A HEAT-STABLE INHIBITOR PROTEIN FOR BOVINE BRAIN CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM *ESCHERICHIA COLI*

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1. Introduction

Calmodulin, which was originally described as an activator of cyclic nucleotide phosphodiesterase (EC 3.1.4.17) [1,2], has been shown to mediate the Ca^{2+}-dependent reactions including activation of several Ca^{2+}-dependent enzymes and demonstrated in various eukaryotes [3–5]. The activation mechanism of Ca^{2+}-dependent enzymes by calmodulin involves the binding of Ca^{2+} to calmodulin followed by the association of the enzyme with the Ca^{2+} · calmodulin complex [3–5]. Inhibitor proteins of Ca^{2+}-dependent phosphodiesterase have been reported in mammalian tissues, and one type of them is heat-stable [6–12]. The inhibitor proteins inhibit the Ca^{2+} · calmodulin-activated phosphodiesterase by forming a complex of inhibitor · Ca^{2+} · calmodulin [6–17]. Namely, the inhibitor proteins are considered to be a kind of calmodulin-binding protein. Furthermore, other kind of calmodulin-binding proteins have also been reported, which functions should be resolved, in various mammalian tissues [13–17]. In other than mammalian tissues, there has been no report concerning a calmodulin-binding protein. We have found calmodulin-like activity and a Ca^{2+}-dependent phosphodiesterase, which is not sensitive to calmodulin, in the soluble fraction of *Escherichia coli* [18,19]. During the course of these studies, a heat-stable and trypsin-sensitive inhibitory factor for bovine brain phosphodiesterase has been found in *E. coli*. Here, the inhibitory factor was partially purified and its physicochemical natures and the mechanism of action in mammalian phosphodiesterase reaction were examined. The results indicate that the inhibitory factor is a protein and combines with calmodulin in the presence of Ca^{2+}, thereby causing an inhibition of phosphodiesterase reaction. The inhibitory factor is tentatively referred to hereafter as the inhibitor protein.

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2. Materials and methods

A strain of *E. coli* (0-143, K-X1) was employed. Calmodulin and calmodulin-deficient phosphodiesterase were prepared from bovine brain by the methods in [20] and [8], respectively. Ca^{2+}-Dependent phosphodiesterase of *E. coli* was purified as in [19]. Calmodulin–agarose affinity column was prepared by the method in [8] employing 18 mg bovine brain calmodulin and 60 ml Sepharose 4B (Pharmacia). *Crotalus atrox* venom (5′-nucleotidase), cyclic AMP, bovine serum albumin, horse heart cytochrome *c*, trypsin, soybean trypsin inhibitor, DNase, RNase and lysozyme were obtained from Sigma. Ovalbumin (twice crystallized) was a product of Nutritional Biochemicals. Other chemicals were obtained from commercial sources.

The inhibitor protein was assayed by its inhibitory activity against Ca^{2+} · calmodulin-activated phosphodiesterase of bovine brain. Prior to assay, the sample was heated for 5 min in a boiling water bath to destroy the endogenous phosphodiesterase activity. The reaction mixture (0.5 ml) contained 40 μmol imidazole–HCl (pH 6.9), 1.5 μmol $MgSO_4$, 150 μmol dithiothreitol, 500 μg bovine serum albumin, 400 nmol cyclic AMP, 500 nmol $CaCl_2$ or 2.5 μmol EGTA, 51 μg

bovine brain calmodulin-deficient phosphodiesterase, 0.33 μg brain calmodulin and the inhibitor protein preparation. The assay was done as in [1]. *E. coli* phosphodiesterase was assayed under the same conditions except that instead of brain phosphodiesterase and calmodulin, 113 μg *E. coli* phosphodiesterase was employed. Protein was determined as in [21]. The M_r-value of the inhibitor protein was estimated by gel filtration on a Sephadex G-100 column (2.5 × 90 cm) as in [22]. The proteins used as standards were bovine serum albumin (M_r 67 500), ovalbumin (M_r 45 000), soybean trypsin inhibitor (M_r 21 600) and horse heart cytochrome *c* (M_r 12 400).

3. Results and discussion

Wet cells (20 g), which were grown aerobically in a nutrient broth and harvested at late logarithmic phase, were sonicated for 5 min with 2 vol. buffer A (20 mM Tris–HCl (pH 7.5), 10 mM 2-mercaptoethanol, 1 mM imidazole, 1 mM magnesium acetate, 0.1 mM EGTA and 0.43 mM phenylmethylsulfonylfluoride). The homogenate was centrifuged for 20 min at 35 000 × *g*. To the supernatant (1634 mg protein) was added a saturated ammonium sulfate solution (pH 7.5) to a final saturation of 55%. The supernatant was dialyzed against 50 vol. buffer A containing 0.2 M NaCl with 3 changes. The sample (356 mg protein) was applied to a DEAE-cellulose (DE-52) column (2.4 × 15 cm) equilibrated with the buffer. After the column was washed with 150 ml buffer, the inhibitor protein was eluted by a 300 ml linear concentration gradient of NaCl (0.2–0.5 M) in buffer A. Fractions 3 ml each were collected. When each fraction of the chromatography was heated and then assayed for inhibitor activity against the Ca^{2+} · calmodulin-activated phosphodiesterase of bovine brain, a single peak of inhibitory activity appeared in the fractions 35–65, as shown in fig.1. These fractions were pooled and concentrated to 12 ml by an Amicon ultrafiltration cell equipped with PM-10 filter. The sample was centrifuged at 100 000 × *g* for 30 min. The supernatant (30 mg protein) was subjected to gel filtration on a Sephadex G-100 column (2.5 × 90 cm) equilibrated with buffer A containing 0.1 M NaCl and 10% sucrose. Elution was performed with the buffer. Fractions 3.3 ml each were collected. The inhibitory activity showed a symmetrical peak on the gel filtration, and the M_r-value was calculated to be ~30 000 (not

