A CALCIUM-DEPENDENT CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM *ESCHERICHIA COLI*

Yasushi IWASA, Kosei YONEMITSU* and Eishichi MIYAMOTO
Department of Pharmacology, Kumamoto University Medical School, Kumamoto 860, Japan

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1. Introduction

Ca^{2+} is considered to have an important role in cellular response to hormonal stimulation; the Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (EC 3.1.4.17) present in many eukaryotic cells [1] plays an important role. In prokaryotes, phosphodiesterases have been reported but are Ca^{2+}-independent [2,3]. The enzyme of *Escherichia coli* requires Fe^{2+} or an activator protein (M_r 90 000) for its activity [2]. Here, we report the occurrence of an additional species of phosphodiesterase in the soluble fraction of *E. coli*. The enzyme is Ca^{2+}-dependent and hydrolyzes both cyclic AMP and cyclic GMP. The existence of Ca^{2+}-dependent phosphodiesterase suggests that, in prokaryotes as well as in eukaryotes, one of the roles of Ca^{2+} in hormonal regulation is acceleration of cyclic nucleotide degradation through the activation of the enzyme.

2. Materials and methods

A strain of *E. coli* (0-143, K-X1) was kindly donated from Dr T. Katsuki (Kumamoto University). The bacterium was cultured at 37°C in a nutrient broth with continuous stirring. After 24 h, the cells were harvested by centrifugation. Calmodulin and calmodulin-deficient phosphodiesterase were purified from bovine brain by the methods in [4] and [5], respectively.

Phosphodiesterase was assayed as in [6]. The standard reaction mixture (0.5 ml) contained 40 μmol imidazole–HCl, (pH 6.9), 1.5 μmol $MgSO_4$, 0.15 μmol dithiothreitol, 500 μg bovine serum albumin, 0.4 μmol cyclic AMP, 0.5 μmol $CaCl_2$ or 5 μmol EGTA and a phosphodiesterase preparation to be assayed. When brain enzyme was assayed, instead of *E. coli* enzyme 1 μg calmodulin and 25.5 μg brain calmodulin-deficient enzyme were contained. One unit of phosphodiesterase activity was defined as that amount of enzyme which hydrolyzed 1 nmol cyclic nucleotide/30 min under standard conditions. Protein was determined as in [7]. The M_r-value of *E. coli* phosphodiesterase was estimated by gel filtration on a Sephadex G-75 column (2.5 × 60 cm) as in [8].

3. Results and discussion

All manipulations were performed at 0–4°C. Wet cells (16 g) were washed 3 times with Tris-buffered saline at pH 7.5, and sonicated for 5 min with 2 vol. buffer A (20 mM Tris–HCl (pH 7.5), 10 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 1 mM imidazole, 0.1 mM EGTA and 0.43 mM phenylmethylsulfonylfluoride). The homogenate was centrifuged for 20 min at 35 000 × *g*. To the supernatant (1290 mg protein) was added a saturated ammonium sulfate solution (pH 7.5) to a final saturation of 55%. The pellet was collected and dissolved in buffer A containing 0.1 M NaCl. The sample was dialysed overnight against 100 vol. same buffer. The dialysate (341 mg protein) was applied to a DEAE-cellulose (DE-52) column (2.5 × 20 cm) equilibrated with the buffer. After the column was washed with 200 ml buffer, elution was performed with a 600 ml linear concentration gradient of NaCl (0.1–0.5 M) in buffer A. Fractions 6 ml each were collected. When each fraction of the chromatography was assayed for phosphodiesterase, two peaks, minor and major, of Ca^{2+}-dependent phosphodiesterase

* Present address: Department of Legal Medicine, Kumamoto University Medical School, Kumamoto 860, Japan

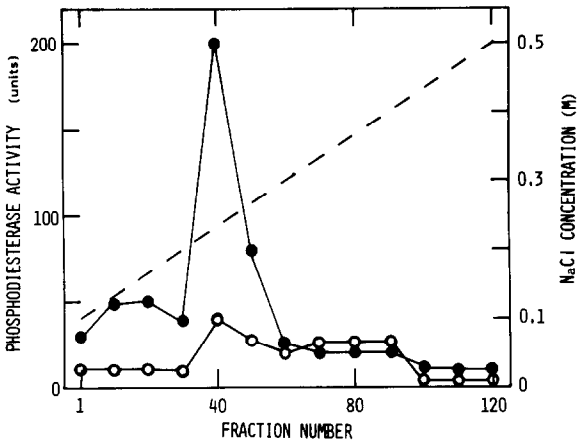

Fig.1. Resolution of Ca^{2+}-dependent phosphodiesterase on DEAE-cellulose column chromatography employing linear concentration gradient of NaCl (----). Detailed experimental coditions are described in the text. Unit of phosphodiesterase activity is defined in the text. A 0.35 ml aliquot of each fraction was assayed under the standard conditions in section 2: (•,○) represent phosphodiesterase activity in the presence of 1 mM $CaCl_2$ and 5 mM EGTA, respectively.

