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2',3'-CYCLIC NUCLEOTIDE 3'-PHOSPHOHYDROLASE IN HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY

2',3'-Cyclic nucleotide 3'-phosphohydrolase has been demonstrated in human erythrocyte membranes. The properties of 2',3'-cyclic nucleotide 3'-phosphohydrolase in erythrocyte membranes were similar to those of the brain enzyme with respect to pH optimum, substrate specificity and effects of metal ions. The estimation of 2',3'-cyclic nucleotide 3'-phosphohydrolase in various stages of membrane preparation showed that the enzyme in erythrocytes is exclusively associated with the membranes.

INTRODUCTION

The presence of 2',3'-cyclic nucleotide 3'-phosphohydrolase* has been reported in various animal tissues[1,4,7,8]. DRUMMOND *et al.*[4] showed that brain and spinal cord have by far the greatest activity. KURIHARA AND TSUKADA[1] found that brain white matter has especially high activity compared with grey matter and enzyme activity is recovered mainly from the myelin fractions of brain homogenates. Since this finding evidence has accumulated showing the association of 2',3'-cyclic nucleotide 3'-phosphohydrolase with myelin in the central nervous system[9–15]. However, it is probable that the enzyme is also located in plasma membranes, because myelin comprises regular layers of plasma membranes from oligodendroglia.

Thus KURIHARA *et al.*[15] proposed a hypothesis that 2',3'-cyclic nucleotide 3'-phosphohydrolase is associated with plasma membranes and their differentiated forms. To examine this hypothesis we have first chosen erythrocyte membranes, because they are the simplest and best-characterized plasma membranes from animal cells. In this paper the occurrence and properties of the enzyme in human erythrocyte membranes are reported.

* The enzyme was named by KURIHARA AND TSUKADA[1] following the *Recommendations (1964) of the International Union of Biochemistry*[2]. The term 2', 3'-cyclic AMP 3'-phosphohydrolase[3] should be avoided, because the enzyme also hydrolyzes 2', 3'-cyclic nucleotides other than adenosine-2', 3' cyclic phosphate[4]. The term ribonucleoside 2', 3'-cyclic phosphate diesterase[5] should also be avoided in order to make a distinction from *Escherichia coli* cyclic phosphodiesterase[6]. We propose a systematic name 2', 3'-cyclic nucleotide 2'-phosphohydrolase for the *E. coli* enzyme.

MATERIALS AND METHODS

Adenosine-2′(3′)phosphate, guanosine-2′(3′)phosphate, uridine-2′(3′)phosphate, cytidine-2′(3′)phosphate (mixed isomers); adenosine, and adenine were obtained from Kohjin Co., Tokyo. Adenosine-3′,5′ cyclic phosphate was purchased from Sigma Chemical Co., St. Louis, Mo. and yeast RNA from Daiichi Chemicals Co., Tokyo. The four 2′,3′-cyclic nucleotides were prepared by the method of SMITH *et al.*[16] from the corresponding 2′(3′)-nucleotides. The barium salts were dissolved in water and converted to the potassium salts by adding excess of 1 M K_2SO_4. The centrifuged supernatant was used as the substrate. Adenosine-2′ phosphate used as standard for paper chromatography was prepared by incubating adenosine-2′,3′ cyclic phosphate with ox brain homogenate for one night under the conditions described previously[1].

The following solvent systems were used for ascending paper chromatography. Solvent 1 (ref. 17): saturated $(NH_4)_2SO_4$–0.5 M sodium acetate–isopropanol (80:18:2, by vol.). Solvent 2 (ref. 18): isopropanol–conc. NH_4OH–water (7:1:2, by vol.).

Preparation of human erythrocyte membranes

Erythrocyte membranes were prepared by the method of DODGE *et al.*[19]. After three washings with 0.018 M phosphate buffer (pH 7.4) the membranes were suspended (approx. 1 mg of protein/ml) in the same buffer and stored at −20° until use. The storage period was no more than a week. There was no activity change during this storage period. The membranes were thawed and centrifuged at 20000 × *g* for 40 min, and the precipitate was suspended in deionized water. This suspension was used as enzyme solution.

