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
Hoffman, 1978) and calibrated it to measure free intracellular Ca (Ca) (Yingst and Hoffman, 1983). We have used this technique because of the very low ratio of free to bound Ca in human red cells, which makes it difficult to measure free Ca directly by atomic absorption. For the same reason, interpreting isotopic measurements can be complex because of the exchange of ⁴⁵Ca with bound Ca and problems of defining specific activities in terms of free Ca. The total Ca 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 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 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. 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 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 four 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 those noted were reagent grade. EDTA (ethylenediaminetetraacetic 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 CaCl₂ from British Drug House (distributed by Gallard-Schlesinger, Carle Place, NY). Before use, MgCl₂·6H₂O 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 Ca₂⁺

Free Ca₂⁺ was calculated from Ca-sensitive changes in the absorbance (ΔA₂⁺) 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 (Ca₂D₂) 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 Ca₂⁺ was calculated by method A (see Appendix). In other experiments where ghosts contained >50 μM free Mg (Fig. 6), free Ca₂⁺ 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

\[ \Delta A_{Ca}^{ghost} = \frac{\Delta A_{Ca}^{solution} \cdot \%HCT}{100}, \]

where \( \Delta A_{Ca}^{ghost} \) 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 (ΔA₂⁺) 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 (ΔA₆⁵₅) is:

\[ \Delta A_{655} = (A_{655} - A_{700})_{Ca} - (A_{655} - A_{700}), \]

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 DT 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, 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 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_{55} in Eq. A2 was calculated by iteration in Eq. A1 knowing the total dye concentration and the free Ca.

**Figure 1.** The measured Ca-sensitive absorbance (ΔA_{55}) of arsenazo III inside ghosts at 655 nm vs. the concentration of free intracellular Ca (Ca). 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 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 was equal to free Ca,, because (a) the reservoir of buffered free Ca, is very large compared with free Ca,, (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). In this measurement it was assumed that free Ca 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 \(\mu\)M free Ca because of the incorporated EGTA. The detection limit of the dye in these experiments is 0.3 \(\mu\)M free Ca. To initiate Ca influx, 10 \(\mu\)l of a concentrated solution of Ca is added to a 3-ml ghost suspension and \(\Delta A^{\text{Ca}}\) is recorded as a function of time. Free Ca, at any subsequent time is calculated from \(\Delta A_{655}^{\text{Ca}}\) 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, and free Ca, accumulation in ATP-depleted ghosts containing 90 \(\mu\)M arsenazo III and <0.1 \(\mu\)M free Ca, 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 by arsenazo III. The free Ca, 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_{2}D_{2}. Free Ca, 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. 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 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, to zero causes Ca, 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, 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
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, 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 of this proposal is that the binding of Ca to these membrane sites would not significantly alter the concentration of free Ca. However, the results presented in Fig. 2 show that free Ca 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, and would make the red cell membrane impermeable to Ca at concentrations of free Ca near 10 μM. How-

![Graph showing the concentration of total (solid symbols) and free Ca (open symbols) in ATP-depleted ghosts after increasing free Ca from 0 to 1 mM at time zero. The free Ca was calculated by method A. The total Ca (Eq. A6, Appendix) is equal to the sum of the free Ca and Ca bound to arsenazo III and does not include Ca that may be bound to cellular constituents. The difference between the free and total Ca 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 to <0.1 μM. At 47.5 min, a final concentration of 5 μM A23187 was added to equilibrate free Ca. 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.](image)
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

The value of Ca 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 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 were incubated in a solution containing Ca and low K 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 and Ca, Ca 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 demonstrate 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 and low K, 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$\infty$. 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 $\mu$M 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$\infty$. 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 $\mu$M arsenazo III was subsequently added. The cuvette was then warmed at 37°C. $\Delta A_{\text{absorbance}}$ was recorded in the dual-wavelength mode, 10 $\mu$l A23187 in absolute ethanol was added at final concentration of 10 $\mu$M, and the subsequent increase in the absorbance was recorded as Ca left the ghosts. Since the concentration of Ca 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 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. 4B). The least dense ghosts contained the same K concentrations as a control group not exposed to Ca during the incubation (Fig. 4A), which indicates that they lost little if any K during the incubation with Ca. The distribution of K present in Fig. 4B 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. 4B) 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, chosen to give 5 μM free Ca, 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. 4B). After this incubation the ghosts were separated by density and the distribution of 45K per ghost was measured. In this case the amount of 45K per ghost in each of the two density fractions was the same (Fig. 4C). The value of 45K per ghost in these fractions was much lower than in the ghosts exposed to Ca in the absence of A23187 (Fig. 4B). 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. 4B) 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**

