BRIEF COMMUNICATION

CHANGES OF INTRACELLULAR Ca^{++} AS MEASURED BY ARSENAZO III IN RELATION TO THE K PERMEABILITY OF HUMAN ERYTHROCYTE GHOSTS

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ABSTRACT A Ca-sensitive dye, arsenazo III, has been incorporated into resealed human erythrocyte ghosts and calibrated to monitor continuously micromolar concentrations of intracellular ionized Ca ($[Ca^{++}]_i$). When the external concentration of Ca is much greater than $[Ca^{++}]_i$, $[Ca^{++}]_i$ increases because of a net balance between Ca influx and efflux. Dynamic changes in $[Ca^{++}]_i$ regulate K efflux, which in turn may influence the rate of Ca influx. A procedure for purifying arsenazo III is also described.

INTRODUCTION

For some time we have been interested in measuring changes in intracellular ionized Ca ($Ca^{++}{}_i$) in human erythrocytes to study the regulation of cation transport (1, 2). For instance, intracellular Ca stimulates net K efflux from erythrocytes (2, 3), but a complete study of the interaction of Ca influx and net K loss has not been possible because of the difficulty of measuring micromolar concentrations of $[Ca^{++}]_i$. The total concentration of Ca in erythrocytes is 16 μM/liter cells (4) and can be measured by atomic absorption, but $[Ca^{++}]_i$ is < 1 μM/liter cells, or $< 6\%$ of the total (5). Thus, one of the problems has been to measure changes of $[Ca^{++}]_i$ against a large background of bound Ca. In addition, erythrocytes have a Ca pump (6) which may be expected to change $[Ca^{++}]_i$ rapidly (5–7). Therefore, to measure changes in $[Ca^{++}]_i$ accurately, it is clearly desirable to have a method that rapidly monitors free Ca, but is unaffected by bound Ca. This paper presents a method for entrapping a Ca-sensitive dye, arsenazo III, into resealed human erythrocyte ghosts and continuously measuring micromolar $[Ca^{++}]_i$. Also described are some interrelationships between $[Ca^{++}]_i$, changes in the rate of Ca influx, and increases in the K permeability of the membrane. The changes in $[Ca^{++}]_i$ in these experiments represent a net balance of Ca influx and efflux, because the direction of change in $[Ca^{++}]_i$ in the pres-

ence of an inward Ca gradient can be reversed by conditions that stimulate the outwardly directed Ca pump.

The use of arsenazo III to detect changes in intracellular Ca^{++} has been previously described for squid axons (8, 9), *Limulus* photoreceptors (10), *Aplysia* neurons (11), and frog muscle (12). Erythrocyte ghosts offer advantages over these preparations in the ease with which the dye and other intracellular constituents can be introduced and manipulated, and the ability to study the ghosts in a single cuvette of a dual wavelength spectrophotometer where conditions can easily be controlled and the response of the dye accurately measured and quantified. For example, our calibration is based directly on the affinity of arsenazo III for Ca, instead, as has previously been the case (9,11), on the properties of a second chelator whose properties may be imperfectly known for the experimental conditions.

MATERIALS AND METHODS

Ghosts were made from fresh, heparinized human erythrocytes, energy depleted for 22 h at 37°C in 155 mM NaCl, 0.1 mM ethyleneglycol-*bis*(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 17 mM *N*-2-hydroxyethylpiperazine – *N'*-2-ethanesulfonic acid (Hepes), pH 7.18 and hemolyzed (1:20) for 5 min at 0°C in 70 μM arsenazo III, 10 mM NaCl, 20 mM Hepes, 1 mM Mg, 20 μM EGTA, and a trace amount of ^{42}K at pH 7.68. Isotonicity was restored with KCl, the ghosts were resealed at 37°C for 1 h, washed by centrifugation in 155 mM NaCl, 17 mM Hepes, 0.1 mM EGTA, pH 7.4 (22°C), centrifuged on a 10% sucrose cushion to reduce heterogeneity (13), washed again with the above solution, and then packed to a hematocrit > 95%.

