ADENOSINE 3′:5′-MONOPHOSPHATE PHOSPHODIESTERASE IN CHICKEN ERYTHROCYTES

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1. Introduction

Work on the turnover of adenosine 3′:5′-monophosphate (cAMP) in chicken erythrocytes indicated that the degradation of the nucleotide in these cells follows a first-order reaction [1]. The decay of cAMP levels in these cells is largely due to intracellular degradation, since the release of the nucleotide to the medium is of relatively small magnitude [2]. The rate of cAMP degradation in these erythrocytes was found to be affected by epinephrine [3]. Longer exposure of erythrocytes to epinephrine resulted in a marked decrease in the rate of the degradation of the cyclic nucleotide [3].

Further understanding of the mode of action of agents influencing the rate of the degradation of cAMP in intact cells requires the characterization of the enzyme that carries out this process, i.e., cyclic nucleotide phosphodiesterase. At present no data is available on the phosphodiesterase activities existing in chicken erythrocytes.

Kinetically distinct phosphodiesterases have been separated from a number of tissues [4–8]. The presence of multiple enzyme forms of phosphodiesterase was also observed in many tissues by gel filtration, anionic exchange chromatography and sucrose density gradients [4,5,9–11].

We report here that in chicken erythrocytes only a single phosphodiesterase of a high affinity to cAMP exists, and define some properties of this enzyme.

2. Materials and methods

2.1. *Preparation of phosphodiesterase*

Heparinized chicken blood was diluted with Krebs-Ringer phosphate buffer (pH 7.4) containing 10 mM glucose and centrifuged at 1000 × *g* for 5 min at 5°C. The supernatant fraction and the upper buffy coat of the precipitate were removed by aspiration. The erythrocytes were washed twice with the same buffer. Lysed erythrocytes were obtained by adding 3 vol. 5 mM Tris–HCl buffer (pH 7.5) containing 5 mM 2-mercaptoethanol and centrifuged at 100 000 × *g* for 30 min. The supernatant was used as the source of soluble phosphodiesterase activity. The particulate fraction was treated by 2 different methods:

(a) The pellet fraction was resuspended in 5 mM Tris–HCl buffer (pH 7.5) containing 5 mM 2-mercaptoethanol and sonicated for 100 s in a New Brunswick sonicator. This was followed by centrifugation at 100 000 × *g* for 30 min at 5°C and the supernatant used as source of particulate phosphodiesterase activity.

(b) The particulate fraction was resuspended and homogenized with 1% Triton X-100 in 5 mM Tris–HCl buffer (pH 7.5) containing 5 mM 2-mercaptoethanol. The supernatant after 100 000 × *g* centrifugation for 30 min at 5°C was used as source of particulate phosphodiesterase.

2.2. *Partial purification and gel filtration chromatography of phosphodiesterase*

Lysed erythrocytes were sonicated and centrifuged at 100 000 × *g* for 30 min at 5°C. The supernatant fraction was freed from hemoglobin by a column of DEAE-cellulose [12] as follows: 75 ml lysate were applied to a 30 ml column of DE-52 (Whatman). The column was washed with 2.5 column vol. 3 mM phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol. The initial wash contained no detectable phosphodiesterase activity. A second wash by 2.5

column vol. 10 mM KCl in the same buffer was also without any phosphodiesterase activity. Phosphodiesterase activity was then eluted by 2.5 vol. 600 mM KCl in Tris–HCl buffer (pH 7.6) containing 5 mM 2-mercaptoethanol. To this fraction ammonium sulfate was added to give 60% saturation. The precipitate was collected by centrifugation at 27 000 × *g* for 10 min and was dissolved in 2 ml 20 mM Tris–HCl buffer (pH 7.6), 5 mM 2-mercaptoethanol and dialyzed overnight against the same buffer. This fraction was applied on Sephacryl S-200 (Pharmacia) column (0.9 × 57 cm). Enzyme activity was eluted in 40 mM Tris–HCl (pH 7.6) containing 5 mM 2-mercaptoethanol at 0.84 ml/5 min flowrate.

2.3. *Phosphodiesterase assay*

Phosphodiesterase activity was determined by the two-step assay in [4].

3. Results

3.1. *Subcellular distribution of phosphodiesterase from chicken erythrocytes*

Comparison of the maximal velocities of phosphodiesterase activities present in the soluble and the particulate fractions of chicken erythrocytes is shown in table 1. Correction for contamination of the particulate extract by the soluble fraction was made by measuring the hemoglobin content of the particulate fractions. As indicated in table 1, the majority of phosphodiesterase activity was present in the soluble fraction. The activity of the enzyme in this fraction was $\gtrsim$2-fold higher than that in the particulate fraction.

