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THE EFFECTS OF IONOPHORE A23187 ON ERYTHROCYTES

RELATIONSHIP OF ATP AND 2,3-DIPHOSPHOGLYCERATE TO CALCIUM-BINDING CAPACITY

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SUMMARY

The divalent cation ionophore, A23187, was employed as a means to load fresh human erythrocytes with calcium, and the capacity for accumulation was characterized. Erythrocytes exposed to A23187 in calcium-containing media rapidly accumulated calcium in millimolar quantities. The final cellular concentration was dependent upon medium calcium concentration and the size of the cellular organophosphate pool. When ATP and 2,3-diphosphoglycerate contents were depleted or repleted, the cellular calcium content changed proportionally. Calcium loading of fresh erythrocytes produced no discernible change in the cellular concentrations of ATP or 2,3-diphosphoglycerate. Calcium accumulation was also accompanied by loss of cellular potassium and H_2O, deterioration of cell filterability, and spheroechinocytic transformation.

INTRODUCTION

It is well known that erythrocytes normally contain very small quantities of calcium and excess intracellular calcium is deleterious to cell function and survival [1]. Calcium accumulation in the erythrocyte, e.g. as a consequence of ATP depletion, has been shown to initiate a series of chemical and structural alterations which include the selective loss of potassium, volume contraction, spherocytic transformation and deterioration of cell membrane deformability [2–5]. The majority of previous studies which have examined these effects have utilized the ATP-depleted, intact cell or the resealed erythrocyte ghost [2–7]. Both methods have inherent limitations and other means to load the erythrocyte with calcium have been sought [8].

In the studies reported here, the divalent cation-selective ionophore, A23187, has been employed as a means to alter the size of the intraerythrocytic pool of calcium. This ionophore has been shown to alter calcium flux and to effect equilibration of calcium across biological and artificial membranes [9, 10]. Consequently, the calcium-

Abbreviation: EGTA, ethyleneglycol bis-(β-aminoethyl)-*N*,*N*'-tetraacetate.

accumulating capacity of the cell and the effects of calcium-loading could be evaluated in the intact cell containing the normal complement of ATP. The capacity for cellular accumulation under the influence of the ionophore is shown to vary directly with the size of the organic phosphate pool. The changes which accompany calcium influx in ATP-depleted cells are observed also in the freshly isolated cells.

MATERIALS AND METHODS

Preparation of erythrocytes

Human erythrocytes were collected by venipuncture in heparinized tubes, separated from the plasma and buffy coat layer and washed with isotonic NaCl. Six-week-old erythrocytes were prepared similarly. The cells were resuspended in isosmotic media of varying calcium ionic content. The standard medium contained 140 mM Na^+, 10 mM K^+, 2.0 mM Mg^{2+}, 1.0 mM Ca^{2+} and 10 mM Tris · HCl. Sodium content was varied to maintain isosmolarity.

In other experiments, freshly collected cells were rapidly depleted of their ATP content by preincubation with iodoacetate [11] and of 2,3-diphosphoglycerate by incubation with $NaHSO_3$ [12]. The 2,3-diphosphoglycerate content of erythrocytes can also be augmented by incubation in media containing inosine, pyruvate and P_i [13]. In this manner, physiologic and supraphysiologic concentrations of 2,3-diphosphoglycerate in outdated and freshly collected erythrocytes, respectively, were prepared as described by Deuticke et al. [14].

Erythrocyte calcium accumulation

The erythrocytic concentrations of calcium were quantified isotopically by the use of tracer amounts of $^{45}Ca^{2+}$ (0.05 μCi/ml) in the medium. The appearance of ^{45}Ca in the cell fraction was measured after extraction 3 times with trichloroacetic acid (final concentration, 5 % w/v). The total calcium content of the media and the washed cell fractions was also measured by atomic absorption spectrophotometry. Good agreement was obtained between the two methods. Because calcium influx was associated with changes in cellular water content and, at times, hemolysis, correction for these factors was made: the degree of hemolysis was estimated according to Palek et al. [5], while the changes in cellular water content were determined with the use of an extracellular marker, [U-^{14}C]sucrose. These methods together with the microhematocrit were used to quantify original cell volumes and their changes.