The rationale behind the use of arsenazo III to measure $[Ca^{++}]_i$ quantitatively is to make dye-containing ghosts with negligible initial $[Ca^{++}]_i$, suspend them in a solution in a cuvette, add Ca to the external solution, and measure by means of arsenazo III the amount of Ca that accumulates in the ghosts. As Ca enters the ghosts, it reacts with the dye forming a Ca-dye complex (CaD), which gives a change in absorbance from which [CaD] is calculated and which in turn, specifies $[Ca^{++}]_i$ by means of a mass action equation. Accordingly, $[Ca^{++}]_i = K_d \times [CaD]/[D]_{free}$ with the assumption that the dye forms a 1:1 complex with Ca (14,15), which we have verified for our experimental conditions. K_d is the dissociation constant of [CaD] and $[D]_{free} = [D]_{total} - [CaD]$. The K_d (1.7×10^{-5}) was measured in solution with an ionic strength (μ) of 0.16 at 37°C, pH 7.18, and does not change when the dye is incorporated into ghosts. [CaD] is calculated from the molar extinction coefficient of the calcium-dye complex (ϵ_{CaD}) determined for the dye in ghosts at pH 7.18, $\mu = 0.16$, 37°C, and 1.67% final hematocrit, which would not be very different from ϵ_{CaD} in solution (2.68×10^4) if the hematocrit were adjusted to 100%. Calculation of ϵ_{CaD} in ghosts assumes that in the the presence of 10 μM A23187 and 1 mM $[Ca]_o$ all of the dye in the ghosts is in the form of CaD.

The solution in the cuvette, and all the reagents employed to make the ghosts, had been previously passed through a Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.) column to remove divalent ion contamination. Also, the cuvettes and glassware utilized in making the ghosts were washed three times with 5 mM EDTA and seven times with deionized distilled water passed through a Chelex column.

Before Ca is added to the suspension of ghosts, $[Ca^{++}]_i$ is <0.1 μM, i.e., below the limit of detection of the dye in this system, because the combined Ca contamination of the Chelex-treated solutions and the dye is low (4 μM or less) and the ghosts contain 20 μM EGTA, a chelator that has a much higher affinity for Ca than does arsenazo III. This concentration of EGTA was experimentally determined so as to be high enough to chelate any initial contaminating Ca present

from making the ghosts, plus the 1 or 2 μM Ca contamination present in the calibrating solutions before additional Ca was added, but low enough to chelate a minimum of the Ca that enters the ghosts from the additions of Ca to the external solution. EGTA does not interfere with the measurement of $[Ca^{++}]_i$, even before its capacity is exceeded, because any changes in $[Ca^{++}]_i$ are reflected in changes of [CaD] that are measured directly and that specify $[Ca^{++}]_i$ by means of the mass action equation. This same argument holds for any intracellular constituents, such as proteins, that might bind Ca and are in equilibrium with free Ca.

Arsenazo III, of course, also binds Ca and under some conditions in these experiments the ratio of [CaD] to $[Ca^{++}]_i$ is as high as 2.5. This is not to argue, however, that arsenazo III interferes with the measurement of $[Ca^{++}]_i$ because [CaD] is simply reflecting the changes in $[Ca^{++}]_i$ with which it is in equilibrium and it is [CaD] from which $[Ca^{++}]_i$ is calculated.

When arsenazo III complexes with Ca, there is an increase in absorbance at 655 nm and only a small change at 700 nm. Arsenazo III also combines with Mg, but the dissociation constant for this complex is at least 300 times greater than for Ca and the molar extinction coefficient for the magnesium-dye complex (MgD) at the wavelength pair 655–700 nm is approximately one-half that for CaD, so that the interference of Mg with Ca at 655–700 nm is small. For instance, at 1 mM $[Mg]_i$, a 20% increase in $[Mg]_i$ would only interfere with the measurement of changes in $[Ca^{++}]_i < 1$ μM. Because arsenazo III is also sensitive to pH, sufficient buffer was incorporated inside the ghosts and added to all solutions to prevent any significant changes in pH during the course of the experiments.

