\text{Phosphodiesterase}^{*}}{\text{Modulator}^{*}\text{-Ca}^{2+}}} \quad (2)$$

The function of the protein modulator may not be restricted to the regulation of the Ca^{2+}-activated phosphodiesterase. Brostrom et al. (14) and later Cheung et al. (15) have demonstrated the activation of a detergent-solubilized brain adenylate cyclase by this modulator. In addition, in many invertebrates and some mammalian tissues, the modulator appears to be in great excess over the Ca^{2+}-activated phosphodiesterase (5,6,16). The observations have led to the suggestion that the modulator has functions in addition to the regulation of cyclic nucleotide metabolism (6, 16).

In this communication, we describe the identification in bovine brain of a protein factor which specifically counteracts the activation of phosphodiesterase by the Ca^{2+}-regulated protein modulator. The possible biological role of this brain protein factor will be discussed.

Materials and Methods

The protein modulator was purified from either bovine brain or bovine heart by a procedure previously described for the heart modulator (17). The preparation of modulator-deficient phosphodiesterase from bovine heart was as previously described (18). A modified procedure was used for the preparation of bovine brain modulator-deficient phosphodiesterase. Frozen (Pel-Freeze) or fresh brains were homogenized in 2½ volumes of 20 mM Tris, 2 mM EDTA, pH 7.5 buffer. The crude extract was purified by the addition of solid $(NH_4)_2SO_4$ to 50% saturation. The pellets were dissolved and dialyzed against a pH 7.0 buffer containing 20 mM Tris, 1 mM imidazole, 1 mM Mg^{2+}, 10 mM β-mercaptoethanol and 0.1 mM EGTA (buffer A). The dialyzed sample was clarified by ultracentrifugation at 40,000 rpm for 1 hr. and then applied to a buffer A-equilibrated DEAE-cellulose column. The column was eluted with a linear NaCl gradient of 0.05 to 0.35 M and the modulator-deficient enzyme was eluted in the NaCl concentration between 0.1 to 0.16 M. Assays of phosphodiesterase and the modulator were as described previously (7, 17).

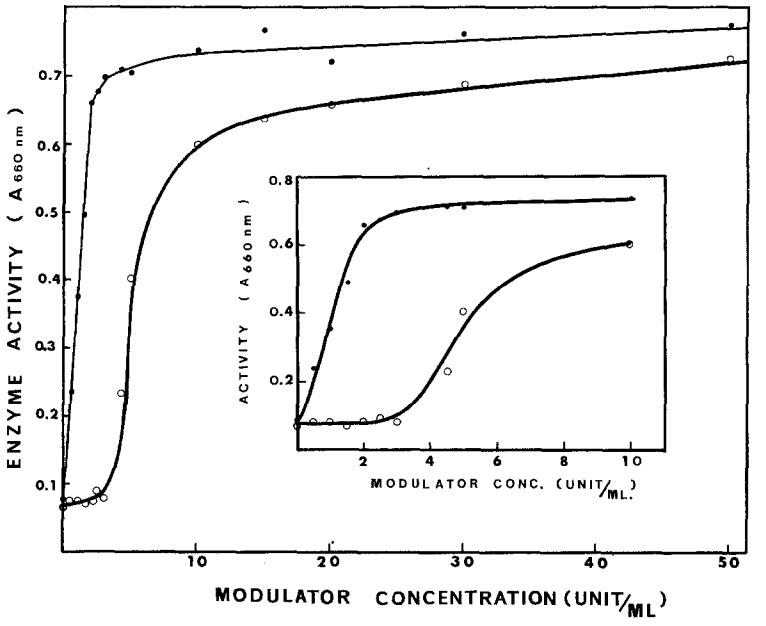

Figure 1 Activities of bovine heart (●) and bovine brain (o) phosphodiesterases were determined in the presence of 0.1 mM Ca^{2+} and varying amounts of the protein modulator purified from bovine brain. Inset: Data at low concentrations of modulator drawn at an expanded scale.

Results and Discussion

Comparison of brain and heart phosphodiesterase activations

Figure 1 compares the dose-response curves for the activation of bovine brain and bovine heart modulator-deficient phosphodiesterase by the Ca^{2+}-regulated protein modulator. The enzyme preparation from brain requires much higher concentrations of the modulator for activation than the heart preparation. In addition, there is a pronounced lag phase in the activation curve of the brain enzyme. The result of Fig. 1 can be accounted for by one of several explanations. Firstly, phosphodiesterases from the two tissues may have different intrinsic properties in terms of their activation by the modulator. Secondly, the brain preparation may contain substances(s) which interferes with the modulator activation of the enzyme. Thirdly, heart preparations may contain factor(s) enhancing the enzyme-modulator interaction.