To ascertain the time course of calcium accumulation, the suspension media and cells were rapidly separated by centrifuging the cells through a layer of silicone fluid of specific gravity 1.004 in an Eppendorf Microfuge. Isotopic counting was performed in Aquasol (New England Nuclear). Internal standardization was used for quench correction.

Biochemical assays

Erythrocyte ATP concentrations were measured by the luciferin-luciferase assay [15, 16]. However, calcium interfered with the assay in direct proportion to the amount of calcium present. Thus the addition of 0.7 μmol of $CaCl_2$ to 0.25 μmol ATP in the 2.3 ml reaction mixture produced a 30 % fall in measured ATP content. The addition of a 2-fold molar excess of EGTA totally prevented the inhibitory effect

of calcium and was itself without effect on the assay. The method of Baginski et al. [17] was used to measure P_i. Measurements of 2,3-diphosphoglycerate were performed enzymatically [18] and hemoglobin was measured spectrophotometrically as cyanmethemoglobin [19].

The association constants of calcium for ATP and 2,3-diphosphoglycerate were determined at an ionic strength of 0.15, pH 7.30 and 37 °C by the addition of increasing amounts of $CaCl_2$ to ATP (1.5 mM) and to 2,3-diphosphoglycerate (4.0 mM) in molar ratios ranging from 1 : 10 to 1 : 1 [20]. The Ca^{2+} concentration of each mixture was measured with a calcium ion-selective electrode (Orion Research, Inc., Model 99-20). For these studies, the sodium and Tris salts of ATP and 2,3-diphosphoglycerate, respectively, were employed.

Reagents

Analytical grade reagents were used in all experiments. $^{45}Ca^{2+}$ and [U-^{14}C] sucrose were purchased from New England Nuclear. The divalent cation ionophore, A23187, was a gift from Eli Lilly and Co. Media and reagents were stored in Nalgene plastic ware and prepared in distilled, deionized water. Stock solutions of A23187 were dissolved in absolute ethanol.

RESULTS

The time course of calcium accumulation. At pH 7.4 and 37 °C, the addition to erythrocyte suspensions of A23187 (dissolved in ethanol) resulted in a time-dependent disappearance of calcium from the medium and its concomitant incorporation into the cell fraction (Fig. 1). Steady-state accumulation was reached in about 15 min. By contrast, calcium uptake did not achieve steady-state at 10 and 4 °C even after 120 and 240 min of incubation, respectively. No discernible calcium accumulation was observed with ethanol alone nor with ionophore concentrations less than 1 μM.

Determination of the erythrocyte's capacity for calcium accumulation. In freshly collected cells exposed to the ionophore, the steady-state calcium content (measured after 120 min of incubation) increased curvilinearly as the external calcium concentration was increased. At concentrations above 2.5 mM, the relationship became linear (Fig. 2). Saturation of calcium influx did not occur even with medium calcium concentrations in excess of 10 mM, at which point significant hemolysis (> 5 %) developed. No discernible differences in rates of calcium influx or the steady-state levels of intracellular calcium were observed when sodium in the medium was replaced with potassium, when isosmotic sucrose was used, or when 5 mM glucose was added to the medium.

