# THE EFFECT OF CALCIUM IONOPHORE A23187 ON THE ATP LEVEL OF HUMAN ERYTHROCYTES

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Summary: Incubation of red cells at 37° with the ionophore A23187 results in a loss of ATP that is dependent on the concentrations of A23187 and $Ca^{2+}$ in the medium. ATP hydrolysis is greatest at micromolar concentrations of $Ca^{2+}$ and decreases as $Ca^{2+}$ in the medium is raised to millimolar levels. The ATP depletion is due to stimulation of calcium ATPase by A23187-mediated $Ca^{2+}$ influx into the cell. The biphasic nature of $Ca^{2+}$-stimulated ATP depletion in whole cells reflects the activity of $Ca^{2+}$-ATPase in membrane preparations at varying $Ca^{2+}$ concentrations. The ionophore can be removed by washing the cells with plasma or bovine serum albumin-containing medium and the ATP levels restored to normal by reincubating with 5 mM adenosine for 1 hr.

Introduction: The ionophore A23187 has been shown to have a variety of effects on erythrocytes. These include intracellular calcium accumulation accompanied by a calcium-dependent extrusion of hydrogen ions, loss of intracellular potassium, and a decrease in cell water and volume (1,2). Magnesium and strontium, as well as calcium, are bound and transported by A23187 (1).

Some investigators have found that red cell ATP levels decrease rapidly in the presence of A23187 (1,3) while others report little or no change (2,4). We report here that A23187 does indeed cause the ATP level of red cells to diminish. The extent of ATP hydrolysis depends on the $Ca^{2+}$ concentration of the medium. At micromolar concentrations of $Ca^{2+}$, the fall in intracellular ATP is associated with the activation of membrane $Ca^{2+}$-ATPase activity. However, at millimolar concentrations of $Ca^{2+}$, the $Ca^{2+}$-ATPase of the red cell membrane is inhibited and ATP levels do not fall as rapidly in the presence of the ionophore.

Methods and Materials: Heparinized blood from normal adult human volunteers was collected fresh each day, washed 2 times with isotonic saline, and the buffy coat removed by aspiration. The cells were then washed in 10 mM potassium phosphate buffer made isotonic (290 mosM) with NaCl, pH 7.4 (phosphate-buffered saline) and kept on ice.





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The incubations were carried out in duplicate or triplicate in phosphate-buffered saline at a final hematocrit of 10-12%. Unless otherwise specified, all components except the ionophore were added and the 1.0 ml incubation mixtures were pre-warmed for 3 min. at 37°. Calcium ionophore A23187 was added as a solution in absolute ethanol. An equivalent amount of ethanol was added to controls. Samples were taken at appropriate times for the determination of ATP. The ionophore solution was stored at -20°C.

ATP assays were carried out by the method of Stanley and Williams (5). Results from each experiment were corrected for the hematocrit of the mixture at the beginning of the experiment. Edmondson and Li (4) reported that calcium interfered with their ATP assay, but we found no such interference, perhaps because the maximal amount of calcium present in our system was only 5 nmoles in the 3 ml assay volume.

Red cell membranes (ghosts) were prepared by washing red cells 3 times in 0.9% NaCl and removing the buffy coat by aspiration. Packed cells were lysed with 20 volumes of cold 10 mM Tris-HCl buffer, pH 7.4, and the membranes were collected by centrifugation at 28,000 g for 20 min. at 0°C. The membranes were washed 4 times with Tris-HCl and frozen at -20°C. They were stored for a maximum of 3 days. Protein was determined by the method of Lowry et al. (6).

$Na^+ - K^+$ and $Ca^{2+}$-ATPases were measured according to Cha, Shin and Lee (7). $MgCl_2$ (4 mM) was included in all incubation mixtures. Inorganic phosphate was measured using the reagents described by Bessman (8), but without automation. The total calcium was determined by atomic absorption spectroscopy and found to be 3 μM in the phosphate-buffered saline.

