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STUDIES ON ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE OF HUMAN ERYTHROCYTE MEMBRANES

KAZUHIRO SUZUKI [a], TADAO TERAO [b], and TOSHIAKI OSAWA [a,*]

[a] *Division of Chemical Toxicology and Immunochemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, and* [b] *Division of Radiochemistry, National Institute of Hygienic Sciences, Setagaya-ku, Tokyo 158 (Japan)*

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Summary

In this work we show the existence of cyclic AMP phosphodiesterase (EC 3.1.4.17) in human erythrocyte membranes and have clarified some properties of the enzyme. In human erythrocytes, about 23% of the total cyclic AMP phosphodiesterase activity is in a membrane-bound form. Although it could be solubilized with Triton X-100 in 5 mM Tris-HCl buffer (pH 8.0), it was not solubilized by a low or high concentration of salt. The enzyme seems to be localized in the cytoplasmic surface, since it is detected in sealed inside-out vesicles of human erythrocyte membranes, but not in intact human erythrocytes. The optimum pH was found to lie between 7.4 and 8.0, and Mg^{2+} was found to be necessary for its activity. Ca^{2+} and calmodulin could not stimulate the activity of this enzyme. Theophylline was a strong inhibitor, but cyclic GMP could not inhibit the enzymic hydrolysis of cyclic [^{32}P]AMP and this membrane-bound enzyme therefore seems to be specific to cyclic AMP.

Introduction

Cyclic nucleotide phosphodiesterase (EC 3.1.4.17) catalyze the hydrolysis of adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate to the corresponding 5′-monophosphates. These enzymes are heterogeneous with respect to tissue distribution, subcellular localization, substrate specificity,

* To whom correspondence should be addressed.
Abbreviations: Cyclic GMP, guanosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

influence of calmodulin (a Ca^{2+}-dependent regulator protein) and other properties [1–5]. Some of these enzymes are found in a membrane-bound form [1–3,6].

Protein kinase activity has been found in human erythrocyte membranes and a number of erythrocyte membrane proteins are found to be phosphorylated by endogenous protein kinases [7–11]. In human erythrocytes, phosphorylation is necessary to keep the morphological shape of erythrocytes [12,13] and phosphorylation of some of these membrane proteins is regulated by cyclic AMP [7,8,11].

In this paper, we describe evidence of the presence of cyclic AMP phosphodiesterase in the cytoplasmic surface of human erythrocyte membranes and also describe some of the properties of the enzyme.

Materials and Methods

Reagents. 5′-Nucleotidase of *Crotalus atrox* venom and neuraminidase of *Vibrio cholerae* were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cyclic [^{32}P]AMP was obtained from New England Nuclear (Boston, MA, U.S.A.), and the Radiochemical Centre (Amersham, U.K.). Cyclic GMP was purchased from Kohjin Co. (Tokyo, Japan).

Concanavalin A-Sepharose 4B was a product of Pharmacia (Uppsala, Sweden). Purified calmodulin [14] was generously donated by Professor S. Kakiuchi (University of Osaka School of Medicine). All other chemicals used were commercial preparations of the highest purity.

Preparation of human erythrocyte membranes, sealed inside-out vesicles and EGTA-treated membranes. Human erythrocyte membranes were prepared from fresh blood of healthy donors according to the method of Dodge et al. [15] using 5 mM Tris-HCl, pH 8.0. Sealed inside-out vesicles were prepared according to the procedure of Steck [16] using 0.5 mM Tris-HCl, pH 8.0. The vesicles were further purified by passing through a concanavalin A-Sepharose 4B column to remove right-side-out vesicles. Unadsorbed vesicles from this column were used as pure inside-out vesicles in these experiments. The purity of the sealed inside-out vesicles was found to be more than 94% by determinations of sialic acid released after neuraminidase treatment and acetylcholinesterase activity. EGTA-treated membranes were prepared by washing the membranes with 1 mM and 100 μM EGTA in 5 mM Tris-HCl, pH 8.0, according to the method of Sobue et al. [17].

