Passive Ca Transport in Human Red Blood Cell Ghosts Measured with Entrapped Arsenazo III

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ABSTRACT The rate of Ca influx into ghosts containing arsenazo III changes with time, being most rapid during the first 5 min after Ca is added to the outside and declining thereafter. The rate of Ca influx is a nonlinear function of extracellular Ca and plateaus as the latter is increased above 1 mM. The rate of Ca influx was measured as a function of the transmembrane gradients of Na and K and changes in the permeability of the membrane to K and Cl produced by valinomycin and SITS (4-acetamido-4′-isothiocyano-stilbene-2-2′-disulfonic acid), respectively. Changes in the rate of Ca influx are consistent with expected effects of these treatments on the membrane potential. Oligomycin (10 μg/ml) and quinidine (1 mM) inhibit the rate of Ca uptake by inhibiting Ca-induced changes in the K permeability. At constant membrane potential, furosemide produced a slight (15%) consistent increase in Ca uptake. Other experiments show that resealed ghosts are heterogeneous in their passive permeability to Ca and that A23187 can be used to effectively eliminate such differences. The results of this paper show that resealed human red cell ghosts containing arsenazo III can be used to continuously monitor intracellular free Ca and to study the factors that influence the permeability of the red cell membrane to Ca.

INTRODUCTION

Increasing free (ionized) Ca in human red cells will initiate a dramatic increase in the K permeability of the membrane (Gardos, 1958; Blum and Hoffman, 1972), inhibit the Na-K pump (Hoffman, 1962; Yingst and Hoffman, 1981), activate the Ca pump (Schatzmann and Vincenzi, 1969), and cause irreversible changes in shape (Weed and Chailley, 1973) and loss of membrane deformability (Weed et al., 1969). Some of these effects may be important for the normal red cell throughout its development, while others may play a role in the etiology of diseases such as sickle cell anemia (Eaton et al., 1973).

In order to study Ca-dependent phenomena in human red blood cells, we have entrapped the Ca chromaphore arsenazo III in resealed ghosts (Yingst and

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Hoffman, 1978) and calibrated it to measure free intracellular Ca (Ca_i) (Yingst and Hoffman, 1983). We have used this technique because of the very low ratio of free to bound Ca_i in human red cells, which makes it difficult to measure free Ca_i directly by atomic absorption. For the same reason, interpreting isotopic measurements can be complex because of the exchange of ^{45}Ca with bound Ca and problems of defining specific activities in terms of free Ca_i. The total Ca_i of normal human red blood cells is ~16 μmol/liter cells, most of which is bound to the plasma membrane (Harrison and Long, 1968). Free Ca_i is estimated to be between 0.3 (Simons, 1976, 1981) and 0.03 μM (Lew et al., 1982), whereas the total extracellular Ca is ~2 mM. The large gradient of Ca across the red cell membrane is maintained by an ATP-driven Ca pump that expels Ca in opposition to a passive influx of Ca across the cell membrane (Schatzmann and Vincenzi, 1969). Free Ca_i can increase as a result of changes in the activity of the Ca pump, an increase in the permeability of the plasma membrane to Ca, and the release of Ca bound to intracellular constituents or to the plasma membrane.

We were interested in elucidating the factors that influence the passive Ca permeability of the red cell membrane because of their potential significance in altering free Ca_i. In this study we used resealed ghosts containing arsenazo III and characterized the pattern of Ca uptake. Information concerning the passive Ca permeability found in this study is applied in the accompanying paper (Yingst and Hoffman, 1984) to analyze the effects of free Ca_i on Ca-stimulated K transport in human red cells.

MATERIALS AND METHODS

Preparation of Resealed Ghosts

Fresh blood was collected in heparin from healthy donors, washed at 4°C with 150 mM NaCl, 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 0.2 mM EGTA [ethyleneglycol-*bis*(β-aminoethyl ether)-*N,N'*-tetraacetic acid], pH 7.4 (23°C). The cells were then suspended at 20% hematocrit in the wash solution, which contained a trace of chloramphenicol (1–2 mg/100 ml) and incubated with gentle shaking at 37°C (pH 7.25) for 24 h to deplete the cells of ATP. After incubation the buffy coat was removed and the cells were washed three times with the aforementioned solution and centrifuged at 12,000 *g* for 3 min, and the supernatant was again removed, leaving packed cells with a hematocrit exceeding 95%.

To make resealed ghosts, 1 vol of packed cells was hemolyzed at 0°C for 5 min in 20 vol of a solution that contained arsenazo III, 22 mM Hepes, 55 μM EGTA, and 1.1 mM Mg titrated to neutrality with Tris hydroxide. The tonicity of the hemolyzing solution was restored to 300 mosmol by the addition of 3 M KCl or NaCl, or a mixture of both, and the ghosts were resealed by incubation at 37°C for 40 min. After resealing, the ghosts were centrifuged at 49,000 *g* and washed four times in 10 vol of 150 mM NaCl, 20 mM Hepes, and 0.1 mM EGTA (pH 7.4 at 23°C), and then separated on a 10% sucrose cushion to reduce heterogeneity (Bodemann and Passow, 1972). After collection from the cushion, the ghosts were washed three more times and packed at 49,000 *g* to a hematocrit exceeding 95%. The final concentrations of the intracellular constituents, based on the composition of the final hemolyzing solution, are given in the appropriate figure legends.

Chemicals

All chemicals except where noted were reagent grade. EDTA (ethylenediamine-tetraacetic acid) and HEDTA (*N*-hydroxyethylethylene-diamine-triacetic acid) were obtained from Sigma Chemical Co. (St. Louis, MO) as disodium salts, and NTA (nitrilotriacetic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). A23187 was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Calcium was purchased as 1-M stock of $CaCl_2$ from British Drug House (distributed by Gallard-Schlesinger, Carle Place, NY). Before use, $MgCl_2 \cdot 6H_2O$ was dried at 60°C and stored over desiccant. The arsenazo III was purified as previously described (Yingst and Hoffman, 1978, 1983) from practical grade arsenazo III obtained from Sigma Chemical Co.