Flux measurements were conducted according to Blum and Hoffman (2) and the percent of ^{42}K released was calculated from: percent $R = ([P_x - P_o]/[WS - P_o]) \times 100$, where, P_x is the counts out at time x, P_o is the counts out at the first time point, and WS is the counts in an equal volume of ghost suspension.

RESULTS AND DISCUSSION

The arsenazo III used in these experiments was first purified to reduce the high level of impurities, the type and amount of which vary with the source of the dye (16). The crude product obtained from Sigma Chemical Co., St. Louis, Mo., was first passed over a column containing the ion-exchange resin Chelex 100 to reduce contamination by divalent ions, and then over a diethylaminoethyl (DEAE) Sephadex (HCO_3^- form) column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.) eluting with a linear gradient of 0.5–2.0 M triethylammonium bicarbonate to separate arsenazo III from its contaminants. Fractions containing primarily arsenazo III, as identified spectrally and by thin-layer chromatography, were recombined and concentrated by evaporation under reduced pressure, leaving the triethylammonium salt of arsenazo III. This compound was converted to the sodium salt on a Dowex 50-W column (The Dow Chemical Co., Midland, Mich.). Finally, this purified product was passed through a Chelex column and its purity was checked by thin-layer chromatography using a *n*-propanol: NH_4OH:H_2O (9:2:9) solvent system on silica gels prewashed with ethylenediamine-tetraacetic acid (EDTA). By this method, arsenazo III could be separated from the three major and a number of minor contaminants present in this batch of dye. This purification procedure appears to give better separation, be more convenient, and allow larger quantities to be processed than the procedure recently published by Kendrick (16), and will be described in more detail elsewhere.