Standard assay conditions for cyclic AMP phosphodiesterase activity. The assay method was essentially the same as that of Raimann and Umfleet [18]. The reaction mixture, in a final volume of 120 μl, contained 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.7 μM cyclic [^{32}P]AMP (2–30 Ci/mmol) and erythrocyte membranes (200 μg protein). After the mixture was incubated for 20 min at 30°C, the reaction was terminated by boiling for 30 s and cooling in an ice bath. 15 μl of 5′-nucleotidase (500 units/ml) in 5 mM Tris-HCl, pH 8.0, were added to the cooled reaction mixture and dephosphorylation of the 5′-[^{32}P]AMP which was formed was accomplished by incubating the mixture for 45 min at 30°C. The inorganic[^{32}P]-phosphate thus liberated was determined on a paper disc (Whatman 3MM

2.4 CMS) according to the method of Raimann and Umfleet [18]. When the effects of various reagents on the enzymic activity were to be studied, they were added to the above reaction mixture at the indicated concentrations.

All the assays were carried out in triplicate. Data are expressed as the mean ± SD. 1 unit is defined as the amount of enzyme which hydrolyzes 1 pmol of cyclic AMP under the standard assay conditions.

Extraction of cyclic AMP phosphodiesterase from human erythrocyte membranes. Packed human erythrocyte membranes (3.6 mg protein/ml) were suspended in 9 vols. of 5 mM Tris-HCl, pH 8.0, containing an appropriate amount of NaCl or 0.33% Triton X-100; or in 9 vols of 1.1 mM EGTA solution containing 5.6 mM 2-mercaptoethanol, at pH 8.0, adjusted with NaOH. Each suspension was stirred gently for 4 h at 4°C, followed by centrifugation at 25 000 × *g* for 60 min to separate the solubilized material from the residue. The residues were then suspended in 5 mM Tris-HCl, pH 8.0, and the supernatants and the resuspended residues dialyzed against 5 mM Tris-HCl, pH 8.0, before assay of cyclic AMP phosphodiesterase activity. When the supernatant contained Triton X-100, it was dialyzed against 5 mM Tris-HCl, pH 8.0, containing 0.3% Triton X-100.

Protein determination. Protein was determined by using the method of Lowry et al. [19] with bovine serum albumin as a standard. When samples contained Triton X-100, a modification of the above method was adopted according to Sugawara [20].

Results

Distribution of cyclic AMP phosphodiesterase activity in human erythrocytes

The distribution of cyclic AMP phosphodiesterase activity in human erythrocytes was determined by measuring the activity in the cytosol and membrane fractions from fresh human erythrocytes. Hemolysates of human erythrocytes in 5 mM Tris-HCl, pH 8.0, were centrifuged as described in Materials and Methods. The supernatants was taken as cytosol fractions and the residues were washed extensively with 5 mM Tris-HCl, pH 8.0, and used as membrane fractions.

About 23% of the total cyclic AMP phosphodiesterase activity of human erythrocytes was present in the membrane fraction as shown in Table I.

The values shown in Table I were obtained when membrane-bound enzymic

TABLE I

DISTRIBUTION OF CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN HUMAN ERYTHROCYTES

Preparation of cytosol and membrane fractions and assay methods were as described in Materials and Methods. Total protein (mg) was determined on 1 ml of packed erythrocytes. Specific activity is expressed as units/mg protein.

Fraction	Total protein	Specific activity	Total units	Distribution (%)
Cytosol	119.3	20.7 ± 0.9	2470 ± 107	77 ± 0.4
Membrane	3.6	205.0 ± 1.2	738 ± 4	23 ± 0.4

activity was measured using intact erythrocyte membranes. When membranes were solubilized with Triton X-100, the activity of the membrane fraction was increased 1.5-fold. The cyclic AMP phosphodiesterase is shown to exist in two forms, one as a membrane-bound form an the other as a soluble form in the cytosol of human erythrocytes. The presence of membrane-bound cyclic nucleotide phosphodiesterase has also been reported previously in some other tissues [1–3,6].