Influence of organic phosphate content on calcium accumulation. Fresh erythrocytes normally contain millimolar amounts of ATP and 2,3-diphosphoglycerate. By incubation with iodoacetate, the cellular ATP content was depleted from 3.11 to 0.75 μmol/g hemoglobin. Calcium accumulation by these cells was significantly lower (Fig. 2). Furthermore, the 2,3-diphosphoglycerate content of erythrocytes may be rapidly depleted by $NaHSO_3$. Erythrocytes depleted of both ATP and 2,3-diphosphoglycerate by prior incubation with iodoacetate and $NaHSO_3$ exhibited a calcium accumulation capacity which was considerably smaller than that produced by ATP depletion alone (Fig. 2). Of importance, is that when both ATP and 2,3-diphospho-

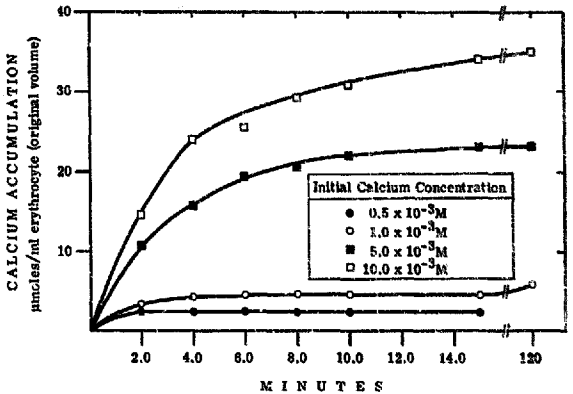

Fig. 1. Time course of A23187-initiated calcium influx into erythrocytes. Freshly isolated erythrocytes (hematocrit, 10%) were incubated at pH 7.4 and 37 °C in media containing the calcium concentrations indicated. The K^+, Mg^{2+}, and Tris concentrations of the media were 10, 2, and 10 mM, respectively. The Na^+ content of the medium, 140 mM, was decreased as the medium calcium content was increased in order to maintain isosmolarity. Cellular calcium accumulation was measured as a function of time after the addition of 10 μM A23187 suspended in a volume of absolute ethanol which was 0.1 % that of the incubation volume.

glycerate were depleted, internal and external calcium concentrations became linearly related over the entire range of calcium concentrations examined.

To further define the relationship between the erythrocyte's calcium accumulation capacity and its organic phosphate pool size, outdated erythrocytes which had been depleted of ATP and 2,3-diphosphoglycerate by storage [21] were studied. The profile of the cellular calcium accumulation capacity of these cells was superimposable on that observed following acute ATP and 2,3-diphosphoglycerate depletion (Fig. 3). Moreover, the organic phosphate stores of erythrocytes can be augmented by incubation with inosine, pyruvate, and P_i [13, 14]. In this manner, the content of organic phosphates in outdated erythrocytes was repleted to normal values and that of fresh cells to supraphysiologic concentrations. Again a predictable and direct relationship between the cellular calcium accumulation capacity and the size of the organic phosphate pool was observed (Table I).

Stability constants of the complexes of calcium with ATP and 2,3-diphosphoglycerate. The stability constants for the interaction of calcium with ATP and 2,3-diphosphoglycerate were measured under conditions mimicking the intracellular environment of the erythrocyte. At pH 7.30 and in a solution of ionic strength 0.15 containing 100 mM K^+, the stability constants of the Ca ATP^{2-} and Ca 2,3-diphosphoglycerate^{3-} complexes were determined to be $4.97 \pm 0.93 \cdot 10^3$ l/mol

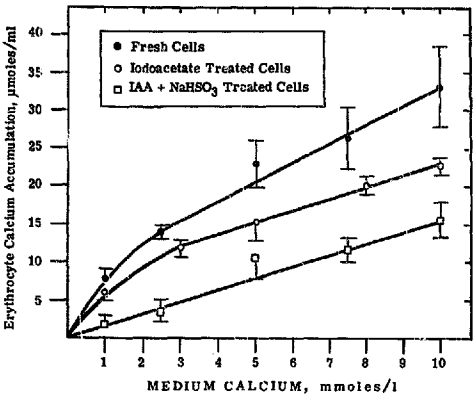

Fig. 2. Cellular calcium accumulation in fresh cells, ATP depleted cells and cells depleted of both ATP and 2,3-diphosphoglycerate. The conditions of incubation are identical to those of Fig. 1. The erythrocyte calcium concentrations, expressed in terms of original cell volumes, represent measurements determined at 120 min of incubation, and are the mean ±S.D. of 5–17 experiments.

