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ON THE SUBSTRATE SPECIFICITY OF THE RED CELL CALCIUM PUMP

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ATP-dependent active calcium transport in inside-out human red cell membrane vesicles is stimulated by magnesium essentially parallel with an increase in MgATP concentration. At a constant, low (1 μM) calcium concentration, increasing ATP and magnesium increase the maximum calcium transport rate irrespective of the constant or decreasing concentrations of CaATP present. K_{Ca} for calcium pumping is practically unchanged at variable ATP and magnesium concentrations. Free magnesium above 1–2 mM inhibits active calcium transport, probably through a direct interaction with the transport enzyme. Based on the experimental findings reported we suggest that the true, physiological substrate of the red cell calcium pump is MgATP.

The energy-donor substrate specificity of the red cell calcium pump has been a subject of controversy in the literature. Experimental findings interpreted to suggest that the substrates of the pump were MgATP [1,2], free ATP [3,4] or CaATP [5,6] have been published. The controversy is mostly based on the variability of the membrane preparations studied and on the interdependent changes in the chelated and free forms of ATP, magnesium and calcium, respectively, under any given experimental conditions. In the present study, by measuring active calcium transport in inside-out red cell membrane vesicles, we report data which consistently support the idea that the physiological substrate of the red cell membrane calcium pump is MgATP.

All the chemicals used were of reagent grade. Inside-out red cell membrane vesicles were prepared and assayed for their sidedness as described in Ref. 7. (These inside-out vesicle are depleted from endogenous calmodulin.) Active calcium uptake was assessed by the rapid filtration of inside-out vesicles and by measuring ^{45}Ca radioactivity in the vesicles, as described in Refs. 4 and 7. Each data point represents triplicate experiments by measuring inside-out vesicle calcium uptake in 3–5 min incubation periods at 37°C. Within this period the calcium uptake was linear in all experimental conditions. Free and chelated concentrations of ATP, calcium and magnesium were calculated by using the following stability constants: $K_{H\text{-}ATP} = 10^{6.9}$; $K_{CaATP} = 10^{3.9}$; $K_{MgATP} = 10^{4.0}$ (based on Refs. 6 and 8).

Fig. 1 shows the rate of active calcium transport in inside-out vesicles as a function of total magnesium concentration in the medium, in the presence of 20 μM ATP and 20 μM calcium. The dashed lines in the figure indicate the calculated concentrations of free- and metal-complexed forms of ATP, respectively, under the same conditions. The rate of calcium transport shows an almost parellel increase with the concentration of MgATP, while the decrease in CaATP has no pronounced effect on calcium uptake. In our previous paper [9] we demonstrated a similar effect of magnesium on

Abbreviations. Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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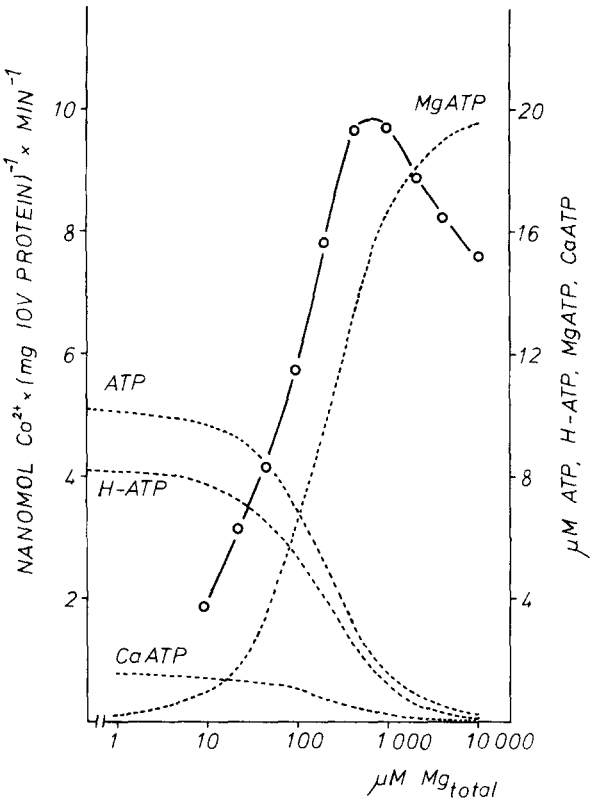