3.2. K_m *of soluble and particulate phosphodiesterase*

Kinetic analysis of cAMP phosphodiesterase activity in the soluble and particulate fractions of lysed erythrocytes showed linear Lineweaver-Burk plots (fig.1). A single low K_m form of phosphodiesterase with K_m value at ~1 μM for cAMP was observed in the soluble as well as the particulate fraction. The particulate phosphodiesterase showed similar kinetic behaviour regardless of the method of the enzyme solubilization; i.e., either by sonication or by Triton X-100 extraction (fig.1b,c). No other phospho-

Table 1
Subcellular distribution of chicken erythrocyte phosphodiesterase

Fraction	Maximal velocity (pmol cAMP hydrolyzed . ml packed cells^{-1} . 10 min^{-1})
Soluble	1142
Particulate:	
Sonicated	486
Triton extract	466

For details on the preparation of the fractions, see section 2

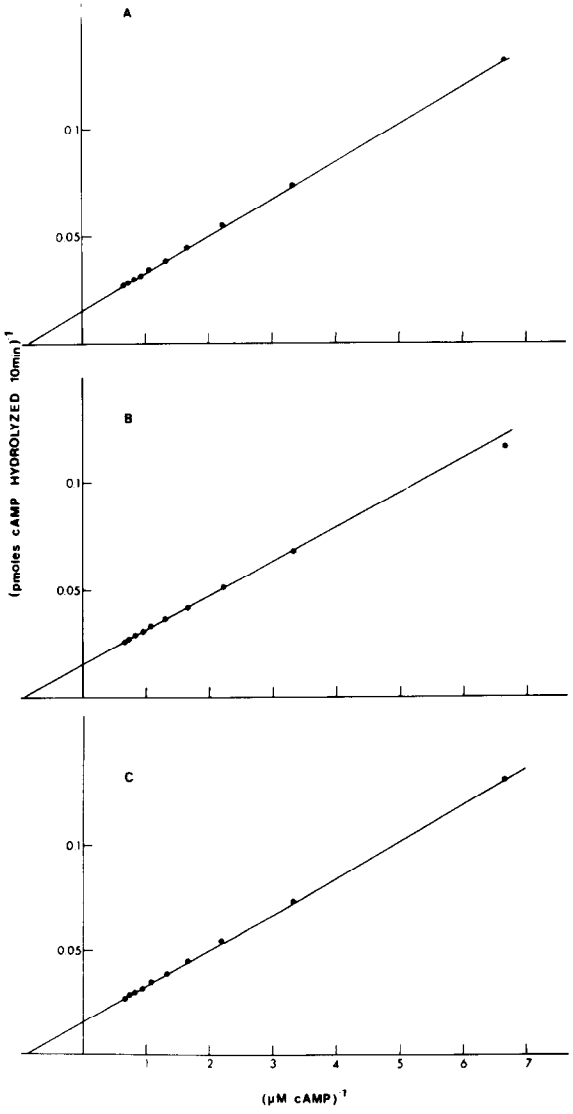

Fig.1. Lineweaver-Burk plot of cAMP hydrolysis catalyzed by subcellular fractions of chicken erythrocytes. (A) Soluble fraction; (B) sonicated particulate fraction; (C) Triton-extractable particulate fraction.