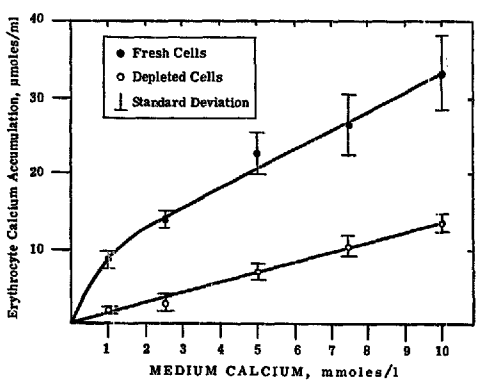

Fig. 3. Cellular calcium accumulation in freshly collected and outdated erythrocytes. The conditions of incubation are those indicated in Fig. 1. The measurements represent determinations at 120 min of incubation, and for outdated erythrocytes are the mean ±S.D. of 5 experiments.

TABLE I

IONOPHORE-INITIATED CALCIUM ACCUMULATION BY ERYTHROCYTES WITH MODIFIED ORGANIC PHOSPHATE CONTENTS

The conditions of incubation are as described for Fig. 1. The results are the mean ±S.D. of 4 experiments.

Medium Calcium (mM)	Cellular Calcium (μmol/ml cell)			
	Fresh	Depleted	Repleted	Supraphysiologic
1	6.03±0.50	1.67±0.10*	6.56±0.44	8.77±0.21*
5	16.60±1.25	8.10±0.44*	15.02±0.88	35.43±1.23*
10	29.00±4.32	14.68±0.39*	24.30±2.14	43.81±1.64*

* $P < 0.001$.

and $7.06 \pm 0.61 \cdot 10^2$ 1/mol, respectively. With the use of these stability constants and the molar concentrations of ATP and 2,3-diphosphoglycerate found in freshly collected erythrocytes, 80 % of the calcium uptake in the non-linear segment of the calcium accumulation profile of freshly collected cells (medium $[Ca^{2+}] < 2.5$ mM, Fig. 2) could be accounted for by complexation of calcium with ATP and 2,3-diphosphoglycerate.

Determination of the location of cellular calcium influx. To substantiate that A23187 was, in fact, effecting the translocation of calcium into cytosol, cells incubated with 10 μM ionophore and 5.0 mM Ca^{2+} were hemolyzed by hypotonic lysis. The calcium recovered with the cell membrane fraction ranged between 0.38 and 0.80 μmol per ml of cells. Thus, less than 1.4 % of the cellular calcium accumulated was membrane-associated. Dialysis of the hemolysate at pH 7.4 confirmed that the calcium was not protein-bound.

Effect of calcium influx on cellular ATP and 2,3-diphosphoglycerate. Ionophore-mediated calcium influx produced only small changes in cellular 2,3-diphosphoglycerate. In 120 min of incubation, ionophore treated cells lost less 2,3-diphosphoglycerate than cells incubated in the absence of A23187, -1.04 ± 0.31 and -2.75 ± 0.35 μmol/g hemoglobin, respectively (mean±S.E., $P < 0.05$). Moreover, over 120

TABLE II

EFFECT OF A23187 ON CELLULAR ATP

Freshly collected erythrocytes were incubated at hematocrit 10 % in standard medium containing 1.0 mM Ca^{2+}. Where indicated, A23187 (10 μM) and EGTA (2 mM) were added to the incubation medium and cellular ATP was measured at the beginning and following 15 min of incubation at 37 °C. The results are expressed as μmol/g of hemoglobin.