Enzyme assay and units

The method of KURIHARA AND TSUKADA[1] (without sonication) was followed for the standared assay using adenosine-2′,3′ cyclic phosphate as substrate. The volume of reaction mixture was doubled (0.4 ml), while the concentration of each component in the mixture remained the same. The amount of acetic acid to be added at the end of incubation was accordingly doubled (0.04 ml). Incubation time was prolonged to 60 min after confirming the linearity of reaction during this period. One unit (U) of enzyme is defined as the amount of enzyme that produces 1 μmole of adenosine-2′ phosphate from adenosine-2′,3′ cyclic phosphate per min at 37° under the standard assay conditions. Specific activity was expressed as U/mg of protein. Protein was determined by the method of LOWRY *et al.*[20] with bovine serum albumin as a standard.

RESULTS

The enzyme reaction proceeded linearly for 60 min and the rate of reaction was proportional to enzyme concentration provided hydrolysis was less than 60 % maximum. The enzyme had a broad optimum pH range between pH 6 and 7.

The enzyme was stable in 25 mM citric acid–50 mM Na_2HPO_4 buffer (pH 6.2). There was no activity change for at least a month when stored at −20° or for 3 weeks at 4° in this buffer. However, when the enzyme in the same buffer was heated at 60° for 15 min, 85 % of the activity was lost.

Identification of reaction product and stoichiometry

As is evident from Fig. 1 the product from adenosine-2′,3′ cyclic phosphate was adenosine-2′ phosphate. No other substance was found on chromatograms developed in Solvent 1 or Solvent 2 (Table I). Furthermore, the conversion of adenosine-2′,3′ cyclic phosphate to adenosine-2′ phosphate was quantitative: the decrease of adenosine-2′,3′ cyclic phosphate (1.20 μmole) was parallel to the appearance of adenosine-2′ phosphate (1.17 μmole). Similarly, the only product from guanosine-2′,3′ cyclic phosphate was guanosine-2′ phosphate.

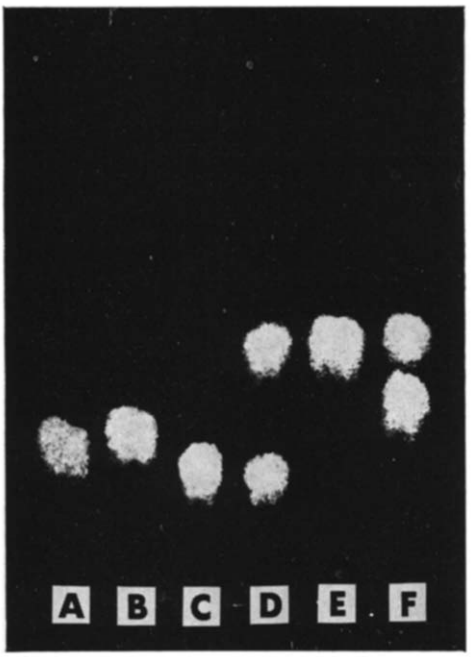

Fig. 1. Identification of reaction product on a paper chromatogram developed in Solvent 1. (A) adenine; (B) adenosine; (C) adenosine-2′,3′ cyclic phosphate incubated without enzyme in the standard assay conditions; (D) adenosine-2′,3′ cyclic phosphate incubated in the standard assay conditions with enzyme solution containing 0.07 mg of protein; (E) adenosine-2′ phosphate; (F) adenosine-2′(3′) phosphate (mixed isomers).