Time course of assay of cyclic AMP phosphodiesterase activity in human erythrocyte membranes

Under the assay conditions, the time course of enzymic hydrolysis of cyclic [^{32}P]AMP was linear at least for 45 min (Fig. 1). Since the incubation time of our standard assay was 20 min, about 6% of cyclic [^{32}P]AMP in the reaction mixture was converted to 5′-[^{32}P]AMP.

Effect of pH on cyclic AMP phosphodiesterase activity of human erythrocyte membranes

A typical pH-activity profile curve is shown in Fig. 2. The optimal pH for maximum enxymic activity was between 7.4 and 8.0, and the enzyme is therefore active under physiological conditions.

Extraction of cyclic AMP phosphodiesterase activity from the membranes

Treatment of the membranes with Triton X-100 enhanced the cyclic AMP phosphodiesterase activity, as mentioned above, and attempts to solubilize the enzyme were therefore carried out under various conditions.

Hypotonic extraction with 1 mM EDTA and 5 mM 2-mercaptoethanol,

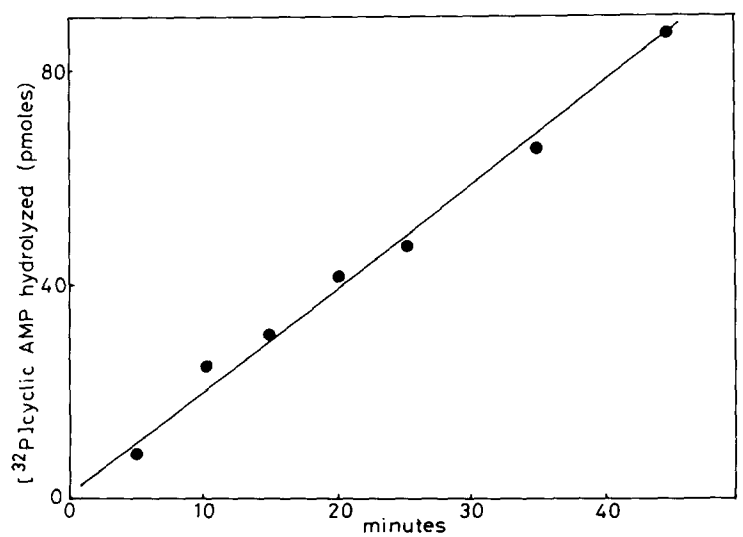

Fig. 1. Time course of assay of cyclic AMP phosphodiesterase on human erythrocyte membranes. The reaction was carried out with a mixture for the standard assay. After appropriate times of incubation, aliquots were withdrawn and the hydrolysis of cyclic [^{32}P]AMP was measured as described in Materials and Methods.

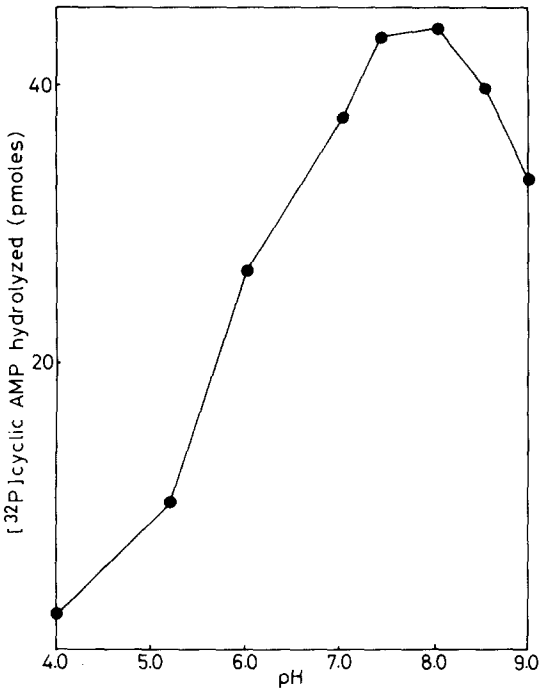

Fig. 2. Effect of pH on cyclic AMP phosphodiesterase of human erythrocyte membranes. The assay as described in Materials and Methods was carried out at various pH values. Buffers used for the assay were as follows: pH 4.0—6.0, 10 mM sodium acetate buffer; pH 7.0—9.0, 10 mM Tris-HCl buffer. Before treatment with 5'-nucleotidase, the pH of each reaction mixture was adjusted to 8.0 with 1 M Tris-HCl buffer.

