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Effect of Ionophore A23187 upon Membrane Function and Ion Movement in Human and Toad Erythrocytes

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Summary. Addition of 0.1–0.3 μM A23187, a divalent cation ionophore, to human erythrocytes suspended in a 1.0 mM $^{45}Ca^{2+}$-containing buffer results in a small (~two fold) increase in $[Ca^{2+}]_i$, a significant decrease in osmotic fragility, and a decrease in intracellular K^+ (100 mmoles/liter of cells to 70 mmoles/liter cells) without significant alteration of intracellular $[Na^+]$. This decrease in $[K^+]_i$ is associated with a significant decrease in packed cell volume and correlates directly with the observed alteration is osmotic fragility. Increasing extracellular K^+ to 125 mM prevents the A23187-induced changes in osmotic fragility, K^+ content and cell volume, but does not prevent the ionophore-induced uptake of $^{45}Ca^{2+}$. Addition of 0.1–0.3 μM A23187 to toad erythrocytes leads to an increase in $^{45}Ca^{2+}$ uptake comparable to that observed in human erythrocytes, but does not alter osmotic fragility, cell volume or K^+ content. Higher concentrations of ionophore (3.0–10.0 μM) cause a 30- to 50-fold increase in $^{45}Ca^{2+}$ uptake and concomitant change in K^+ content, cell volume and osmotic fragility. These changes in cell properties can be prevented by increasing extracellular $[K^+]$ to 90 mM. The difference in sensitivity of the two cell types to A23187 is attributed to the presence of additional intracellular calcium pools within toad erythrocytes that prevent an increase in cytoplasmic Ca^{2+} until Ca^{2+} uptake is increased substantially at the higher concentrations of A23187.

The intracellular calcium content of the human erythrocyte has a profound influence upon many of the physical properties of the cell membrane. Osmotic fragility, cell size and shape, viscosity, deformability, membrane potential and monovalent cation transport are all altered by changes in erythrocyte calcium content (Gardos, 1958; Hoffman, 1962, 1966; Weed, LaCelle & Merrill, 1969; Palek, Curby & Lionetti, 1971; Dunn, 1974; Lassen, Pape, Vestergaard-Bofind & Bengtson, 1974). The calcium content of the intact erythrocyte is controlled by the inward diffusion of calcium across the plasma membrane and the outward extrusion of Ca^{2+} by a highly efficient active Ca^{2+}-pump coupled to ATP hydrolysis (Ca^{2+}-sensitive ATPase) present in the plasma membrane (Schatzmann, 1975). Under the usual incubation conditions in the pres-

ence of 1.0 mM free extracellular calcium ion concentration, intracellular calcium concentration is no greater than 10^{-6} M (Schatzmann, 1975). Thus, in order to increase intracellular calcium ion concentration, either erythrocytes must be lysed and resealed in the presence of high extracellular calcium or cells must be depleted of ATP to inhibit calcium extrusion and allow net calcium uptake. In the present investigation we have utilized a specific divalent cation ionophore, A23187 (Reed & Lardy, 1972) to explore the relationship between small changes in calcium content of intact erythrocytes with normal ATP content and the functional properties of these cells. Our results extend the earlier observations of Reed and Lardy (1972), White (1974), Steer and Levitzki (1975), and Ferreira and Lew (1976). They indicate that the addition of low concentrations of A23187 to erythrocytes induces a Ca^{2+}-dependent increase in the K^+ permeability of the cell membrane that leads to a decrease in intracellular K^+. This decrease in K^+ content leads to a decrease in cell volume that results in a marked decrease in osmotic fragility of the cell.

Materials and Methods

Human blood was obtained by venipuncture from normal adult donors. Toad blood (*Bufo marinus*, National Reagents, Bridgeport, Connecticut) was collected by cardiac puncture. Heparin (10 units/ml) was employed as anticoagulant. Human erythrocytes were separated from plasma and buffy coat by centrifugation at room temperature (IEC Model CL) and washed five times in a buffer containing (in mM): NaCl, 140; KCl, 5; $MgSO_4$, 1; $CaCl_2$, 1; NaH_2PO_4, 1; Tris, 10; and glucose, 5 pH 7.4. In some experiments $[K^+]$ was increased to 125 mM by substitution of KCl for NaCl (high-K buffer). For toad blood NaCl was 102 mM, KCl, 2.5 mM; the other components were as above. For investigations of toad erythrocytes in high-K buffer, $[K^+]$ was increased to 90 mM by substitution of KCl for NaCl. This lower K^+ concentration was used because the osmolarity to toad plasma is 60–70% that of human plasma. After the final wash, packed cells were resuspended in washing buffer at a hematocrit of 20% v/v. Osmotic fragility was measured by adding aliquots (50 μl) of the cell suspension to 2 ml of a series of hemolyzing solutions of decreasing osmolarity prepared by diluting the standard washing buffer. After a 15-min incubation at room temperature the degree of hemolysis was determined by measuring optical density (576 nm) of the supernatant following centrifugation to sediment unhemolyzed cells. An equal aliquot was added to distilled water to obtain the optical density corresponding to 100% hemolysis.

To determine $^{45}Ca^{2+}$ uptake, erythrocytes were resuspended in standard buffer containing 1 mM $^{45}CaCl_2$ (20 mCi/mmole). Aliquots (200 μl) were removed at desired time intervals and added to 10 ml of standard buffer at 4 °C that contained 2×10^{-5} M $LaCl_3$. Cells were collected by centrifugation for 5 min at $500 \times g$ in a refrigerated centrifuge and washed three times with $LaCl_3$-containing buffer. Cold 10% trichloroacetic acid (1.5 ml) was added to the final cell pellet, the mixture rapidly mixed on a Vortex mixer, allowed to stand 15 min on ice, and then centrifuged at $2000 \times g$ for 10 min. The final clear supernatant was added directly (200 μl/10 ml) to a toluene methylcellosolve (5:3) scintillation fluid and

counted in a Packard Tricarb scintillation counter. Intracellular Na^+ and K^+ was determined by atomic absorption spectroscopy (Perkin-Elmer Model 290) of hemolysates prepared by rapidly washing cells four times in 110 mM $MgCl_2$ at 4 °C and dilution (1:150) in deionized water.

To investigate the relationship between osmotic fragility and $^{45}Ca^{2+}$ uptake or intracellular K^+ content, aliquots of cell suspension were taken simultaneously for hemolysis, and determination of $^{45}Ca^{2+}$ uptake or intracellular K^+ content 10 min after addition of A23187. Osmolarity of the hypotonic buffer required to lyse 50% of the cell population (a measure of osmotic fragility) was determined from the hemolysis curve for each sample, and correlated with K^+ content or $^{45}Ca^{2+}$ uptake after correction for uptake in the control sample. ATP was assayed by luciferin-luciferase (Stanley & Williams, 1969), utilizing a crude firely lantern extract (Sigma) and a Model 3385 Packard Tricarb liquid scintillation counter. The tonicity of all buffers and hemolyzing solutions was measured on an osmette Model 2007 osmometer. The A23187 was a gift from Dr. Robert Hamill of Eli Lilly and Company. A stock solution of the ionophore (2×10^{-3} M) was prepared in 50% ethanol and stored at −2 °C. Final ethanol concentration in control and experimental samples was 0.5% by volume.

