Vanadate-induced Movements of Ca\textsuperscript{2+} and K\textsuperscript{+} in Human Red Blood Cells*

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Fresh human red blood cells become highly labeled with \textsuperscript{45}Ca\textsuperscript{2+} when exposed to 0.5 mM vanadate. The effect of vanadate requires its penetration into the cell, and is attributed to the inhibition of the outwardly directed Ca\textsuperscript{2+}-pumping ATPase which would otherwise "mask" the uptake of \textsuperscript{45}Ca\textsuperscript{2+}. Since the inhibition of the Ca\textsuperscript{2+} pump by vanadate is not complete, a transmembrane Ca\textsuperscript{2+}-Ca\textsuperscript{2+} exchange can be detected. The influx leg of the exchange is inhibited by verapamil, quinidine, and Ca\textsuperscript{2+}. This, as well as additional (kinetic) evidence, indicates that the influx of Ca\textsuperscript{2+} is a carrier-mediated process.

Experiments in which the transmembrane K\textsuperscript{+} gradient has been abolished or decreased with isophanes, or by increasing the K\textsuperscript{+} concentration in the medium, suggest that the K\textsuperscript{+} gradient may play a role in the influx of Ca\textsuperscript{2+}. The vanadate-induced accumulation of Ca\textsuperscript{2+} by red cells promotes a massive efflux of K\textsuperscript{+}, indicating the activation of a Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channel.

The results indicate the occurrence of a slow cycling of Ca\textsuperscript{2+} across the red cell membrane. The influx leg of the cycle occurs through a verapamil-sensitive channel, and is possibly driven by the discharge of the transmembrane K\textsuperscript{+} gradient. The efflux leg of the cycle consists of the Ca\textsuperscript{2+}-pumping ATPase.

The extrusion of Ca\textsuperscript{2+} ions from red blood cells is accomplished by a Ca-ATPase (1). The functional properties of this enzyme in red cell membrane preparations have recently been reviewed by several authors (2-4). This enzyme has recently been extracted from the membrane, purified to homogeneity (5), and reconstituted in liposomes, where it pumps Ca\textsuperscript{2+} with optimal efficiency (6, 7).

The system responsible for the influx of Ca\textsuperscript{2+} into red blood cells, on the other hand, has not been characterized. The matter is routinely condensed in a short reference to the extremely low permeability of the red blood cell membrane for Ca\textsuperscript{2+}. A problem here and in other cells as well, could be represented by the Ca\textsuperscript{2+}-ejecting ATPase, since its function would result in the underestimation, or even in the total "disappearance," of the Ca\textsuperscript{2+} that has penetrated into the red blood cells under usual experimental conditions. In an attempt to circumvent the problem, some authors have studied the influx of Ca\textsuperscript{2+} in Ehrlich ascites (8), or liver cells (9) at low temperature, i.e. under conditions which should inhibit the Ca-ATPase. This procedure indeed unmasked the influx of Ca\textsuperscript{2+}.

The study of the mechanism(s) for the inward transport of Ca\textsuperscript{2+} into human red blood cells is of a particular interest. These cells apparently lack functions mediated by the influx of Ca\textsuperscript{2+}, yet possess an efficient Ca pump, which is normally used far below its full capacity (10). Intact red blood cells containing a normal amount of ATP do not take up added \textsuperscript{45}Ca\textsuperscript{2+} in amounts exceeding 0.1 to 1.0 pmol/liter of packed cells. After ATP depletion, however, they accumulate Ca\textsuperscript{2+} up to a concentration 50 times higher than in the normal state (see Ref. 10, for a review). This suggests that the activity of the Ca-ATPase, rather than the "absolute" impermeability of the cell membrane is the cause of the negligible uptake of \textsuperscript{45}Ca\textsuperscript{2+} usually observed in the red blood cells.

In the present study, we have used an alternative approach to the study of the influx of \textsuperscript{45}Ca\textsuperscript{2+} in human red blood cells. In intact erythrocytes, we have induced Ca\textsuperscript{2+} influx by preincubation with vanadate, a compound that inhibits the Ca-ATPase in red cell membranes (11) and in the purified state (7), and the (Na,K)-ATPase in intact red blood cells after permeating through the membrane via the anion channel (12). The results have provided support for the concept that the influx of Ca\textsuperscript{2+} into red blood cells is a carrier-mediated process. The sensitivity of the Ca\textsuperscript{2+} influx to verapamil suggests that the transport system in red blood cells is similar, or identical, to the slow Ca\textsuperscript{2+} channel of excitable tissues. Moreover, the results suggest that the gradient of K\textsuperscript{+} ions may play a role in the influx of Ca\textsuperscript{2+}.

