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THE LOCALIZATION OF THE MM ISOZYME OF CREATINE PHOSPHOKINASE ON THE SURFACE MEMBRANE OF MYOCARDIAL CELLS AND ITS FUNCTIONAL COUPLING TO OUABAIN-INHIBITED (Na^+,K^+)-ATPase

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Summary

A rat heart plasma membrane preparation isolated in a sucrose medium and some of its enzymatic properties have been investigated.

It has been shown that a rat heart plasma membrane fraction contains high creatine phosphokinase activity which can not be diminished by repeated washing with sucrose solution. Creatine phosphokinase extracted from a plasma membrane fraction with potassium chloride and 0.01% deoxycholate solution is electrophoretically identical to MM isoenzyme of creatine phosphokinase. Under the conditions where (Na^+,K^+)-ATPase is activated by addition of Na^+, K^+ and MgATP, creatine phosphokinase of plasma membrane fraction is able to maintain a low ADP concentration in the medium if creatine phosphate is present. The rate of creatine release is dependent upon MgATP concentration in accordance with the kinetic parameters of the (Na^+,K^+)-ATPase and is significantly inhibited by ouabain (0.5 mM). The rate of creatine release is also dependent on creatine phosphate concentration in conformance with the kinetic parameters of MM isozyme of creatine phosphokinase,

It is concluded that in intact heart cells the plasma membrane creatine phosphokinase may ensure effective utilization of creatine phosphate for immediate rephosphorylation of ADP produced in the (Na^+,K^+)-ATPase reaction.

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Introduction

Recent studies have revealed the important role of creatine phosphokinase isozymes in energy transport processes in the heart cells [1—10]. Thus, it has been shown that about 30% of total cellular activity of creatine phosphokinase is localized in mitochondria [4,5]. The close functional coupling between mitochondrial isozyme of creatine phosphokinase and ATP-ADP translocase may ensure complete conversion of chemical energy of the ATP molecules into chemical energy of creatine phosphate molecules in heart mitochondria under physiological conditions (in the presence of creatine and creatine phosphate) [6,7]. Due to the localization of MM isozyme of creatine phosphokinase in the myofibrils creatine phosphate can be effectively used for rephosphorylation of ADP formed during contraction cycle [4,8,9]. According to the data obtained by several authors a part of MM isozyme of creatine phosphokinase is bound to the membranes of the heart sarcoplasmic reticulum where it apparently supplies energy from creatine phosphate for Ca^{2+}-dependent ATPase [11,12].

As it has been indicated by Langer about 5% of myocardial energy would be expended on the $(Na^+ + K^+)$-pump in the surface membrane of cells to maintain the Na^+ gradient across the cell membrane [13]. It was of significant interest therefore to study the question whether this energy could be supplied by the creatine phosphate-creatine phosphokinase system of the heart cells.

Methods

Preparations of myocardial plasma membrane were isolated from rat hearts by a method of Kidway et al. [14], which includes the procedures of homogenization of heart tissue in sucrose medium and separation of cellular structures on the sucrose density gradient.

Extraction of creatine phosphokinase for electrophoretic analyses was performed by resuspension of a membrane pellet in a solution containing 0.3 M KCl, 0.1 M potassium phosphate, pH 7.2, and 0.01% sodium deoxycholate. After dialysis for 24 h against 0.1 M potassium phosphate solution, pH 7.2, a suspension was centrifuged for 10 min at 15 000 $\times g$. A supernatant obtained was concentrated on Amicon filters and used in experiments. Electrophoretic analyses were carried out as described earlier [5]. 1 μl of enzyme solution was applied to a 12 × 150 mm cellulose acetate strip. Creatine phosphokinase electrophoresis was carried out at 4°C in a Gelman Instrument Company model 51159 apparatus for a period of 100 min at 150—170 V and current of 1 mA in Tris barbital buffer, pH 8.8 [15], containing 0.05 mM dithiothreitol. Development of the electrophoretogram was carried out under conditions of the creatine phosphokinase reaction in the direction of creatine formation (reverse reaction). For this procedure, 15 min before ending electrophoresis, a cellulose acetate strip was saturated with a solution containing all components of the incubation and indicator mixtures [16]. The strip was blotted lightly with filter paper and, after the end of electrophoresis, was laid solidly on the cellulose acetate strip from electrophoresis. These double strips were incubated in a thermostat at 37°C for 10 min. The developed strips were scanned in a microdensitometer (Chromoscan MK II, Joyce Loebl) at a wavelength of 575 nm.