Results

Effects of A23187 upon Osmotic Fragility of Human Erythrocytes

Dependence upon [Ca^{2+}] and [A23187]. The effects of increasing extracellular calcium concentration upon the percent hemolysis of human erythrocytes incubated in the absence or presence of increasing concentrations of A23187 are shown in Fig. 1. In this study a standard hemolyzing solution (147 mOsM) is employed that produces approximately 25% hemolysis of normal washed cells incubated for 10 min in the 1.0 mM $CaCl^2$ buffer without ionophore. As shown in this figure, increasing the extracellular calcium concentration from 0 to 10.0 mM, in the absence of ionophore, has no effect upon osmotic fragility. When cells are incubated in the absence of external calcium, low doses of ionophore (0.1–0.2 μM) have no effect upon hemolysis, and higher doses cause only a slight increase in hemolysis. In contrast, when cells are incubated with 0.1 μM ionophore, increasing the external calcium concentration results in a progressive and very significant decrease in osmotic fragility. Thus, at 0.1 and 0.2 mM Ca^{2+} this concentration of ionophore has no effect, but from 0.5 to 5.0 mM [Ca^{2+}] there is a progressively greater decrease in percent hemolysis. When higher concentrations of ionophore (1.0 μM) are employed the decrease in percent hemolysis is maximal when [Ca^{2+}] is between 0.1 and 0.5 mM. As ionophore concentrations are increased the [Ca^{2+}] concentration required to induce a maximal decrease in percent hemolysis is, therefore, decreased. When the concentration of ionophore is 1.0 μM, concentrations of [Ca^{2+}] above 0.5 mM cause a reversal

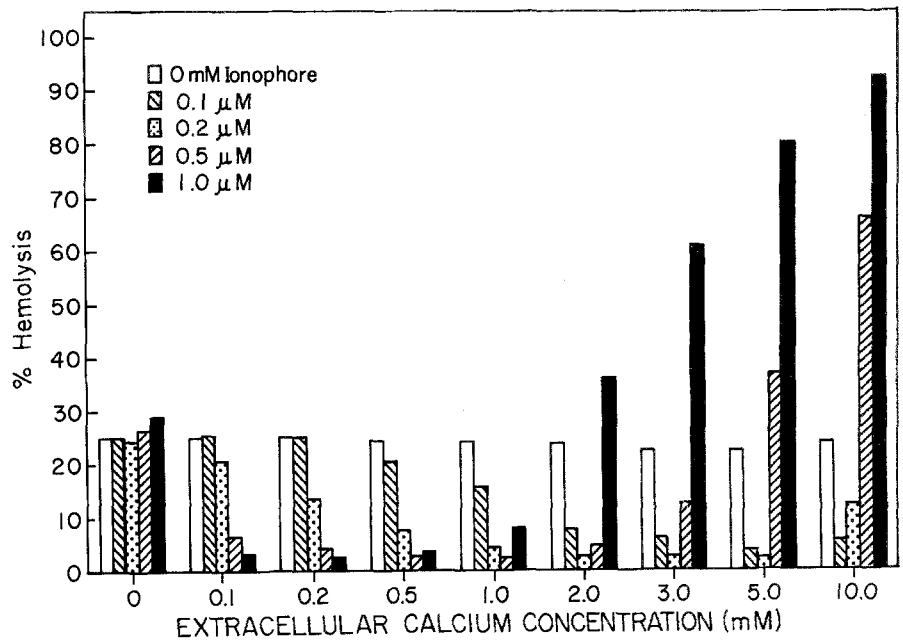

Fig. 1. Osmotic fragility of washed human erythrocytes in a standard hemolyzing solution as a function of extracellular calcium concentration. Human erythrocytes were washed five times in standard Ca^{2+}-containing buffer (1 mM) and once in a calcium-free buffer containing EGTA (1.6 μM). After the final wash cells were collected, suspended to 20% v/v in the EGTA buffer and divided into 2-ml aliquots. The desired final [$CaCl_2$] was obtained by addition of $CaCl_2$ (20 μl) from a series of stock solutions in distilled water. Cell suspensions were incubated for 10 min in the presence of 0 (□), 0.1 μM (▧), 0.2 μM (▣), 0.5 μM (▨), or 1 μM (■), A23187 before removal of an aliquot (50 μl) for hemolysis in 2 ml of the standard hemolyzing solution. Percent hemolysis was determined as described in Materials and Methods

of the effect upon hemolysis, and under these conditions (e.g., ionophore – 1.0 μM, and [Ca^{2+}] – 10 mM) there is a very striking increase in percent hemolysis.

Dependence upon Time. The effect of ionophore is not only [Ca^{2+}]- and [A23187]-dependent, but time-dependent as well. When cells are treated with 0.5 μM ionophore in the presence of 1.0 mM [Ca^{2+}], there is an initial, very marked decrease in percent hemolysis that then becomes progressively less marked if the incubation time is extended for more than 60 min (Fig. 2). In the absence of external calcium there is no change in hemolysis with time. Consequently, a standard incubation time of 10 min and 1.0 mM [Ca^{2+}] was utilized in the present study unless otherwise indicated.

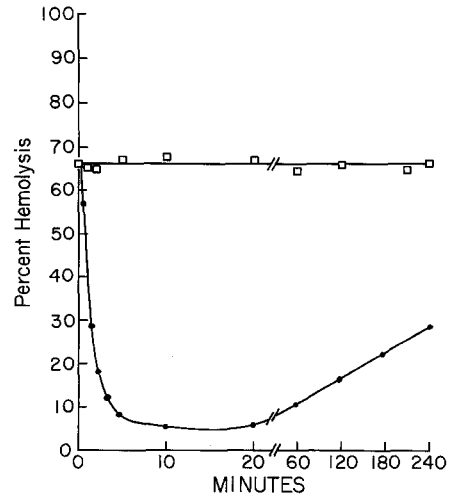

Fig. 2. Effect of A23187 upon the osmotic fragility of washed human erythrocytes in a standard hemolyzing solution as a function of time. At $t=0$ cells suspended in 1 mM Ca^{2+} containing buffer were treated with 0 (□), or 0.5 μM A23187 (●). Aliquots (50 μl) were removed at timed intervals for hemolysis in 2 ml of the standard hemolyzing solution. Percent hemolysis was determined as described in Materials and Methods

Effect of Magnesium upon the Ionophore Response. The effect of extracellular magnesium ion concentration upon the osmotic fragility of human erythrocytes, in the absence of extracellular calcium and in the absence or presence of A23187, is shown in Fig. 3*a*. In this experiment an osmotic fragility curve for the entire cell population is obtained by addition of aliquots of the cell suspension to a series of hypotonic hemolyzing solutions of decreasing tonicity prepared by dilution of the standard buffer. In the absence of extracellular calcium, addition of 0.3 μM A23187 has no effect upon the osmotic fragility of cells suspended in media containing either 1.0 or 10.0 mM [Mg^{2+}]. In contrast, in media containing 1.0 mM [Ca^{2+}] and 1.0 mM [Mg^{2+}] (Fig. 3*b*) the osmotic fragility of the entire cell population is decreased dramatically, and hemolysis does not occur until cells are added to hemolyzing solutions of much lower tonicity. The tonicity of hemolyzing solution required to hemolyze 50% of the control cell population (T_{50}) is 134 mOsM, while the T_{50} for ionophore-treated cells in the presence of 1.0 mM Mg^{2+} and 1.0 mM Ca^{2+} is 99 mOsM. When the magnesium ion concentration in the media is increased, such that [Mg^{2+}] = 10.0 mM and [Ca^{2+}] = 1.0 mM, the change in osmotic fragility observed after treatment with 0.3 μM A23187 is less marked ($T_{50}=120$ mOsM), but is still significantly reduced when compared to the osmotic fragility of control cells (Fig. 3*b*).