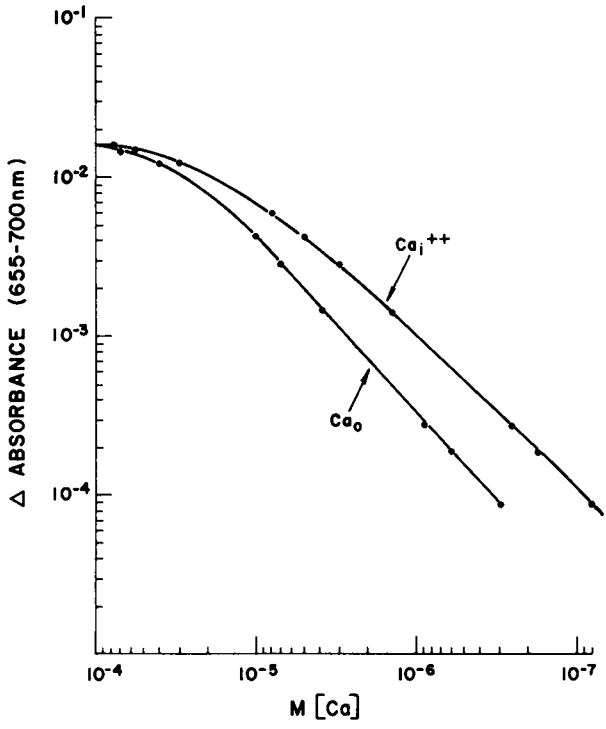

FIGURE 1 Calibration curve of arsenazo III in resealed ghosts. The curve labeled Ca_o is the measured Δ absorbance for given changes in the Ca concentration of the external solution in the presence of A23187. The curve labeled $Ca^{++}{}_i$ is the calculated concentration of $[Ca^{++}]_i$ determined from the measured Δ absorbance by means of a mass action equation as described in Methods. Ghosts, prepared to contain 70 μM arsenazo III, 140 mM KCl, 10 mM NaCl, 20 mM Hepes, 1 mM $MgSO_4$, and 20 μM ETGA, pH 7.4 at 22°C, and <0.1 μM $[Ca^{++}]_i$, were added at 1.67% final hematocrit to a stirred solution of 155 mM KCl, and 17 mM Hepes at pH 7.18 (37°C) in a single cuvette of a dual wavelength spectrophotometer (Aminco DW-2, American Instrument Co., Inc., Silver Springs, Md.) and allowed to equilibrate at 37°C for 5 min. After mixing, the Δ absorbance (655–700 nm), originally adjusted to zero, increased to 8.7×10^{-3}. The divalent ionophore, A23187, was then added to give a final concentration of 10 μM, which decreased the Δ absorbance to 5.5×10^{-3} as Mg^{++} left the ghosts and equilibrated with the external solution. Next, a small volume of $MgSO_4$ was added to the cuvette giving a final concentration of 1 mM, which increased the Δ absorbance to 8.9×10^{-3} and restored $[Mg^{++}]_i$ to its original concentration. These three absorbances are the means of two measurements. At this point the Δ absorbance was adjusted to zero and small volumes of concentrated $CaCl_2$ solutions were added to change the Ca concentration of the external solution. With each addition of Ca there was a rapid increase in the Δ absorbance which stabilized in <1 min as a result of the presence of A23187. The Δ absorbance in Fig. 1 refers to the changes from the base line established when the Δ absorbance (655–700 nm) was zeroed before the first Ca addition.

To illustrate the above points: when the Ca concentration of the external solution was increased over its original value by 3×10^{-6} M, the Δ absorbance was 10^{-3}, as shown by the curve labeled Ca_o, and this Δ absorbance is equal to a calculated $[Ca^{++}]_i$ of 10^{-6} M as shown by the curve labeled $Ca^{++}{}_i$. The Ca concentration of the external solution is changed during the calibration only as a device to introduce Ca into the ghosts and there is no assumption that $[Ca^{++}]_o$ necessarily equals $[Ca^{++}]_i$. To use the calibration in the subsequent experiments to find $[Ca^{++}]_i$, one measures the Δ absorbance, finds the value on the ordinate, reads across to the curve labeled $Ca^{++}{}_i$ and reads $[Ca^{++}]_i$ on the abscissa without reference to the curve labeled Ca_o.

Each point is the mean of two measurements, and from 6×10^{-7} M to 4×10^{-5} M $[Ca]_o$ each measurement was within 7% of the mean value. Outside of this range values were within 15% of the mean. Furthermore, this calibration is very similar to numerous other curves obtained from different batches of ghosts.