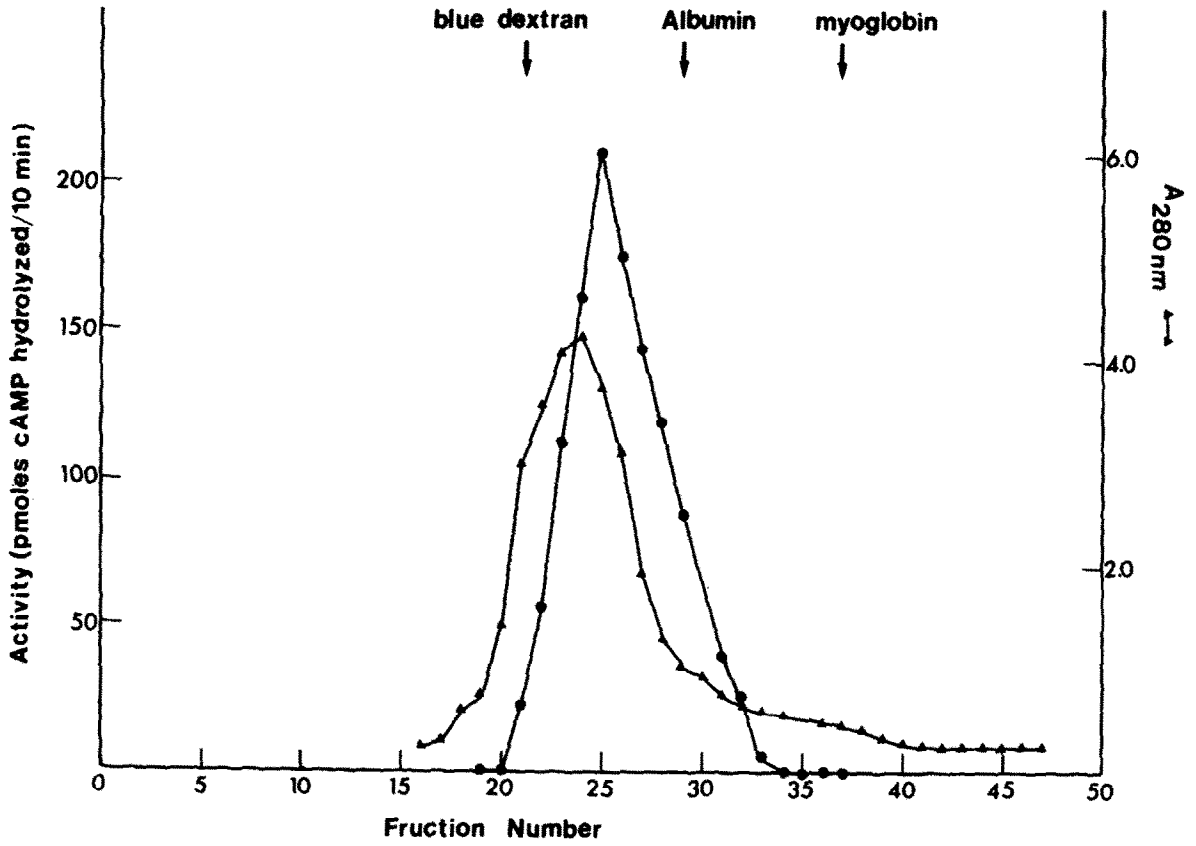

Fig.2. Elution profile of cAMP phosphodiesterase on Sephacryl S-200. Column chromatography was performed as in section 2. cAMP in the assay was 1 μM.

diesterase activity could be detected in any of the fractions when higher cAMP concentrations were used (10–20 μM).

3.3. *Gel filtration chromatography*

A sonicated lysed erythrocyte preparation, in which soluble and particulate phosphodiesterase were present, was partially purified as in section 2 and separated on a Sephacryl S-200 column. The phosphodiesterase activity was consistently eluted as a single peak (fig.2). The enzyme had est. mol. wt ~90 000.

3.4. *Heat stability of the chicken erythrocyte phosphodiesterase*

Soluble and sonicated extracts of the 100 000 × *g* particulate fraction were incubated at 40°C or 45°C for different times. As shown in fig.3, phosphodiesterase activity was found to be very heat labile. The rate of

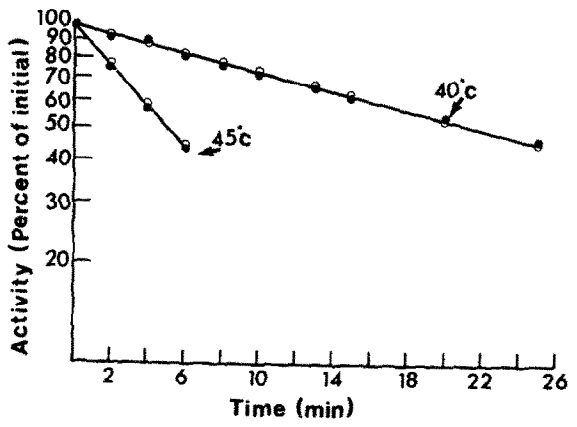

Fig.3. Heat stability of phosphodiesterase present in the soluble or particulate fractions. The soluble (•——•) or particulate (○——○) fractions were incubated at 40°C or 45°C for different times and then transfered to 0°C. The activity of phosphodiesterase in these preparations was assayed with 1 μM cAMP.