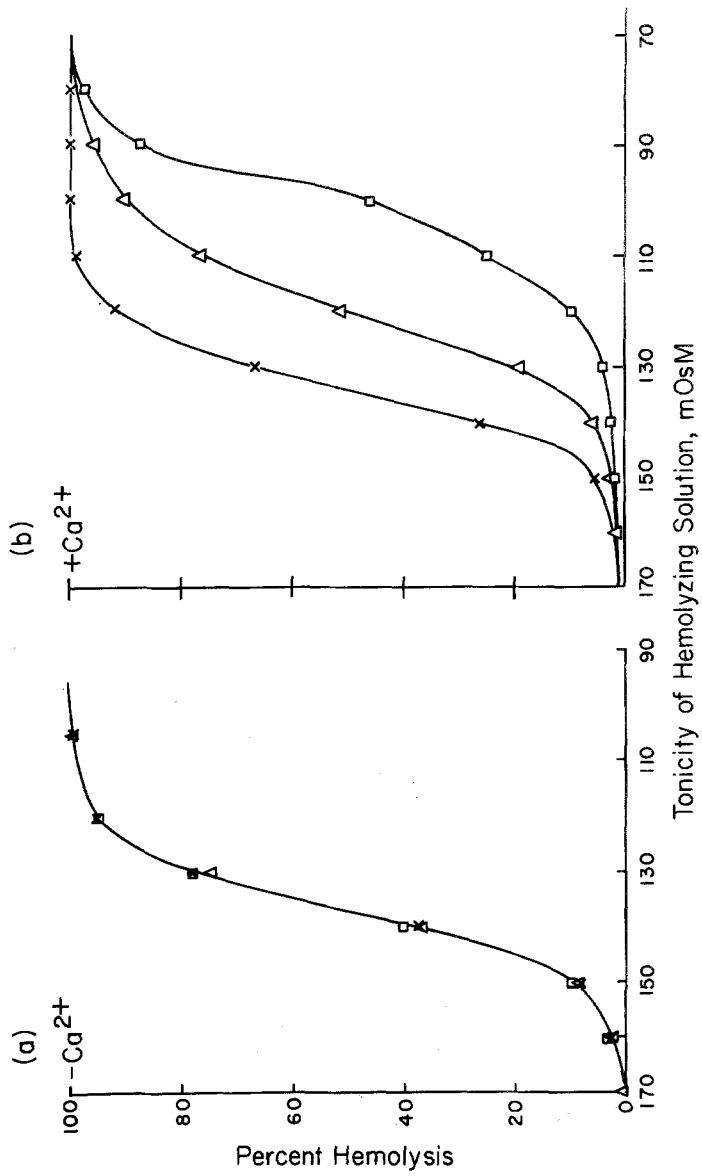

Fig. 3. Effect of extracellular magnesium ion concentration upon the osmotic fragility of washed human erythrocytes incubated in the absence (*a*) or presence (*b*) of extracellular calcium (1.0 mM) and in the absence or presence of 0.3 μM A23187. Cells suspended in buffer containing 1.0 mM (×, □) or 10.0 mM (△) magnesium were incubated in the presence of 0 (×), or 0.3 μM A23187 (□, △) for 10 min. An osmotic fragility curve was obtained by addition of aliquots of the cell suspension to a series of hypotonic hemolyzing solutions of decreasing tonicity prepared by dilution of the standard buffer

Effect of Extracellular K^+ Concentration upon the Ionophore Response. The effect of increasing concentrations of A23187 (0.1–0.3 μM) upon the osmotic fragility of human erythrocytes incubated in standard (5.0 mM) or high-potassium (125 mM) buffers containing 1.0 mM Ca^{2+} and 1.0 mM Mg^{2+} is shown in Fig. 4. When cells are suspended in standard buffer, addition of increasing amounts of ionophore causes a progressive decrease in the osmotic fragility of the cells. The tonicity of

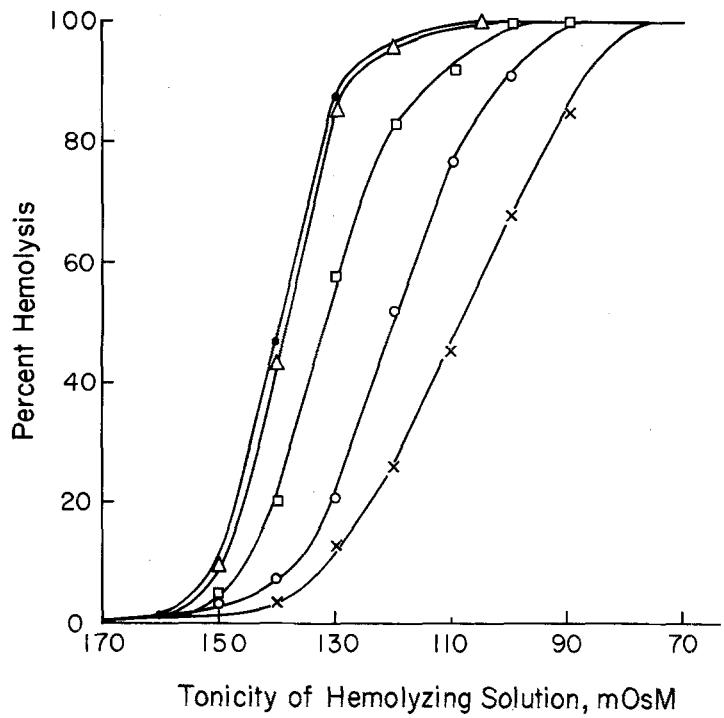

Fig. 4. Effect of increasing concentrations of A23187 upon the osmotic fragility of washed human erythrocytes suspended in standard buffer (5 mM) or a high-potassium (125 mM) buffer containing 1.0 mM extracellular Ca^{2+}. Cells were incubated for 10 min in standard buffer K^+ with 0 (△), 0.1 μM (□), 0.2 μM (○), or 0.3 μM (×) A23187 or in high-K^+ buffer (125 mM K^+) with 0.3 μM (●) A23187. Percent hemolysis was determined as described in Materials and Methods

hemolyzing solution required to hemolyze 50% of the control cell population is 139 mOsM, and after treatment with 0.1 μM A23187 the T_{50} is 132 mOsM. After treatment with 0.2 and 0.3 μM ionophore T_{50} is decreased to 120 and 109 mOsM, respectively. When the monovalent cation composition of the incubation media is altered, such that $Na^+ = 15$ mM and $K^+ = 125$ mM, the addition of 0.3 μM A23187 has no effect upon osmotic fragility (control cells, $T_{50} = 130$ mOsM; ionophore-treated cells, $T_{50} = 139.5$ mOsM).