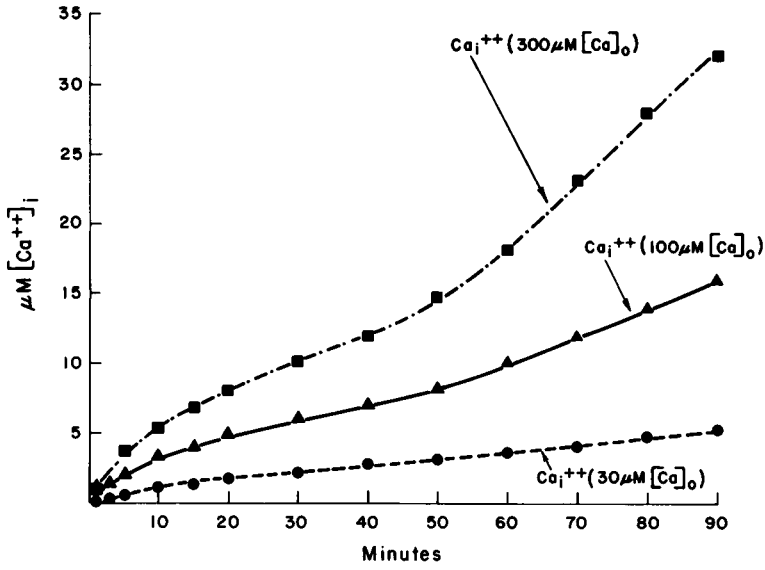

FIGURE 2 Ca influx into ghosts at various external Ca concentrations ($[Ca]_o$). To make the measurements, ghosts which were from the same preparation as in Fig. 1 were pipetted to a final hematocrit of 1.67% into Chelex-treated 155 mM NaCl, 2 mM K, 1 mM iodoacetic acid, 17 mM Hepes, pH 7.18 (37°C), and allowed to temperature-equilibrate for 7 min at 37°C. The Δ absorbance (655–700 nm) was adjusted to zero and small volumes of concentrated $CaCl_2$ were added to give specific final concentrations at time zero. Subsequent changes in absorbance (655–700 nm) were recorded continuously, and $[Ca^{++}]_i$ at any given time was calculated directly from these changes in absorbance by means of the curve labeled $Ca^{++}{}_i$ in Fig. 1. During the first 10 min the rate of Ca influx is maximum. This initial rapid phase may in part result from a residual subpopulation of ghosts that are tight to dye but leaky to Ca, because the magnitude of the fast phase shown here after purification on a sucrose cushion (13) is considerably less than with ghosts not centrifuged on the cushion. Also, there is almost no leakage of the dye out of the ghosts. Rates of Ca^{++} influx were then calculated for the linear portions of the Ca^{++} influx curves, ignoring the initial rapid phase. The rates of Ca^{++} influx at different external Ca concentrations were: 0.05 μmol/liter·min (15–90 min) at 30 μM $[Ca]_o$; 0.13 μmol/liter·min (15–50 min), 0.20 μmol/liter·min (50–90 min) at 100 μM $[Ca]_o$; and 0.21 μmol/liter·min (15–40 min), 0.3 μmol/liter·min (40–60 min), 0.48 μmol/liter·min (60–90 min) at 300 μM $[Ca]_o$. Within the drift of the recorder ($<5 \times 10^{-4}$ OD/h or <0.5 μMOL Ca^{++}/liter·h), there was no change in $[Ca^{++}]_i$ for solutions with no added $[Ca]_o$. Repeat measurements of Ca influx showed only minor differences.

In erythrocyte ghosts, changes of intracellular ionized Ca $[Ca^{++}]_i$ from <0.1 μM to ≅50 μM can be measured with incorporated arsenazo III in the presence of 1 mM Mg under the conditions described in Fig. 1. These ghosts may be made with <0.1 μM $[Ca^{++}]_i$ by using a trace (20 μM) of EGTA and removing background Ca contamination from reagents with Chelex 100. Further increases in $[Ca^{++}]_i$ may then be quantified by measuring the change in absorbance at 655 nm minus the absorbance at 700 nm in a single cuvette using a dual wavelength spectrophotometer (Fig. 1).

When ghosts containing <0.1 μM $[Ca^{++}]_i$ are placed in solutions containing varying amounts of Ca, Ca influx during the first 10–15 min is nonlinear and, as explained in Fig. 2, may be entering ghosts that are tight to dye but leaky to Ca. After this phase

