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OCCURRENCE OF CREATINE KINASE ACTIVITY IN HUMAN ERYTHROCYTE MEMBRANE

SHIRO MAWATARI * and NOBUE SHINNOH

Health Administration Center, Kyushu Institute of Technology, and Department of Neurology, Neurological Institute, Faculty of Medicine, Kyushu University, Fukuoka 812 (Japan)

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Summary

Some evidences for creatine kinase activity in normal human erythrocyte membrane were presented. The creatine kinase was indicated to be a constituent of the integral proteins of erythrocyte membrane or to be tightly bound to the membrane, and was contrasted to the results obtained with adenylate kinase. Isoenzyme distribution of the erythrocyte creatine kinase by electrophoresis was identical to MM-creatine kinase from rabbit muscle.

The presence of creatine in normal human erythrocytes has been known for a long time [1], but physiological role of the erythrocyte creatine remains unclear [2—6]. Only creatine kinase (EC 2.7.3.2) is known in enzyme of human tissues for which phosphocreatine and creatine are substrate [7], however, presence of phosphocreatine-creatine kinase system in human erythrocyte has been uncertain [3—6]. This paper described evidences for occurrence of creatine kinase activity in normal human erythrocyte membranes.

Venous blood from healthy adults was drawn into heparinized tubes and immediately put on ice. The erythrocytes were separated by centrifugation at $1000 \times g$ for 10 min, and plasma and buffy coat were removed. The erythrocytes were washed three times with 0.85% NaCl and buffy coat was removed at each washing. After hemolysis of the washed erythrocytes in 8 vol. of 10 mM Tris-HCl (pH 7.4), the membranes were washed five times with the same solu-

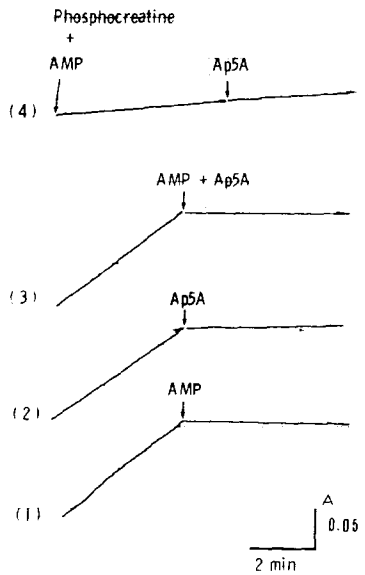
* Correspondence should be addressed to: Dr. Shiro Mawatari, Department of Neurology, Neurological Institute, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan.

tion by centrifugation at 25 000 × *g* for 20 min. Opaque button at the bottom of centrifuge tubes which is considered to be aggregated leukocytes [8] was removed each time. All procedures were done at 2 to 4°C. The white, creamy erythrocyte membranes were suspended in the washing solution to make protein concentration about 4 mg/ml and stored at −80°C until use. Purity of the erythrocyte membranes was assessed by SDS-acrylamide gel electrophoresis according to the methods described by Fairbanks et al. [8].

Creatine kinase activity of the erythrocyte membranes was determined using a coupled enzyme assay in the direction; phosphocreatine + ADP → creatine + ATP. To examine possible influences of adenylate kinase (or other enzymes) on the assay system for creatine kinase, adenylate kinase activity was initially assayed in the direction 2 ADP → ATP + AMP. The reaction mixture for adenylate kinase consisted of 100 mM Tris-acetate, pH 7.0, 10 mM magnesium acetate, 1 mM ADP, 20 mM glucose, 0.8 mM/NADP, 1.5 units/ml of hexokinase, 1.5 units/ml of glucose-6-phosphate dehydrogenase and 200 to 400 μg of membrane protein. After equilibration at 37°C, the reaction was followed by recording absorbance at 340 nm. The reaction was linear for more than 20 min and proportional to the membrane protein concentration up to 700 μg. This reaction was completely blocked by addition of either 10 mM AMP, or 10 μM P^1,P^5,-di(adenosine-5′)pentaphosphate (Ap_5A), or both to the reaction mixture (Fig. 1). Ap_5A has been known as a specific inhibitor of adenylate kinase [9]. Therefore, these facts indicated that the activity by this assay system represented only that of adenylate kinase. Creatine kinase activity was measured at 37°C by adding 30 mM disodium phosphocreatine (pH 7.0) and 10 mM AMP to the assay system for adenylate kinase, and the reaction was followed by recording at 340 nm. Despite the presence of 10 mM AMP in the reaction mixture an activity was observed, indicating that phosphocreatine-creatine kinase system was working. Addition of 10 μM Ap_5A to this reaction mixture did not block the activity, in contrast to the case of adenylate kinase (Fig. 1). The reaction was linear for more than 30 min.

Creatine kinase activity of the erythrocyte membranes was also confirmed by measuring creatine formation in the reaction mixture consisted of 100 mM Tris-acetate, pH 7.2, 10 mM magnesium acetate, 10 mM disodium phosphocreatine, 1 mM ADP, 10 μM Ap_5A, and 200 to 400 μg membrane protein. After incubating at 37°C for 30 min, the creatine formed was measured colorimetrically by the naphthol-diacetyl reaction [10,11]. Creatine kinase activity by this method was 0.8 ± 0.2 nmol/min per mg of membrane protein (four experiments).