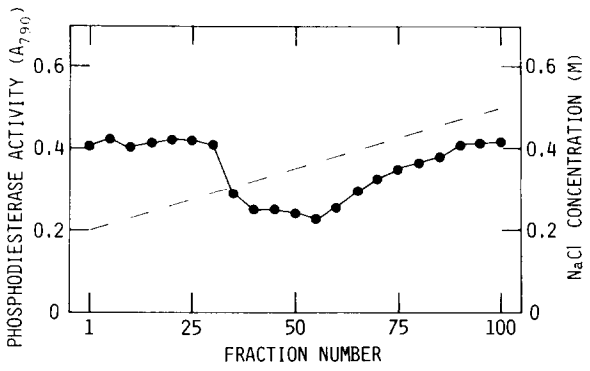

Fig.1. Resolution of heat-stable inhibitor protein on DEAE-cellulose column chromatography employing linear concentration gradient of NaCl (– – –). Detailed experimental conditions are described in the text. A 0.35 ml aliquot of each fraction was boiled for 5 min and then assayed for its inhibitory activity against Ca^{2+} · calmodulin-activated bovine brain phosphodiesterase under the standard conditions in section 2 except that 25.5 μg phosphodiesterase was employed. The enzyme activity in the presence of 1 mM $CaCl_2$ is indicated by closed circle.

shown). Active fractions were pooled and concentrated by the ultrafiltration to 2 ml (7.1 mg protein). This purified inhibitory factor was employed for the following studies. To examine the chemical nature of the inhibitory factor, the purified sample was preincubated with various enzymes. The heat-stable inhibitory factor appeared to be a protein since its activity was readily destroyed upon preincubation with trypsin and not with other enzymes such as DNase, RNase and lysozyme (table 1). As shown in fig.2, the inhibitor protein appeared to inhibit the phosphodiesterase of bovine brain in the presence of both Ca^{2+} and calmodulin, and increasing the amount of the inhibitor protein resulted in increased inhibition of the reaction. The rate of the maximally inhibited reaction approached that of the basal activity of the enzyme in the presence of EGTA. The enzyme activity in the absence of Ca^{2+} (Ca^{2+} was chelated with EGTA) was not affected by the inhibitor protein. A calmodulin-independent enzyme, Ca^{2+}-dependent phosphodiesterase of *E. coli* was not also inhibited by the inhibitor protein. Moreover, an increase in the reaction mixture of Ca^{2+} from 0.1–2 mM did not affect the inhibition of brain phosphodiesterase by the inhibitor protein (not shown). However, high concentration of calmodulin could reverse the inhibition of brain phosphodiesterase by the inhibitor protein. Fig.3 shows the effect of the inhibitor protein on the dose–response curve

Table 1
Nature of the inhibitory factor from *E. coli*

Additions	Treatment of inhibitor	Phosphodiesterase activity (A_{790})
None		0.467
Inhibitor	None	0.042
Inhibitor	Boiled for 2 min	0.049
Inhibitor	Boiled for 5 min	0.049
Inhibitor	Boiled for 10 min	0.046
Inhibitor	Trypsin	0.411
Inhibitor	RNase	0.035
Inhibitor	DNase	0.045
Inhibitor	Lysozyme	0.040

The inhibitory factor (25.6 μg protein) was preincubated in the presence of 50 mM Tris–HCl (pH 7.5), 1mM $MgCl_2$ and where indicated, trypsin (1 mg/ml), RNase (5 mg/ml) or lysozyme (5 mg/ml), in a total volume of 0.2 ml, for 60 min at 30°C. Trypsin inhibitor (soybean) was then added at a final concentration of 1.2 mg/ml to the trypsin-treated sample. The inhibitory factor remaining after these treatments was assayed for its ability to inhibit the activity of bovine brain phosphodiesterase (25.5 μg) supported by brain calmodulin (0.45 μg) and 1 mM $CaCl_2$ under the standard conditions in section 2

of the activation of brain phosphodiesterase by calmodulin. In the presence of the inhibtor protein, the dose–response curve for the enzyme shifted to the right. The amount of calmodulin required to give half-maximal activation of the enzyme increased from