MATERIALS AND METHODS

Red Blood Cells—Fresh citrate-treated human blood was provided by a local blood bank, stored under sterile conditions at 0-4 °C, and used within 3 weeks.

Red blood cells were prepared immediately before the experiment. Blood was centrifuged (5 min, 2500 g). The plasma and the buffy layer were sucked off, and the red blood cells were suspended in a medium containing 50 mM Na-Hepes, pH 7.2, 0.9% NaCl, 0.5 mM KCl, 0.3 mM MgCl\textsubscript{2}, and 15 mM glucose. The suspension was centrifuged as above, and the washing procedure was repeated four times. The washed cells were resuspended in the same medium at a concentration of 30%.

\textsuperscript{45}Ca\textsuperscript{2+} Uptake Experiments—The suspension of cells (hematocrit 30%) was preincubated 15 min with 0.5 mM Na-ortho-vanadate from a 0.5 M stock solution in 0.5 M Na-Hepes, pH 7.2. \textsuperscript{45}CaCl\textsubscript{2} (specific activity about 2500 cpm/nmol) was then added to a final concentration of 2.5 mM, unless indicated otherwise. After the addition of Ca\textsuperscript{2+}, 0.5-mi aliquots of the suspension were withdrawn at 0, 50, 100, 150, and 300 s.

The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPP\textsuperscript{+}, tetrathylphosphophonium; di-S-C\textsubscript{3}-(5), dipropythiodiocarbocyanine iodide; DIDS, 4,4-diisothiocyano-2,2-di-sulfonic acid stilbene; FCCP, carbonyl cyanide p-trifluoromethoxyphenyl hydrazone.

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min (unless indicated otherwise), diluted to 1.5 ml with medium containing 0.5 mM vanadate, and centrifuged 0.5 min in an Eppendorf 3200 microcentrifuge. The supernatants were discarded and the pellets suspended in 1 ml of the medium and centrifuged as above. The washing procedure was repeated four times. Finally, the pellets were resuspended with 0.5 ml of 10% (w/v) trichloroacetic acid containing 20 mM LaCl₃. After removal of the precipitate by centrifugation, 0.5 ml of the supernatant was used for the measurement of radioactivity in a scintillation counter using Biofluor New England Nuclear as a scintillation fluid. The experiments were performed at 25 °C. The control samples were treated as above but without vanadate in the suspension, or in the washing medium. The order of addition of the compounds tested is indicated in the figures.

"Ca²⁺ Efflux Experiments"—The cells were loaded with "Ca²⁺" as described above (1-h incubation with "Ca²⁺"), and then washed four times at 0 °C with standard medium containing 0.5 mM vanadate. The washing procedure reduced the radioactivity of the medium practically to the background level. The suspension was then adjusted to the original volume, divided into aliquots, supplemented with the compounds to be tested, and incubated at 25 °C. 0.9 ml aliquots were withdrawn at the times indicated in the figures, and centrifuged in an Eppendorf 3200 microcentrifuge. After careful separation of the supernatants from the pellets, the latter were precipitated with trichloroacetic acid, and counted as above. No correction was made for the supernatant trapped in the extracellular space.

"K⁺ Efflux Experiments"—The incubation of the suspension with vanadate and Ca²⁺ was performed as described for the "Ca²⁺" uptake experiments except that nonradioactive Ca²⁺ was used. The aliquots withdrawn at the time indicated in the figures were centrifuged, and the supernatants separated from the pellets. The content of K⁺ in the supernatants was measured by atomic absorption in samples diluted 100 times.

ATP Content of Red Blood Cells—The conditions were the same as for the "K⁺" efflux experiments except that the aliquots of the suspensions were added to the test tubes containing 40 µl of 70% HClO₄. ATP was measured in the supernatant after the removal of the precipitated proteins by a coupled enzyme assay method (Boehringer Mannheim AG).

Concentration of Free Ca²⁺—The concentration of added Ca²⁺ was 50 and 300 µM, in the medium used for the Ca²⁺ uptake experiments.