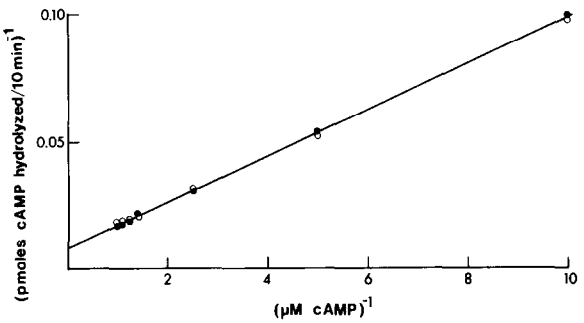

Fig.4. Effect of exposure of intact cells to epinephrine on particulate phosphodiesterase activity. Erythrocytes were preincubated in the absence (●———●) and presence (○———○) of 3 × 10^{-5} M epinephrine for 45 min at 37°C. The cells were lysed and particulate Triton extractable phosphodiesterase was then prepared as in section 2.

enzyme inactivation followed linear first-order kinetics, indicating the presence of a single component. Furthermore, exactly similar rates of enzyme inactivation were found with phosphodiesterases of either soluble or particulate origin.

The half-life times of the heat inactivation of either soluble or particulate phosphodiesterase were around 23 min at 40°C and 5 min at 45°C (fig.3).

3.5. *Effect of epinephrine on phosphodiesterase activity in vitro*

The effect of exposure of intact erythrocytes to epinephrine on the activity of particulate phosphodiesterase in vitro was examined. Erythrocytes were incubated with 3 × 10^{-5} M epinephrine for 45 min, the cells were then lysed and the sonicated extract of the particulate fraction was kinetically analyzed. As shown in fig.4, no hormonal effect on the in vitro phosphodiesterase activity could be detected. Inclusion of 0.1 mM Ca^{2+} or EDTA in the phosphodiesterase assay was without any effect on the enzyme activity in all fractions.

4. Discussion

This study indicates that in chicken erythrocytes only a single species of phosphodiesterase exists. This is based on kinetic analysis, gel filtration and heat stability measurements of soluble as well as particulate fractions of lysed erythrocytes.

In addition to chicken erythrocytes, human lymphocytes and monocytes are the only cell types known at present to contain only one phosphodiesterase form [13]. This is in contrast to the widespread phenomenon of multiple enzyme forms present in many mammalian tissues [5]. In all cell types where a single phosphodiesterase form exists, the enzyme shows high affinity for its substrate and thus resembles the so called low K_m enzyme form. The enzyme in these 3 cell types shows apparent Michaelis-Menten kinetics. This is in contrast to the negative cooperativity of the low K_m form present in tissues consisting of multiple enzyme forms [8]. The K_m value for cAMP of the erythrocyte phosphodiesterase falls within the range of the physiological cellular levels of cAMP.

The phosphodiesterase activity of chicken erythrocytes is eluted in 1 peak on gel filtration with est. mol. wt ~90 000. A large variation of molecular weights of phosphodiesterases from different tissues was reported in [5]. The estimated molecular weight for the low K_m phosphodiesterase in the different tissues is 45 000–200 000 [5,9,13,14].

In frog erythrocytes where 2 forms of phosphodiesterase exist [9], the major entity of the enzyme has the same molecular weight as was found for the chicken erythrocytes phosphodiesterase. However, the frog enzyme shows low affinity for the cAMP substrate.

The phosphodiesterase activity in chicken erythrocytes was found to be located in the soluble as well as the particulate fraction. The activity found in the soluble fraction was twice as much as in the particulate fraction. This was confirmed by eluting the particulate enzyme in 2 different methods: sonication or Triton extraction. There is no evidence at present to tell whether in situ the phosphodiesterase is located only in the particulate fraction or distributed among the particulate as well as the soluble fraction of chicken erythrocytes. The low K_m form of phosphodiesterase was reported to be in the particulate fraction in most tissues [8].

The possible role of epinephrine in regulating phosphodiesterase activity was examined since epinephrine was found to affect the rate of the degradation of cAMP in intact chicken erythrocytes [3]. Unlike the particulate phosphodiesterase from adipose tissue [15] and in accord with observations on the hepatic low K_m enzyme [14], no effect could be observed on particulate Triton-extractable phosphodiesterase activity obtained from eryth-

rocytes preincubated with epinephrine.

In tissues consisting of multiple phosphodiesterase forms, the role of the different enzyme entities in the degradation of cAMP in situ is still questionable. Up to now, when only a single form exists in the cells, it was found to be the low K_m form. It is of interest whether cell types with only high K_m enzyme do exist. A comparison of the in situ degradation of cAMP in cells consisting of single or multiple forms of phosphodiesterase might serve also as a useful tool in understanding the physiological role of the different forms.

Acknowledgement

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