Results are given for duplicate or triplicate incubation mixtures from typical experiments since absolute ATP values varied somewhat from day to day, perhaps due to the use of cells from different donors.

A23187 was a gift from Eli Lilly Co. Firefly tails, adenosine and EGTA* were from Sigma Chemical Co.

Results and Discussion: The effect of 5 μM A23187 on the ATP level of human erythrocytes is shown in Figure 1. In the absence of added divalent cations, the ATP fell progressively until, at 20 min., its concentration was below the level of detection of the assay (0.06 mM). These results confirm the reports of Reed (1) and Allan et al. (3). Control ATP levels were occasionally increased over zero time values, presumably due to metabolism of 2,3-diphosphoglycerate since there was no exogenous energy source. When 2 mM $CaCl_2$ was included, ATP levels did not drop as quickly as with ionophore alone. $Ca^{2+}$ had little or no effect on ATP levels in the absence of ionophore (Figure 1). ATP remaining after 20 min. of incubation with 5 μM ionophore and 2 mM $Ca^{2+}$

*Abbreviation: EGTA: ethylene-glycol-bis-(β-amino-ethyl ether)N,N'-tetra-acetic acid

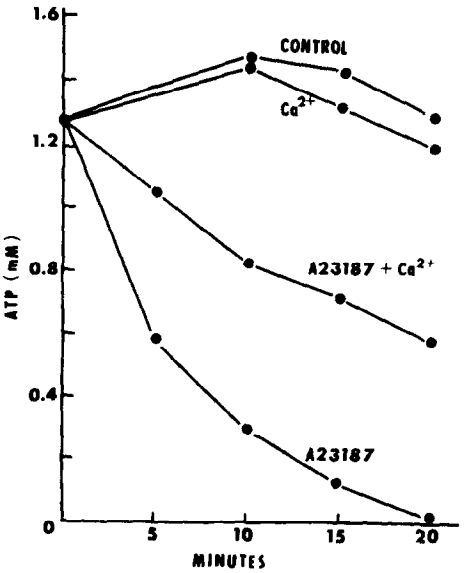


Figure 1: The Effect of Ionophore and Added Calcium on ATP Concentration of Erythrocytes. Incubation and ATP analysis were carried out as described in Methods and Materials. $CaCl_2$ concentration was 2 mM and ionophore concentration was 5 μM. Total calcium of the buffer was 3 μM.

TABLE I

EFFECT OF IONOPHORE CONCENTRATION ON ATP CONCENTRATION IN ERYTHROCYTES

Experimental details for incubations and ATP analysis are given under Methods and Materials. ATP concentration of cells was 1.03 ± .06 mM at zero time. Incubation was for 20 min. without added $Ca^{2+}$.

| Ionophore Concentration μM | Red Cell ATP | |
|---|---|---|
| | mM | % of control after incubation |
| 0 | 0.86 ± 0.12 | 100 |
| 1.25 | 0.57 ± 0.03 | 66 |
| 2.5 | 0.10 ± 0.05 | 12 |
| 5.0 | nd (< 0.06) | < 7 |
| 10 | nd (< 0.06) | < 7 |