	0 min	15 min
No additions	5.1	5.2
A23187+EGTA	5.1	5.0
A23187	5.2	1.6
A23187*	5.4	5.3

* Measured with 1 μmol EGTA in the 2.3 ml assay mixture.

min of incubation, the rate of release of P_i was not significantly different in ionophore treated and untreated cells (1.5 and 1.3 μmol/ml cell/h, respectively). In accordance with this observation, there was no perceptible difference in cellular ATP content. It is noteworthy that there was inhibition by Ca^{2+} of the assay of ATP which can be obviated by the inclusion of EGTA in the assay (Table II).

DISCUSSION

These studies demonstrate that very high intracellular calcium concentrations are attainable in erythrocytes with A23187. A recent report by Kirkpatrick et al. [22] had also examined the effects of this ionophore on fresh erythrocytes. However, their study was confined to extracellular concentrations of Ca^{2+} of 0.5 mM or less and the full calcium accumulating potential of the cells was not investigated. The studies reported here not only document the effects of A23187 on cellular calcium uptake in greater detail and over a wider calcium concentration range but also provide an explanation for the unique calcium accumulating properties of the fresh erythrocyte.

In erythrocytes which were depleted of their organic phosphate stores, the relationship of intra- to extracellular calcium concentration was linear (Figs. 2,3), a finding which is consistent with the known action of the ionophore, which is to increase the permeability of the cell membrane to Ca^{2+}. However, in freshly collected cells or in cells whose 2,3-diphosphoglycerate stores had been augmented, the profile of cellular accumulation as a function of extracellular calcium concentration became curvilinear (Fig. 2) and the steady-state concentrations of intracellular calcium were higher than those of the depleted cells (Table I). These results suggested that complexation of calcium by ATP and 2,3-diphosphoglycerate contributed in a significant manner to calcium accumulation. This conclusion was substantiated by the compleximetric titrations of ATP and 2,3-diphosphoglycerate with Ca^{2+}. While the stability constants indicate low affinity ($4.9 \cdot 10^3$ l/mol and $7.0 \cdot 10^2$ l/mol, respectively), their presence in millimolar concentrations in fresh erythrocytes would render their effect appreciable. Thus 70–80 % of the calcium uptake in the non-linear segments of the various calcium accumulation profiles could be readily accounted for by complexation with ATP and 2,3-diphosphoglycerate.

Schatzmann had previously observed that erythrocytes loaded with calcium by ATP-depletion and subsequently repleted of their energy stores contained a calcium buffer [23]. This unidentified, nonprotein buffer accounted for the binding of 50 % of the intracellular calcium. Based upon the data in this report, it may be concluded that the organic phosphate compounds are this buffer.

When compared with untreated erythrocytes, the ionophore, either in the presence or absence of calcium, was found not to significantly alter the cellular content of 2,3-diphosphoglycerate and ATP. These results are at variance with those of Kirkpatrick et al. [22], who observed that the ionophore itself depleted cellular ATP. However, the experimental conditions employed in the two studies differ considerably. Moreover, it was found that calcium was an inhibitor in the luciferin-luciferase assay for ATP (Table II), and its removal with EGTA was necessary before reliable ATP measurements could be made.

In accordance with the observations of Kirkpatrick et al. [22], A23187 and Ca^{2+} were found to cause K^+ efflux, decrease in cell water content and membrane

rigidity. With 1.0 mM Ca^{2+} and 10 μM A23187 in the medium, cell potassium decreased from 99 to 80 mequiv/l, cell water decreased 20 ‰, and filterability decreased markedly. Moreover, the cells underwent spheroechinocytic transformation, as visualized by stereoscanning electron microscopy. All these changes were prevented by the addition of 2 mM EGTA to the medium and were largely independent of the extracellular Ca^{2+} concentration. Such findings are not surprising in view of the fact that the amount of intracellular calcium achieved here was markedly in excess of that necessary to produce these changes.

ADDENDUM

Since the submission of this paper, a report has appeared which used the ionophore to study cytoplasmic Ca^{2+} buffering and Ca^{2+} pump activity [24].

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