at pH 8.0, as previously used for the solubilization of peripheral proteins of human erythrocyte membranes such as spectrin [21], or high-salt extraction with 0.5 or 1.5 M NaCl [22], did not solubilize the enzyme from the membranes. When the membranes were treated with 0.3% Triton X-100, however, the enzymic activity was solubilized into the supernatant solution.

The results shown in Table II indicate that the cyclic AMP phosphodiesterase of human erythrocyte membranes is an 'intrinsic' enzyme which probably interacts with membrane lipids by hydrophobic interaction [23]. The total activity of the cyclic AMP phosphodiesterase solubilized by the Triton X-100

TABLE II

EXTRACTION OF CYCLIC AMP PHOSPHODIESTERASE ACTIVITY FROM HUMAN ERYTHROCYTE MEMBRANES

Extraction and assay of the enzyme were as described in Materials and Methods. These values are calculated as the distribution (%) of total activity.

Fraction	EDTA (1 mM) + 2-mercaptoethanol (5 mM)	NaCl (0.5 M)	NaCl (1.5 M)	Triton X-100 (0.3%)
Extract	5.3 ± 0.9	6.7 ± 0.7	7.6 ± 0.7	81.9 ± 0.5
Residue	94.7 ± 0.9	93.3 ± 0.7	92.4 ± 0.7	18.1 ± 0.5

TABLE III

ORIENTATION OF CYCLIC AMP PHOSPHODIESTERASE ACTIVITY ON HUMAN ERYTHROCYTE MEMBRANES

Specific activity is expressed as the mean of units/mg protein. Assay of the enzyme was as described in Materials and Methods except that reaction mixtures contained 100 mM NaCl. In the case of intact erythrocytes, 40 μl of packed erythrocytes (previously washed with 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl) were used and after incubation, the reaction mixture was centrifuged at 180 × *g* for 1 min at 0°C. 5′-AMP in the supernatant was determined as described in Materials and Methods.

Sample	Membranes	Inside-out vesicles	Intact erythrocytes
Specific activity	197.1 ± 1.0	119.4 ± 1.2	0.0

treatment was about 150% of the original activity in the membranes. The other treatments did not change the activity of the enzyme.

Orientation of cyclic AMP phosphodiesterase activity in human erythrocyte membranes

The orientation of cyclic AMP phosphodiesterase in the membranes was determined using intact erythrocytes, unsealed membranes and purified sealed inside-out vesicles.

Enzymic activity was detected only in unsealed erythrocyte membranes and inside-out vesicles, as shown in Table III.

The results shown in Table III strongly suggest that membrane-bound cyclic AMP phosphodiesterase activity of human erythrocytes is localized in the cytoplasmic surface rather than in the external surface.

TABLE IV

EFFECTS OF DIVALENT METAL IONS AND CHELATING AGENTS ON CYLIC AMP PHOSPHODIESTERASE ACTIVITY ON HUMAN ERYTHROCYTE MEMBRANES

The assay conditions were the same as the standard assay (see Materials and Methods) except for the absence of 10 mM $MgCl_2$. 5′-Nucleotidase treatments were carried out in the presence of 10 mM $MgCl_2$. Each metal ion used in this assay was in the chloride form. The values are expressed as the mean of units ± S.D. (see Materials and Methods). The values in parentheses are % of control.