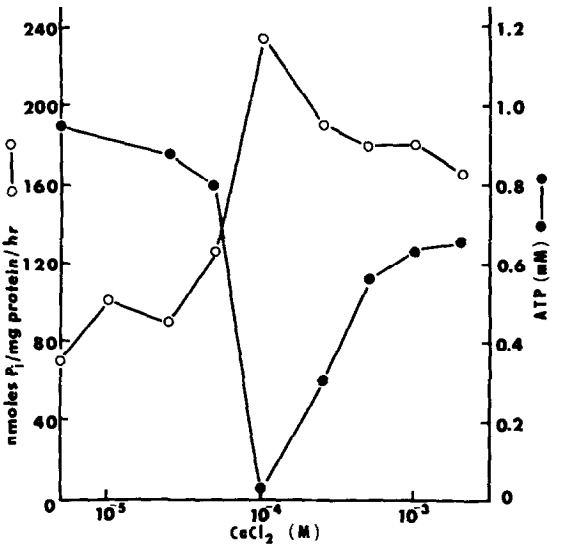


Figure 2: The Effect of $Ca^{2+}$ on $Ca^{2+}$-ATPase Activity of Membranes and on A23187-Induced ATP Loss in Intact Red Cells. ATP concentrations (o—o) were measured in red cells exposed to 80 μM EGTA, 5 μM A23187 and indicated concentrations of $CaCl_2$ for 10 min. at 37°C. Total calcium of the buffer was 3 μM. A23187 was added to cells, EGTA and buffer; the mixtures were incubated for 30 sec. at 37° and $CaCl_2$ was added. After 10 min., samples were removed for ATP analysis. $Ca^{2+}$-ATPase activity (•—•) was measured in red cell ghosts. Buffer, EGTA (final conc. 80 μM) and $CaCl_2$ were mixed, ghost protein (0.08 mg) was added, and the mixtures preincubated for 3 min. The reaction was started with ATP. Details of procedures are given in Methods. ATP at the start of the reaction was 1.09 mM.

varied from 30-78% of control values in 5 experiments. Although not shown, the fall in ATP slowed with decreasing temperature.

The fall in ATP over a 20 min. period was dependent on the concentration of ionophore (Table I). The decrease in ATP levels was accompanied by a corresponding increase in inorganic phosphate. For example, in two experiments the difference in ATP concentrations in ionophore-treated and control cells was 1.1 and 1.2 mM while the corresponding increases in phosphate were 1.5 and 1.7 mM. Therefore, the decrease in ATP concentration in the presence of ionophore can be attributed to hydrolysis of ATP.

It seemed likely that the decrease in ATP levels was due to activation of $Ca^{2+}$-ATPase by the $Ca^{2+}$ entering the cell when A23187 was present. That the presence of $Ca^{2+}$ is essential for the drop in ATP is illustrated in Figure 2. When 80 μM EGTA was present to chelate the free $Ca^{2+}$ ($\leq$ 3 μM) found in the buffer, the ATP fell only 0.13 mM in 10 min. in the presence of 5 μM A23187. Compare this to Figure 1 where no EGTA was present and the drop in ATP for the same time period was about 1 mM. It can be seen in Figure 2 that as increasing amounts of $Ca^{2+}$ were added to the incubation mixtures containing cells, 5 μM A23187 and 80 μM EGTA, ATP hydrolysis was maximally stimulated and then decreased progressively. If the effective formation constant for the Ca-EGTA complex is assumed to be between 6 and 7 for the conditions used (9,10), then free $Ca^{2+}$ would be about 1 μM at the point of maximal ATP hydrolysis. This is in the range of the $Ca^{2+}$ concentration ($\leq$ 3 μM) found to be present in the phosphate buffered saline in which all of the whole cell experiments were carried out. It is also near the free $Ca^{2+}$ concentration of the red cell ($\leq$ 1 μM) (3) and the $K_d$ of the high affinity $Ca^{2+}$-ATPase for $Ca^{2+}$ (4 μM) (9).

When ghosts were prepared and the $Ca^{2+}$-ATPase activity measured in the presence of 80 μM EGTA (no A23187 was present), a pattern of stimulation by $Ca^{2+}$ was seen which was very similar to that found in whole cells (Figure 2). The exact pattern of stimulation appeared to be very sensitive to the conditions of isolation of the membranes and length of time of their storage; in some preparations the point of maximal stimulation was shifted slightly to the right, and the inhibition at high $Ca^{2+}$ concentrations was greater than that shown in the Figure. Although it is well established that $Ca^{2+}$ concentrations at the millimolar level are inhibitory (10,11), the maximal stimulation observed in these studies is at a lower free $Ca^{2+}$ concentration than usually seen.