(Na^+,K^+)-ATPase activities were determined by a coupled enzyme system by a method of Schwartz [17]. The reaction medium contained 25 mM Tris · HCl, pH 7.4, 0.14 mM NADH, 3.0 mM ATP, 5 mM $MgCl_2$, 0.8 mM phospho*enol*-pyruvate, 100 mM NaCl, 10 mM KCl, 2 I.U. of pyruvate kinase and 2 I.U. of lactate dehydrogenase per ml. The rate of ATPase reaction was determined by recording the change in absorbance at 340 nm on a Perkin-Elmen spectrophotometer (model 402) after addition of a plasma membrane preparation to the final protein concentration of 0.06—0.08 mg per ml.

Creatine phosphokinase activities were assessed by the reverse reaction as indicated earlier [5,9]. The reaction medium contained 25 mM Tris · HCl, pH 7.4, 0.6 mM NADP, 2 mM glucose, 3.3 mM $MgCl_2$, 0.3 mM dithiothreitol, 5.0 mM AMP, 2 I.U. hexokinase and 2 I.U. glucose-6-phosphate dehydrogenase per ml, 1.0 mM ADP, 15 mM creatine phosphate and 0.06—0.08 per ml of a plasma membrane protein. The reaction was started by addition of creatine phosphate and followed by recording of absorbance at 340 nm.

The rate of creatine phosphate splitting coupled to the sarcolemmal (Na^+,K^+)-ATPase reaction was measured in a medium containing 0.05 M Tris/acetate, pH 7.4, 5 mM magnesium/acetate, 0.5 mM dithiothreitol, 4 mM potassium phosphate. Plasma membrane fraction was added to give a final protein concentration of 0.05—0.08 mg per ml and the reaction mixture was incubated for 15 min at 30° C without or with oubain (0.5 mM if added). This incubation procedure in presence of Mg^{2+} and phosphate was carried out to complete enzyme-oubain complex formation [18,19]. After 15 min of incubation KCl was added to the final concentration of 10 mM, sodium/acetate to 100 mM, creatine phosphate and ATP in concentrations given in legends to figures. The reaction was started by addition of ATP. After 4 and 8 min of reaction, aliquots of 2 ml were withdrawn from the mixture, mixed with 0.4 ml of cold 6% perchloric acid and neutralized by addition of 2.5 M K_2CO_3 in the presence of methyl orange. In these samples creatine concentration was measured by a colorimetric method [20] and ADP concentration was assessed by a coupled enzyme assay [6] in Aminco-Change double beam dual wavelength spectrophotometer.