Effect of A23187 upon the Osmotic Fragility of Toad Erythrocytes in Standard or High-K^+ Buffer

To study the effects of the ionophore upon nucleated as well as nonnucleated cells, its effects upon toad erythrocytes were examined.

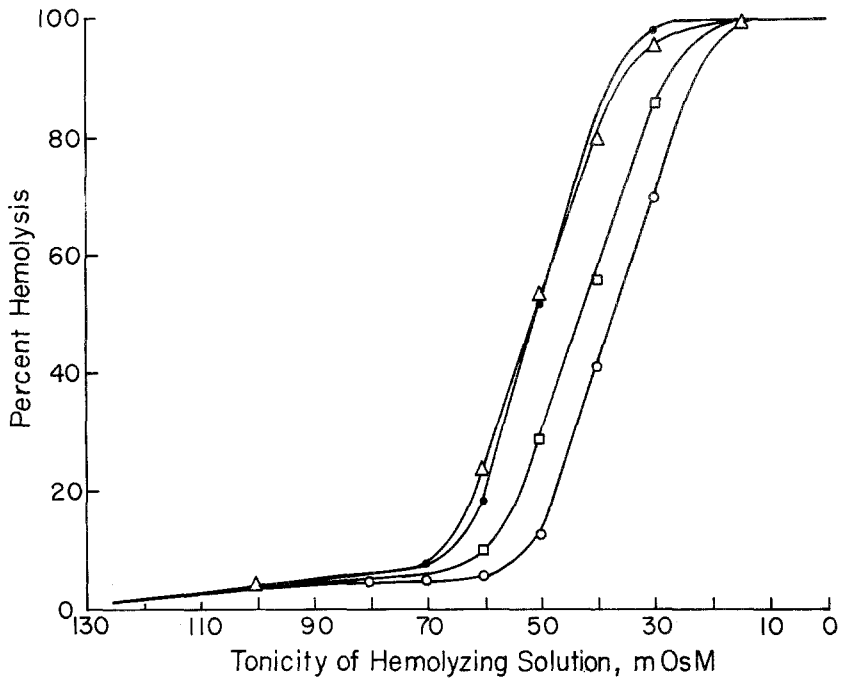

Fig. 5. Effect of increasing concentrations of A23187 upon the osmotic fragility of washed toad erythrocytes suspended in low-potassium (2.5 mM) or high-potassium (90 mM) buffer containing 1.0 mM extracellular Ca^{2+}. Cells were incubated for 10 min in standard buffer with 0 (△), 3.0 μM (□), or 5.0 μM (○) A23187 or in high-K^+ buffer with 5.0 μM (●) A23187. Percent hemolysis was determined as described in Materials and Methods

The effect of increasing concentrations of A23187 upon the osmotic fragility of toad erythrocytes suspended in standard (2.5 mM) or high-potassium (90 mM) buffer containing 1.0 mM Ca^{2+} is shown in Fig. 5. In the absence of ionophore the osmolarity of hemolyzing solution required to lyse toad erythrocytes is lower than that required for human erythrocytes (toad cells, $T_{50}=51$ mOsM; human cells, $T_{50}=139$ mOsM).

When toad erythrocytes in standard buffer are treated with 0.1–2.0 μM A23187 a concentration range that causes a marked change in the osmotic fragility of human erythrocytes, no change in the osmotic fragility of toad cells occurs. However, as shown in Fig. 5, addition of 3.0 μM A23187 to toad erythrocytes does produce a decrease in the osmotic fragility of these cells. Under these conditions T_{50} is reduced from 51 mOsM in control cells to 43 mOsM in cells treated with 3.0 μM ionophore. Addition of 5.0 μM ionophore results in a further decrease in osmotic fragility ($T_{50}=37$ mOsM). When toad cells are suspended in high-K^+ buffer (90 mM) no decrease in osmotic fragility occurs after treatment with 5.0 μM A23187 (Fig. 5).

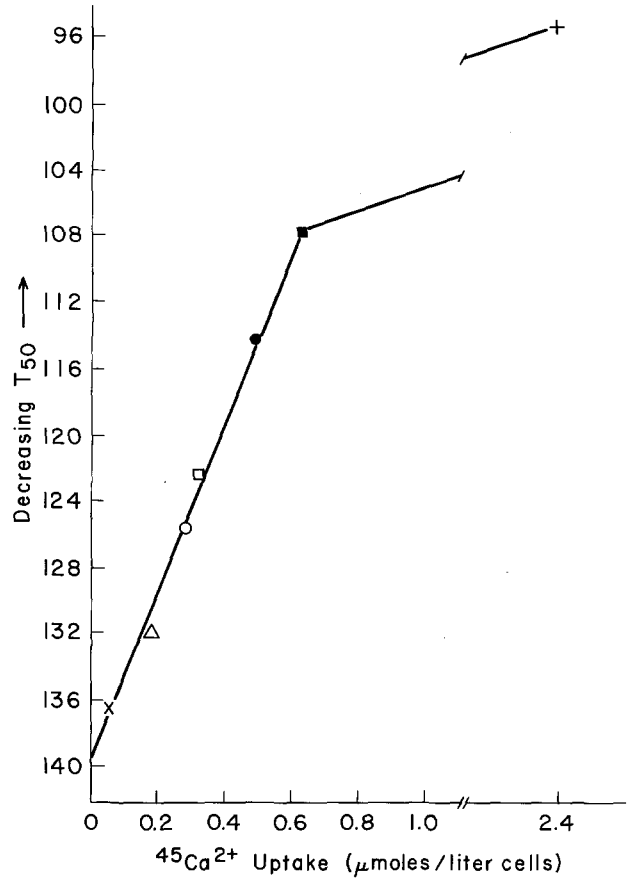

Fig. 6. Correlation between osmotic fragility and net $^{45}Ca^{2+}$ uptake of washed human erythrocytes 10 min after addition of A23187. Cells were preincubated with 1.0 mM $^{45}CaCl_2$ (20 mCi/mmole) for 10 min and divided into eight equal aliquots. At $t=0$ samples were treated with ethanol or various concentration of ionophore in ethanol (×, 0.05 μM; △, 0.075 μM;○, 0.10 μM; □, 0.125 μM; ●, 0.175 μM; ■, 0.20 μM; +, 0.30 μM A23187). At $t=10$ min aliquots were removed simultaneously for determination of $^{45}Ca^{2+}$ uptake and hemolysis. The T_{50} was determined from the osmotic fragility curve for each sample as described (*see* text) and correlated with $^{45}Ca^{2+}$ uptake after correction for uptake in the control sample. Final ethanol concentration was 0.5% v/v

$^{45}Ca^{2+}$ Uptake in A23187-Treated Human Erythrocytes

A study of the relationship between net $^{45}Ca^{2+}$ uptake and osmotic fragility (T_{50}) of human erythrocytes suspended in standard buffer is shown in Fig. 6. In this experiment human cells were preincubated for 10 min with 1.0 mM $^{45}CaCl_2$ and divided into eight equal aliquots. At $t=0$, samples were treated with various concentrations of ionophore

(0–0.3 μM), as indicated in the Figure legend. At $t = 10$ min aliquots were removed simultaneously for determination of $^{45}Ca^{2+}$ uptake and hemolysis. The T_{50} was determined from the hemolysis curve for each sample and correlated with $^{45}Ca^{2+}$ uptake after correction for uptake in control cells.