These experiments suggest that A23187 causes a fall in red cell ATP levels by increasing the free $Ca^{2+}$ concentration of the cell, thereby activating $Ca^{2+}$-ATPase. Therefore, the ATP level of red cells treated with A23187 will depend

TABLE II

REPLETION OF ATP IN IONOPHORE-TREATED RED CELLS

Cells containing 1.62 mM ATP were incubated for 20 min. without added $Ca^{2+}$ in the presence of 5 μM ionophore as described in Methods and Materials. At the end of the incubation with ionophore, red cell ATP was < .07 mM. After incubation, the supernatants were removed and the cells were washed 2 times with a) phosphate buffered saline, b) phosphate buffered saline containing 1% bovine serum albumin, or c) plasma. A fourth set of incubation mixtures was not washed. The cells were then incubated with 5 mM adenosine in phosphate buffered saline and aliquots were taken for ATP estimation after 1 and 2 hr. of incubation.

| Washing Medium | Red Cell ATP (mM) Minutes of Incubation with Adenosine | |
|---|---|---|
| | 60 | 120 |
| Phosphate Buffered Saline | 1.07 | 1.04 |
| Phosphate Buffered Saline & 1% BSA | 1.61 | 2.00 |
| Plasma | 1.69 | 1.82 |
| None | 1.04 | 1.04 |

not only on ionophore concentration and the time and temperature of incubation, but also on the $Ca^{2+}$ concentration of the medium. The addition of $Mg^{2+}$ would also be expected to affect the $Ca^{2+}$-ATPase and therefore the ATP concentration.

ATP depletion was found to be reversible if the medium was removed from the ionophore-treated cells and the cells subsequently reincubated with 5 mM adenosine for 2 hr. (Table II). Results were the same if the cells were washed with phosphate-buffered saline before adenosine treatment. However, as illustrated in Table II, washing ionophore-treated cells with plasma or bovine serum albumin and incubating with 5 mM adenosine restored the ATP to starting levels within 1 hr. It has been shown previously that washing cells with bovine serum albumin removes A23187 (11), and presumably the plasma serves the same function.

Cells in which ATP has been reduced to low levels are useful for the study of energy-dependent processes. The usual method of metabolic depletion involves incubating red cells for 10-15 hr. at 37° in the absence of substrate for glycolysis (12). In this method, ATP repletion is accomplished after 2 hr. in the presence of 30 mM adenosine. Inhibition of glycolysis will also allow depletion of ATP to occur. For example, treating red cells with 25 mM NaF for 80 min. results in an ATP concentration of 0.1 mM (13). However, reversal of this type of inhibition would be expected to be difficult, if not impossible. A23187 can serve as a useful means for depleting cells of ATP rapidly and reversibly.

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REFERENCES

1. Reed, P.W. (1976) J. Biol. Chem., 254, 3489-3494.
2. Kirkpatrick, F.H., Hillman, D.G. and LaCelle, P.L. (1975) Experientia, 31, 653-654.
3. Allan, D., Watts, R. and Michell, R. (1976) Biochem. J., 156, 225-232.
4. Edmondson, J.W. and Li, T.K. (1976) Biochem. Biophys. Acta, 443, 106-113.
5. Stanley, P.E. and Williams, S.G. (1969) Analyt. Biochem., 29, 381-392.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265-275.
7. Cha, Y.N., Shin, B.C. and Lee, K.S. (1971) J. Gen. Physiol. 57, 202-214.
8. Bessman, S.P. (1974) Analyt. Biochem., 59, 524-532.
9. Schatzmann, H.J. and Rossi, G.L. (1971) Biochim. Biophys. Acta, 241, 379-392.
10. Scharff, O. (1972) Scand. J. Clin. Lab. Invest., 30, 313-320.
11. Sarkadi, B., Szász, I. and Gárdos, G. (1976) J. Membrane Biol., 26, 357-370.
12. Weed, R.I., LaCelle, P.L. and Merrill, E.W. (1969) J. Clin. Invest., 48, 795-809.
13. Lichtman, M.A., Miller, D.R. and Weed, R.I. (1969) Trans. Assoc. Amer. Physicians, 82, 331-343.