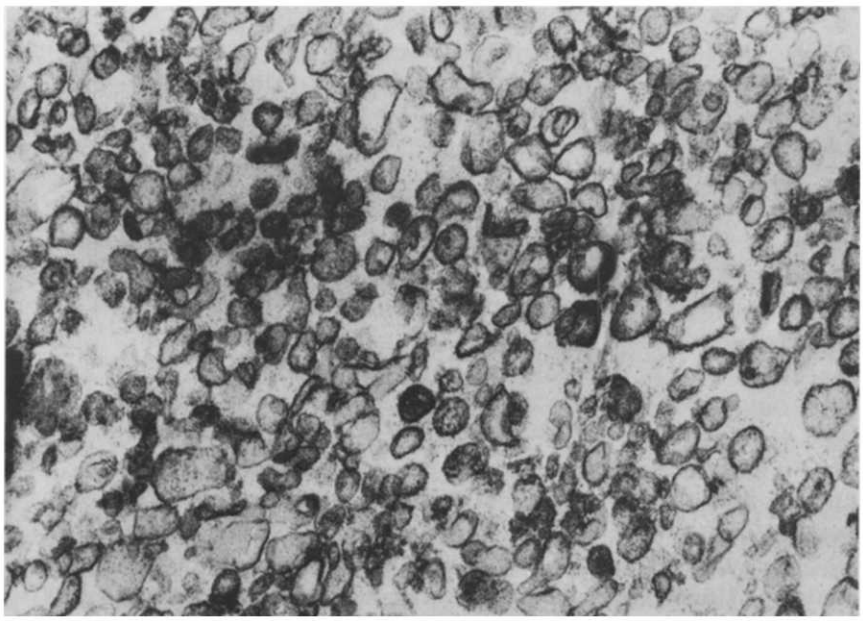
Concentrations of ADP, ATP and creatine phosphate in stock solutions were determined before experiments by enzymatic methods [6]. Protein concentration was determined by a biuret method in the presence of deoxycholate [21].

Results

1. Some properties of myocardial plasma membrane preparations

An electron micrograph of a plasma membrane preparation isolated by a method of Kidwai et al. [14] is presented in Fig. IA. It is evident that this preparation consists of closed vesicles having similar sizes. No mitochondrial contaminations could be seen by an electron microscopic investigations of preparations. Spectrophotometric determinations [5] also did not reveal any cytochrome content in this plasma membrane fraction.

(Na^+,K^+)-ATPase activity of plasma membrane preparation is inhibited by oubain to 50—60% of its normal value (Table I). This activity was not inhibited by ethyleneglycol bis(aminoethyl)-tetraacetate (2.5 mM) which may be taken



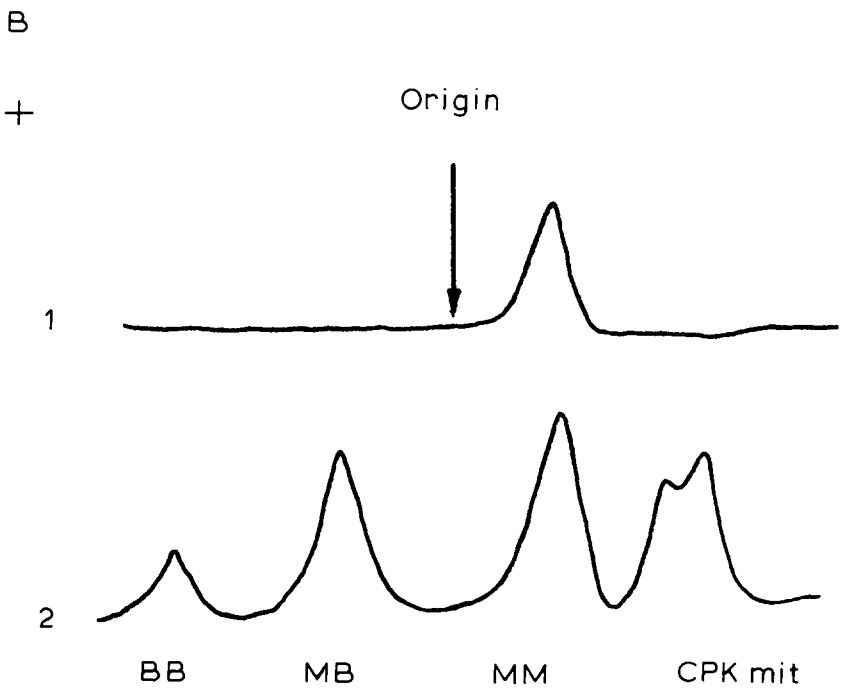


Fig. 1. (A) Electron photomicrograph of sarcolemmal preparation. Osmium fixation. X 30 000. (B) Creatine phosphokinase (CPK) electrophoretogram of KCl-deoxy-cholate extract of sarcolemmal preparation (1) and of total extract of cardiac tissue (2). Total extract was prepared as indicated earlier [5]. Isoenzyme patterns were vizualized by activity staining.

to indicate the absence of Ca^{2+}-dependent ATPase activity of the sarcoplasmic reticulum.