Removal of interfering factor(s) from the brain enzyme

When the modulator-deficient brain phosphodiesterase was chroma-

tographed on a Sephadex G-200 column, the elution profile of the brain enzyme depended on the amount of protein modulator used in the assay of the eluents. Figure 2 contrasts the elution profiles obtained by assaying the enzyme using 3 and 60 units of the protein modulator. The fractions containing enzyme activity could be divided into two regions: regions A and B. Phosphodiesterase in region A was almost fully activated by 3 unit modulator, similar to the enzyme from bovine heart whereas that in region B was activated only slightly at this low level of modulator, characteristic of the original brain enzyme. The result is in agreement with the suggestion that the original brain preparation contained inhibitory substance(s).

Identification of the protein factor(s)

Since the trailing side of the phosphodiesterase peak from the G-200 Sephadex column appeared to contain the inhibiting factor(s) (Fig. 2), fractions eluted after the peak fractions (Fig. 2, region C) were pooled, concentrated by ultrafiltration using an Amicon PM 10 membrane and then analyzed for phosphodiesterase inhibiting activity. Figure 3 shows that the

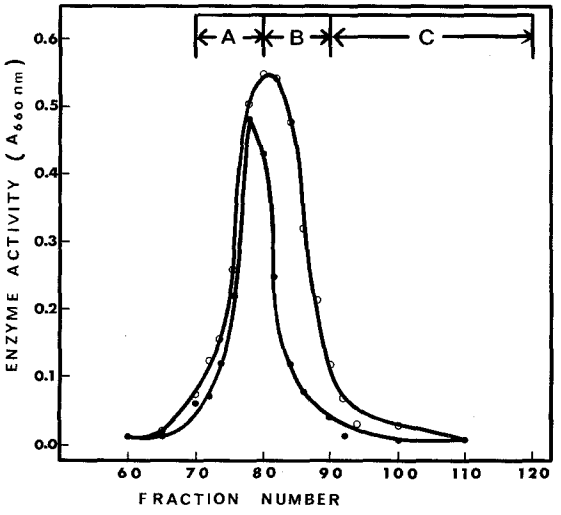

Figure 2 Sephadex G-200 column eluents of the brain modulator-deficient phosphodiesterase were assayed in the presence of 0.1 mM $CaCl_2$ and 3 (●) or 60 (o) units of the modulator.

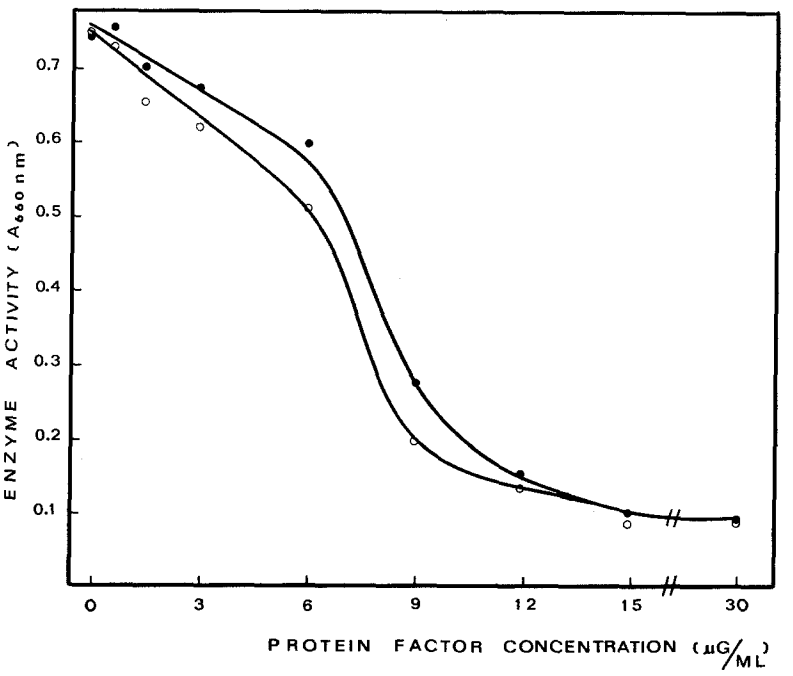

Figure 3 Brain phosphodiesterase from region A of Sephadex G-200 column was assayed in the presence of 3 units of modulator and varying concentrations of the protein factor in the presence of either 0.1 (o) or 1 (●) mM $CaCl_2$. Basal enzyme activity (assayed at 0.1 mM EGTA) was 0.075 $A_{660\ nm}$

