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Calcium Dependent ATP Losses in Intact Red Blood Cells without Cellular Accumulations of Calcium

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Summary. Intact human red blood cells incubated with ionophore A23187 and calcium develop a depletion of ATP that is dependent upon the concentrations of both A23187 and Ca. Incubations of fresh cells with 0.5 μM A23187 and concentrations of Ca at or below 70 μM produce a depletion of ATP without a net cellular uptake of Ca. In contrast, ATP-depleted cells display an ionophore-dependent cellular uptake of Ca, under identical conditions. A hypothesis is proposed that relates these ionophore-produced ATP depletions to active Ca extrusion by the Ca ATPase.

The low concentration of calcium in human red blood cells is thought to be maintained by the low permeability of the membrane to calcium (Ferreira & Lew, 1975) and by the extrusion of calcium through an ATP-dependent pump (Schatzman, 1973). The divalent cation ionophore, A23187, has been used to increase selectively the permeability of this membrane to calcium (Reed & Lardy, 1972; Sarkadi, Szasz & Gardos, 1976). Previous work has shown that the effect of calcium on the permeability of the erythrocyte to potassium is markedly enhanced in the presence of this ionophore (Reed, 1976; Plishker & Gitelman, 1976). In addition, exposure to calcium and ionophore is accompanied by a substantial depletion of the cellular ATP in fresh, intact erythrocytes (Kirkpatrick, Hillman & LaCelle, 1975). The present study further evaluates the influence of calcium and ionophore on the process of ATP depletion.

Materials and Methods

Blood was collected with heparinized syringes, and, except for the studies done with metabolically depleted cells, all experiments were begun within 15 min of venipuncture. The plasma and buffy coat were removed before the cells were washed twice in a 300 mos-

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mol/kg solution containing 5 mM Tris, pH 7.4, and tetramethylammonium (TMA) chloride. Cells were then washed once and resuspended in the solutions which contained 100 mM sodium chloride, 1 mM potassium chloride, 40 mMTMA chloride, and 20 mM HEPES-Tris buffer pH 7.4. Appropriate amounts of calcium chloride were added to this cell suspension. The indicated extracellular concentrations of calcium (Ca_{EXT}) were measured at the end of the incubations. All incubations were performed at 20 °C with moderate shaking. A cell to medium ratio of approximately 1:20 was maintained in all experiments. For cell Ca determinations the cells were packed by the centrifuge method previously described by Parker (1971). Since erythrocytes do not accumulate Ca in the absence of ionophore, we used the Ca values of nonionophore-treated samples to correct for the trapping of extracellular fluid. As expected, we observed that the Ca values of the pellets of these nonionophore-treated cells were linearly related to the concentration of Ca in the supernates. We established this linear relationship with nonionophore-treated cells within each experiment. With the ionophore-treated groups, we used this relationship and their supernatant concentrations of Ca to calculate their trapped Ca values. These values were subtracted from the Ca contents of the pellets to obtain the cellular Ca contents (Ca_{INT}). Calcium measurements were done with an atomic absorption spectrophotometer equipped with a heated graphite atomizer as previously described (Parker, Gitelman, Glosson & Leonard, 1975). The ATP determinations were accomplished by the semi-automated procedure of Dufresne and Gitelman (1970), which is not affected by the concentrations of Ca employed in our studies. Hemoglobin concentrations were determined by the method of VanKampen and Zijlstra (1961). Cellular concentrations are expressed in terms of hemoglobin so the units of measure are independent of any alterations in cell volume. Cell water determinations were done by drying to constant weight (Parker 1971). Red cell ghosts were prepared by hemolyzing cells in a cold 0.1 mM EDTA solution, followed by repeated washes in 15 mM sodium chloride solutions containing 1.7 mM tris buffer, pH 7.4. The ATPase activity of these ghosts was determined by measuring the release of inorganic phosphate. Incubations were performed at 37 °C in the presence of 60 mM sodium chloride, 20 mM potassium chloride, 1 mM ouabain, 1.6 mM magnesium chloride, 0.8 mM ATP, and 25 mM tris buffer, pH 7.45.