Concentration of TPP⁺—The concentration of TPP⁺ was measured by a TPP⁺-selective electrode (13), in the standard medium. The concentration of added TPP⁺ was 10 µM, the concentration of red blood cells 0.2%.

Fluorescence Changes of dis-C₃(5)—The fluorescence measurements were performed according to the method of Sims et al. (14). The concentration of cells was 0.5%, that of the dye, 0.33 µM. The total concentration of methanol was 0.17%.

Chemicals—Na-ortho-vanadate was obtained from ICN Pharmaceuticals, Plainview, NY. The solutions were prepared based on a M₉, of 400. "CaCl₂" was obtained from the Radiochemical Centre; nigericin and monensin, from Lilly; valinomycin from Calbiochem; DIDS, Na-salt, and quinidine sulfate from Sigma; Arsenazo III from Fluka, Buchs, Switzerland; verapamil from Knoll, Ludwigshafen, West Germany, trifluoperazine from Smith, Kline and French Laboratories, Philadelphia, PA. R 24571 was a kind gift of Dr. Van Belle, Janssen Pharmaceutica, Beerse, Belgium; diS-C₃(5) was a kind gift of Dr. A. Waggoner, Amherst College, Amherst, MA.

RESULTS

Ca²⁺ Uptake by the Red Blood Cells

Red blood cells incubated with "Ca²⁺" became labeled only to an extent corresponding to their low Ca²⁺ content, which is in the range of 0.1 to 1.0 µmol/liter of packed cells. This is in agreement with previous observations made in several laboratories (see Ref. 9 for a review). The preincubation of the cell suspension with vanadate, however, dramatically changed this pattern. The cells took up "Ca²⁺" in a manner dependent on the concentration of added vanadate. In a series of several experiments, the threshold was at about 0.1 mM, and saturation was approached at about 6 mM (Fig. 1A). It was necessary to buffer heavily the vanadate solution to prevent alkaliniza-

![Fig. 1. Dependence of the vanadate-induced "Ca²⁺" influx on the concentration of vanadate (A) and "Ca²⁺" (B).](http://www.jbc.org/)

Mechanism of the Vanadate-induced "Ca²⁺" Uptake

As shown by Cantley et al. (12), vanadate inhibits the (Na,K)-ATPase of red blood cells from the inner side of the membrane. Since, in our experiments, we have used similar conditions, it could be expected that the Ca-ATPase would also be inhibited, thus unmasking the uptake of "Ca²⁺". The
vanadate is inside the cell, the anion channel is no longer tested by measuring Ca$^{2+}$ efflux from red blood cells loaded outwardly directed Ca$^{2+}$ pump. The hypothesis has been that vanadate induces "uptake" of Ca$^{2+}$ by inhibiting the uptake. Experiments with radioactive vanadate have showed with $^{45}$Ca$^{2+}$ by vanadate. $^{45}$Ca$^{2+}$ efflux by $^{40}$Ca$^{2+}$ approached saturation at about the anion channel to stimulate the uptake of Ca$^{2+}$. Once this indicates that vanadate must cross the membrane via the anion channel DIDS (Fig. 3). Preincubation of red cells with DIDS prior to the addition of vanadate inhibited substantially Ca$^{2+}$ uptake. Experiments with radioactive vanadate have showed that red blood cells indeed take up vanadate via the anion channel DIDS (Fig. 3). Preincubation of red cells with DIDS for 5 min. Then, $0.5$ mm vanadate was added. After 10 min of further incubation, the uptake was started by the addition of $^{45}$Ca$^{2+}$ (2.5 mm). A second portion of the suspension (C) was pretreated for 10 min with vanadate and then for 5 min with DIDS, before the uptake was started with $^{44}$Ca$^{2+}$. Another portion of the suspension (C) was preincubated as the first portion, but dimethyl sulfoxide was used instead of DIDS (final concentration 0.5%). The control suspensions were treated in the same way but without vanadate (closed symbols). B, red blood cells were loaded with $^{40}$Ca$^{2+}$ as described under "Materials and Methods" and preincubated with $0.58$ mm DIDS for 5 min at 0°C. Then $2.5$ mm $^{45}$Ca$^{2+}$ was added (time 0) (>). The control (C) was treated in the same way but with 0.5% (v/v) of dimethyl sulfoxide instead of DIDS. ■, labeled suspensions to which no $^{40}$Ca$^{2+}$ was added.
increased intracellular Ca\(^{2+}\) level, because the Ca\(^{2+}\)-Ca\(^{2+}\) exchange in Ca\(^{2+}\)-loaded cells was also saturable with in the same concentration range. The concept of a Ca\(^{2+}\) carrier is supported also by the finding that the influx of Ca\(^{2+}\) was inhibited by several compounds, and appears to be specific for Ca\(^{2+}\).