Measurement of Free Ca_i

Free Ca_i was calculated from Ca-sensitive changes in the absorbance (ΔA^{Ca})[1] of entrapped arsenazo III with knowledge of the total dye concentration, the hematocrit, and the dissociation constants and molar extinction coefficients for 1:1 (CaD) and 2:2 (Ca_2D_2) complexes of Ca and dye (Yingst and Hoffman, 1983). In experiments where the ghosts contained <50 μM free Mg (Figs. 1, 2, and 5), the concentration of free Ca_i was calculated by method A (see Appendix). In other experiments where ghosts contained >50 μM free Mg (Fig. 6), free Ca_i was calculated by method B (see Appendix).

Both methods are based on the observation that the absorbance of the dye packaged inside ghosts that are suspended in a solution is equal to the absorbance of that same concentration of dye and free Ca dissolved directly in a bulk solution, when the pathlengths of light through the dye in the ghosts and in the bulk solution are the same (Yingst and Hoffman, 1983). In other words, if the absorbances of the dye in the ghosts and in the bulk solution are measured in a cuvette with the same pathlength, then

$$\Delta A^{Ca}_{ghosts} = \frac{\Delta A^{Ca}_{solution} \cdot \%HCT}{100}, \tag{1}$$

where ΔA^{Ca}_{ghosts} is a Ca-sensitive change in absorbance of the dye package inside ghosts suspended in a solution, and $\Delta A^{Ca}_{solution}$ is a Ca-sensitive change of the dye dissolved directly in a bulk solution at the same concentrations of total dye and free Ca that are present inside the ghosts. The value of $\Delta A^{Ca}_{solution}$ at hypothetical values of total dye and free Ca can be predicted from the previously developed model describing the change in the absorbance of the dye as a function of free Ca (Yingst and Hoffman, 1983).

A Ca-sensitive change in absorbance (ΔA^{Ca}) is defined as the absorbance of a sample with a given concentration of free Ca and arsenazo III minus the absorbance of an identical sample with no free Ca. A Ca-sensitive change of arsenazo III at 655 nm (ΔA^{Ca}_{655}) is:

$$\Delta A^{Ca}_{655} = (A_{655} - A_{700})_{Ca} - (A_{655} - A_{700}), \tag{2}$$

where A_{655} is the absorbance at 655 nm, A_{700} is the absorbance at the reference wavelength of 700 nm, $(A_{655} - A_{700})_{Ca}$ is the difference in absorbance between these wavelengths in a sample containing arsenazo III and Ca, and $A_{655} - A_{700}$ is a similar difference in absorbance in a sample with the same D_T and no free Ca. Measurements of the dye in ghosts were carried out at 1.67% hematocrit in a cuvette with a 1.0-cm pathlength using the dual-wavelength mode of a DW-2 spectrophotometer (American Instruments Co., Silver Spring, MD) as previously described (Yingst and Hoffman, 1983).

[1] The abbreviation for absorbance (A) is synonymous with "AB" in Yingst and Hoffman, 1983.

The relationship between the measured absorbance of arsenazo III in resealed ghosts as a function of free Ca_i is shown in Fig. 1. The solid line in this figure was calculated from Eq. A2 (Appendix) after determining the total dye concentration in the ghosts (Yingst and Hoffman, 1983) and assuming that free Ca_i was equal to that in which the ghosts were suspended and that the Ca in the ghosts was evenly distributed throughout the ghost population. The concentration of free dye (D) needed to calculate ΔA_{655}^{Ca} in Eq. A2 was calculated by iteration in Eq. A1 knowing the total dye concentration and the free

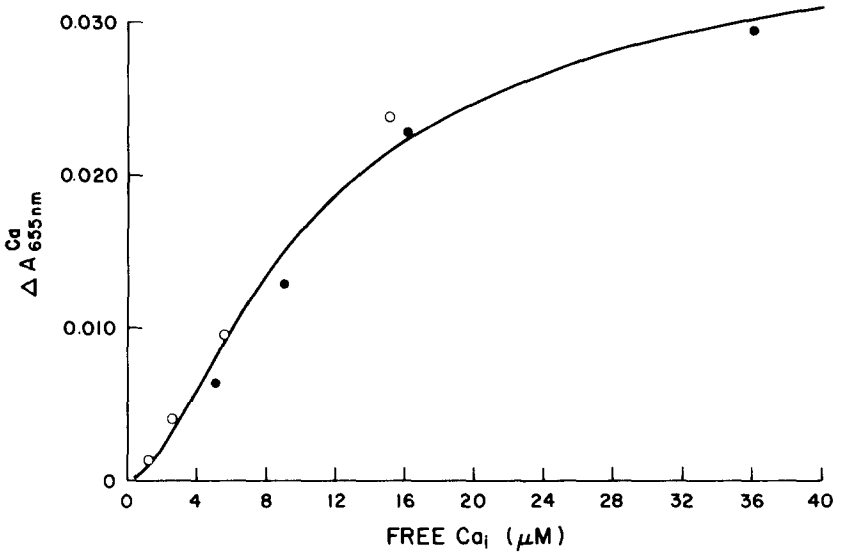


FIGURE 1. The measured Ca-sensitive absorbance (ΔA_{655}^{Ca}) of arsenazo III inside ghosts at 655 nm vs. the concentration of free intracellular Ca (Ca_i). The solid line is the theoretical prediction calculated from Eq. A2 (Appendix), with a total dye concentration of 110 μM, assuming that the free Ca_i was equal to the extracellular free Ca (Yingst and Hoffman, 1983). The concentration of free Ca was buffered on the outside of the ghosts with either HEDTA (○) or NTA (●). It was assumed that free Ca_i was equal to free Ca_o, because (*a*) the reservoir of buffered free Ca_o is very large compared with free Ca_i, (*b*) the membrane is freely permeable to free Ca because of the A23187, (*c*) the membrane potential should be close to zero, and (*d*) the ghosts are depleted of ATP, which could fuel the outwardly directed Ca pump. The composition of the external solution was 145 mM KCl, 20 mM Hepes, 4.3 mM HEDTA (open symbols) or 4.6 mM NTA (solid symbols), 0.51–3.66 mM Ca, and 5 μM A23187. The internal composition of the ghosts was 110 μM arsenazo III, 145 mM KCl, 5 mM NaCl, and 20 mM Hepes. The procedures followed in making these measurements have been outlined in detail in Yingst and Hoffman (1983).