Additionally, we have found that this preparation possesses high creatine phosphokinase activity (Table I) comparable to its (Na^+,K^+)-ATPase activity. Repeated washing (three times) with sucrose solution did not diminish the creatine phosphokinase activity of plasma membrane fractions. Electrophoretically creatine phosphokinase bound to the sarcolemmal membrane is identical to MM isozyme (Fig. IB).

2. The functional coupling between sarcolemmal creatine phosphokinase and (Na^+,K^+)-ATPase

The results of an analysis of the reaction mixture under conditions when (Na^+,K^+)-ATPase was activated by addition of Na^+ (100 mM), K^+ (10 mM), Mg^{2+} (5 mM) and different amounts of ATP in the presence of creatine phosphate (6.6 mM) are shown in Fig. 2. It can be seen that at any concentration of ATP only the concentration of creatine increases linearly with the time of the reaction (Fig. 2A), the ADP concentration remaining constant (Fig. 2B). These data indicate that under the conditions described ADP produced in the (Na^+,K^+)-ATPase reaction is effectively rephosphorylated in the creatine phosphokinase reaction.

On the basis of data similar to those given in Fig. 2, the dependence of the creatine formation (creatine phosphate splitting) rate on the MgATP concentration in the presence of Na^+ and K^+ was determined for the case when oubain was not added (curve 1, Fig. 3A) and for the case when oubain was added up to 0.5 mM (curve 2, Fig. 3A). One can see that the addition of ouabain to the reaction medium significantly decreases the rate of creatine production (Fig. 3A). Very similar dependences have been observed when ATP concentration was kept constant at 0.3 mM and the concentration of creatine phosphate was changed (Fig. 3B). The rate of creatine production enhances with increase in the creatine phosphate concentration in the medium and is sensitive to inhibition by oubain (Fig. 3B).

The linearization of the dependences given in Fig. 3A on Lineweaver-Burk plots ($1/v$ versus $1/[MgATP]$) is shown in Fig. 4 and gives the apparent K_m value for this substrate in the creatine formation reaction equal to 0.09 mM. In Fig. 4 the dependences from Fig. 3B are also linearized in coordinates $1/v$ ver-

TABEL I

SOME ENZYMATIC PROPERTIES OF RAT MYOCARDIAL PLASMA MEMBRANE FRACTION AT 30°C, pH 7.4

Conditions of measurements of enzymatic activities are given in Methods. In the Table the mean values and S.D. are given for 5 determinations.

Activity	Specific activity, (μmol · min^{-1} · mg^{-1} without ouabain)	Specific activity (μmol · min^{-1} · mg^{-1} with oubain, 0.5 mM)
1. (Na^+, K^+)-ATPase	0.5 ± 0.1	0.26 ± 0.12
2. Creatine phosphokinase	0.62 ± 0.15	0.63 ± 0.14

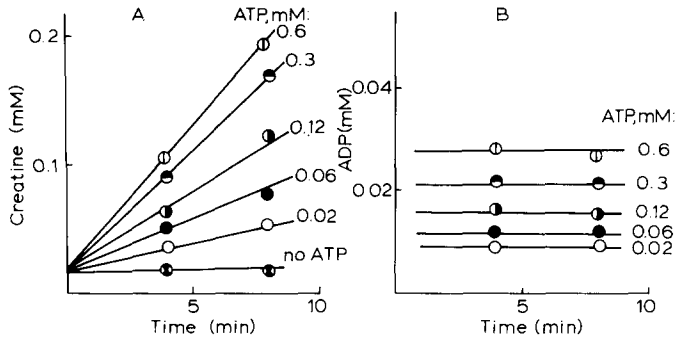


Fig. 2. Changes in creatine (A) and ADP (B) concentrations with time under the conditions of (Na^+,K^+)-ATPase reaction in the presence of creatine phosphate and plasma membrane fraction. The conditions of the reaction are described in Methods. Creatine phosphate concentration was constant and equal to 6.6 mM.