Fig 1 Magnesium concentration dependence of active calcium transport in inside-out red cell membrane vesicles (IOV) The incubation media contained 120 mM KCl, 17 mM Hepes (pH 7 0), 20 μM $CaCl_2$ (including ^{45}Ca tracer), 20 μM ATP, and the magnesium concentrations indicated The inside-out vesicle concentrtion was 20 μg protein/ml medium, temperature 37°C O——O, inside-out vesicle calcium uptake, nmol (mg IOV protein)$^{-1}$ min^{-1}, ------, calculated concentrations of ATP, H-ATP, MgATP and CaATP, respectively

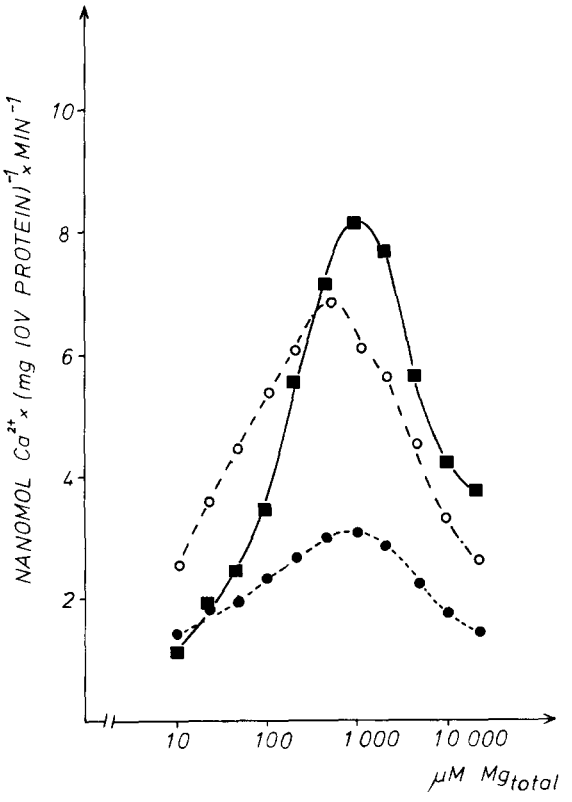

Fig 2 Magnesium and ATP concentration dependence of active calcium transport in inside-out vesicles (IOV) The incubation media contained 120 mM KCl, 17 mM Hepes (pH 7 0), 100 μM EGTA, 70 μM $CaCl_2$ including ^{45}Ca tracer (free calcium concentration 1 μM), and the ATP and magnesium concentrations indicated ●------●, 5 μM ATP; O – – O, 100 μM ATP, ■——■, 1 mM ATP The inside-out vesicle concentration was 20 μg/ml medium, temperature 37°C

inside-out vesicle calcium transport in the presence of 1 μM free (EGTA-buffered) calcium and 1 μM ATP. All these findings indicate that the substrate of the in situ calcium pump is MgATP, rather than free ATP or CaATP. At high magnesium concentrations active calcium transport is inhibited, and the smaller the calcium concentration the more pronounced is this inhibition (Penniston, J.T., personal communication). As we are going to show below, magnesium inhibition is probably due to a direct effect of this ion on the pump enzyme. In a previous paper [9] we showed that addition of calmodulin does not significantly alter the characteristics of magnesium stimulation of active calcium transport in inside-out vesicles. In the present experiments we studied the functioning of the calmodulin-depleted enzyme, in order to avoid any possible effect of the calcium-magnesium competition on the calmodulin molecule.