There is a direct correlation between net $^{45}Ca^{2+}$ uptake and the decrease in T_{50} until net $^{45}Ca^{2+}$ uptake exceeds 0.6 μmoles/liter of cells. An ionophore concentration of 0.2 μM is required to produce a $^{45}Ca^{2+}$ uptake of 0.6 μmoles/liter of cells in 10 min. When the ionophore concentration is increased to 0.3 μM an additional fourfold increase in net $^{45}Ca^{2+}$ uptake occurs (from 0.6 μmoles/liter of cells to 2.4 μmoles/liter of cells), but the decrease in T_{50} in response to this increase in $^{45}Ca^{2+}{}_i$ is significantly less than that observed after initial small changes in $^{45}Ca^{2+}{}_i$. Treatment of cells with 0.05 μM ionophore produces a net $^{45}Ca^{2+}$ uptake of 0.06 μmoles/liter of cells in 10 min which is sufficient to produce an observable decrease in osmotic fragility of human erythrocytes. When human cells, suspended in high-K^+ buffer (125 mM), are treated with 0.3 μM ionophore, net $^{45}Ca^{2+}$ uptake is not altered (2.4 μmoles/liter of cells) when compared to $^{45}Ca^{2+}$ uptake by cells suspended in the standard buffer. However, no decrease in osmotic fragility occurs after ionophore treatment in high-K^+ buffer (Fig. 4).

$^{45}Ca^{2+}$ Uptake in A23187-Treated Toad Erythrocytes

The relationship between net $^{45}Ca^{2+}$ uptake and osmotic fragility of toad erythrocytes is shown in Fig. 7. Addition of 0.2 μM A23187 to toad erythrocytes results in a $^{45}Ca^{2+}$ uptake comparable to that observed in human erythrocytes (0.55 ± 0.02 μmoles $^{45}Ca^{2+}$/liter toad cells *vs.* 0.62 ± 0.02 μmoles/liter of human cells). However, unlike human erythrocytes, there is no concomitant effect of this concentration of ionophore upon osmotic fragility of toad cells.

When toad cells are treated for 10 min with ionophore concentrations between 0.2 and 2.0 μM, the $^{45}Ca^{2+}$ uptake is much less than that observed in human cells after treatment with the same concentration of ionophore. Thus, $^{45}Ca^{2+}$ uptake in human erythrocytes 10 min after treatment with 0.5 μM A23187 is 60 μmoles/liter of cells (data not shown), while $^{45}Ca^{2+}$ uptake in toad erythrocytes under the same conditions is 1.2 μmoles/liter of cells (Fig. 7). A net $^{45}Ca^{2+}$ uptake of 1.2 μmoles/liter of cells is associated with a dramatic decrease in the osmotic fragility of human cells. In contrast, no change in the osmotic

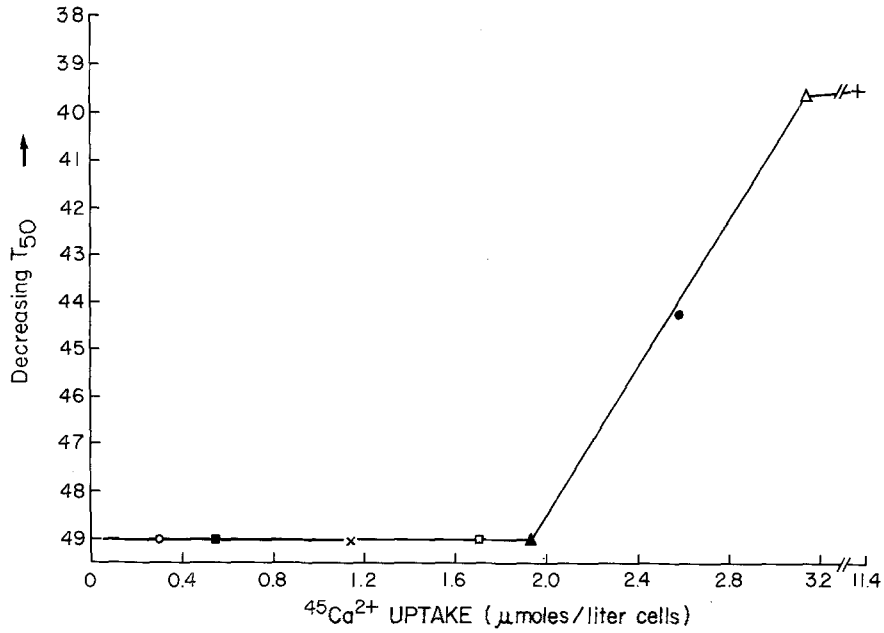

Fig. 7. Correlation between osmotic fragility and net $^{45}Ca^{2+}$ uptake of washed toad erythrocytes 10 min after addition of A23187. Cells were preincubated with 1.0 mM $^{45}CaCl_2$ (20 mCi/mmole) for 10 min and divided into nine equal aliquots. At $t=0$ samples were treated with ethanol or various concentrations of ionophore in ethanol (○, 0.1 μM; ■, 0.2 μM; ×, 0.5 μM; □, 1.0 μM; ▲, 2.0 μM; ●, 3.0 μM; △, 5.0 μM; +, 10.0 μM). The experiment was performed as described in Fig. 6

fragility of toad erythrocytes is observed until net $^{45}Ca^{2+}$ uptake exceeds 2.0 μmoles/liter of cells; i.e., 30- to 40-fold greater than is required to produce a detectable decrease in the osmotic fragility of human cells (0.06 μmoles/liter of cells). When $^{45}Ca^{2+}$ uptake in toad erythrocytes is between 2.0 and 3.15 μmoles $^{45}Ca^{2+}$/liter of cells, there is a direct correlation between net $^{45}Ca^{2+}$ uptake and the decrease in T_{50}. Ionophore concentrations between 2.0 and 5.0 μM produce a net $^{45}Ca^{2+}$ uptake of 2.0 to 3.15 μmoles/liter of cells in 10 min in toad erythrocytes. Increasing the concentration of ionophore to 10.0 μM results in a further fourfold increase in $^{45}Ca^{2+}$ uptake, but only a slight additional decrease in osmotic fragility.