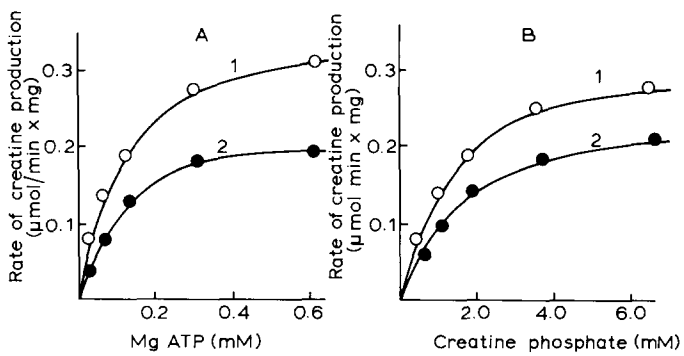


Fig. 3. The dependence of the rate of creatine formation on MgATP concentration (A) and creatine phosphate concentration (B) in the absence (1) and in the presence (2) of ouabain (0.05 mM). The dependences were calculated from data given in Fig. 2 or similar data. In (A) creatine phosphate concentration was 6.6 mM. In (B) MgATP concentration was 0.3 mM.

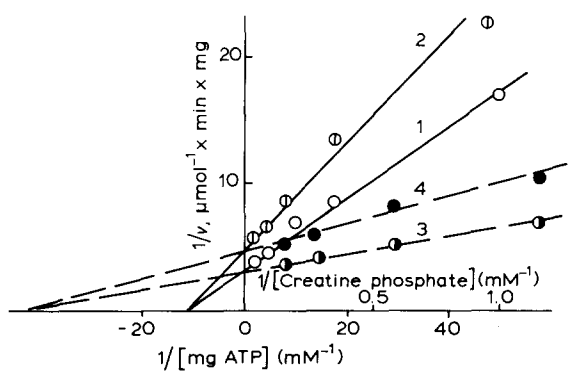


Fig. 4. Linearization of data from Fig. 3A and 3B on Lineweaver Burk plots. Solid lines: $1/v$ versus 1/[MgATP] calculated from Fig. 3A. 1, without ouabain; 2, with ouabain (curves 1 and 2 in Fig. 3A correspond). Dashed lines: $1/v$ versus 1/[creatine phosphate] calculated from Fig. 3B. 3, without ouabain; 4, with ouabain (curves 1 and 2 in Fig. 3B correspond). v, the rate of creatine formation (creatine phosphate splitting).

TABLE II

THE VALUES OF KINETIC CONSTANTS OF ENZYMATIC REACTIONS RELATED TO THE RAT MYOCARDIAL PLASMA MEMBRANES

The values of constants for reactions 1 and 2 were determined in separate experiments as described in the text. The constants for reaction 3 are obtained from Fig. 4. CP, creatine phosphate.

Reaction	Kinetic constant	The numeral value of constant
1. (Na^+, K^+)-ATPase	K_m^{MgATP}, mM	0.05
2. Creatine phosphokinase	K_m^{CP}, mM	1.5
	K_m^{MgADP}, mM	0.08
3. The reaction of creatine phosphate splitting coupled to (Na^+, K^+)-ATPase	K_m^{MgATP}, mM	0.09
	K_m^{CP}, mM	1.25

sus 1/[creatine phosphate] that gives the apparent K_m value for this substrate equal to 1.25 mM. The values of the maximal reaction rates calculated from both dependences are very close and are changed to the same degree after addition of oubain to the reaction mixture (Fig. 4, lines 2 and 4). As it can be seen from Fig. 4 oubain does not change the K_m values neither for MgATP nor creatine phosphate.