Fig. 2 is to show the effect of increasing magnesium concentrations on the rate of active calcium transport at a constant, low calcium (1 μM, buffered with EGTA) and at variable ATP concentrations. The most important findings in these experiments are that maximum calcium transport rate is greater at greater ATP concentrations, although CaATP is limited by the 1 μM calcium available and that magnesium stimulates calcium

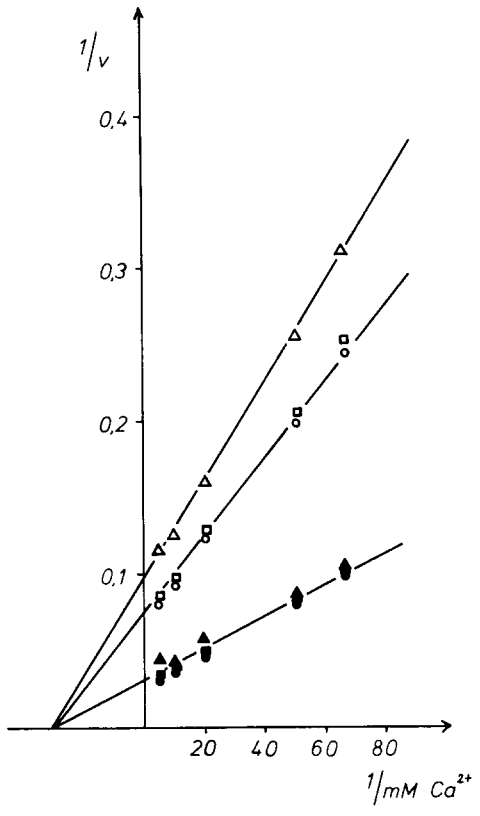

Fig 3 Calcium concentration dependence of active calcium transport in inside-out vesicles effects of ATP and magnesium The incubation media contained 120 mM KCl, 17 mM Hepes (pH 7 0), variable concentrations of unbuffered calcium (including ^{45}Ca tracer), and the ATP and magnesium concentrations indicated Inside-out vesicle concentration 20 μg/ml medium, temperature 37°C □——□, 20 μM ATP+0 5 mM magnesium, ○——○, 20 μM ATP+2 0 mM magnesium; △——△, 20 μM ATP+20 0 mM magnesium, ■——■, 500 μM ATP+2 mM magnesium, ●——●, 500 μM ATP+5 mM magnesium, ▲——▲, 500 μM ATP +20 mM magnesium Double reciprocal representation v is expressed as nmol Ca (mg IOV protein)$^{-1}$ min^{-1}

transport in spite of a strong decrease in [CaATP]. These data speak against the basic role of CaATP as a substrate for the pump. As has been shown by pump phosphorylation-dephosphorylation experiments [10,11] and by experiments on the effects of metals on the calcium transport kinetics [9], free magnesium is required for calcium transport in the micromolar concentration range and cannot affect the maximum calcium transport rate in the present experiments. The relative inhibition of calcium transport by higher [ATP] at low [Mg^{2+}] is probably explained by the chelation of free magnesium or by an inhibitory effect of free ATP on the transport system.

As seen from Fig. 2, magnesium above 1–2 mM inhibits calcium transport with about the same apparent $K_{I,Mg}$ at the three ATP concentrations tested, indicating a direct effect of magnesium ions on the pump activity, rather than an inhibition by the formation of MgATP. The observed magnesium-inhibition of ATPase activity in the isolated calcium pump [5,6] is probably caused by an increased susceptibility of the purified pump protein to this magnesium effect seen in inside-out vesicles.

In the following experiments we analyzed the effect of magnesium and ATP on the calcium kinetics of the calcium pump in inside-out vesicles. As is shown in Fig. 3, the value of K_{Ca} is unchanged at the various ATP and magnesium concentrations tested. The data indicate that the availability of MgATP is a limiting factor in the calcium transport, but the apparent calcium affinity of the pump is unaltered in this range of ATP and magnesium concentrations. In these experiments we did not observe a competitive-type inhibition by higher [Mg^{2+}], thus, if there is a calcium-magnesium competition, it is probably not at the calcium transport site of the enzyme.

Based on the data reported above we conclude that the substrate of the red cell membrane calcium pump is MgATP, while high concentrations of free magnesium (and possibily also free ATP) inhibit active calcium transport.

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