In experiments not presented here, we have found that the addition of up to 8 mM Sr\(^{2+}\) or Ba\(^{2+}\) had only a negligible inhibitory effect on 45Ca\(^{2+}\) uptake. Co\(^{2+}\) ions, however, inhibited strongly (IC\(_50\) at about 150 \mu M) (Fig. 6A). Since even at the highest concentration used (1.5 mM) Co\(^{2+}\) did not stimulate the influx of 45Ca\(^{2+}\) from loaded red cells (Fig. 6B), the inhibition of the 45Ca\(^{2+}\) uptake was not due to the activation of the Ca-ATPase (e.g., by displacement of vanadate). The "Ca\(^{2+}\) antagonist" verapamil (20, 21) inhibited the vanadate-induced 45Ca\(^{2+}\) uptake (Fig. 7) in the range 10 to 100 \mu M (IC\(_50\) at about 70 \mu M). Even at the highest concentration tested (200 \mu M), verapamil did not stimulate the activity of the Ca\(^{2+}\) pump (Fig. 7B). Concentrations of verapamil in excess of 0.2 mM (e.g., 0.5 mM) induced less inhibition and very high conc.

### Table I

**Rates of influx and efflux of Ca\(^{2+}\) in vanadate-treated cells**

The initial rates were calculated from the difference of the uptake (release) of 45Ca\(^{2+}\) between the zero time and the first experimental point. The time interval between these points was 20 to 25 min for the uptake and 5 min for the release. Where indicated, the pooled values are expressed as mean ± S.E. Other experimental details as under "Materials and Methods."

| Initial rate of influx \(\mu\text{molCa}/\text{litercells/h}\) | Initial rate of efflux \(\mu\text{molCa}/\text{litercells/h}\) |
|----------------|----------------|
| 22.12 ± 12.73 (n = 16) | 13.4 ± 5.5 (n = 8) |
| (range 7.4-60.0) | (range 9.5-25.3) |

### Factors Influencing the Uptake of Ca\(^{2+}\)

**Effect of Divalent Cations and of Inhibitors of Ca\(^{2+}\)**

Influx—The inward movement of 45Ca\(^{2+}\) in vanadate-treated red blood cells could occur on a specialized carrier, or by defects in the membrane structure. Several observations support the existence of a carrier-mediated uptake. One is the saturability of the uptake (Fig. 1B), which agrees with what observed in ATP-depleted cells (19). This fact cannot be attributed to the activation of the Ca\(^{2+}\)-ATPase by calmodulin due to the

### Fig. 5.

**Effect of the anticalmodulin agents R 24571 and trifluoperazine on the vanadate-induced uptake of 45Ca\(^{2+}\).** Red cells were preincubated with vanadate as described under "Materials and Methods." 0.5-ml aliquots were transferred to Eppendorf tubes containing 1.6 \mu g of nigericin (\(\alpha\)) or 0.8 \mu l of methanol (C). After 2 min, R 24571 was added to the concentration indicated in the figure and the same volume of DMSO (0.5%) was added to the control tubes. After 60 min, the reaction was stopped. Control tubes (A\(\alpha\)) were treated identically except that vanadate was omitted.