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, during two different time periods. Fig. 5 shows that the rate of free Ca uptake is a nonlinear function of Ca, and begins to plateau when Ca 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 (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. 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 45Ca uptake into intact cells also appears to saturate as a function of Ca 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 Ko (150 mM), the rate of net Ca influx in energy-depleted intact cells during the first 30 min after the addition of 2 mM Cao is ~40 μmol/liter cells per hour (Ferreira and Lew, 1978).

Effect of Membrane Potential (Em)

As noted above, increasing Ca(o) 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 Em (Hunter, 1971, 1977; Hoffman and Laris, 1974). The presumed effect of Em 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 Em 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 not shown).
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. 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

\[ \text{mg/ml} \] 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 \( K_o \), 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

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**Figure 6.** The effects of SITS and valinomycin (Val) on the time course of free Ca; accumulation in ghosts containing 147 mM K, 20 mM Hepes, 20 \( \mu \)M EGTA, 1.1 mM Mg, and 140 \( \mu \)M arsenazo III. At time zero, 1.1 mM Ca was added to the extracellular solution and the subsequent concentration of free Ca; 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 \( \mu \)M, respectively. All measurements were carried out at 37°C, pH 7.25, and 1.67% hematocrit.
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, and free Ca, 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, from $\Delta A^{Ca}$ 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 $\mu$M free Mg (Figs. 1, 2, and 5), the concentration of free Ca 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$_2$D$_2$:

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

where $K_{d,CaD}$ and $K_{d,Ca_2D_2}$ are the dissociation constants defined in Table 1. At a given D$_T$, the Ca-sensitive change in absorbance at 655 nm ($\Delta A^{Ca}_{655}$) is:

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

where $\Delta A^{Ca}_{655}$ and $\Delta A^{Ca}_{655}$ are molar extinction coefficients for a change in absorbance for CaD and Ca$_2$D$_2$ at 655 nm, respectively (Table 1), multiplied by the percent hematocrit and divided by 100. Solving Eq. A2 for D yields

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

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:

$$[\text{free } Ca] = W / \left(2 \cdot \Delta A^{Ca}_{655} \cdot (D_T - W / (2 \cdot \Delta A^{Ca}_{655} \cdot K_{d,CaD}) - W^2 / (2 \cdot \Delta A^{Ca}_{655} \cdot K_{d,Ca_2D_2})) \right). \quad (A4)$$
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 $\Delta A_{655}$ is

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

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          | $K_d$         | $\Delta A_{600}$ | $\Delta A_{650}$ | $\Delta A_{655}$ |
|---------------------|---------------|------------------|------------------|------------------|
| $K_{CaD}$           | $[\text{free Ca}]/[D]$ | $7 \times 10^{-3}$ M | $1.61 \times 10^4$ | $1.54 \times 10^4$ | $1.66 \times 10^4$ |
| $K_{Ca_2D_2}$       | $[\text{free Ca}^2]/[D]_2$ | $2 \times 10^{-14}$ M$^2$ | $5.94 \times 10^4$ | $2.55 \times 10^4$ | $4.96 \times 10^4$ |
| $K_{MgD}$           | $[\text{free Mg}]/[D]$ | $1.25 \times 10^{-5}$ M$^2$ | $1.60 \times 10^4$ | $1.63 \times 10^4$ | $9.2 \times 10^3$ |

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$_2$D$_2$, 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$_2$D$_2$, 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

$$[Ca_T] = [\text{free Ca}] + [CaD] + 2 [Ca_2D_2].$$

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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