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KINETIC PROPERTIES OF THE (Ca^{2+} + Mg^{2+}) –ATPASE BOUND TO AND EXTRACTED FROM THE PIG ERYTHROCYTE MEMBRANE

The activity of the pig erythrocyte membrane (Ca^{2+} + Mg^{2+})–ATPase was investigated. Two kinds of enzyme preparations were used: enzyme bound to the fragments of membrane and extracted from them. Both enzyme preparations exhibited biphasic aubstrate curves displaying the existence of two functional active sites with high and low affinity to ATP. Also the relationship between the activity of bound enzyme and Ca^{2+} concentration was biphasic. The activity reached maximum at about 20 μM then dropped progressively as the Ca^{2+} concentration was raised. Obtained data indicate that the dependence of the pig erythrocyte membrane (Ca^{2+} + Mg^{2+})–ATPase on both ATP and free Ca^{2+} concentration as well as kinetic parameters of the enzyme are similar to those obtained for human erythrocytes.

1. INTRODUCTION

(Ca^{2+} + Mg^{2+})–ATPase from erythrocyte was the first discovered enzyme of the plasma membrane acting as a calcium pump [1]. It is now established that low level of calcium ions inside human erythrocyte [2] is maintained by an active extrusion of them [3]. The energy necessary for this process is supplied by the hydrolysis of ATP located on the internal surface of the membrane [3].

(Ca^{2+} + Mg^{2+})–ATPase of the human erythrocyte has been well characterized [4], but there are little information on the properties of the enzyme of pig red cell membrane. In the present work the basal (i.e. independent of calmodulin) activity of pig erythrocyte membrane (Ca^{2+} + Mg^{2+})–ATPase was investigated. Two kinds of membrane preparations were used: the enzyme bound to fragments of membrane and extracted from them by means of detergent.

2. MATERIALS AND METHODS

The method of Au [5] was employed to obtain pig erythrocyte membranes containing (Ca^{2+} + Mg^{2+})–ATPase. Briefly, washed erythrocytes were lysed with 8 vol. of 10 mM Tris, 1 mM EDTA (pH 7.4), then washed five times with the same buffer before three more washes with 10 mM Tris–HCl (pH 7.4). Membrane suspensions were adjusted to about 2 mg protein/ml and were kept at –75°C before use. When an extracted enzyme was used, the membrane fragments were treated for 10 min at 4°C with 50 mM imidazole, 100 mM NaCl, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X–100, 1% Tween 20, 0.1% phosphatidylcholine (pH 7.4). After the treatment, the mixture was centrifuged at 100,000 g for 60 min to sediment residual membranes and the supernatant containing the ATPase was concentrated by ultrafiltration.

Protein was estimated by the protein-dye binding method of Bradford [6] with bovine serum albumin as the standard.

The activity of (Ca^{2+} + Mg^{2+})–ATPase was determined spectrophotometrically on the basis of amount of inorganic phosphate (P_i) released during an enzymatic hydrolysis of ATP [7]. Briefly, membrane preparations were preincubated with 0.1 mM EGTA for 60 min at 37°C and washed three times with 20 mM Hepes buffer (pH 7.4). The washed membrane were incubated for 30 min at 37°C in a buffer containing 2 mM ATP, 55 mM Tris–HCl (pH 7.2), 66 mM NaCl, 1 mM $MgCl_2$, 0.1 mM oubain, 0.1 mM EGTA plus sufficient amount of $CaCl_2$ to give a final free Ca^{2+} concentration of 19.1 μM. The concentrations of both ATP and free calcium were changed when required. At the end of the incubation the reaction was stopped by the addition of the colour developing reagent. The activity of the enzyme was expressed in μmol P_i released per hour per mg of protein. From the total amount of P_i released during the incubation period the amount of P_i resulting from Ca^{2+}-independent hydrolysis of ATP was subtracted.

Inorganic phosphorus was determined by the sensitive malachite green method [8] as described by Lanzetta et al. [9].

Free calcium levels in the presence of EGTA and ATP, and MgATP concentrations were calculated using the binding constants given by Rooney and Lee [10].

One-way analysis of variance was used in the statistical analysis. The differences between means were compared by the Scheffe's multiple comparison test [11].