Results

Figure 1 displays representative time courses for ATP depletions performed in the presence of 0.5 μM A23187 with either 50 or 500 μM external concentration of calcium (Ca_{EXT}) in the absence of glucose. For the first 30 min of incubation, the rates of change of ATP concentrations are linear for both curves, and are clearly influenced by the Ca_{EXT}. After 30 min the slopes of the two curves decrease with time and are less influenced by the Ca_{EXT}. Figure 2 is the composite results of six experiments with 30 min incubations with different Ca_{EXT} in the presence of either 0.5 or 1 μM A23187. In order to evaluate the results from cells of different donors, the measured changes in ATP content within each study have been expressed as a percentage of each initial ATP concentration. The figure shows that the degree of ATP depletion is dependent upon the concentration of both the ionophore and the external Ca. A doubling of the A23187 concentration more than doubles the rate of ATP decline. The shape of these curves suggests that this depletion

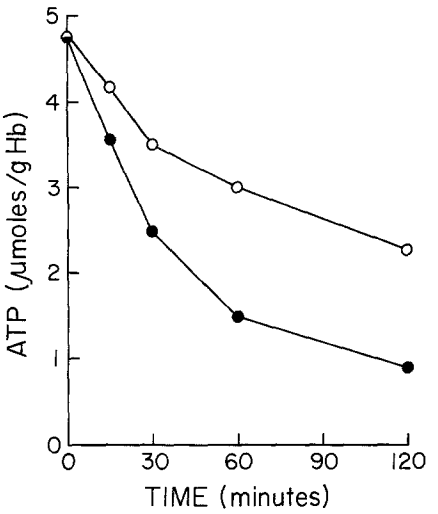

Fig. 1. Intracellular ATP as a function of time in the presence of two external concentrations of Ca. Erythrocytes were incubated at 20 °C with 0.5 μM A23187. The incubation media contained (in mM): 100 NaCl, 1 KCl, 40 Tetramethylammonium chloride, 20 HEPES (N-2 hydroxyethylpiperazine-N′-2-ethanesulfonic acid)–Tris buffer at pH 7.4 and either 50 (○) or 500 (●) μM $CaCl_2$

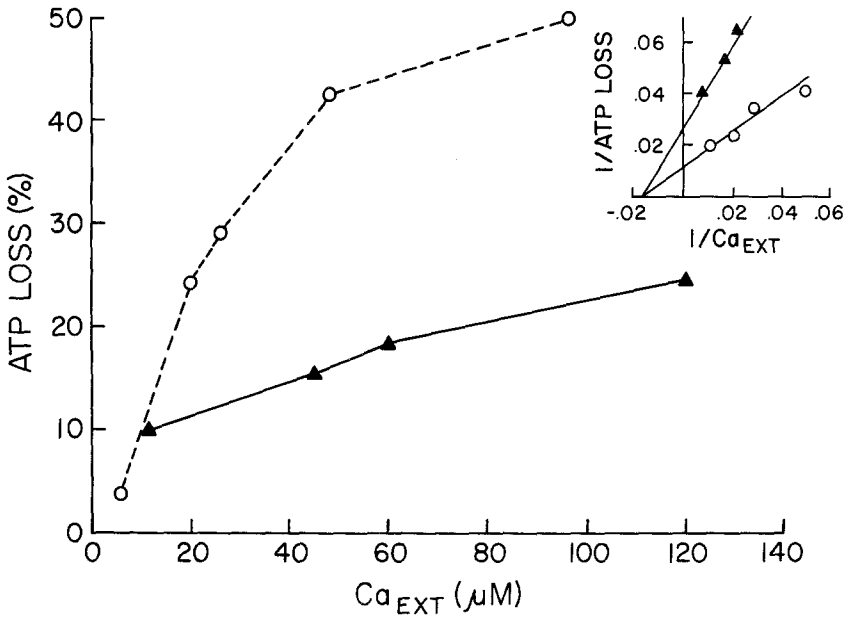

Fig. 2. Changes in the cellular content of ATP as a function of the extracellular concentration of Ca, in the presence of two concentrations of ionophore. Erthrocytes were incubated for 30 min under the same conditions described in Fig. 1. The closed symbols represent incubations in the presence of 0.5 μM A23187. The open symbols represent incubations in the presence of 1 μM A23187. The change in ATP is expressed as a percentage of the cells' original content of ATP. The figure insert shows a double reciprocal plot of the data at or above 20 μM Ca_{EXT}

Table 1. Lack of effect of ouabain on ATP depletion

Ca_{EXT} (μM)	ATP Loss (%)	
	No ouabain	2×10^{-4} M ouabain
50	10.3±3.2	10.3±2.5
100	13.3±3.8	14.7±5.7

30-min incubations were performed under the same conditions described in Fig. 1 with A23187 at 0.5 μM. Each value represents the results from 3 experiments with standard errors. The change in ATP within each experiment is expressed as a percentage of the original content of ATP.

