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REGULATION OF THE Ca^{2+}-PUMP BY CALMODULIN IN INTACT CELLS

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ATP-enriched human red cells display high rates of Ca^{2+}-dependent ATP hydrolysis (16 mmol·litre cells^{-1}·h^{-1}) with a high Ca^{2+} affinity ($K_{0.5} \sim 0.2$ μM). The finding suggests a mechanism for regulation of cell Ca^{2+} levels, involving highly-cooperative stimulation of active Ca^{2+} extrusion following binding of calmodulin to the ($Ca^{2+} + Mg^{2+}$)-ATPase.

Recent studies have demonstrated the presence of calmodulin-regulated, ATP-fuelled Ca^{2+} pumps in the plasma membrane of many cells [1–8]. This pump has been assumed to be involved in maintaining the low intracellular Ca^{2+} concentration in intact cells [9]. In isolated membrane systems, calmodulin-bound Ca^{2+} pumps show a high Ca^{2+} affinity and high turnover rates [10–12]. But measurements of Ca^{2+} pump activity in intact red cells have cast doubt on the physiological significance of Ca^{2+}-pump regulation by calmodulin in vivo, since only low Ca^{2+} affinity and low turnover rates have been observed [13–15]. Depletion of ATP [16–18] and the presence of inhibitory concentration of calcium ions in red cells used in previous work, could have altered the properties of the pump [19]. Therefore, we have measured Ca^{2+} pump activity in intact red cells enriched with ATP and containing low, controlled Ca^{2+} concentrations. We find a high Ca^{2+} affinity and high turnover rate of the Ca^{2+} pump in the cells. On the basis of this finding we propose that binding or dissociation of calmodulin to the Ca^{2+} pumps is critically affected by the cytoplasmic free Ca^{2+} ions concentration, providing a mechanism for rapid restoration of cytoplasmic Ca^{2+} levels raisedby an increaed membrane permeability to Ca^{2+}. This mechanism could apply to all cells containing calmodulin-activated Ca^{2+} pumps.

Fig. 1 shows a time-course of phosphate release in intact red cells treated with a high concentration of divalent metal ionophore A23187, in media containing Mg^{2+} or Mg^{2+} plus Ca^{2+}. The free Ca^{2+} concentration in the cells reached equilibrium with that in the medium which is determined by the presence of EGTA. Measurements ot total calcium in the cells showed that in our conditions a constant level was achieved within 30 seconds after treatment with A23187 (100 μM), and remained constant during the test incubation (not shown). The medium contained a high K^+ concentration in order to minimize changes in membrane potential due to activation of the Ca^{2+}-dependent K^+ permeability channel (see Ref. 20). Assuming equilibration of Ca^{2+} and Mg^{2+} and an unchanged membrane potential of −8 mV, the concentration of the free ions in the cells will be 1.9-fold greater than in the medium. With the concentration of Mg^{2+} (4.75 mM) and Ca^{2+} (19 μM) used in this experiment essentially all of the cellular ATP should be MgATP and the con-

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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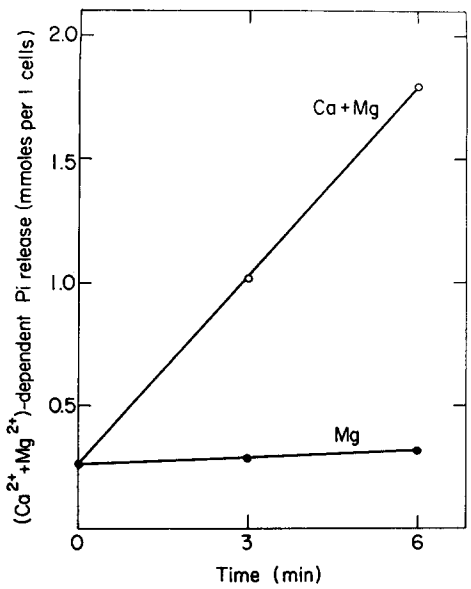

Fig. 1. $(Ca^{2+} + Mg^{2+})$-dependent P_i release in ATP enriched intact red cells.

(1) Preparation of ATP loaded red blood cells. The procedure wasbased on the method of Whittam and Wiley [21] with the following modifications: Cells wwere washed three times (by centrifugation at 5000× *g* for 5 min) in a solution composed of 60mM NaCl, 75 mM KCl, 0.2 mM $MgCl_2$ and 10 mM Hepes (pH 7.4 at 37°C) (solution A). Then the packed cells were resuspended in (~10% hematocrit) the same solution containing 15 mM adenosine, 10 mM K_2PO_4 (pH 7.4), 0.2 mM EGTA and 0.1 mM ouabain. Following incubation for 60 min at 37°C the cells were washed three times at room temperature with solution A, and resuspended and incubated for a further 30 min at 37°C in solution A containing 10 mM inosine, 0.2 mM EGTA and 0.1 mM ouabain. At the end of this incubation the cells were washed five times with solution A containing 0.05 mM ouabain (with 5 min incubation at room temperature between each wash in order to allow efflux of inosine).