Ca. In this measurement it was assumed that free Ca_i was equal to the extracellular free Ca buffered with either HEDTA or NTA, because the membrane had been made freely permeable to Ca by the addition of A23187, the membrane potential should be close to zero, the ghosts had been depleted of ATP to fuel the outwardly directed Ca pump, and the reservoir of buffered free Ca in the extracellular solution was very large compared with the volume of the intracellular Ca (Fig. 1).

Ca Influx Experiments

The influx of Ca into resealed ghosts was measured by monitoring the absorbance of arsenazo III trapped inside the ghosts. The ghosts were suspended at 1.67% hematocrit in 3 ml of a stirred solution in a cuvette that had previously been warmed to 37°C and that contained at least 0.1 mM EGTA, in addition to various concentrations of salts as given in the appropriate figure legends. A few minutes after temperature equilibration, the baseline absorbance of the sample was measured at $A_{655} - A_{700}$ nm in the dual-wavelength mode of the spectrophotometer. This absorbance is equal to the absorbance of the dye in the ghosts containing <0.05 μM free Ca because of the incorporated EGTA. The detection limit of the dye in these experiments is 0.3 μM free Ca. To initiate Ca influx, 10 μl of a concentrated solution of Ca is added to a 3-ml ghost suspension and ΔA^{Ca} is recorded as a function of time. Free Ca_i at any subsequent time is calculated from ΔA^{Ca}_{655} as outlined above.

Calculation of Extracellular Free Ca

Extracellular free Ca was calculated from the concentration of total Ca and the appropriate buffer, which was either NTA, HEDTA, or EDTA, according to published procedures (Yingst and Hoffman, 1983).

RESULTS AND DISCUSSION

Pattern of Ca Uptake

The time course of total Ca_i and free Ca_i accumulation in ATP-depleted ghosts containing 90 μM arsenazo III and <0.1 μM free Ca_i is shown in Fig. 2. The maximum rate of Ca influx immediately follows the addition of Ca to the extracellular solution, and over the next few minutes the rate of Ca accumulation declines markedly. In Fig. 2 the quantity labeled "total Ca" is equal to the sum of the free Ca and the Ca bound to arsenazo III (Eq. A6, Appendix). This value is presumably somewhat lower than the amount of Ca that would be determined by atomic absorption. The difference between the concentrations of total and free Ca represents the degree of buffering of free Ca_i by arsenazo III. The free Ca_i as depicted in Fig. 2 is in equilibrium with Ca bound to arsenazo III and in fact is calculated directly from the absorbance of CaD and Ca_2D_2. Free Ca_i is also in equilibrium with the Ca bound elsewhere within the cell.

As Ca moves on and off of these binding sites, there will be subsequent changes in free Ca_i. The addition of arsenazo III to the interior of the cell may change the ratio of free to total cellular Ca as measured by atomic absorption, but it would presumably not alter the relationship between free Ca_i and the amount of Ca bound to specific cellular sites. As shown in Fig. 2, the addition of sufficient EDTA to reduce the free Ca_o to zero causes Ca_i to decline only slightly. However, all of the Ca that has accumulated in the ghosts is exchangeable, as demonstrated by the rapid efflux of Ca_i after the permeability of the membrane to Ca was increased by the addition of A23187 (Fig. 2), an ionophore for divalent ions (Reed, 1976).

The pattern of Ca influx observed in Fig. 2, in which the rate of Ca influx is initially rapid and then declines, is similar to that observed when measuring ^{45}Ca uptake in resealed ghosts (Porzig, 1972) or assaying total Ca and ^{45}Ca in intact

cells (Ferreira and Lew, 1978). To explain this pattern, Porzig (1972) proposed that the rate of Ca influx is controlled by the amount of Ca bound to two different classes of binding sites on the red cell membrane. The first class of binding sites has a large capacity for Ca, equilibrates with Ca_o, and would be involved in the initial rapid phase of Ca influx and the release of accumulated Ca when the inward transmembrane gradient of Ca is reversed. A key feature

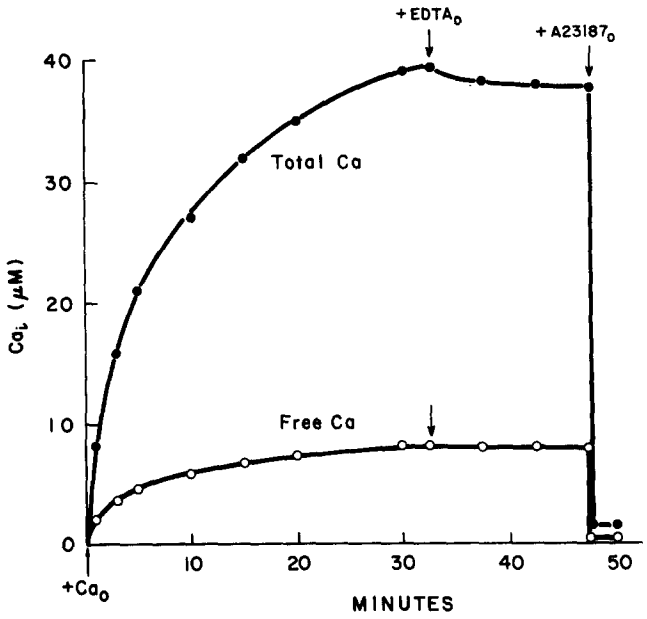


FIGURE 2. The concentration of total (solid symbols) and free Ca_i (open symbols) in ATP-depleted ghosts after increasing free Ca_o from 0 to 1 mM at time zero. The free Ca_i was calculated by method A. The total Ca_i (Eq. A6, Appendix) is equal to the sum of the free Ca_i and Ca_i bound to arsenazo III and does not include Ca that may be bound to cellular constituents. The difference between the free and total Ca_i represents the degree of buffering by arsenazo III. EDTA was added to a final concentration of 3 mM at 32.5 min to reduce free Ca_o to <0.1 μM. At 47.5 min, a final concentration of 5 μM A23187 was added to equilibrate free Ca_i. The internal composition of the ghosts before the addition of Ca was 150 mM KCl, 0.5 mM Na, 20 mM Hepes, 40 μM EDTA, and 90 μM arsenazo III. The initial extracellular solution contained 150 mM KCl, 20 mM Hepes, and 0.1 mM EGTA. The measurements were carried out at 1.67% hematocrit at 37°C.