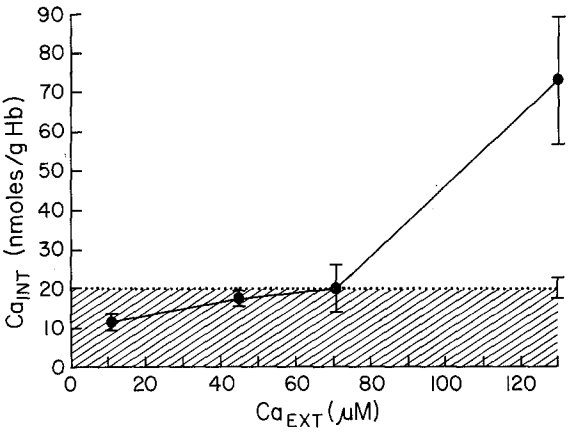

Fig. 3. Cellular Ca as a function of extracellular Ca in the presence of 0.5 μM A23187. The results are from 4 experiments. Standard errors are shown. The division line between the shaded and unshaded regions represents the Ca value of cells not treated with ionophore. The erythrocytes were incubated for 30 min under the same conditions described in Fig. 1

process is a saturable function of the Ca_{EXT}. A double reciprocal plot of the points at or above 20 μM Ca_{EXT} (see figure insert) agrees with this interpretation and suggests that the ATP-depletion processes at both ionophore concentrations have similar affinity constants for calcium (60 μM). In contrast, in experiments not shown when the cells are pre-incubated with 10 mM glucose for 30 min at 20 °C, ATP depletion does not occur with 0.5 μM A23187 and Ca_{EXT} as high as 100 μM.

ATP depletion produced by A23187 and calcium is insensitive to ouabain. Table 1 shows a comparison of ATP losses at two concentrations of calcium in the presence and absence of 2×10^{-4} M ouabain. No detectable differences occurred whether ouabain was present or not during the incubations.

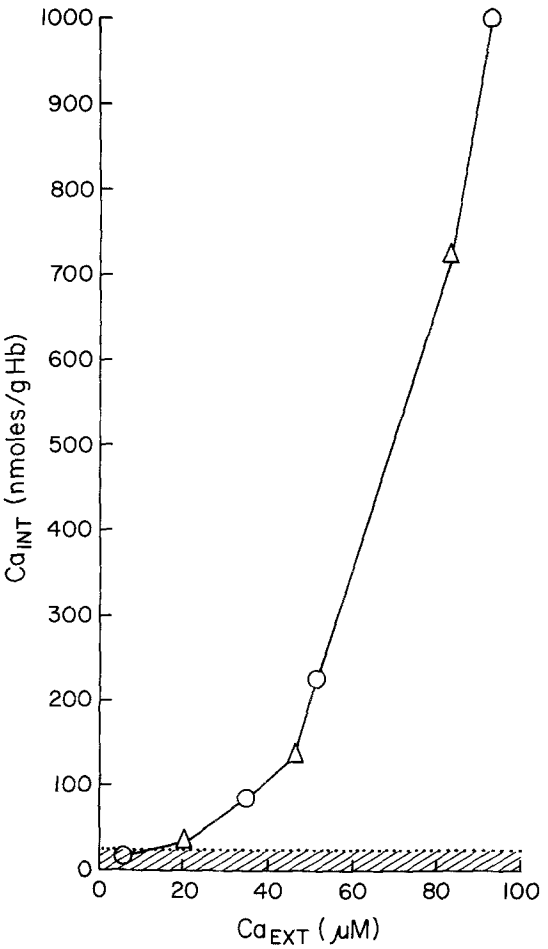

Fig. 4. Cellular Ca as a function of extracellular Ca in the presence of 1 μM A23187. The results in the presence of A23187 are from two experiments, and are distinguished by different symbols. The division line between the shaded and unshaded regions represents the cellular Ca value of cells not treated with ionophore. The erythrocytes were incubated for 30 min under the same conditions described in Fig. 1

Figure 3 shows the internal amount of calcium (Ca_{INT}) at the end of 30-min incubations with different external Ca in the presence of 0.5 μM A23187. In comparison with the nonionophore-treated control value, represented by the division line between the shaded and nonshaded regions, the 0.5 μM A23187-treated cells have lower Ca_{INT} when the external calcium is less than 70 μM. In experiments not shown, we observed with 0.5 μM A23187 and 50 μM Ca_{EXT} that the Ca_{INT} was stable for 1 hr. Since these intracellular calciums are expressed in terms of hemoglobin, these values are independent of cellular volume. To establish whether