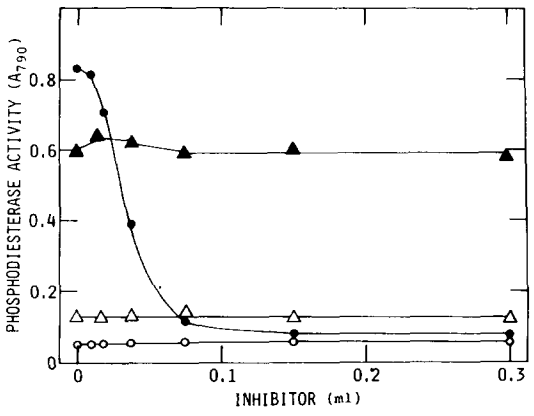

Fig.2. Effect of the inhibitor protein on the activities of bovine brain and *E. coli* phosphodiesterases. Phosphodiesterases were assayed under the standard conditions in the presence of various concentrations of the inhibitor protein as indicated. Circles and triangles indicate bovine brain and *E. coli* phosphodiesterase activities, respectively, in the presence of 1 mM $CaCl_2$ (●,▲) or 5 mM EGTA (○,△).

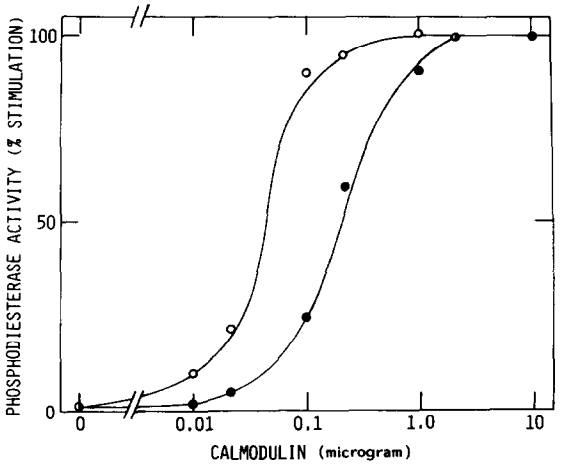

Fig.3. Effect of calmodulin concentration on the inhibition of bovine brain phosphodiesterase by the inhibitor protein. Phosphodiesterase activity in the presence (●) and absence (○) of 1.36 μg inhibitor protein was assayed at various concentrations of calmodulin indicated.

45–170 ng. With a maximum amount of calmodulin, the final activities in the enzyme system were comparable whether the inhibitor protein was present or not. Fig.4 shows the formation of the inhibitor protein · calmodulin complex in the presence of Ca^{2+}. The inhibitor protein was applied to a calmodulin–agarose

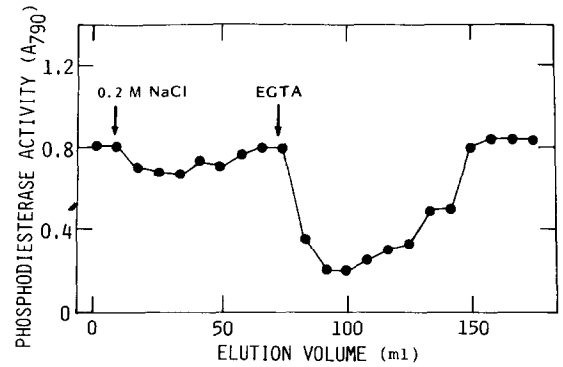

Fig.4. Ca^{2+}-dependent formation of the inhibitor protein · calmodulin complex in a calmodulin–agarose affinity column. The inhibitor protein (1.8 mg) was applied to a calmodulin-coupled Sepharose 4B column (2 × 16 cm) equilibrated with buffer A containing 1 mM $CaCl_2$. After the column was washed by a 60 ml buffer A containing 1 mM $CaCl_2$ and 0.2 M NaCl, as indicated by the arrow, the elution buffer was changed to buffer A containing 5 mM EGTA and 0.2 M NaCl. Fractions 4 ml each were collected. A 0.35 ml aliquot of each fraction was assayed for the inhibitor activity under the standard conditions except that instead of 1 mM $CaCl_2$, 5 mM $CaCl_2$ was employed.

affinity column. In the presence of Ca^{2+}, the inhibitor protein was retained by the column. When the column was washed with a buffer containing EGTA, the inhibitor protein was promptly released from the column. This experiment indicated that in the presence of Ca^{2+}, the inhibitor protein interacted with calmodulin to form the inhibitor protein · calmodulin complex, and that chelating Ca^{2+} with EGTA dissociated the complex into its two components.

These results indicate that the inhibitor protein inhibits the activity of bovine brain phosphodiesterase supported by calmodulin with forming the inhibitor protein · calmodulin · Ca^{2+} complex. The purified inhibitor protein had no activity of phosphodiesterase or myosin light chain kinase, but it is plausible that the protein is another Ca^{2+} · calmodulin-dependent enzyme. Although the exact physiological function of this protein remains unknown at this time, it is clear that the inhibitor protein is a reaction site of calmodulin in *E. coli*. Further extensive studies are now in progress to obtain more detailed information regarding physicochemical properties and physiological significance of the inhibitor protein in *E. coli*.

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