### Fig. 6.

**Effect of Co\(^{2+}\) ions on the vanadate-induced 45Ca\(^{2+}\) influx and on the 45Ca\(^{2+}\) efflux.** A, red cells were preincubated with vanadate, as described under "Materials and Methods," and 0.5-ml aliquots were transferred to Eppendorf centrifuge tubes containing amounts of "Ca\(^{2+}\)" and Co\(^{2+}\) adequate to yield a final concentration of 2.5 mM Ca\(^{2+}\) and the concentrations of Co\(^{2+}\) indicated in the figure. The suspensions were incubated for 60 min (C). Controls (C) were treated identically but without vanadate. B, loading and washing of cells were performed as described under "Materials and Methods." Aliquots of the washed suspension were supplemented with (C\(\Delta\)) or without (C\(\alpha\)) 2.5 mM Ca\(^{2+}\) and with (\(\alpha\Delta\)) or without (\(\alpha\alpha\)) 1.5 mM Co\(^{2+}\).
The uptake decreased at prolonged presence of the latter, the decrease at longer incubation times with isotonic sucrose in the medium had, however, no inhibitory effects. Instead, the uptake of Ca\(^{2+}\) was strongly increased if Ca\(^{2+}\) enters the cell by means of simple diffusion or of an amphiphilic substance (see Ref. 15). The enhanced influx of Ca\(^{2+}\) could account for only a minor portion of the effect of FCCP. Therefore, the influx component of the Ca\(^{2+}\) exchange is also inhibited. In principle, this could be due to the dissipation of the H\(^{+}\) gradient, or to the generation of a membrane potential as a result of the increase of the H\(^{+}\) permeability of the membrane. This explanation is, however, difficult to reconcile with the generally accepted assumption that H\(^{+}\) are distributed passively across the red cell membrane. Control experiments on this point indeed agreed with this assumption. Under conditions similar to those of the Ca\(^{2+}\) uptake experiment was performed as described in Fig. 6A, except that the suspension was incubated for 2 min with verapamil (concentration as indicated) before the addition of 45Ca\(^{2+}\). Control samples were treated identically but without verapamil. The concentration of verapamil was 0.4% (w/v) in all samples. B, the experiment was performed as described in Fig. 6A, except that 200 µM verapamil was added 2 min before the addition of CaCl\(_2\). C, control treated with 0.4% methanol instead of verapamil.

Effect of Monovalent Ions—Since the uptake of Ca\(^{2+}\) must be electrically compensated by the movement of other ions, the lack of suitable co- or counter-ions should restrict the uptake of Ca\(^{2+}\) and thus lead to inhibition of the uptake. Because of the unusual permeability properties of the red blood cell membrane for monovalent ions one could predict a strong inhibition of the Ca\(^{2+}\) uptake by the removal of C\(^{-}\) for impermeable anions. Nonspecific damages of the membrane at these concentrations have already been described for a number of amphiphilic substances (see Ref. 15 for a review). It is of interest for the discussion below that quinidine, an inhibitor of the K\(^{+}\)-sensitive K\(^{+}\) channel (22), was found to inhibit the uptake of Ca\(^{2+}\) at the concentration usually employed for the inhibition of the channel (not shown).

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ments, FCCP did not change the pH of the suspension even when the buffer was omitted from the medium (not shown). In other experiments, no changes in the transmembrane movement of the lipid-soluble cations TPP, and dis-c3(5) were seen after the addition of FCCP (not shown). Furthermore, the inhibition by FCCP was still observed in media containing 100 mM Na-gluconate instead of NaCl and 0.5 mM vanadate; O, no additions; A, 2.5 mM Ca2+; C, 0.5 mM vanadate; O, 2.5 mM Ca2+ and 0.5 mM vanadate; C, 4.4 mM nigericin; V, 2.5 mM Ca2+, 0.5 mM vanadate, and 0.4 mM DIDS dissolved in 0.5% dimethyl sulfoxide; V, 2.5 mM Ca2+, 0.5 mM vanadate, and 0.5% dimethyl sulfoxide. V and W were taken from an independent experiment.

The Transmembrane K+ Gradient in the Uptake of 45Ca2+

The possibility that the transmembrane K+ gradient is involved in the process of Ca2+ uptake has been investigated. Preincubation of vanadate-treated cell with nigericin or monensin (4.4 and 5.3 μM, respectively) was exchanged for Na+ and K+ for H+ and thus collapse Na+ and K+ gradients (27) strongly inhibited the influx of Ca2+ (Fig. 10A). The ionophores had no effect on control suspensions not treated with vanadate nor did they change the pH of the suspension. Nigericin did not stimulate the influx of 45Ca2+ from Ca2+-loaded cells (Fig. 10B), but promoted a rapid release of K+ (Fig. 10C). The inhibition of the Ca2+ influx by nigericin (and monensin) is likely to be due to the collapse of the K+ gradient, since the collapse of the Na+ gradient should activate rather than inhibit, the Ca2+ influx, if the latter were linked to Na+ by known transport mechanisms (Na/Ca exchange). The role of H+ is made unlikely by the data mentioned above. The role of the K+ gradient is supported by the decreased uptake of Ca2+ in media of increasing K+ concentration under conditions where Na+ was decreased correspondingly, and the concentration of CI- (and thus the osmolarity) was kept constant (Fig. 11A). The time course of Ca2+ uptake in suspensions exposed to various K+ concentrations varied. At low K+, the amount of Ca2+ taken up declined after prolonged incubation, possibly due to the opening of K+ channels by the increased concentration of cell Ca2+