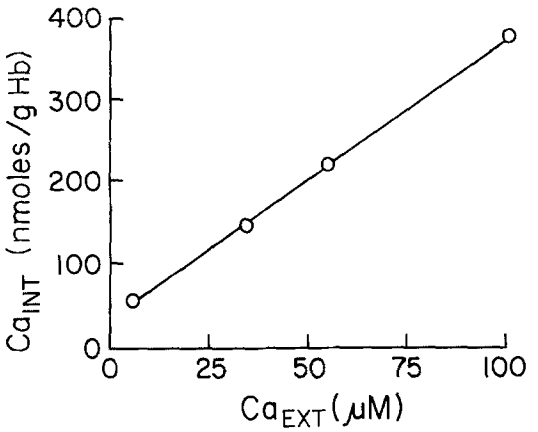

Fig. 5. Cellular Ca of metabolically depleted cells as a function of extracellular Ca. Cells were preincubated for 24 hr at 37 °C in the presence of isotonic NaCl solution. They were then washed and incubated for 30 min in the manner described in Fig. 1

these decreases in Ca are associated with a decrease in the concentration of Ca in cell water, we compared the water content of cells incubated for 30 min with or without 0.5 μM A23187 in the presence of 30 μM Ca_{EXT}. The ionophore-treated cells contained 1854 ± 4 g of water per kg dry solid, while the nontreated cells contained 1996 ± 4 g of water per kg dry solid. This 7% decrease in cell water would be associated with a 25% decrease in total Ca (*cf.* Fig. 3). Figure 4 shows that the relationship between Ca_{EXT} and Ca_{INT} can be altered by changing the concentration of the ionophore. In contrast to the results of Fig. 3, when the concentration of ionophore is doubled to 1 μM significant accumulations of Ca occur at all Ca_{EXT} at or above 20 μM.

Figure 5 shows that metabolically depleted cells accumulate Ca in the presence of 0.5 μM A23187 under the same conditions in which metabolically replete cells maintain low Ca_{INT} (Fig. 3). Incubations in the absence of ionophore show a trivial accumulation of Ca.

Figure 6 displays the results of four separate experiments performed on cells from the same donor. The change in ATP content is plotted as a function of the internal Ca in the presence of either 0.5 (closed symbols) or 1 μM A23187 (open symbols). At the higher internal Ca (>80 nmoles/g Hb), the amount of ATP depletion is similar for the two ionophore concentrations. The greatest difference in these two groups comes at the lower internal Ca (<40 nmoles/g Hb). The cells treated with the lower concentration of A23187 appear to have greater sensitivity to or apparent affinity for Ca, than the cells treated with

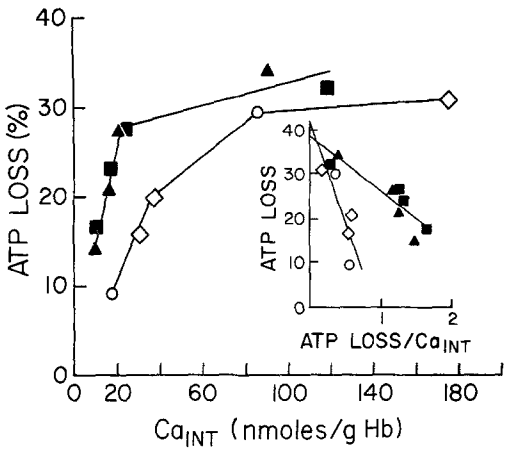

Fig. 6. Changes in the cellular content of ATP as a function of the intracellular Ca in the presence of two concentrations of ionophore. Measurements were done after 30-min incubations in media containing different concentrations of Ca. The closed symbols represent incubations in the presence of 0.5 μM A23187. The open symbols represent incubations in the presence of 1 μM A23187. The different symbols represent the different experiments. The figure insert is a Hofstee plot of the data

Table 2. Lack of effect of A23187 on ATPase of broken cell preparation

Ca (μM)	Ca-ATPase (nmole/mg/h)	
	No A23187	0.5 μM A23187
15	77.9	74.8
100	124.7	121.6
150	130.9	124.7
250	118.4	118.4
750	84.2	79.8

Stroma were prepared by hemolyzing the cells in a cold 0.1 mM EDTA solution, followed by repeated washes in a 15 mM NaCl solution containing 1.7 mM Tris buffer, pH 7.4. The ATPase activity of this preparation was determined at 37 °C in the presence of (in mM) 60 NaCl, 20 KCl, 1 ouabain, 1.6 $MgCl_2$, 0.8 ATP, and 25 Tris buffer, pH 7.45. Ca ATPase activity is the increment in inorganic PO_4 release produced by the addition of Ca to this basal salt solution. The units of activity are expressed as nmoles Pi/mg dry wt./h.

twice the ionophore concentration. A Hofstee plot of the data (*see* the figure insert) supports these observations and reveals that the maximum depletion of ATP in 30 min would be approximately 38% of the starting concentration. The apparent affinity of the process for Ca in the presence of 0.5 and 1 μM ionophore is 12 and 43 nmoles Ca per g Hb, respectively.