of this proposal is that the binding of Ca to these membrane sites would not significantly alter the concentration of free Ca_i. However, the results presented in Fig. 2 show that free Ca_i does increase during the initial phases of Ca accumulation, so that rapid Ca uptake cannot simply be explained in terms of membrane binding. The second class of membrane sites (Porzig, 1972) has a small capacity and equilibrates with free Ca_i and would make the red cell membrane impermeable to Ca at concentrations of free Ca_i near 10 μM. How-

ever, as shown in Fig. 2, there is a gradual decline in the rate of Ca uptake rather than a marked change at some threshold value of free Ca_i.

The value of Ca_i in Fig. 2 (and Figs. 5 and 6 below) was calculated by assuming that Ca was evenly distributed in all the ghosts, as is the case when A23187 is present to equilibrate the Ca (Fig. 1). The results of Fig. 3 to be discussed below show that this assumption needs to be modified when the permeability is not uniform throughout the population of ghosts, and some ghosts may consequently accumulate more Ca than others. In the absence of A23187, the calculated free Ca_i represents a composite value that can be fully interpreted when the distribution of Ca throughout the ghost population has been established.

Heterogeneity

If all the ghosts used in the experiment depicted in Fig. 2 had a uniform permeability to Ca, the initial changes in the rate of Ca accumulation might indicate that the ghosts were becoming less permeable to Ca with time. Since such a Ca-stimulated decrease in permeability might provide insight into the regulation of the Ca permeability, it was important to determine whether all the ghosts were equally permeable to Ca. To evaluate the relative Ca permeability of the ghosts, ATP-depleted resealed ghosts containing high K_i were incubated in a solution containing Ca and low K_o to load the cells with Ca. More permeable ghosts would be expected to accumulate Ca at a greater rate and to contain a higher concentration of Ca at the end of a finite incubation period than the less permeable ghosts. It was expected that an increase in intracellular Ca would increase the K permeability of the membrane (Gardos, 1958), which in the presence of an outwardly directed K gradient would then lead to a net loss of K, subsequent shrinkage, and an increase in buoyant density. If the ghosts that accumulated more Ca lost more KCl, they would have a higher buoyant density and could be separated by centrifugation. Therefore, after incubating the ghosts in the solution containing low K_o and Ca_o, Ca_o was removed, the ghosts were fractionated according to subsequent differences in buoyant density, and the Ca content of each density fraction was measured. The results of such an experiment show that some ghosts accumulate more Ca than others and demonstate that the ghosts that accumulate the most Ca are the most dense (Fig. 3). Since these ghosts lacked ATP to fuel the outwardly directed Ca pump, this unequal distribution of Ca suggests that these ghosts have varying permeabilities to Ca. Since Ca would be expected to enter more permeable ghosts at a faster rate, heterogeneity in the Ca permeability of the ghosts could account for changes in the rate of Ca accumulation as seen in Fig. 2. Similar rapid initial rates of Ca influx have been observed in resealed ghosts (Porzig, 1972) and in intact cells depleted of ATP (Ferreira and Lew, 1978).

To test whether the most dense ghosts also had the lowest K, a preparation of ghosts containing high K was incubated in a solution containing sufficient Ca_o and low K_o and then separated by centrifugation, as was done for the experiment shown in Fig. 3. After centrifugation the ghosts were divided according to density into three equal fractions and the K content of each fraction was measured. The results showed that the least dense fractions contained the most