K+ Movements Induced by the Influx of Ca2+

That Ca2+ can trigger the loss of K+ from erythrocytes has been shown by Gardos in 1958 (28). The incubation of red blood cells with vanadate alone did not induce a significant loss of K+ (Fig. 10B), although the (Na, K)-ATPase is markedly inhibited under these conditions (12). This is in agreement with the extremely low monovalent cation permeability of the red cell membrane (29). Incubation with Ca2+ alone had no effects on the K+ movements, but the presence of both Ca2+ and vanadate greatly enhanced the influx of K+ (30). Maximal release was reached in 15 to 20 min, and the plateau after about 60 min. Since the content of Ca2+ in cells, albeit increasing under these conditions, usually remained in the micromolar range, the affinity of the K+ channel for Ca2+ evidently is in this range. This is in agreement with the findings on sealed ghosts (30), but is at variance with data obtained with ionophore-loaded red blood cells, where substantially higher values were found (31).

The influx of K+ was efficiently blocked by DIDS (Fig. 10B). This suggests net efflux of K+, with chlorides leaving the cell via the anion channel. Since DIDS did not inhibit the influx of 45Ca2+ (Fig. 3), the Ca2+-activated K+ channel is not directly involved in the inward translocation of Ca2+. Thus, the decreased Ca2+ uptake in high K+ media was not due to the inhibition of the Ca2+-activated K+ channel (32, 33).

DISCUSSION

The experiments presented here indicate quite compellingly that the loading of the red cells with 45Ca2+ in the presence of...
vanadate is due to the inhibition of the outwardly directed Ca\(^{2+}\) pump. The inhibition results from the interference of vanadate with the Ca\(^{2+}\) pump, and not, as could in principle also be suggested, from the exhaustion of the intracellular ATP store. Indeed, the level of intracellular ATP did not deviate significantly from the starting concentration of about 0.6 mM throughout the entire duration of the Ca\(^{2+}\) uptake experiments.

The uptake of Ca\(^{2+}\) after inhibition of the Ca\(^{2+}\) pumping of the plasma membrane seems to be a widely distributed phenomenon. It can be revealed by the exposure of Ehrlich ascites tumor cells (8) or liver slices (9) to low temperature, of liver slices to anaerobiosis (9), or by ATP depletion of human red blood cells (28). In dog red blood cells, uptake of Ca\(^{2+}\) can be induced by the removal of external Na\(^+\) ions (34), which indicates the existence of a Na\(^+\)/Ca\(^{2+}\) exchange system. These observations suggest that, in resting cells, a slow cycling of Ca\(^{2+}\) across the plasma membrane occurs. Since verapamil inhibits the uptake of Ca\(^{2+}\) in red blood cells, it seems probable that Ca\(^{2+}\) uptake in erythrocytes and during activation of excitable cells is mediated by the same structural components of the plasma membrane.

In the present work, the rate of Ca\(^{2+}\) cycling could be derived from the initial rates of Ca\(^{2+}\) influx and efflux, and from the degree of the inhibition of the Ca\(^{2+}\)-ATPase by vanadate. Assuming that saturating concentrations of vanadate inhibit the ATPase 100\%, the dependence of Ca\(^{2+}\) uptake on the vanadate concentration suggests that at 0.5 mM vanadate the inhibition approaches 40\%. The average data for influx and efflux of Table I suggest a turnover of Ca\(^{2+}\) at 25 °C of between 40 and 200 µmol/liter of packed cells/h. This exceeds the value estimated by Lew and Ferreira (10) from the difference in ATP consumption in iodoacetamide-poisoned red blood cells in the presence and in the absence of Ca\(^{2+}\). The difference in temperature between our conditions and those of Lew and Ferreira (10) cannot completely explain the difference in the estimated turnover of Ca\(^{2+}\), which differ by a factor of between 3 and 15, assuming a Ca/ATP stoichiometry of 1. Our data suggest a higher turnover than that of the capacity of the Ca\(^{2+}\) pump and estimate it at about 1%. This low utilization value could explain the requirement for very high concentrations of vanadate to unmask the uptake of Ca\(^{2+}\), since in intact cells the Ca\(^{2+}\) pump must be inhibited more than by 99\%.