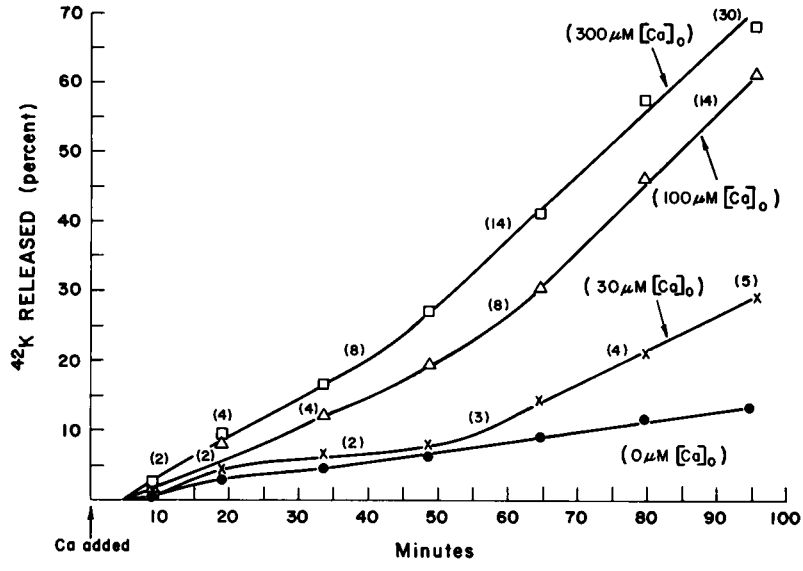

FIGURE 3 Percent ^{42}K released versus time in ghosts incubated at different external calcium concentrations ($[Ca]_o$) at 1.1% hematocrit in 155 mM NaCl, 2 mM K, 1 mM iodoacetic acid, 17 mM Hepes, and pH 7.18 at 37°C. Points are the mean of three separate samples and the results are representative of a number of similar experiments. Ghosts are the same ones used in Figs. 1 and 2. The micromolar concentration of $[Ca^{++}]_i$ at given times are in parentheses and were calculated from the rates of Ca influx from Fig. 2 for the appropriate $[Ca]_o$ and time period, except for the values during the first 15 min after Ca is added. These values were calculated from the first period for each $[Ca]_o$ when the rate of Ca influx was linear (Fig. 2). Note that for $[Ca]_o$ = 30 μM, it makes little difference if one uses the rate of Ca influx to calculate $[Ca^{++}]_i$ or determines $[Ca^{++}]_i$ directly from the curve for $[Ca^{++}]_i$ in Fig. 2, because when $[Ca]_o$ is 30 μM there is a negligible rapid phase in the first few minutes. At the beginning of the fluxes the $[Ca^{++}]_i$ was below the limit of detection by the dye and was therefore neglected.

is an interval of linear Ca influx which is a function of $[Ca]_o$ and whose duration also depends on $[Ca]_o$, as described in the legend of Fig. 2. At 100 and 300 μM $[Ca]_o$ these linear rates later increase.

The rate of net ^{42}K loss is stimulated by 1–3 μM $[Ca^{++}]_i$ at all $[Ca]_o$ tested (Fig. 3), but at least 8 μM $[Ca^{++}]_i$ is necessary to produce a maximum rate of K efflux in this preparation of ghosts. It is evident that the time required for K efflux to reach a maximum rate was inversely related to $[Ca]_o$. Also, it was shortly after these maximum rates were achieved that the rates of Ca influx at the two higher $[Ca]_o$'s increased over their initial linear rates (see Fig. 2). Such increases could be caused by an augmented inward driving force for Ca, if the rate of K efflux is sufficient to hyperpolarize the membrane.

The correlation between K efflux and $[Ca^{++}]_i$ presented here is in reasonable agreement with the estimate of Blum and Hoffman (2), who found that 9 μM Ca trapped in resealed ghosts was almost sufficient to stimulate K efflux maximally. These results also qualitatively agree with the high affinity pattern observed by Lew and Ferreira (17)

in erythrocytes, although their published data are not sufficiently detailed at $[Ca^{++}]_i$ in the micromolar range to allow a quantitative comparison. On the other hand, we observed nothing similar to their low affinity pattern. Others using ghosts with Ca chelators, such as EGTA, trapped inside to buffer $[Ca^{++}]_i$ have found that 0.2 μM $[Ca^{++}]_i$ is sufficient to stimulate either the rate of K:K exchange (18) or net K efflux (4) over control rates, and that the maximum rates of these processes were achieved at 2–4 μM $[Ca^{++}]_i$. Such a discrepancy between the apparent Ca sensitivity of the K efflux mechanism may in part be due to the difference between having a net Ca influx during the course of the K efflux as opposed to having a fixed buffered concentration of $[Ca^{++}]_i$. By using Ca buffers, however, these authors were also forced to calculate $[Ca^{++}]_i$ from dissociation constants originally determined under different experimental conditions (4) or to use ionic strengths below physiological levels (18).