Metal	Experiment 1		Experiment 2		
	Concentration (mM)	Cyclic AMP phosphodiesterase activity	Chelating agent	Concentration (mM)	Cyclic AMP phosphodiesterase activity
None		17.6 ± 0.2 (100)	None		24.6 ± 0.6 (100)
Mg^{2+}	1	25.2 ± 0.2 (143)	EDTA	0.01	21.8 ± 0.8 (89)
	10	38.6 ± 0.3 (219)		0.1	20.4 ± 0.6 (83)
Ca^{2+}	1	20.6 ± 0.1 (117)		1.0	13.2 ± 0.5 (54)
	10	21.7 ± 0.1 (123)		10.0	0.0 ± 0.6 (0)
Co^{2+}	1	35.4 ± 0.5 (201)	EGTA	0.01	25.0 ± 0.5 (102)
Fe^{2+}	1	29.8 ± 0.3 (169)		0.1	22.4 ± 0.1 (91)
Mn^{2+}	1	25.8 ± 0.2 (147)		1.0	16.6 ± 0.5 (67)
Cu^{2+}	1	33.4 ± 0.1 (190)		10.0	12.6 ± 0.4 (51)
Zn^{2+}	1	27.6 ± 0.1 (157)			

Effects of divalent metal ions and chelating agents on cyclic AMP phosphodiesterase activity of human erythrocyte membranes

Effects of various divalent metal ions and chelating agents were investigated and the results are shown in Table IV.

This enzyme was activated by various metal ions, among these, Co^{2+} and Cu^{2+} were powerful activators. Although the presence of 1 mM Mg^{2+} activated the enzyme moderately, 10 mM Mg^{2+} enhanced its activity more than 2-fold, while Ca^{2+} did not affect the activity.

Chelating agents inhibited its activity at rather high concentrations, and the activity was completely abolished in the presence of 10 mM EDTA, while EGTA at the same concentration inhibited the activity by only about 50%.

Effects of various reagents on cyclic AMP phosphodiesterase activity of human erythrocyte membranes

Effects of various reagents; theophylline, cyclic GMP, calcium and calmodulin (a calcium-dependent regulator), were investigated. The results are shown in Table V.

Theophylline is known as a specific inhibitor of cytoplasmic cyclic AMP phosphodiesterases. This compound also inhibited the membrane-bound cyclic AMP phosphodiesterase of human erythrocytes. In the presence of 20 mM theophylline, inhibition was almost complete. The effect of cyclic GMP on the enzymic hydrolysis of cyclic [^{32}P]AMP was tested to investigate whether or not this enzyme had any affinity with cyclic GMP. The presence of 200 μM cyclic GMP in the reaction mixture showed no inhibition of the hydrolysis of cyclic [^{32}P]AMP by this enzyme. The concentration of cyclic GMP was 280-times higher than that of the substrate in the mixture, it is therefore concluded that cyclic AMP phosphodiesterase of human erythrocyte mem-

TABLE V

EFFECTS OF VARIOUS REAGENTS ON CYCLIC AMP PHOSPHODIESTERASE ACTIVITY OF HUMAN ERYTHROCYTE MEMBRANES

The standard assay as described in Materials and Methods was performed. The values are expressed as mean of units ± S.D. (see Materials and Methods). The values in parentheses are % of control.

Reagent	Experiment 1		Experiment 2		
	Concentration (mM)	Cyclic AMP phosphodiesterase activity	$CaCl_2$ (μM)	Calmodulin (μg/ml)	Cyclic AMP phosphodiesterase activity
None		38.6 ± 0.3 (100)	0	0	33.0 ± 0.2 (100)
Theophylline	1	32.4 ± 0.1 (84)	100	10	34.6 ± 0.2 (105)
	6	19.4 ± 0.1 (50)			
	10	4.8 ± 0.8 (12)			
	20	0.3 ± 0.1 (1)	0	0	31.9 ± 0.1 * (100)
Cyclic GMP	0.01	37.7 ± 0.5 (98)	100	25	33.6 ± 0.3 * (105)
	0.1	39.0 ± 0.3 (100)			
	0.2	37.4 ± 0.1 (97)			

* These values were obtained when EGTA-treated membranes (see Materials and Methods) were used instead of intact membranes. In this case the reaction mixture contained 50 μM EGTA.

branes has almost no affinity to cyclic GMP.