(2) Measurement of P_i release. The ATP loaded, washed cells were suspended (at 25% hematocrit) into solution A that contained 1 mM EGTA, 0.1 mM ouabain, 2.5 mM $MgCl_2$ without (○) and with (●) 1004 μM $CaCl_2$ added, giving 10 μM external free Ca^{2+}. ATP hydrolysis was initiated by the addition of the ionophore A23187 to a final concentration of 100 μM. After various times at 37°C, 0.5 ml of cells suspension was transferred into Eppendorf tubes (containing 10 μl of $LaCl_3$ stopping solution, 10 mM) which were imersed in an ice-cold water bath. After completion of the experimental incubations, the cells were lysed with 0.8 ml water. Protein was precipitated by the addition of 100 μl of perchloric acid 70% and removed by centrifugation at 12000 rpm for 3 min (using the Eppendorf centrifuge). 0.8 ml of clear supernatant was removed for P_i determination, measured by the sensitive assay described in Ref. 14. ATPase activity was calculated as mmol·(litre cells)$^{-1}$ ·h^{-1}.

TABLE I

$(Ca^{2+} + Mg^{2+})$-DEPENDENT ATPase ACTIVITY, MEASUREMENT OF EITHER ATP CONSUMPTION OR P_i RELEASE

Loading of cells with ATP and all experimental incubations were as described in the legend to Fig. 1, except the wash solution A was replaced by one consisting of 130 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$ and 10 mM Hepes (pH 7.4 at 37°C). The ATP concentrations inthe red cells were measured as described in Ref. 22.

$[Ca^{2+}]$ (μM)	Iodo-acetamide (6 mM)	$(Ca^{2+} + Mg^{2+})$-ATPase activity (mmol·(litre cells)$^{-1}$·h^{-1})	
		ATP consumption	P_i release
25	–	–	11.99
25	+	–	12.13
50	+	10.63	–

centration of Ca ATP should be negligible. Mg^{2+}-dependent P_i release is slow (0.4 mmol·(litre cells)$^{-1}$·h^{-1}), but addition of only 19 μM free Ca^{2+} raised the rate by 40-fold to 16 mmol·(litre cells)$^{-1}$·h^{-1}. For incubation times of up to 6 min, Ca^{2+}-dependent P_i release was linear, at all Ca^{2+} concentrations used in the present experiments.

In order to observe the very high rate of P_i release in Fig. 1 it has been found essential to preload the cells with ATP. This was achieved by preincubation with adenosine and phosphate which raised ATP levels from about 0.6 to 2.5 mM. Subsequent treatment with inosine lowered excess phosphate to about 0.25 mM. Measurement of Ca^{2+}-dependent P_i release in unfed red cells showed both a low activity (≃4 mmol·(litre cells)$^{-1}$·h^{-1}) and low Ca^{2+} affinity ($K_{0.5} \simeq 300$ μM) as reported before [13–15].

In order to confirm that the Ca^{2+}-dependent P_i release is due to ATP hydrolysis, rather than that of other phosphate compounds, we have made also parallel estimates of ATP consumption (this must be done in the presence of iodoacetamide to prevent glycolytic regeneration of ATP). The rates of P_i appearance and ATP disappearance are essentially equal (Table I).

Fig. 2 shows both a very high Ca^{2+} affinity ($K_{0.5} \simeq 0.5$ μM) and activity of Ca^{2+}-dependent P_i release when measured in intact red cells by the

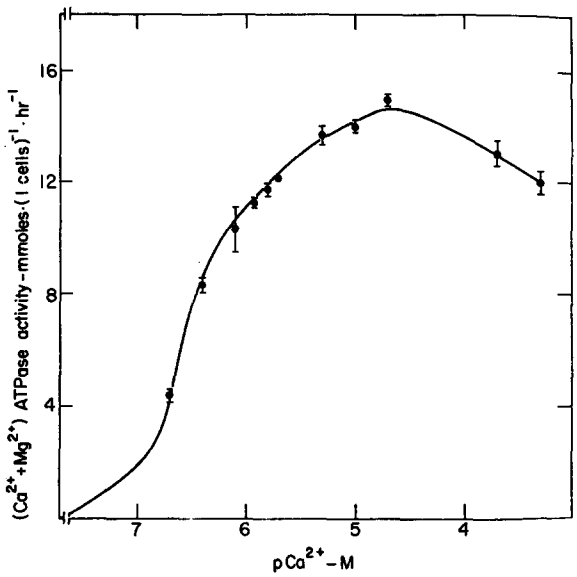

Fig. 2. Ca^{2+} dependence of the $(Ca^{2+}+Mg^{2+})$-ATPase activity in intact red cells. All the experimental procedures were essentially as described in the legend to Fig. 1 except that the reaction media contained the appropriate amounts of $CaCl_2$ to give the indicated free Ca^{2+} concentrations. The $(Ca^{2+}+Mg^{2+})$-ATPase activities were measured at 37°C for 3 and 6 min at all Ca^{2+} concentrations and the Mg^{2+}-dependent ATPase activity was subtracted from the total activities measured in the presence of $(Ca^{2+}+Mg^{2+})$.

method described in Fig. 1. Inhibition of activity became apparent at Ca^{2+} concentrations of 200 μM or higher and this is due probably to the presence of appreciable concentrations of Ca ATP (see Ref. 19). The high Ca^{2+} affinity and high activity is very similar to that observed both in resealed ghosts [23] and inbroken membranes [24], when activated by calmodulin. from measurements of steady-state Ca^{2+} distribution between the medium and ionophore-treated intact red cells, Ferreira and Lew [25] calculated, indirectly, very similar kinetic parameters of $K_{0.5}$ for $Ca^{2+} \simeq 0.7$ μM and $V_{max} \simeq 12$ mmol · (litre cells)$^{-1}$ · h^{-1}.