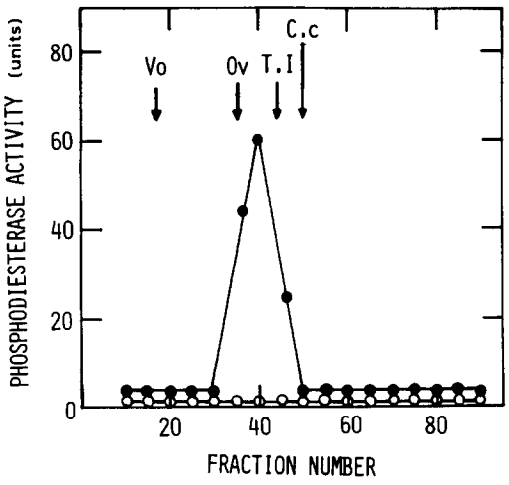

Fig.2. Elution of phosphodiesterase from Sephadex G-75 column. Detailed experimental conditions are described in the text. Symbols are the same as shown in legend for fig.1. Elution positions of 4 markers including blue dextran (V_O, void volume), ovalbumin (Ov, M_r 45 000), soybean trypsin inhibitor (TI, M_r 21 600) and horse heart cytochrome *c* (C. *c*, M_r 11 700) are indicated by arrows.

were found as plotted separately in fig.1. The first minor peak was not studied here. The major peak, fractions 31–54, were pooled and concentrated to 8.5 ml by an Amicon ultrafiltration cell equipped with PM-10 filter. This preparation (178 mg protein) was subjected to a Sephadex G-75 column (2.5 × 60 cm) equilibrated with buffer A containing 0.1 M NaCl and 10% sucrose. Elution was performed with the same buffer, and fractions 3.3 ml each were collected. When each fraction was assayed, a single symmetrical peak was found at a elution volume of 135 ml and calculated to have M_r ~31 000 (fig.2). Active fractions were pooled and concentrated to 5.7 ml (3.5 mg protein) by ultrafiltration. This partially purified enzyme was employed for the following studies. By these purification procedures, the Ca^{2+}-dependent phosphodiesterase was purified 30-fold with an overall recovery of ~4%. Poor purification and recovery were probably due to the removal of activators* in the course of purification as shown in mammalian enzyme [6,9]. The phosphodiesterase required Ca^{2+} for its activity, and effect of varying the concentration of this cation is shown in fig.3. The maximum activity was obtained at 1 mM and app. K_a-value (the concentration needed for half maximum activation) was 5×10^{-5} M. This value was higher than that of brain enzyme. Furthermore, Fe^{2+} and Co^{2+} were 2 times as active as Ca^{2+}

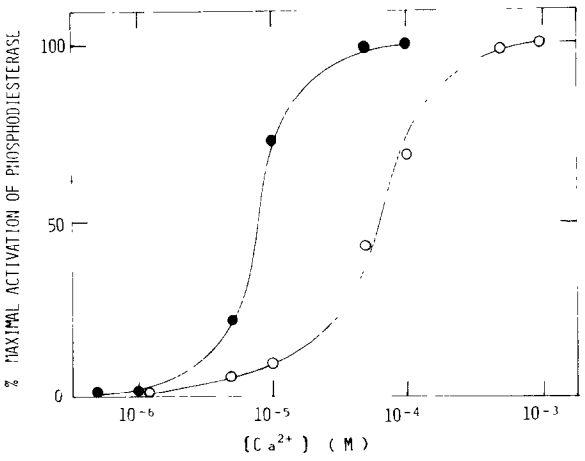

Fig.3. Effect of various concentration of Ca^{2+} on activation of bovine brain (•) and *E. coli* (○) phosphodiesterases. Phosphodiesterases were assayed under the standard conditions except that 80 mM imidazole–HCl (pH 6.8) and different concentrations of Ca^{2+} were employed. When *E. coli* phosphodiesterase was assayed, 186 μg purified enzyme was used. Free Ca^{2+} concentration was varied by use of Ca^{2+}/EGTA buffer [10].

* Two types of Ca^{2+}-dependent activators have been found. One showed an est. M_r 42 000, the other est. M_r 500 000. Details of the activators will be described elsewhere

when tested at 1 mM. Other cations tested such as Zn^{2+}, Mn^{2+}, Ni^{2+}, Sr^{2+}, Sn^{2+}, Ba^{2+}, La^{3+} and Fe^{3+} were inactive. The enzyme hydrolyzed both cyclic AMP and cyclic GMP, and showed a lower app. K_m for cyclic AMP (0.3 mM) than for cyclic GMP (1 mM). The enzyme also showed a greater maximum reaction velocity for cyclic AMP than for cyclic GMP. When brain calmodulin was employed to stimulate the activity of the enzyme, calmodulin could not increase the activity over 2.5–200 μg/ml. One of the major forms of phosphodiesterase from eukaryotic sources is Ca^{2+}-dependent enzyme which reacts with both cyclic AMP and cyclic GMP. The Ca^{2+}-dependent cyclic nucleotide phosphodiesterase of *E. coli* presented here resembles, but is distinguished from, eukaryotic enzymes in several respects, including K_a for Ca^{2+}, cation effect, K_m for cyclic GMP, calmodulin effect and M_r. Nevertheless, this enzyme may be a working point of Ca^{2+} in cellular response to hormonal stimulation in *E. coli*.

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