activity of a modulator-activated phosphodiesterase was inhibited by increasing amounts of the concentrated sample to approach the non-activated enzyme activity. In a separate experiment, it was found that the sample had little effect on phosphodiesterase activity in the absence of the protein modulator. The results suggest that the sample contains a factor(s) which specifically counteracts the modulator activation of phosphodiesterase. The enzyme inhibition is not due to the chelation of Ca^{2+} by the factor since similar inhibition curves were obtained using either 0.1 or 1 mM $CaCl_2$ in the enzyme assay (Fig. 3).

The inhibitory substance(s) appears to be a protein factor(s), since incubation of the sample at 30° with 10 µg/ml of trypsin or chymotrypsin for 30 min. completely eliminated the inhibitory activity. In these expe-

riments, the protease activity was terminated after the preincubation by the addition of 0.1 mg/ml of lima bean inhibitor which inhibits both trypsin and chymotrypsin. In control experiments while the protease inhibitor was added prior to the addition of the proteases, no effect on the inhibitory activity of the sample was observed. Other hydrolases which included pancreatic RNase, DNase and amylase (DFP-treated) had no effect on the inhibitor activity.

The protein factor was also characterized by gel filtration on a G-200 Sephadex column. Figure 4 shows that the inhibitor was eluted in a single peak with an elution volume slightly greater than that of the enzyme.

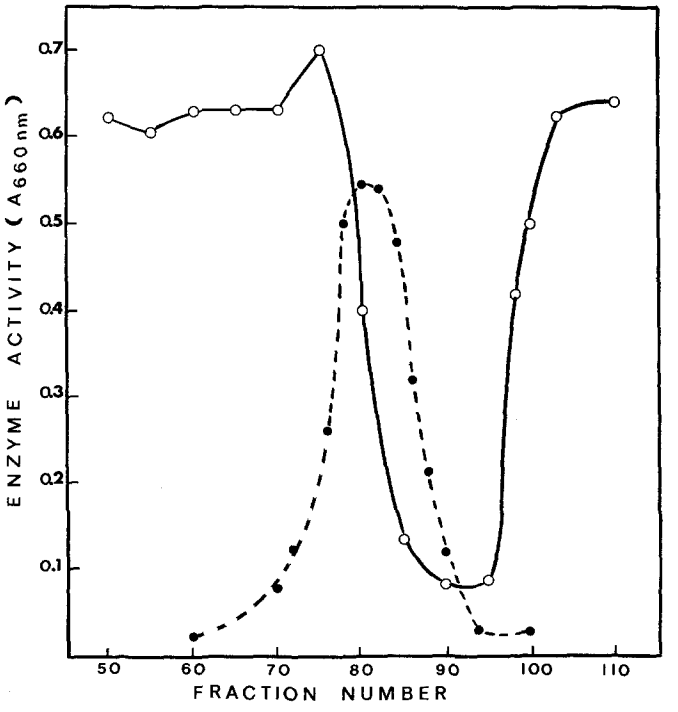

Figure 4 A sample of the pooled and concentrated region C fractions (Fig. 2) was chromatographed on a Sephadex G-200 column and eluents were assayed for inhibitor activity (o—o). Inhibitor assay was carried out as described in Fig. 3. Separate gel filtration was carried out for a sample of the brain phosphodiesterase on the same column and eluents were assayed for phosphodiesterase activity using 60 units of the modulator (•---•).

Molecular weight of the protein factor was estimated on a calibrated G-200 Sephadex column to be approximately 110,000.

In summary, a protein factor which specifically inhibits the activation of phosphodiesterase by the Ca^{2+}-regulated protein modulator has been shown to exist in bovine brain extract. The biological function of the factor is not clear. One possibility is that the protein represents an additional regulatory agent for the Ca^{2+}-activated phosphodiesterase. Alternatively, the factor may be another protein modulator-regulated enzyme or protein which competes with phosphodiesterase for the modulator. Work is now in progress to distinguish these possibilities.

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