Some of the cytoplasmic cyclic nucleotide phosphodiesterases have been shown to be regulated in their activities by calmodulin (a calcium-dependent regulator), and the activities of these enzymes are completely dependent on the Ca^{2+} · calmodulin complex [1–4,14,24]. Thus, the effect of calmodulin on the erythrocyte membranous cyclic AMP phosphodiesterase was investigated in the presence of Ca^{2+}. As Sobue et al. [17] found that the removal of Ca^{2+} and calmodulin from membranes by EGTA treatment was essential to investigate the effect of calmodulin on membrane bound (Ca^{2+} + Mg^{2+})-ATPase, we used EGTA-treated membranes in this study. As shown in Table V, even when EGTA-treated membranes were used, the Ca^{2+} · calmodulin complex seemed to have had very little effect on the phosphodiesterase activity.

Discussion

In this paper, we have shown the presence of cyclic AMP phosphodiesterase in human erythrocyte membranes. This enzyme appears to be an intrinsic protein since it could be solubilized by Triton X-100 and its activity was shown to be localized in the cyctoplasmic surface. These properties are similar to those of the cyclic AMP-dependent protein kinase of human erythrocyte membranes [25,26] and this phosphodiesterase may regulate the cyclic AMP-dependent endogenous phosphorylation of membrane proteins by the cyclic AMP-dependent membranous protein kinase [7,8,11].

Two types of cyclic nucleotide phosphodiesterase, i.e., membrane-bound and soluble phosphodiesterases are known to exist in various tissues [1–3,6], and soluble cyclic nucleotide phosphodiesterases are also reported to exist in multiple forms [1–3,5,27]. Gain and Appleman [2] have reported that the cyclic nucleotide phosphodiesterases of particulate fractions from rat liver and rabbit heart were relatively specific to cyclic AMP. In the case of the cyclic nucleotide phosphodiesterase of human erythrocyte membranes, as reported here, specificity to cyclic AMP by this enzyme is not inhibited by a large excess of cyclic GMP to the substrate. Under the assay conditions, as used in the present work, the concentration of substrate (0.7 μM cyclic [^{32}P]-AMP) was low enough to study the specificity of cyclic nucleotide phosphodiesterase [2,27,28]. Therefore, we consider that cyclic nucleotide phosphodiesterase of human erythrocyte membranes is specific to cyclic AMP.

It has been reported that membrane-bound cyclic nucleotide phosphodiesterase of rat liver [2], brain [29] and rabbit heart [2] were not activated by calcium and calmodulin. Our present results also indicate that the membrane-bound cyclic nucleotide phosphodiesterase of the human erythrocyte is also insensitive to calmodulin in the presence of Ca^{2+}. It is known that calmodulin is present at cytoplasmic surface of human erythrocyte membranes and that it activated the (Ca^{2+} + Mg^{2+})-ATPase of the membranes [30–32], but it does not seem to play a regulatory role for the cyclic AMP phosphodiesterase described here, the K_m value of this membranous cyclic AMP phosphodiesterase was 3–5 μM, so the enzyme may be classified as low-K_m cyclic AMP phosphodiesterase of multiple forms [1–3,5,27]. The order of the

K_m value was consistent with that of particulate cyclic AMP phosphodiesterase of various tissues [1—3], but the precise K_m value should be measured after purification, since the human erythrocyte membranes have other cyclic AMP-binding systems, such as cyclic AMP-dependent protein kinase [7,8,11] or the cyclic AMP-transporting system [33].

The biological function of this enzyme, especially with respect to the protein-phosphorylating systems of the human erythrocyte membranes, remains unclear at the present time, and to investigate further this point, some purification of the enzyme will have to be performed.

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