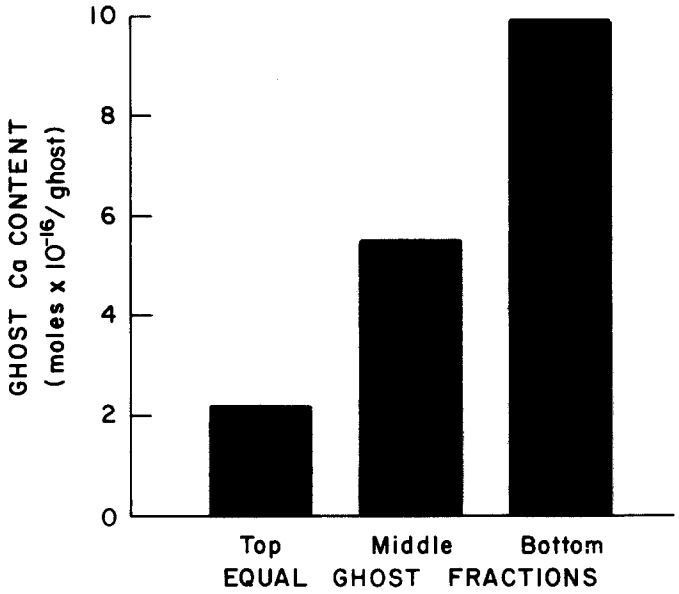


FIGURE 3. The distribution of Ca in three equal subpopulations of ghosts of different buoyant density after exposure to Ca_o. The experiment was carried out by suspending ghosts containing 147 mM K, 0.5 mM Na, 1.1 mM Mg, 20 mM Hepes, and 30 μM EGTA at 3% hematocrit in 150 mM NaCl, 3 mM KCl, 20 mM Hepes, 1 mM iodoacetic acid, and 0.1 mM EGTA, and equilibrating them at 37°C for 10 min. A small volume of concentrated Ca was then added to half of the flasks to give a final concentration of 1.0 mM free Ca and the incubation was continued for another 45 min. The ghosts in each flask were then washed three times with 10 vol of 153 mM KCl and 20 mM Hepes to remove Ca_o. They were then transferred to and centrifuged for 30 min at 12,000 *g* in a specially designed tube containing a tapered region in the center of the bottom in which ghosts would collect in a vertical column and distribute themselves from bottom to top according to their relative buoyant densities. After centrifugation, the resultant column of ghosts was 2–4 cm high and contained 0.4–0.9 ml of packed ghosts. Without disturbing this vertical distribution, the top, middle, and bottom fractions of the ghosts were removed and individually added at 4–8% hematocrit to a cuvette containing a solution of 150 mM KCl and 20 mM Hepes which had been previously passed through a Chelex column (Bio-Rad Laboratories, Richmond, CA) and to which 100 μM arsenazo III was subsequently added. The cuvette was then warmed at 37°C, $\Delta A^{Ca}_{solution}$ was recorded in the dual-wavelength mode, 10 μl A23187 in absolute ethanol was added at final concentration of 10 μM, and the subsequent increase in the absorbance was recorded as Ca left the ghosts. Since the concentration of Ca_o before the addition of A23187 was negligible, the total Ca released from the ghosts is equal to the Ca concentration of the extracellular solution after equilibration times the volume of the solution. The total Ca_o is in turn equal to the sum of the free Ca as calculated by method A and the Ca bound to arsenazo III (Eq. A6, Appendix). To correct for possible differences in the number of ghosts in each suspension, an aliquot of each solution was removed and diluted, and the number of ghosts was determined by means of a Coulter Counter (Hialeah, FL). The final results are then given in terms of total Ca per ghost. Each value is the mean of two separate measurements made on separate incubation flasks.

K and the most dense fractions contained the least K (Fig. 4*B*). The least dense ghosts contained the same K concentrations as a control group not exposed to Ca during the incubation (Fig. 4*A*), which indicates that they lost little if any K during the incubation with Ca. The distribution of K present in Fig. 4*B* is what would be expected on the basis of the Ca distribution of Fig. 3 and the effect of Ca on K loss; i.e., ghosts that have accumulated the most Ca might be expected to have lost the most K.

To test directly the idea that the unequal distributions of Ca (Fig. 3) and K (Fig. 4*B*) were due to differences in the Ca permeabilities of the ghosts as suggested above, the same ghosts used in Fig. 4, *A* and *B*, were incubated in the presence of 3 μM A23187 and 4 μM Ca_o chosen to give 5 μM free Ca_i, as calculated from the absorbance of entrapped arsenazo III. The absorbance of the entrapped arsenazo III in this case was approximately the same as that measured under the conditions of the incubation without A23187 (Fig. 4*B*). After this incubation the ghosts were separated by density and the distribution of ^{42}K per ghost was measured. In this case the amount of ^{42}K per ghost in each of the two density fractions was the same (Fig. 4*C*). The value of ^{42}K per ghost in these fractions was much lower than in the ghosts exposed to Ca in the absence of A23187 (Fig. 4*B*). Only two fractions were collected because the volume of ghosts decreased substantially after exposure to Ca and A23187. The results of this experiment show that there is no detectable difference in the Ca-dependent loss of K when the Ca permeability is increased by the addition of A23187. This result, therefore, supports the conclusion that the differences in the distribution of Ca (Fig. 3) and K (Fig. 4*B*) were due to underlying heterogeneity in the passive permeability of the ghosts to Ca. Equalization of Ca-dependent K loss through the use of the A23187 suggests that this ionophore can effectively be used to remove Ca heterogeneity.

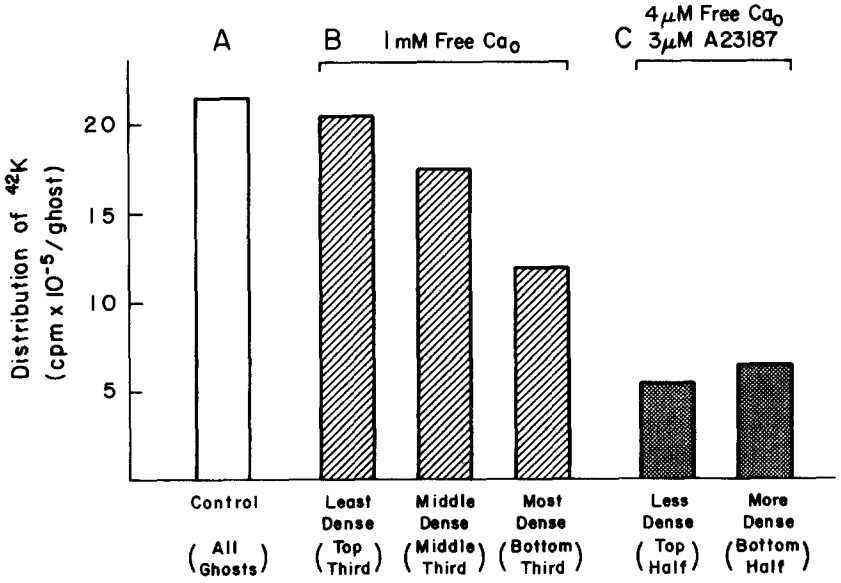
Ca Uptake as a Function of Ca_o

On the basis of the results of the experiments shown in Figs. 3 and 4, the initial rate of net Ca influx in Fig. 2 is probably due to Ca preferentially entering the more permeable ghosts. To test whether Ca was entering all the ghosts in a similar manner and to help elucidate the nature of the transport process, the rate of Ca influx was measured as a function of Ca_o during two different time periods. Fig. 5 shows that the rate of free Ca uptake is a nonlinear function of Ca_o and begins to plateau when Ca_o is increased above 1 mM. In agreement with the influx pattern observed in Fig. 2, the rate of Ca accumulation during the first 5 min after the addition of Ca_o (upper curve) is much higher than that in the later period (lower curve). If the higher apparent influx rate during the first 5 min represents influx into the more permeable ghosts and the lower rate reflects influx into the less permeable ghosts, then it appears that the underlying transport mechanism in all ghosts shows a similar dependency on Ca_o. The nonlinear rate of Ca accumulation during both time periods indicates that the influx of Ca is complicated and may proceed by more than one mechanism. The rate of ^{45}Ca uptake into intact cells also appears to saturate as a function of Ca_o with a half-maximal value between 0.5 and 1 mM Ca (Ferreira and Lew, 1978). The rate of net Ca accumulation in resealed ghosts appears to be about twice

that measured in intact cells under comparable conditions. Thus, for the conditions described in Fig. 5 the average rate of total Ca accumulation (that is, the sum of free Ca and Ca bound to arsenazo III) during the first 30 min after the addition of 2 mM Ca is ~92 μmol/liter ghosts per hour. Under comparable conditions and at the same K_o (150 mM), the rate of net Ca influx in energy-depleted intact cells during the first 30 min after the addition of 2 mM Ca_o is ~40 μmol/liter cells per hour (Ferreira and Lew, 1978).