Sarkadi suggested that ionophore may interact with the Ca^{2+} pump and raise the apparent Ca^{2+} affinity [9]. This kind of explanation of the high Ca^{2+} affinity observed in Fig. 2 cannot be true. Firstly, as mentioned above, ionophore treated intact cells which were not enriched with ATP show low activity, low Ca^{2+} affinity, ATPase activity. Also the high Ca^{2+} affinity evident in intact cell and resealed ghosts experiments, is found with broken membranes either treated or untreated with ionophore (Ref. 17; and Muallem, S. and Karlish, S.J.D., unpublished data).

The passive Ca^{2+} permeability of intact red cells at 37°C is no greater than 10 μmol · (litre cells)$^{-1}$ · h^{-1} [26]. Assuming a pump-leak steady-state for the Ca^{2+} distribution, the Ca^{2+} pumping rate can be no higher than this value. If one knew the precise concentration of free Ca^{2+} in the red-cell cytoplasm one could deduce unequivocably (from the curve in Fig. 2) whether or not the pumps were bound with calmodulin. In the absence of such information, a rate of pumping as low as 10 μmol · (litre cells)$^{-1}$ · h^{-1} implies that the free ion concentration of Ca^{2+} is much lower than $2 \cdot 10^{-7}$ M (i.e. the lowest concentration tested) although precisely, how much lower cannot be predicted (e.g. 10^{-8} or 10^{-9} M etc.), because we do not know the dependence of pumping on Ca^{2+} levels below $2 \cdot 10^{-7}$ M. The activation of the pump by Ca^{2+} concentrations below 10^{-7} could be affected by several factors. Calmodulin binds 3–4 calcium ions with affinities in the range 10^{-7}–10^{-6} M [27] and binding of Ca^{2+} to calmodulin is necessary for binding of calmodulin to the pump [28,29]. The actions of so-called calmodulin-binding proteins [11,30,31] could also affect the Ca^{2+} dependence of pumping below $2 \cdot 10^{-7}$ M, and explain the very low rate of pumping in normal cells. At all events it is very unlikely that calmodulin is bound to the pump under steady-state physiological conditions. When the internal Ca^{2+} concentration is raised, as in the experiment of Fig. 2, the very large increase in rate and Ca^{2+} affinity of $(Ca^{2+}+Mg^{2+})$-ATPase suggest that calmodulin must become bound to the pump.

One may ask what is the advantage to the cell of a Ca^{2+}-pump with a maximal turnover rate 3 orders of magnitude greater than is normally required? Consider the possibility of a rapid and transient influx of calcium ions through a membrane, across which there is a very large concentration gradient. This will rapidly raise the internal Ca^{2+} concentration by a very large factor. Since a rise in cytoplasmic Ca^{2+} affects many important functions of the cell and in time may cause irreversible damage [9] the primary necessity for a high-capacity pump should be to extrude incoming

Ca^{2+} as quickly as possible. A rapid increase in Ca^{2+} concentration to say 10^{-7}–10^{-6} M or above will cause calmodulin to bind the Ca^{2+} and activate the Ca^{2+} extrusion mechanism in a manner which is highly cooperative with respect to the Ca^{2+} concentration. This will be achieved by the requirement for 3–4 calcium ions to be bound, giving a large increase in apparent Ca^{2+} activation affinity. The degree of non-linearity is compounded by the combination of the rise in Ca^{2+} affinity and in turnover rate of the pump. We have shown that Mg ATP stimulates the Ca^{2+} pump in a negatively cooperative fashion in calmodulin-activated membranes [32]. This feature, too, can be seen to contribute to maintenance of a high rate of Ca^{2+} pumping for as long as necessary, because as ATP is consumed the rate will fall more slowly than would be the case if, for example, activationby ATP were hyperbolic.

In red cells specific Ca^{2+} permeation mechanism havenot yet been described. However, in many other cells Ca^{2+} permeation mechanisms exist, for example voltage-sensitive Ca^{2+} channels, Ca^{2+}-Na^{+} exchange mechanisms, hormone-sensitive Ca^{2+} entry etc. [33]. Many cells having specific Ca^{2+} permeation mechanisms have now been shown to actively extrude Ca^{2+} by means of high Ca^{2+} affinity, calmodulin-sensitive ATP dependent Ca^{2+}-pumps [2–8]. Therefore, the mechanism proposed above for adjusting Ca^{2+} pumping rates to rapid increases in cytoplasmic Ca^{2+} may be particularly relevant to the case of such cells.

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