TABLE I

R_F VALUES OF REACTION PRODUCT AND AUTHENTIC SAMPLES

Compound	R_F *in Solvent 1*	R_F *in Solvent 2*
Adenine	0.15	0.75
Adenosine	0.18	0.72
Adenosine-2′(3′) phosphate (mixed isomers)	0.29 0.21	0.23
Adenosine-2′ phosphate	0.29	0.23
Adenosine-2′,3′ cyclic phosphate	0.13	0.48
Adenosine-3′,5′ cyclic phosphate	0.14	0.51
Reaction product	0.29	0.23

Substrate specificity

Table II shows the relative activity of the enzyme with various cyclic nucleotide substrates. The relative activity decreased in the following order: adenosine-2',3' cyclic phosphate > guanosine-2',3' cyclic phosphate > cytidine-2',3' cyclic phosphate > uridine-2',3' cyclic phosphate. The enzyme did not hydrolyze adenosine-3',5' cyclic phosphate. There was no activity on yeast RNA when tested by the method of KURIHARA[21] without *p*-chloromercuribenzoate in a reaction mixture.

TABLE II

RELATIVE ACTIVITY ON VARIOUS CYCLIC NUCLEOTIDES

The activity was determined under the standard assay conditions using the cyclic nucleotides as substrates, except that Solvent 2 in place of Solvent 1 was employed for the chromatographic separation of products.

Substrate	*Relative activity* (%)
Adenosine-2',3' cyclic phosphate	100
Guanosine-2',3' cyclic phosphate	41
Cytidine-2',3' cyclic phosphate	28
Uridine-2',3' cyclic phosphate	13
Adenosine-3',5' cyclic phosphate	0

TABLE III

EFFECT OF VARIOUS COMPOUNDS ON ENZYME ACTIVITY

Compound	*Concentration* (*mM*)	*Relative activity* (%)
None	—	100
$MgCl_2$	2	105
$MnCl_2$	2	102
$CoCl_2$	2	96
$ZnCl_2$	2	86
$CuCl_2$	2	25
EDTA	10	108
	1	102
p-Chloromercuribenzoate	0.2	2

Effects of metal ions and other compounds

As shown in Table III, the enzyme requires no metal ions. EDTA (10 mM or 1 mM) was without effect or slightly accelerative. Zn^{2+} (2 mM), Cu^{2+} (2 mM), *p*-chloromercuribenzoate (0.2 mM) caused 14, 75 and 98 % inhibition, respectively.

2',3'-Cyclic nucleotide 3'-phosphohydrolase in various stages of membrane preparation

In order to determine the localization in erythrocytes, 2',3'-cyclic nucleotide 3'-phosphohydrolase was estimated in varouis stages of membrane preparation (Table IV). 2',3'-Cyclic nucleotide 3'-phosphohydrolase was found exclusively in the membrane fraction (haemolysed precipitate). There was no enzyme activitv in cytoplasm (haemolysed supernatant).

TABLE IV

2′,3′-CYCLIC NUCLEOTIDE 3′-PHOSPHOHYDROLASE AND PROTEIN IN VARIOUS STAGES OF MEMBRANE PREPARATION.

Packed erythrocytes (10 ml) were haemolysed with 30 vol. of 0.018 M phosphate buffer (pH 7.4). After 30 min the suspension was centrifuged at 20000 × *g* for 40 min. The haemolysed precipitate was washed three times with 50 ml of 0.018 M phosphate buffer (pH 7.4). The precipitate of the last washing was suspended in the same buffer and stored at −20°. The frozen suspension was thawed on the following day and centrifuged at 20000 × *g* for 40 min to give the pellet of final membrane preparation.

Preparation	*Total protein (mg)*	*Total activity (U)*	*Specific activity (U/mg of protein)*
Intact erythrocytes	2960	3.6*	0.0012
Haemolysate	2940	5.6	0.0019
Haemolysed supernatant	2840	0.0	0.0000
Haemolysed precipitate	144	5.37	0.0373
Membranes washed once	65.5	9.85	0.150
Membranes washed twice	37.5	10.35	0.276
Membranes washed three times	37.0	11.00	0.297
Final membrane preparation	31.6	9.24	0.292

* The activity value for intact erythrocytes was not reproducible and varied between 0 and 6.

The specific activity of the membrane fraction increased greatly during the first two washings, possibly by the removal of haemoglobin. However, the total activity also increased gradually suggesting that 2′,3′-cyclic nucleotide 3′-phosphohydrolase in the haemolysed precipitate might have been partially masked and that the unmasking might have occurred during these washings.