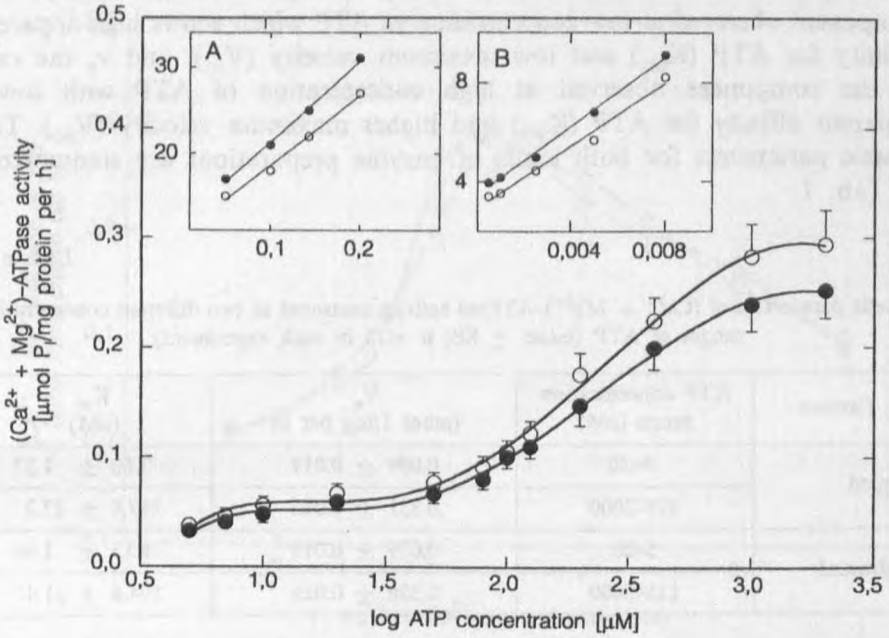

Fig. 1. Pig erythrocyte membrane $(Ca^{2+} + Mg^{2+})$-ATPase activity as a function of ATP concentration. Open symbols – bound enzyme, solid symbols – extracted enzyme. Error bars denote SE, each experimental point is the mean of five replications. Insets – Lineweaver-Burke plots for ATP concentrations in the range 5–20 μM (A) and 0.125–2 mM. Regression lines were calculated by means of the least square method. The R values were equal to 0.986 and 0.991 for bound and extracted enzyme respectively

3. RESULTS AND DISCUSSION

The activity of $(Ca^{2+} + Mg^{2+})$-ATPase was measured as the function of ATP concentration in the range 5 μM–2 mM. Results (Fig. 1) show that the response of the enzyme to ATP is biphasic. Reciprocal plots of $(Ca^{2+} + Mg^{2+})$-ATPase activity against ATP concentration (Fig. 1, insets) in the range 5–20 μM and 0.125–2 mM yield straight lines for both kinds of enzyme preparations. The substrate curves in Fig. 1 can therefore be represented by the sum of two Michaelis – like equations

$$v = v_1 + v_2 = \frac{V_{m1}}{1 + \frac{K_{M1}}{[S]}} + \frac{V_{m2}}{1 + \frac{K_{M2}}{[S]}}$$

where [S] is the concentration of ATP, v_1 represents the rate of the component observed at low concentration of ATP which shows high-apparent affinity for ATP (K_{M1}) and low maximum velocity (V_{m1}), and v_2 the rate of the component observed at high concentration of ATP with lower apparent affinity for ATP (K_{M2}) and higher maximum velocity (V_{m2}). The kinetic parameters for both kinds of enzyme preparations are summarized in Tab. 1.

Table 1

Kinetic parameters of (Ca^{2+} + Mg^{2+})–ATPase activity measured at two different concentration ranges of ATP (mean ± SE; n = 5 in each experiment)

Enzyme	ATP concentration range (μM)	V_m (μmol 1/mg per h)	K_M (μM)
Bound	5–20	0.099 ± 0.019	7.60 ± 1.37
	125–2000	0.331 ± 0.043	217.4 ± 27.2
Extracted	5–20	0.079 ± 0.015	8.13 ± 1.46
	125–2000	0.286 ± 0.026	194.6 ± 21.4

Biphasic ATP activation curves for the (Ca^{2+} + Mg^{2+})–ATPase has also been found in human erythrocytes [12–14], membrane preparations from rat pancreatic islet cells [15], human lymphocytes [16] and rabbit sarcoplasmic reticulum [17].

Richards et al. tested human erythrocyte membranes for Ca^{2+} + Mg^{2+})–ATPase activity within a range of ATP concentration from 0.5 to 4000 μM [18]. They obtained values of V_m equaled 0.0343 and 0.375 μmol/mg/h for high and low affinity active site, respectively. K_M was equal to 2.46 and 143 μM. These values are of the same order as obtained in this work.