Erythrocyte Na^+ and K^+ Content

Increasing concentrations of A23187 cause a progressive depletion of the intracellular K^+ content of human erythrocytes (Fig. 8*a*). Simul-

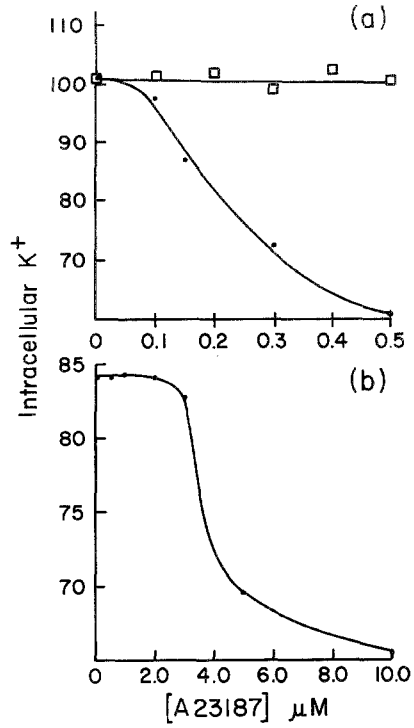

Fig. 8. The effect of A23187 upon the K^+ content (mmoles K^+/liter of original cell volume) of washed human (*a*) and toad (*b*) erythrocytes. Human erythrocytes were incubated for 10 min with A23187 in the presence (●) or absence (□) of calcium (1.0 mM). Toad erythrocytes were incubated for 10 min in the presence of 1.0 mM $[Ca^{2+}]_o$. K^+ content was determined as described in Materials and Methods

taneous measurement of Na^+ content shows no significant change (control, 5.4 ± 0.2 mmoles/liter of cells; 0.3 μM A23187, 5.6 ± 0.2 mmoles/liter of cells) while ionophore induces a fall in K^+ content of the same cells from 100 to 70 mmoles/liter of original cell volume (Fig. 8*a*). When cells are suspended in calcium-free buffer, no change in K^+ content occurs after treatment with 0.1–0.5 μM ionophore.

The relationship between intracellular K^+ content and T_{50}, obtained by incubating cells with increasing concentrations of ionophore, is shown in Fig. 9. There is a linear correlation between K^+ content and the osmotic fragility such that a decrease in K^+ content is associated with a decrease in the T_{50}.

In high-K^+ buffer, however, the K^+ content of human erythrocytes treated with 0.3 μM A23187, is increased slightly when compared to control cells, (96.7 ± 0.9 mmoles/liter of cells in control *vs.* 103.6 ± 0.9/liter of cells in A23187-treated cells).

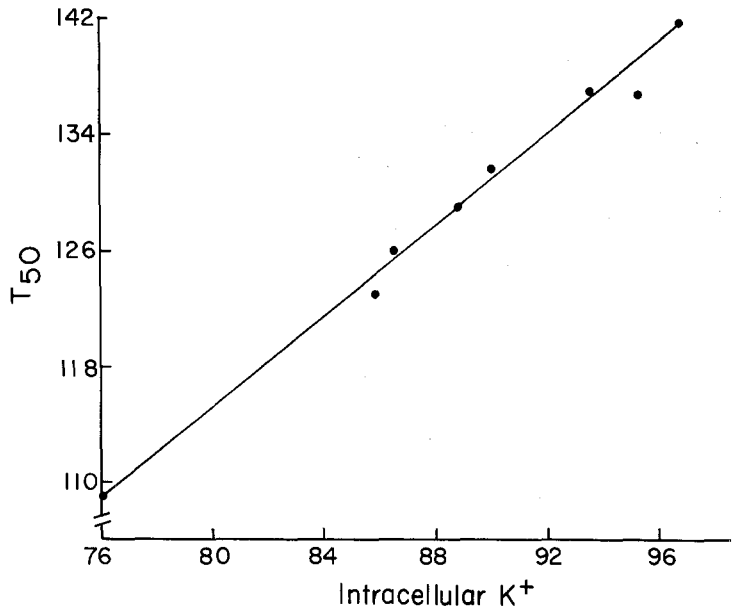

Fig. 9. Correlation between osmotic fragility and cellular K^+ content. Human erythrocytes were incubated for 10 min with A23187 (0.075–0.3 μM) and aliquots taken simultaneously for determination of osmotic fragility and intracellular K^+. The T_{50} was determined from the fragility curve for each sample and correlated with cellular K^+ content (expressed as mmoles K^+/liter of original cell volume)

The concentration of A23187 required to produce a detectable decrease in K^+ content of toad erythrocytes is 40-fold higher than is required in human cells (4.0 μM *vs.* 0.1 μM). The response of toad erythrocytes, in terms of K^+ content, to increasing concentrations of A23187 is shown in Fig. 8*b*. Addition of 4.0 μM ionophore to a suspension of toad cells results in a decrease in K^+ content from 84 to 72 mmoles/liter of cells in 10 min. Increasing the concentration of ionophore to 10.0 μM produces a further decrease in K^+ content to 65.4 mmoles/liter of cells.

Effect of A23187 on Packed Cell Volume

The effect of increasing concentrations of A23187 upon the packed cell volume of human erythrocytes in standard buffer is shown in Fig. 10. The packed cell volume of the control cell suspension is 19%. When cells are treated with increasing concentrations of ionophore a progressive decrease in packed cell volume is observed. After treatment with 0.2 μM ionophore for 10 min, packed cell volume is decreased to 17.5%. Addition

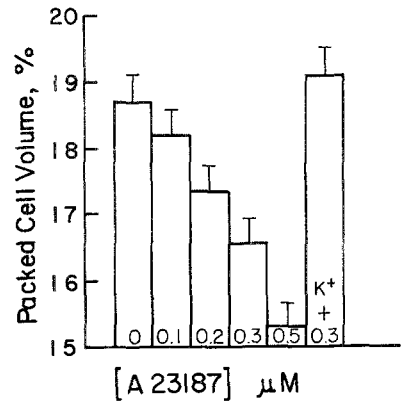

Fig. 10. The effect of A23187 upon the packed cell volume of washed human erythrocytes. Cells were incubated for 10 min in standard buffer with various concentrations of A23187 (0–0.5 μM) or in high-K^+ calcium containing buffer with 0.3 μM A23187

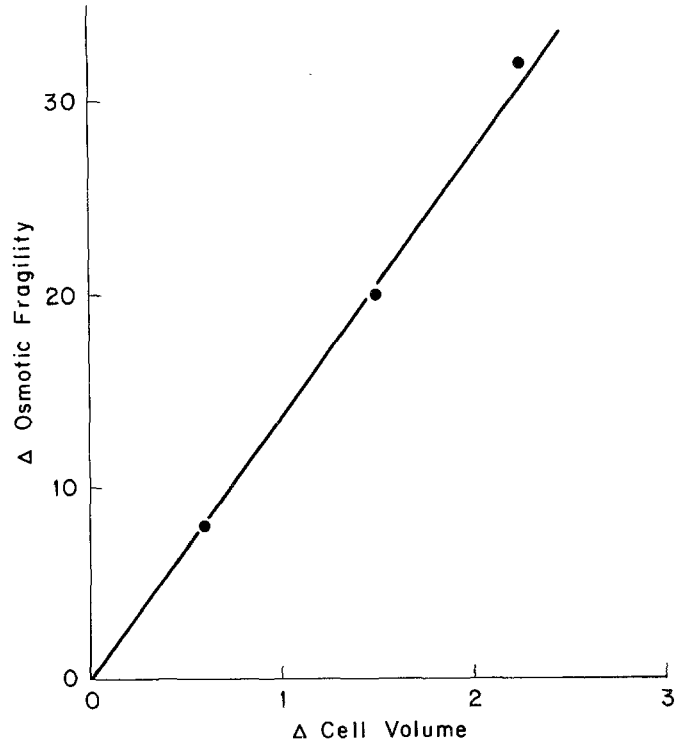

Fig. 11. The change in ionophore-induced reduction of osmotic fragility as a function of ionophore-induced decrease in cell volume. The measure of osmotic fragility employed was the tonicity of the salt solution necessary to produce 50% hemolysis of suspensions of red cells incubation with 0, 0.1, 0.2 and 0.3 μM A23187. The Δ osmotic fragility is the difference between the tonicity of the solution required to produce 50% hemolysis in the presence of a given ionophore concentration and that required to produce 50% hemolysis in the absence of ionophore. The Δ volume is the difference between the volume of packed cells in the presence and absence of a given ionophore concentration