For the possible activation of enzyme the haemolysed precipitate was treated with sodium deoxycholate[13] (Table V). 2′,3′-Cyclic nucleotide 3′-phosphohydrolase in the haemolysed precipitate was activated about 3-fold by this treatment, whereas no stimulation occurred in the final membrane preparation. This means that repeated washings of the membranes caused the unmasking of enzyme. Deoxycholate treatment was effective for obtaining liberated activity in the crude membrane fraction of erythrocytes, as in the brain homogenates and their fractions[13,15]. Judging from the specific activity after the treatment (Table V), three washings with phosphate

TABLE V

EFFECT OF DEOXYCHOLATE ON 2′,3′-CYCLIC NUCLEOTIDE 3′-PHOSPHOHYDROLASE IN DIFFERENT STAGES OF MEMBRANE PREPARATION

Haemolysed precipitate and final membrane preparation (see Table IV) were treated with sodium deoxycholate following the procedure of KURIHARA *et al.*[13].

Preparation	*Total protein (mg)*	*Activity before treatment*		*Activity after treatment*	
		Total activity (U)	*Specific activity (U/mg of protein)*	*Total activity (U)*	*Specific activity (U/mg of protein)*
Haemolysed precipitate	149	5.29	0.0355	14.57	0.0978
Final membrane preparation	31.6	9.21	0.292	9.42	0.298

buffer not only caused the unmasking of enzyme, but also increased the enzyme content in the membrane fraction. This increase seems to be correlated with the removal of attached haemoglobin from the membrane fraction.

DISCUSSION

The results show the presence of 2′,3′-cyclic nucleotide 3′-phosphohydrolase associated with erythrocyte membranes. The reported absence[4] in intact erythrocytes can be attributed to the extremely low specific activity of intact erythrocytes compared with the activities of brain and other tissues, and even with the activity of erythrocyte membranes. The presence of 2′,3′-cyclic nucleotide 3′-phosphohydrolase in erythrocytes suggests that the enzyme is more widely distributed than it has previously been thought. Our unpublished data indicate its presence in leucocytes, thymus cells and a variety of cultured glial cells.

The properties of 2′,3′-cyclic nucleotide 3′-phosphohydrolase in erythrocyte membranes were remarkably similar to those of the brain enzyme[4]. The optimum pH range and the relative activity on four 2′,3′-cyclic nucleotides were the same within the experimental errors. The effects of metal ions were also similar. The virtually complete inhibition by *p*-chloromercuribenzoate is of interest because the importance of -SH groups in erythrocyte membranes has been suggested[22].

2′,3′-Cyclic nucleotide 3′-phosphohydrolase was found exclusively in the membrane fraction. The repeated washings of the membranes had the effect of increasing enzyme content as well as causing the unmasking of enzyme. These observations indicate that the enzyme in erythrocytes is exclusively associated with the membranes. They support the hypothesis[15] that 2′,3′-cyclic nucleotide 3′-phosphohydrolase is associated with plasma membranes and their differentiated forms.

It is well known that 2′,3′-cyclic nucleotide 3′-phosphohydrolase in brain is firmly bound to insoluble particulate material[4,21,23]. In fact, no one has succeeded in obtaining pure enzyme, though LUNDBLAD AND MOORE[23] have reported the solubilization by "sulphitolysis". It is quite likely that the enzyme in erythrocyte membranes is of the same nature. The observations in various stages of membrane preparation are in favour of this view. Therefore, we suggest that 2′,3′-cyclic nucleotide 3′-phosphohydrolase in erythrocytes is, like acetylcholinesterase (EC 3.1.1.7)[24] and ($Na^+ + K^+$)-stimulated ATPase (EC 3.6.1.3)[25], firmly bound to the membranes and may be useful as a membrane marker.

The physiological role of 2′,3′-cyclic nucleotide 3′-phosphohydrolase is unknown. The presence in erythrocyte membranes suggests that the enzyme may play a more fundamental role than previously supposed[1], presumably in relation to membrane function.

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