By using incorporated arsenazo III, we have also found (data not shown) that the stimulation of K efflux caused by increasing external [K] from 0.6 mM to 2 mM probably does not cause an increase in the rate of Ca influx, as has been suggested by Plishker and Gitelman (19) and considered by others (20). Moreover, the results presented in Fig. 3 suggest that the delay in the onset of the maximum rate of K efflux observed when depleted erythrocytes are placed in saline plus Ca (21) is probably a measure of the time it takes $[Ca^{++}]_i$ to reach levels necessary to trigger K efflux.

Ca that has entered the ghosts after incubation in a medium containing Ca and phosphate will be extruded from the ghosts when they are provided with adenosine

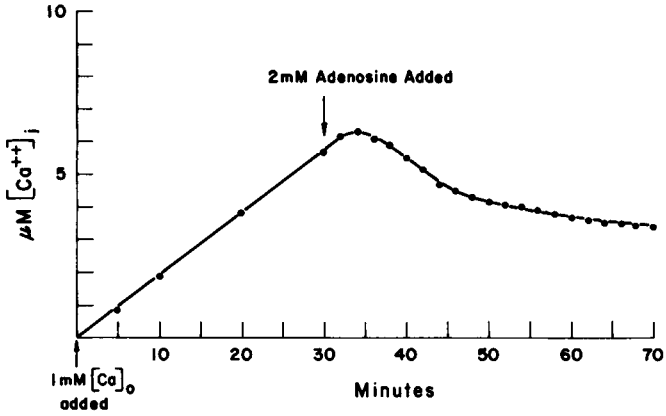

FIGURE 4 Increase in $[Ca^{++}]_i$ with time in ghosts incubated in 155 mM NaCl, 17 mM Hepes, 1 mM Ca, and 2 mM phosphate, pH 7.18 (37°C), and subsequent decrease in $[Ca^{++}]_i$ upon the addition of 2 mM adenosine. Ghosts were made from fresh cells which had not been energy depleted, but otherwise were prepared as previously described. The influx part of the experiment was conducted as described in Fig. 2 and the $[Ca^{++}]_i$ at any given time was calculated from the influx rate between 15 and 30 min, 0.19 μmol/liter·min. The rate of the stimulated net Ca^{++} efflux from 34 to 44 min was 0.16 μmol/liter·min which considering the rate of net influx gives a total net efflux rate of 0.35 μmol/liter·min. Each point is a single measurement, but these results are representative of four similar experiments.

(Fig. 4) from which erythrocytes can synthesize ATP (22). The maximum rate of the stimulated net Ca^{++} efflux under these conditions was 0.16 μM/liter · min, which, considering the rate of Ca influx, gives a total net Ca^{++} efflux rate of 0.35 μM/liter · min. The latter is much less than the rate measured for the Ca pump at comparable $[Ca^{++}]_i$ using Ca chelators (23), and details of this interrelationship are still to be established. Yet, insofar as this activity represents the Ca pump, these studies suggest a way of investigating the Ca pump at known $[Ca^{++}]_i$ in the in vivo physiological range under unbuffered conditions where $[Ca^{++}]_i$ can change.

We thank Dr. J. Kaplan and Dr. B. Forbush for suggesting the method of dye purification, Sigma Chemical Co. for recommending the thin-layer chromatography solvent system, Dr. R. Hamill and the Eli Lilly and Co., Indianapolis, Ind. for the A23187, and Mrs. A. Downey and Mr. P. Gilligan for technical assistance.

This research was supported by a National Institutes of Health Postdoctoral Fellowship 1-F32-AM-05173 to D. R. Yingst and U.S. Public Health Grant AM-17433 and HL-09906.

Received for publication 21 February 1978 and in revised form 11 May 1978.

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