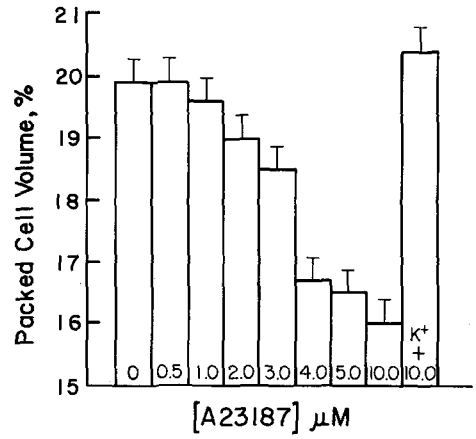

Fig. 12. The effect of A23187 upon the packed cell volume of washed toad erythrocytes. Cells were incubated for 10 min in standard calcium-containing buffer with various concentrations of A23187 (0–10 μM) or in high-K^+ calcium-containing buffer with 10 μM A23187

of 0.3 μM ionophore results in a further decrease in packed cell volume to 16.6%. When cells are suspended in high-K^+ buffer an increase (19 to 19.3%) in packed cell volume occurs after treatment with 0.3 μM A23187. This contrasts with the decrease (16.6%) observed after treatment of cells in the standard buffer. The decrease in cell volume (Fig. 10) correlates directly with the decrease in osmotic fragility (Fig. 4) as shown in Fig. 11.

The effect of increasing concentrations of A23187 upon the packed cell volume of toad erythrocytes is shown in Fig. 12. The packed cell volume of the control cell suspension is 20%. Treatment of toad cells suspended in standard buffer with ionophore concentrations below 2.0 μM produces no significant change in packed cell volume. Addition of 3.0 or 5.0 μM ionophore decreases the packed cell volume to 18.7 and 16.7%, respectively, after a 10-min incubation. A further decrease in packed cell volume occurs after treatment with 10.0 μM ionophore (16.2%). When cells are suspended in high-K^+ buffer, treatment with 10.0 μM ionophore results in an increase in packed cell volume to 21%.

Effect of A23187 on ATP Content

Concentrations of A23187 as high as 2 μM have no effect upon ATP content of human erythrocytes when measured 10 min after addition of ionophore (control: 1.6 ± 0.1 mmoles/liter of cells; 2 μM A23187: 1.6 ± 0.1 mmoles/liter of cells).

Effect of Ca^{2+} Upon Cellular Uptake Ionophore

To determine if Ca^{2+} is required for insertion of ionophore into the plasma membrane, human erythrocytes were preincubated with A23187 (0.3 μM) for 10 min, separated from the incubation medium by centrifugation, and washed extensively in Ca^{2+}-free buffer. Buffer separated from cells incubated with A23187 in the absence of Ca^{2+} had no effect upon the osmotic fragility of a previously unexposed cell population after addition of calcium (1.0 mM) and incubation for 10 min (percent hemolysis in 147 mOsM hemolyzing solution: control, 26%; cells in preincubation buffer, 26%). In contrast, the percent hemolysis of cells preincubated with A23187 in the absence of calcium, collected by centrifugation, and washed extensively in Ca^{2+}-free buffer is decreased significantly 10 min after resuspension in 1.0 mM Ca^{2+} buffer (control cells, 26% hemolysis; preincubated cells, 5% hemolysis). These data indicate the ionophore is taken up and retained by the cell even in the absence of calcium.

Discussion

The influence of changes in intracellular calcium content of human erythrocytes on the properties of this cell has been extensively documented. Dramatic changes in cellular and membrane deformability, cell size and shape, viscosity, membrane potential and cation transport occur when intracellular $[Ca^{2+}]$ is altered (Gardos, 1958; Hoffman, 1966; Weed *et al.*, 1969; Palek *et al.*, 1971; Romero & Whittam, 1971; Dunn, 1974; Lassen *et al.*, 1974; White, 1974; LaCelle & Kirkpatrick, 1975; Reed, 1976). What is not clear, however, is how these various changes are related. Do they represent different effects of Ca^{2+}, or is there an initial effect of calcium upon some critical membrane function which when altered leads to the other changes?

One documented effect of a change of $[Ca^{2+}]_i$ is a change in monovalent cation permeability, but even here there is not unanimity of opinion. Blum and Hoffman (1972) and Passow (1963) have reported that increased Ca_i^{2+} affects only K^+ and not Na^+ permeability, but Romero and Whittam (1971), Porzig (1973), Dunn (1974) and Kirkpatrick, Hillman and LaCelle (1975) have also observed that increased Ca_i^{2+} induces changes in Na_i^+. This apparent conflict probably reflects differences in $[Ca^{2+}]_i$. Romero and Whittam (1971) utilized ATP-depleted high-$[Ca^{2+}]_i$ erythrocytes for their studies while Kirkpatrick *et al.* (1975)

observed an increase in Na_i^+ from 9 mM to 12–16 mM after treatment of erythrocytes with 10 μM A23187 for 2 hr. Dunn (1974), using trinitrocresol to increase $[Ca^{2+}]_i$, reports an increase in Na^+ influx when $[Ca^{2+}]_i$ exceeds 0.2 mM. In all of these studies $[Ca^{2+}]_i$ is relatively high. In contrast, Porzig (1973) finds that $[Ca^{2+}]_i$ in the range of 10^{-6} to 10^{-5} M causes a significant increase in K^+ permeability of erythrocyte ghosts and Blum and Hoffman (1972) report that the effect on K^+ permeability is maximal at 10 μM $[Ca^{2+}]_i$. Our present studies support the conclusion of Passow (1963), Blum and Hoffman (1972), and more recently Reed (1976), that a small rise in intracellular calcium causes a specific increase in the K^+ permeability of the human red cell membrane.

A controlled change in $[Ca^{2+}]_i$ of human red cells can be induced by the addition of very small amounts of the divalent cation ionophore A23187 (Fig. 6) and this change causes not only an increase in K^+ efflux (Fig. 8) but also a decrease of osmotic fragility (Figs. 1–4).

Furthermore, our results show that A23187 induces a marked change in the osmotic fragility of both nonnucleated (human) and nucleated (toad) erythrocytes and that the presence of extracellular Ca^{2+} is essential for producing this effect (Figs. 1, 2, 4, 5). The effect of ionophore upon cell fragility is not seen if low external Ca^{2+} is replaced with low or high external Mg^{2+} (Fig. 3*a*). In fact, high external Mg^{2+} partially inhibits the effect of Ca^{2+} (Fig. 3*b*) presumably because Ca^{2+} and Mg^{2+} compete for binding and transport with A23187 (Pfeiffer, Reed & Lardy, 1974).