Attempt to extract the creatine kinase was done by suspending 1 ml of the membrane solution in a 5-ml solution containing 0.01% deoxycholate, 0.3 M KCl and 0.1 M potassium phosphate, pH 7.4, and was then followed by dialysis against 0.1 M potassium phosphate (pH 7.4) [12]. Supernate was obtained by centrifugation at 25 000 × *g* for 30 min. However, only a part of enzyme activity appeared in the supernate. Creatine kinase isoenzymes were separated on a thin agarose gel. 1—2 μl of the supernate from the extraction by deoxycholate and KCl, which was concentrated about 50-times by Amicon filter (Minicon B-15, Amicon Co., U.S.A.), was run for 30 min at 150 V in veronal buffer (pH 8.6, ionic strength 0.06) at 2 to 5°C. Enzymatically active components were



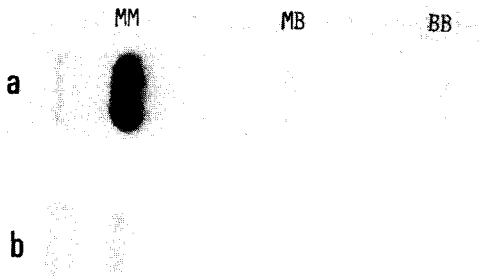


Fig. 1. Representation of actual recording of creatine kinase and adenylate kinase activity of the erythrocyte membranes. Adenylate kinase activity was completely blocked by 10 mM AMP (1), 10 μM Ap_5A (2), and 10 mM AMP + 10 μM Ap_5A (3). When 30 mM phosphocreatine and 10 mM AMP were included in the assay mixture for adenylate kinase, an activity indicating occurrence of creatine kinase activity was observed (4), and 10 μM Ap_5A did not block the activity (4), contrasting with the case of adenylate kinase. Membrane protein was 320 μg in all experiments. ↓, addition of the indicated substances to the assay system for adenylate kinase.

Fig. 2. Creatine kinase isoenzyme from the erythrocyte membranes by agarose gel electrophoresis. (Agarose films were purchased from Pfizer Co., U.S.A.) (a) Control isoenzymes from rabbit tissues (purchased from Sigma Chem Co., U.S.A.). (b) The enzyme from normal human erythrocyte membranes. MM, MM-creatine kinase, MB, MB-creatine kinase, BB, BB-creatine kinase. The dye mixture for creatine kinase consisted of Tris-acetate (0.1 M, pH 6.7), ADP (2 mM), phosphocreatine (22.2 mM), AMP (13 mM), Ap_5A (10 μM), NADP (0.8 mM), hexokinase (1 unit/ml), glucose-6-phosphate dehydrogenase (1 unit/ml), glucose (20 mM), $MgCl_2$ (12 mM), reduced glutathione (9 mM), diaphorase (1.8 units/ml) and nitrotetrazolium blue (0.37 mM). Agarose gel was incubated in the dye mixture at 37°C for 30—40 min.

detected by incubation of the gel at 37°C in a coupled enzyme dye mixture (composition of the dye mixture was listed in the legend for Fig. 2). Electrophoretically, the creatine kinase of the erythrocyte membranes showed only one band and was identical to MM-creatine kinase from rabbit muscle (Fig. 2). The same result was observed by electrophoresis on cellulose acetate membrane according to the method of Turner et al. [13].

To obtain some insight into the location of the enzyme in the erythrocyte membrane, the membranes were further washed with 0.1 mM EDTA [8]. The washing procedures were described in the footnote of Table I, and the washing caused marked reduction of spectrin bands and disappearance of actin band on SDS-acrylamide gel electrophoretogram with Coomassie-blue staining in our study [14]. The washed membranes showed an increase (about 30%) of the specific activity of creatine kinase (Table I). On the other hand, the specific activity of adenylate kinase of the washed membranes was about half of that of the original membranes and a high activity of the enzyme was noted in the supernate (Table I). These results indicate that the erythrocyte membrane crea-

TABLE I

CREATINE PHOSPHOKINASE AND ADENYLATE KINASE ACTIVITY OF HUMAN ERYTHROCYTE MEMBRANE

Enzyme activities were expressed as nmol/min per mg of membrane protein. Assay methods of the enzymes were described in text. Protein concentration was measured as described by Lowry et al. [15].

	n	Creatine phosphokinase	Adenylate kinase
Original membrane	4	2.87 ± 1.02	35.6 ± 5.6
EDTA-washed membrane *	4	3.78 ± 0.72	16.9 ± 1.8
EDTA-washed supernate *	4		99.6 ± 15.0

* The original membranes were incubated at 37°C for 20 min in 9.5 vol. of 0.1 mM EDTA (disodium salt) (pH 8.0) and centrifuged at 40 000 × *g* for 30 min at 4°C. After separation of the supernate, the precipitates (membranes) were washed again in 10 vol. of distilled water by the same centrifugation and was resuspended in the water.

tine kinase is a constituent of the integral proteins of the membrane or is tightly bound to the membrane, and that adenylate kinase is loosely bound to the membrane in similar manner to spectrin and actin.

The creatine kinase activity of human erythrocyte membrane is relatively low when it is compared to that of adenylate kinase (Table I). However, the activity is comparable to that of $(Na^+ + K^+)$-ATPase in our study [14]. (The ATPase activity was 3.3 ± 0.2 nmol/min per mg of membrane protein, pH 7.4, at 37°C.) Although the physiological function of creatine kinase in the erythrocyte membrane remains to be elucidated, it is conceivable that the enzyme is playing a role by associating with ATP-utilizing systems of the membranes. The presence of creatine kinase in plasma membrane of rat heart has been indicated [7,12].

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