In order to interpret the kinetic data obtained from Fig. 4 the values of the kinetic constants of sarcolemmal creatine phosphokinase have been determined in separate experiments in the presence of creatine phosphate and MgADP by a method used in ref. 6, and the value of K_m for MgATP in the (Na^+,K^+)-ATPase reaction has been determined directly from the dependence of the rate of ATP splitting upon MgATP concentration. The results of these experiments are given in Table II. As it can be seen from this table the apparent K_m values for MgATP and creatine phosphate determined from the creatine formation reaction coupled to (Na^+,K^+)-ATPase are very close to those in the separately functioning (Na^+,K^+)-ATPase and creatine phosphokinase reactions, respectively.

Discussion

The procedure proposed by Kidwai et al. [14] for isolation of a plasma membrane fraction from the heart without application of concentrated salt solutions allows us to preserve the intact complexes of enzymes on the membrane surface. The results of this study show that one of those complexes is that containing MM isozyme of creatine phosphokinase and (Na^+,K^+)-ATPase. The data obtained allow us also to conclude that there is a close functional coupling of creatine phosphokinase with (Na^+,K^+)-ATPase on the surface membrane of myocardial cells.

It has been shown earlier that there are three isozymes of creatine phosphokinase (MM, MB and BB) in the cytosol of heart cells [4,5]. In plasma membrane fractions we have found only MM isozyme which could not be removed by repeated washing with sucrose solution, but could be extracted by deoxycholate and KCl solutions. These data make it unlikely that creatine phosphokinase found in plasma membrane fractions results from nonspecific binding of

cytosolic isozymes during the isolation procedure.

As it has been shown in Fig. 2 creatine phosphokinase bound to the sarcolemmal membrane is very effective in the reaction of rephosphorylation of ADP formed in the (Na^+,K^+)-ATPase reaction and is able to maintain a low and constant steady-state ADP concentration in the presence of creatine phosphate. The rate of creatine phosphate splitting is dependent on the MgATP concentration in accordance with the kinetic parameters of (Na^+,K^+)-ATPase and is sensitive to oubain (Figs. 3 and 4). Thus, the rate of creatine phosphate splitting quantitatively reflects the rate of the (Na^+,K^+)-ATPase reaction. As it has been shown in Table I, the activities of plasma membrane creatine phosphokinase and (Na^+,K^+)-ATPase at saturating substrate concentrations are similar. Thus, creatine phosphokinase cannot be considered to be present in excess and to act as a usual soluble coupling enzyme not limiting the overall reaction rate. In fact, as is shown in Fig. 3B, the rate of creatine formation in the coupled reaction is dependent on the creatine phosphate concentration in the medium in conformance with the kinetic parameters of MM isozyme of creatine phosphokinase.

As is shown in Fig. 4 and Table II the behavior of the creatine phosphokinase (Na^+,K^+)-ATPase system in plasma membranes can be described kinetically as one enzyme complex having separate binding sites for creatine phosphate and MgATP, the catalytic properties (V) of which are changed by ouabain.

The results of this study allow us to suppose that in intact heart cells creatine phosphate is used by plasma membrane creatine phosphokinase for immediate rephosphorylation of ADP produced in the (Na^+,K^+)-ATPase reaction, the latter being involved in active transport of cations across the cell membrane [22]. In this coupled system the rate of energy supply needed for active transport of Na^+ may be determined mainly by the creatine phosphate concentration in the cells.

More than ten years ago Caldwell and Hodgkin showed that the Na^+ efflux from the giant axon cells may be maintained by splitting of arginine phosphate in the arginine phosphokinase system [23,24]. As has been demonstrated in this study a similar system may be actively functioning in the mammalian heart cells, arginine phosphate, arginine phosphokinase being replaced by the creatine phosphate-creatine phosphokinase system.

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