High concentrations of ionophore will cause $[Ca^{2+}]$ across the erythrocyte membrane to reach equilibrium (Hamill, Gorman, Gale, Higgins & Hoehm, 1972; Reed & Lardy, 1972), and the effects of A23187 upon $[Ca^{2+}]_i$ are dependent upon the extracellular calcium concentration, time of exposure (Ferreira & Lew, 1976) and the ionophore concentration (Figs. 1, 6 and 7), (Ferreira & Lew, 1976). When $[Ca^{2+}]$ is 0.5 mM, addition of 10 μM ionophore to a suspension of human erythrocytes results in total cell calcium content of 3 mmoles/liter of cells under steady-state conditions, with the fraction of ionized calcium between 30 and 50% of the total calcium content (Ferreira & Lew, 1976). However, in the present study, the increase in $[Ca^{2+}]_i$ required to protect cells from hemolysis is much less than this. An increase in $[Ca^{2+}]_i$ of less than 0.2 μmoles/liter of cells will protect the cells from hemolysis. The importance of this aspect of the ionophore effect is illustrated by the observation that incubation of human cells for 10 min at low concentrations of $[Ca^{2+}]$ or [A23187], where a small increase in $[Ca^{2+}]_i$ occurs,

results in a marked decrease in osmotic fragility (Fig. 1). Furthermore, as shown in Fig. 1, combination of higher ionophore and external Ca^{2+} concentrations cause a reversal of the specific effect on osmotic fragility due to a massive accumulation of Ca^{2+} by the cells.

Since we wished to investigate the effects of small changes in $[Ca^{2+}]_i$ on cellular and membrane properties, we utilized a standard incubation time of 10 min and 1 mM $[Ca^{2+}]$ for the present work. Under these conditions there is a linear relationship between ionophore-induced $^{45}Ca^{2+}$ uptake and the osmolarity of standard hemolyzing solution required to lyse 50% of the cell population until $^{45}Ca^{2+}$ uptake exceeds 0.6 μmoles/liter of original cell volume (Fig. 6). Since $[Ca^{2+}]_i$ in human erythrocytes is probably less than 1 μM (Schatzmann, 1975), it can be seen that very small changes in $[Ca^{2+}]_i$ caused very marked changes in osmotic fragility.

We have also investigated the effect of A23187 on osmotic fragility and $^{45}Ca^{2+}$ uptake of the nucleated toad erythrocyte. These cells are of interest because they possess additional intracellular calcium pools (nucleus and mitochondria) not found in human erythrocytes, and thus, allow an investigation of the importance of these pools in controlling $[Ca^{2+}]_i$-induced changes in cellular and membrane properties. A23187 decreases osmotic fragility of these cells (Fig. 5) in the presence of 1 mM $[Ca^{2+}]$, but no response is observed at ionophore concentrations below 3 μM; i.e., the ionophore concentration must be 30-fold higher in these nucleated cells in order to alter their osmotic fragility. Uptake of $^{45}Ca^{2+}$ under these conditions is also 30- to 40-fold higher than those observed in human erythrocytes (Fig. 7). Addition of lower concentrations of A23187 (0.1–0.3 μM) to toad erythrocytes results in an increase in $^{45}Ca^{2+}$ uptake comparable to that observed in human erythrocytes, thus indicating that the difference in response of the two cell types is not due to differences in the ability of A23187 to induce the transport of calcium across the plasma membrane. The difference probably reflects the presence of additional intracellular calcium reservoirs in nucleated cells whose capacity is sufficient to prevent an increase in cytoplasmic calcium when $^{45}Ca^{2+}$ uptake is increased by A23187 at lower concentrations. At higher [A23187], however, the increase in $^{45}Ca^{2+}$ uptake would exceed the capacity of these reservoirs and produce the change in cytoplasmic Ca^{2+} necessary to induce changes in membrane properties. It is possible that in these nucleated cells, after addition of large amounts of ionophore, some of the drug enters the cell and becomes incorporated into one or more subcellular membrane and thereby further influences the $[Ca^{2+}]$

of the cell cytosol. It is necessary to point out that the present studies in osmotic fragility were all carried out 10 min after ionophore addition. It is quite possible that in toad cells exposed for longer periods of time, the ionophore could gain access to intracellular (e.g., mitochondrial) membranes and cause a release of calcium from these internal stores. This might, in turn, cause a rise in $[Ca^{2+}]$ in the cell cytosol and an increase in K^+ efflux. This aspect of the effect of ionophore on toad erythrocytes remains to be investigated.

The results with the toad cells indicate that it is the calcium ion content of the cytosol, and not some superficial membrane calcium pool that is responsible for the change in K^+ efflux after ionophore addition. This conclusion is based on the observation that low doses of ionophore cause Ca^{2+} uptake into these cells but no change in fragility.

The basis of the Ca^{2+}-induced decrease in osmotic fragility seen after A23187 addition is apparently due to a specific Ca^{2+}-dependent increase in the K^+ permeability (Figs. 8 and 9). This leads to a loss of intracellular electrolyte, and a decrease in cell volume (Figs. 10–12). Because initial cell volume is a major determinant of osmotic fragility (Ponder, 1948), these small K^+-depleted cells are more resistant than normal to osmotic lysis. Support for this proposed sequence is provided by the observations that when, in either human or toad cells, the Ca^{2+}-dependent K^+ depletion is prevented by incubating ionophore-treated cells in high K^+ media, there is no change in osmotic fragility even though Ca^+ uptake is stimulated. Also, there is a direct correlation between K^+ content and osmotic fragility (Fig. 9), and a direct correlation between the change in initial cell volume and change in osmotic fragility when 0.1 to 0.3 μM ionophore is added to human cells (Fig. 11). These two correlations indicate that the decrease in cell volume is a direct function of the decrease in K^+ content and that K^+ does not exchange to any appreciable extent with external cations, but that the K^+ loss is accompanied by loss of anion to maintain electrical neutrality. It seems likely that this anion is Cl^- because Reed (1976) has shown that agents which decrease the Cl^- permeability of the membrane inhibit ionophore-induced K^+ loss.

From our data it is not possible to conclude that a small rise in $[Ca^{2+}]_i$ alters the intrinsic deformability of the membrane of the human erythrocyte although the data of Weed *et al.* (1969) and LaCelle and Kirkpatrick (1975) suggest that this is the case. It is possible, for example, that the results of LaCelle and Kirkpatrick (1975) are due either to the fact that larger concentrations of ionophore were used in their studies,

or that a reduction in cell volume *per se* led to a change in 'intrinsic membrane deformability' as measured by their technique. In either case, it would be of great interest to study the intrinsic membrane deformability of cells treated with low concentrations of ionophore in a high K^+ medium in order to determine whether small changes in $[Ca^{2+}]_i$ do alter membrane deformability independent of Ca^{2+}-induced change in K^+ efflux and cell volume.

The mechanism by which an alteration in $[Ca^{2+}]_i$ changes K^+ permeability is not known. It is possible that a calcium-dependent change in the state of aggregation of the spectrin complex leads to a reorientation of membrane proteins and thus to changes in K^+ permeability, or that a Ca^{2+} interaction with the membrane phospholipids changes the lipid environment of the ion channel and thereby the rate of K^+ flux. Further experiments are necessary to determine which of these is the more likely.

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