Effect of Membrane Potential (E_m)

As noted above, increasing Ca_i increases the permeability of the membrane to K (P_K). P_K increases without a concomitant change in the permeability to Na (P_{Na}) or Cl (P_{Cl}), which has the effect of hyperpolarizing the membrane when K_i is greater than K_o (Glynn and Warner, 1972; Hoffman and Knauf, 1973). This effect is similar to that of valinomycin in increasing the ratio of P_K relative to P_{Cl} with resultant changes in E_m (Hunter, 1971, 1977; Hoffman and Laris, 1974). The presumed effect of E_m on Ca uptake can be seen clearly in Fig. 6, which shows the uptake of Ca into ghosts containing 147 mM K_i. The control rate of Ca uptake where K_o and K_i are equal is shown by the dotted line (Fig. 6). In this circumstance E_m should be close to zero and should not change as Ca enters. When K_o is lowered to 6 mM, the cell hyperpolarizes and the rate of Ca uptake increases (Fig. 6). At 6 mM K_o the rate of Ca uptake can be further augmented either by the addition of SITS, which would increase P_K/P_{Cl} by inhibiting P_{Cl} (Hoffman and Knauf, 1973), or by the addition of valinomycin, which increases P_K relative to P_{Cl} (Fig. 6). On the other hand, if valinomycin is applied when K_o is 147 mM, so that the membrane cannot hyperpolarize even with an elevated P_K/P_{Cl}, then the rate of Ca uptake is the same as without valinomycin (Fig. 6).



These results are similar to those obtained in intact cells that have been depleted of endogenous ATP (Gardos et al., 1980).

The manipulations presented in Fig. 6 for ghosts with K_i were also carried out under the same conditions on ghosts with the same constituents, except that K_i was lowered to 6 mM through replacement with Na. In ghosts with 6 mM K_i, neither SITS nor valinomycin alters Ca uptake when K_o is also 6 mM, and increasing K_o to 147 mM does not significantly inhibit the rate of Ca influx (data

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FIGURE 4. The effect of Ca and A23187 on the distribution of K between ghosts separated by buoyant density after incubation in a solution containing either: (*a*) no Ca and no A23187, (*b*) 1 mM Ca_o and no A23187, or (*c*) 4 μM Ca_o and 3 μM A23187. The incubation period in which Ca stimulated the loss of K was 18 min. This period was begun by adding the appropriate volume of concentrated Ca to a 30-ml solution at 37°C containing 150 mM Na, 3 mM K, 20 mM Hepes, 1 mM iodoacetic acid, 0.1 mM EGTA, either 30 μl of A23187 in absolute alcohol or 30 μl of alcohol alone, and ghosts at 1.67% hematocrit. The ghosts initially contained 140 μM arsenazo III, 147 mM K, 0.5 mM Na, 1.1 mM Mg, 20 mM Hepes, 30 μM EGTA, and a trace of ^{42}K. Note that the concentration gradient for K favors an efflux of K when the permeability to K is increased by Ca_i. Measurements with entrapped arsenazo III showed that the free Ca_i in the flasks containing A23187 equilibrated to 5 μM in <1 min after the addition of Ca_o and remained constant throughout the incubation. In flasks to which 1 mM Ca and no A23187 had been added, the average free Ca_i increased over the course of the incubation from <0.1 μM to 12 μM, reaching 5 μM ~5 min after the addition of Ca_o. At the end of the 18-min flux, the free Ca_i in all the ghosts was reduced to <0.1 μM by adding a final concentration of 3 mM EDTA to each of the flasks, followed by the addition of 3 μM A23187 to the flasks containing no ionophore. The contents of each incubation flask were then added to a separate centrifuge tube and centrifuged at 30,000 *g* for 5 min, the supernatant was removed, and the ghosts were washed twice in 20 vol of ice-cold 150 mM K, 20 mM Hepes, and 0.1 mM EGTA, pH 7.26. After the second wash the packed ghosts from each tube were suspended in 5 vol of the same solution and each suspension was added to a separate centrifuge tube tapered on the inside to a narrow, straight-walled chamber in the bottom that was ~0.8 cm in diameter and 2 cm high. These tubes were then centrifuged for 15 min at 25,000 *g* at 4°C in a swinging bucket rotor (Sorvall HB-4, Newtown, CT). At the end of this centrifugation the ghosts were confined to the narrow chamber with the more dense ghosts at the bottom and least dense at the top. For ghosts exposed to Ca and no A23187 during the measurement of K efflux, the top, middle, and bottom thirds of each column of ghosts were carefully removed without disturbing the vertical separation and the amount of ^{42}K per ghost in each third was determined. Since the overall ghost volume was much smaller for those exposed to both Ca and A23187, the ghosts in these tubes were removed in two sections, a top and a bottom half, and the amount of ^{42}K per ghost in each of these sections was determined. The control ghosts that were not exposed to Ca or A23187 were washed and processed the same as the other ghosts; the value of ^{42}K per ghost for the control shown above is the average for ghosts of all densities. Each of the values is the mean of two independent measurements. The ghosts in a given sample were diluted and counted in a Coulter Counter.

not shown). The latter result suggests that the inhibitory effect of high K_o on Ca influx is dependent on K_i.

The results presented in Fig. 6 are not significantly altered by the shrinkage of the ghosts that may have occurred when K_o was reduced to 6 mM and P_K was elevated because of either increased Ca_i or valinomycin. This conclusion is based on measurements of ghosts that were made to contain a fixed amount of Ca and arsenazo III and were shrunk under conditions similar to those described in Fig.