When the dependence of the activity of (Ca^{2+} + Mg^{2+})–ATPase on the concentration of free Ca^{2+} ions was tested the concentration of ATP was equal to 2 mM, whereas free Ca^{2+} concentration varied from 0.5 μM to 5 mM.

The results are displayed in Fig. 2. The curve relating (Ca^{2+} Mg^{2+})–ATPase activity to free Ca^{2+} is also biphasic, the activity reaches the maximum centered at around 20 μM free Ca^{2+}, and then drops to almost zero as Ca^{2+} concentration rises. Similar dependence was obtained in the research on the human red cell membrane [19–21]. In order to explain the results of the inhibition of the (Ca^{2+} + Mg^{2+})–ATPase from plasma membrane, it was considered that the inhibitory species could be either Ca^{2+} or CaATP complex.

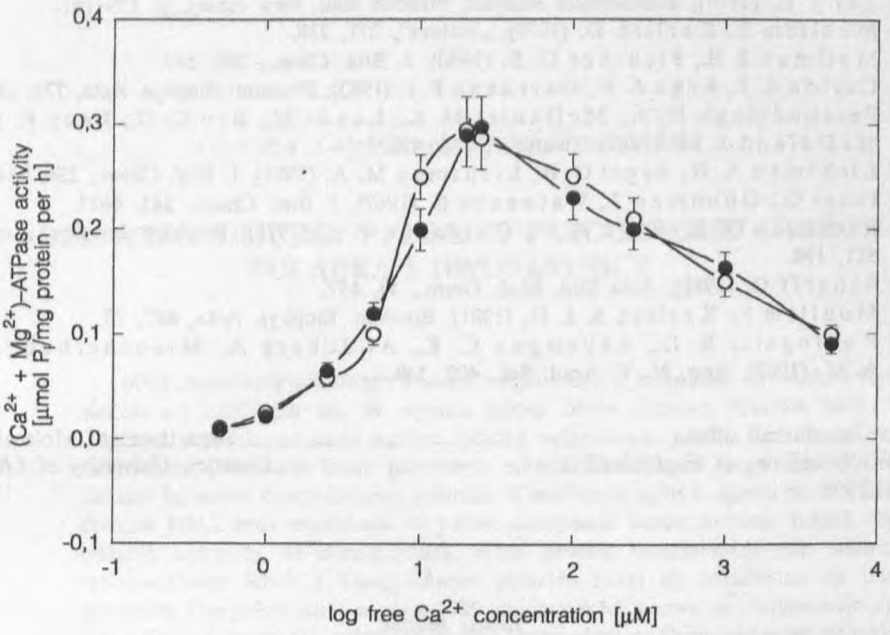

Fig. 2. The activity of the (Ca^{2+} + Mg^{2+})-ATPase bound to (open symbols) and extracted from (solid symbols) pig erythrocyte membrane as a function of free Ca^{2+} concentration. The ATP concentration was 2 mM in either case. Each point represents the mean of five replications. Errors bars denote SE

Data obtained in this work indicate that the dependence of the activity of the pig erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase on both ATP and free Ca^{2+} is similar to that obtained for human erythrocytes. Also kinetic parameters of the enzyme from pig red cells are close to the values typical for human being.

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WŁASNOŚCI KINETYCZNE (Ca^{2+} + Mg^{2+})-ATPAZY ZWIĄZANEJ Z BŁONAMI ERYTROCYTÓW ŚWINI I EKSTRAHOWANEJ Z NICH

Badano aktywność (Ca^{2+} + Mg^{2+})-ATPazy z błon erytrocytów świni. Używano dwóch rodzajów preparatów: enzymu związanego z fragmentami błon oraz enzymu ekstrahowanego z błon za pomocą detergentu. Obydwa preparaty enzymatyczne charakteryzowały się dwufazową zależnością aktywności od stężenia substratu, świadczącą o istnieniu dwóch funkcjonalnych centrów aktywnych: o niskim i wysokim powinowactwie do ATP. Również zależność aktywności enzymu od stężenia wolnych jonów Ca^{2+} miała charakter dwufazowy. Aktywność osiągała maksimum dla stężenia około 20 μM, a następnie stopniowo malała, gdy stężenie Ca^{2+} rosło. Otrzymane rezultaty świadczą, że zależność aktywności (Ca^{2+} + Mg^{2+})-ATPazy błon erytrocytów świni od stężenia ATP i Ca^{2+}, jak również charakteryzujące ją parametry kinetyczne są podobne do otrzymanych dla enzymu z błon erytrocytów ludzkich.