The Ca-stimulated ATPase activity of broken red cell preparations is not affected by the presence of ionophore. As shown in Table 2, 0.5 μM A23187 has no apparent effect on the sensitivity of the enzyme to Ca or on the maximal rate of hydrolysis.

Discussion

Kirkpatrick *et al.* (1975) first reported ATP depletions with A23187 in intact red blood cells. Their observations, performed at 37 °C, included a significant cellular accumulation of Ca. Our studies reveal further characteristics of the depletion process: (a) under the conditions used in our studies the rates of ATP depletion are constant for the first 30 min of incubation; (b) during this 30-min period of incubation the extent of depletion is dependent upon the concentrations of both ionophore and external Ca; (c) under certain conditions these depletions occur without a net cellular accumulation of Ca. Since these ATP depletions can be prevented by preincubation with glucose, we believe the loss of ATP in the absence of glucose reflects an increased utilization of ATP.

In terms of current theory, A23187 enhances Ca influx across the membrane by forming a complex with Ca which traverses the plasma membrane (Reed, 1976). If the ionophore acts in this manner in human eryhtrocytes, we would predict that our observed Ca-dependent ATP loss should be related to the influx of Ca produced by A23187. The relationship between the depletion process and the concentrations of both ionophore and external Ca agrees with this theory. As shown in Fig. 2, the depletion is dependent upon the concentration of ionophore. Furthermore, as expected with a carrier system, the process appears, at different ionophore concentrations, to have a fixed affinity for external Ca, and appears to saturate with respect to Ca_{EXT}.

Under these circumstances, we expected that some net accumulation of Ca would be necessary to initiate the increased utilization of ATP. In contrast, under special conditions, we observe marked ATP losses in the absence of Ca accumulations (*cf.* Figs. 2 and 3). Although the water loss from these cells would contribute to a higher intracellular concentration of Ca in cell water, the change does not appear to be sufficient to raise the concentration above the normal value. Thus, the initiation of the ATP loss cannot be totally explained by a change in cell volume. It seems unlikely that this phenomenon can be attributed to a sensitization of the ATP hydrolysis system by ionophore, since

ionophore has no effect on the Ca activated ATPase system in stromal preparations (Table 2). Alternatively, the initiation of the loss of ATP in the absence of any net accumulation of Ca could be explained by a redistribution of Ca within the cell that enhances the delivery of the ion to those areas involved with Ca dependent ATP hydrolysis.

If we are correct in assuming that A23187 enhances Ca influx to the cells, it follows that the ionophore-treated cells maintain their low Ca_{INT} by transporting Ca out against a gradient. Since the red cell is thought to have a Ca transport ATPase, it is also reasonable to suggest that this active Ca extrusion and the observed ATP depletion are interrelated. As long as the amount of Ca influx does not exceed the outward transport capabilities of the ATPase, the cells do not accumulate Ca. At the same time the rate of ATP hydrolysis remains a function of the amount of Ca in the external solution, since under these conditions, the external concentration of Ca, the influx of Ca and the efflux of Ca must all be related. At the concentrations of Ca employed in these experiments this equilibrium is obtained only in the presence of 0.5 μM A23187. Doubling the concentration of ionophore increases Ca influx to the point that it exceeds the rate of Ca extrusion and the cells therefore accumulate Ca (Fig. 4). A similar relationship between the concentrations of both A23187 and Ca has been suggested previously in erythrocyte studies on the basis of changes in either the permeability of these cells to potassium (Reed, 1976) or their internal steady state of Ca (Ferreira & Lew, 1976).

Our hypothesis that ATP depletion may be related to ionophore-mediated Ca influx is also consistent with our other findings. The insensitivity of the system to ouabain (Table 1) agrees with this hypothesis, since ouabain does not inhibit the Ca activated ATPase. The marked accumulation of Ca in metabolically depleted cells supports the correlation between the hydrolysis of ATP and the maintenance of low Ca_{INT}. Finally, the apparent affinity of the ATP depletion process for internal Ca (Fig. 6) has the same order of magnitude as has been reported for the Ca ATPase in reconstituted red cells (Schatzmann, 1973).

Further proof that the loss of ATP is related to the outward transport of Ca might be obtained by measuring the relationship between Ca efflux and ATP loss. Unfortunately, technical limitations prevent us from obtaining precise unidirectional flux data for this system at this time.

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