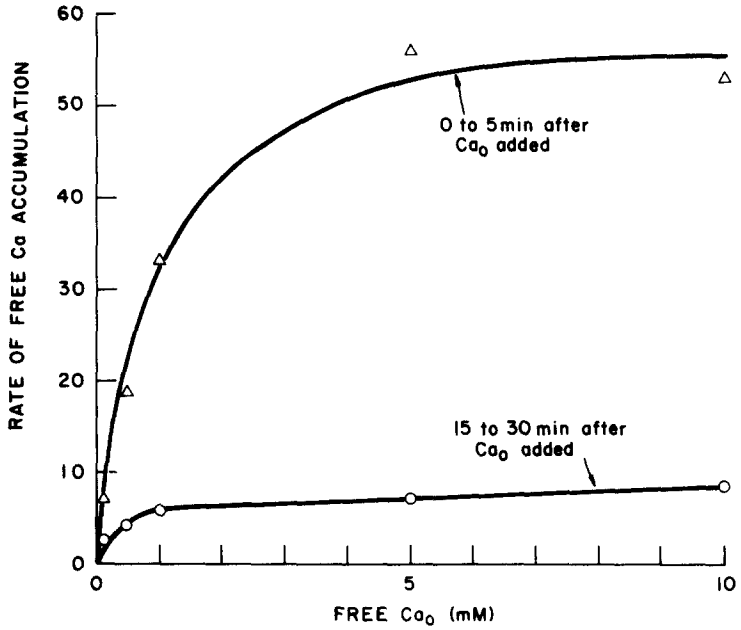

FIGURE 5. The average rate of free Ca_i accumulation as a function of Ca_o during an initial period when rapid influx occurs (0–5 min after the addition of Ca_o at time zero) and later when the rate of influx had slowed (15–30 min after the addition of Ca_o). The ghosts were made to contain 150 mM K, 0.5 mM Na, 20 mM Hepes, 40 μM EGTA, 100 μM arsenazo III, and <0.1 mM Mg. The external solution contained 150 mM K, 20 mM Hepes, 0.1 mM EGTA, and free Ca_o as indicated. The measurements were at 37°C, pH 7.25, and 1.67% hematocrit. Each point is the mean of two separate measurements carried out at a given concentration of free Ca_o on one preparation of ghosts. Units on the ordinate are micromoles per liter ghosts per hour.

6 (data not shown). Possible effects of shrinkage on the calculated free Ca_i would be minimized by compensating changes. First, shrinkage of the ghosts concentrates the total dye and total Ca equally, producing only minor shifts in the buffering of free Ca by arsenazo III. Second, the decrease in volume that increases these concentrations decreases the effective pathlength of the dye through the ghosts by an equal amount. Scattering artifacts that could accompany shrinkage are reduced by the dual-wavelength recording method (Yingst and

Hoffman, 1983). For these reasons, the effects of K_o in the experiment shown in Fig. 6 are primarily due to alterations in the membrane potential.

Effect of Inhibitors

The effects of agents that inhibit the Ca-stimulated K transport system of red cells were tested on the passive Ca permeability of red cell ghosts. Under the conditions outlined for the experiments presented in Fig. 6, both oligomycin (10

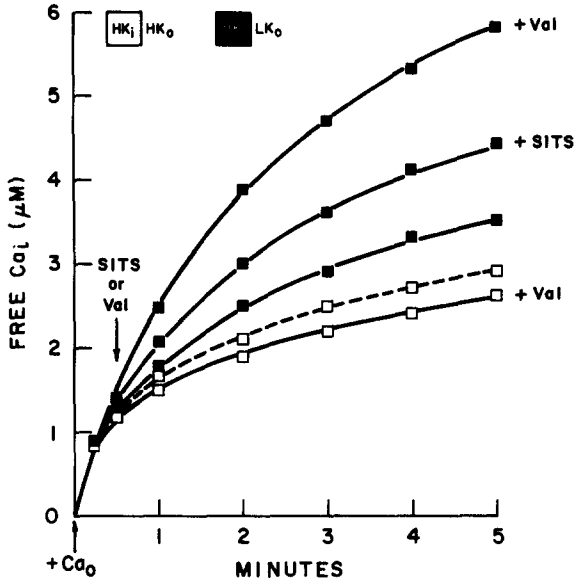

FIGURE 6. The effects of SITS and valinomycin (Val) on the time course of free Ca_i accumulation in ghosts containing 147 mM K, 20 mM Hepes, 20 μM EGTA, 1.1 mM Mg, and 140 μM arsenazo III. At time zero, 1.1 mM Ca was added to the extracellular solution and the subsequent concentration of free Ca_i was measured with entrapped arsenazo III. The extracellular solution contained either 147 mM K and 0.5 mM Na (open symbols) or 6 mM K and 142 mM Na (closed symbols) plus 20 mM Hepes, 1.1 mM Mg, 1 mM iodoacetic acid, and 0.1 mM EGTA. A small volume of SITS and Val was added as indicated to give final concentrations of 0.1 mM and 10 μM, respectively. All measurements were carried out at 37°C, pH 7.25, and 1.67% hematocrit.

μg/ml) and quinidine (1 mM) reduced Ca influx into ghosts containing 147 mM K_i when they were suspended in solutions containing 6 mM K_o. At 147 mM K_i and 6 mM K_i, the percent reduction of Ca influx caused by these agents was the same as that achieved by raising K_o from 6 to 147 mM. Thus, these agents probably slow Ca uptake by inhibiting the Ca-stimulated increase in P_K. At 147 mM K_i, 0.1 mM ouabain does not alter the rate of Ca uptake at 6 or 147 mM K_o. Furosemide (1 mM), on the other hand, produces a slight (15%), consistent increase in Ca influx at 147 mM K_i and 147 mM K_o. In testing the effects of

quinidine on Ca uptake, we found that this drug interacts directly with arsenazo III by decreasing the absorbance at 655 nm by 30%. The above effects of quinidine on Ca uptake were made after multiplying all the absorbances measured in the ghosts by 1.4 and assuming that quinidine does not change the affinity of arsenazo III for Ca.

Summary

This paper shows that arsenazo III can be used to study the factors that influence the rate of Ca influx into human red blood cell ghosts. The factors studied in this paper include the kinetics of Ca influx, the effects of Ca_o and free Ca_i, and the influence of E_m. The ghosts used in this study were prepared using a sucrose cushion to eliminate the most permeable ghosts (Bodemann and Passow, 1972). Nevertheless, the remaining ghosts still demonstrate varying permeabilities to Ca. Ca preferentially enters the more permeable ghosts and they in turn make a larger contribution to the measured absorbance than the ghosts containing less Ca. The results of Fig. 4 emphasize that A23187 can be used to eliminate differences in the passive Ca permeability and the K heterogeneity that can result from a heterogeneous distribution of Ca (Fig. 3). As a consequence of these findings, we have used A23187 in all of the experiments in the accompanying paper (Yingst and Hoffman, 1984), which examines the effect of Ca on cation transport in resealed ghosts containing arsenazo III.

APPENDIX

Calculation of Free Ca_i from ΔA^{Ca} by Methods A and B

The following section is based on the data and conclusions that are presented in Yingst and Hoffman (1983). In experiments where the ghosts contained <50 μM free Mg (Figs. 1, 2, and 5), the concentration of free Ca_i was calculated by method A. In this case the concentration of dye bound to Mg is small and can be neglected, so that D_T is equal to the sum of the free dye (D) and the dye bound to Ca in the form of CaD and Ca_2D_2:

$$[D_T] = [D] + \frac{[\text{free Ca}]\cdot[D]}{K_{d,CaD}} + \frac{2\cdot[\text{free Ca}]^2\cdot[D]^2}{K_{d,Ca_2D_2}}, \tag{A1}$$

where $K_{d,CaD}$ and K_{d,Ca_2D_2} are the disassociation constants defined in Table I. At a given D_T, the Ca-sensitive change in absorbance at 655 nm (ΔA^{Ca}_{655}) is:

$$\Delta A^{Ca}_{655} = \frac{\Delta\epsilon_{CaD}\cdot[\text{free Ca}]\cdot[D]}{K_{d,CaD}} + \frac{\Delta\epsilon_{Ca_2D_2}\cdot[\text{free Ca}]^2\cdot[D]^2}{K_{d,Ca_2D_2}}, \tag{A2}$$

where $\Delta\epsilon_{CaD}$ and $\Delta\epsilon_{Ca_2D_2}$ are molar extinction coefficients for a change in absorbance for CaD and Ca_2D_2 at 655 nm, respectively (Table I), multiplied by the percent hematocrit and divided by 100. Solving Eq. A2 for D yields

$$[D] = ([\Delta\epsilon_{Ca_2D_2}\cdot\Delta A^{Ca}_{655}\cdot K_{d,Ca_2D_2}\cdot 4 + K^2_{d,Ca_2D_2}\cdot\Delta\epsilon^2_{CaD}]^{1/2} - K_{d,Ca_2D_2}\cdot\Delta\epsilon_{CaD})/(2\cdot\Delta\epsilon_{Ca_2D_2}\cdot[\text{free Ca}]). \tag{A3}$$

If the terms inside the first parentheses in Eq. A3 are equal to W, and Eq. A3 is substituted into Eq. A1, the resultant expression can be solved for free Ca;

$$[\text{free Ca}] = W/\{2\cdot\Delta\epsilon_{Ca_2D_2}\cdot(D_T - W/[2\cdot\Delta\epsilon_{Ca_2D_2}\cdot K_{d,CaD}] - W^2/[2\cdot\Delta\epsilon^2_{Ca_2D_2}\cdot K_{d,Ca_2D_2}])\}. \tag{A4}$$

In other experiments where ghosts contained >50 μM free Mg (Fig. 6), free Ca was calculated by method B. This procedure is similar to method A except that the concentration of dye-bound Mg, [MgD], was measured before the addition of Ca and it was assumed that [MgD] stayed constant throughout the experiment. Free Ca was then calculated by means of Eq. A4, where D_T is now equal to $[D_T] - [MgD]$ and ΔA_{655}^{Ca} is

$$\Delta A_{655}^{Ca} = (A_{655} - A_{700})_{Ca\text{ and }Mg} - (A_{655} - A_{700})_{Mg}, \quad (A5)$$

where the subscript Ca and Mg denotes a sample with Ca and Mg and the subscript Mg is a similar sample with the same D_T and free Mg, but no free Ca.

TABLE I

Equilibria	K_d	Molar extinction coefficients for a change in absorbance		
		$\Delta\epsilon_{600}$	$\Delta\epsilon_{630}$	$\Delta\epsilon_{655}$
			liter/mol·cm	
$K_{d,CaD} = \frac{[\text{free Ca}]\cdot[D]}{[CaD]}$	7×10^{-5} M	1.61×10^4	1.54×10^4	1.66×10^4
$K_{d,Ca_2D_2} = \frac{[\text{free Ca}]^2\cdot[D]^2}{[Ca_2D_2]}$	2×10^{-14} M^3	5.94×10^4	2.55×10^4	4.96×10^4
$K_{d,MgD} = \frac{[\text{free Mg}]\cdot[D]}{[MgD]}$	1.25×10^{-3} M	1.60×10^4	1.63×10^4	9.2×10^3

The dissociation constants (K_d) and molar extinction coefficients for a change in absorbance for arsenazo III with Ca and Mg at 37°C, 0.17 M, and pH 7.26. The first column shows the equilibria (reactants/products) described by the dissociation constant, where D is free dye, CaD, Ca_2D_2, and MgD are the bound forms of the dye, and the brackets denote concentrations. Free dye is equal to the total dye minus the dye bound as CaD, Ca_2D_2, and MgD and includes dye complexed with protons and any other constituents present in the calibrating solution, which contained 145 mM KCl, 20 mM Hepes (Tris), and EDTA, NTA, or HEDTA. The molar extinction coefficients are for the Ca- or Mg-sensitive changes in absorbance, which are equal to the difference in absorbance between a sample with dye plus Ca (or Mg) minus the absorbance of the same concentration of dye without Ca (or Mg).

Calculation of Total Ca

The total Ca that is detected by the arsenazo III is the sum of the free Ca and that bound to the dye, which is

$$[Ca_T] = [\text{free Ca}] + [CaD] + 2\,[Ca_2D_2]. \quad (A6)$$

This value of $[Ca_T]$ could be different from that measured by atomic absorption because it would not include Ca bound to cellular constituents.

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