The results of Lew and Ferreira (10) on ATP-depleted red cells, and the present results on vanadate-treated cells, have shown that the influx of Ca\(^{2+}\) from previously loaded cells is stimulated by the addition of Ca\(^{2+}\). It is clear from the data presented that, in vanadate-treated cells, the entry and exit legs of this Ca\(^{2+}\) exchange are mediated via independent routes, the former involving the verapamil-sensitive component, the latter the Ca\(^{2+}\)-pumping ATPase. Possibly, Ca\(^{2+}\) is lost by the cells during the washing procedure, decreasing its concentration below the level at which the pump is saturated. The cold calcium added will penetrate into the cells, restore the original Ca\(^{2+}\) level, and activate the pump. The observation that several inhibitors of the influx of Ca\(^{2+}\) have no effect on the influx of Ca\(^{2+}\) from the loaded cells may be rationalized by recalling that the influx component of the Ca\(^{2+}\)-Ca\(^{2+}\) exchange process is in excess of the requirements for optimal rates of the Ca\(^{2+}\)-Ca\(^{2+}\) exchange (see Table I). Thus, its inhibition, even if substantial, may well have no effect on the exchange.

Concerning the route of Ca\(^{2+}\) influx, one possibility is a leak through which Ca\(^{2+}\) is driven by its large transmembrane gradient. However, the previously mentioned observation on the saturability of Ca\(^{2+}\) and on the inhibition of the uptake by verapamil and Ca\(^{2+}\), as well as the experiments of Lew and Ferreira (10, 19), favor the possibility of a carrier-mediated transport. Although not all criteria for it were verified, the experimental data obtained would be difficult to interpret in terms of membrane leaks.

The present study has shown that the transmembrane gradient of K\(^{+}\) is important in the maintenance of the uptake of Ca\(^{2+}\). This is somewhat surprising, since the Ca\(^{2+}\) concentration at the two sides of the membrane differ by a factor of 1000, and that of K\(^{+}\) by a factor of only 20. The concentration (activity) of Ca\(^{2+}\) at the membrane surface could, however, be lower than in the bulk aqueous phase, emphasizing the possible role of the cell surface in the translocation of Ca\(^{2+}\) as suggested by Langer (35) for the activation of heart muscle cells.

At the present state of knowledge, it cannot be decided whether the electrical or the concentration components of the K\(^{+}\) gradient play a role in the inward movement of Ca\(^{2+}\). The membrane of red blood cells in the "resting" state does not discriminate between Na\(^{+}\) and K\(^{+}\) (28) but becomes selectively permeable to K\(^{+}\) after the activation of the K\(^{+}\) channel. This could lead to membrane hyperpolarization, and it could drive electropheretically Ca\(^{2+}\) into the cells. This interpretation would explain the experiments with nigericin-treated red blood cells, and those on the uptake of Ca\(^{2+}\) in media with varying K\(^{+}\) concentrations, and is supported by the observation made in our laboratory that valinomycin stimulates slightly the uptake of Ca\(^{2+}\). However, following this interpretation, the depolarization of the membrane by substitution of chlorides in the medium with impermeable anions, or sucrose, should also lead to the inhibition of the Ca\(^{2+}\) uptake, a prediction which was not supported by the experimental results (see Fig. 8). The postulate of an electrogenic Ca\(^{2+}\)/K\(^{+}\) antiporter, analogous to the electrogenic Ca\(^{2+}\)/H\(^{+}\) antiporter suggested by Hinnen et al. (8) could, however, accommodate all experimental results obtained.

Irrespective of the mechanism of the Ca\(^{2+}\)/K\(^{+}\) interaction in the process of the Ca\(^{2+}\) uptake, there is a link between the difference of the K\(^{+}\) levels at the two sides of the membrane and the extent and the velocity of the Ca\(^{2+}\) uptake (Fig. 11). The activation of the Ca\(^{2+}\)-sensitive K\(^{+}\) channel will lead to the dissipation, or to the decrease, of the K\(^{+}\) gradient, decreasing the uptake of Ca\(^{2+}\) and contributing to the establishment of its low steady state intracellular level. The decreased levels of cell Ca\(^{2+}\) during the later stages of the uptake in low K\(^{+}\) media, as well as the observation that this decrease is not seen in media of higher K\(^{+}\) content, support the involvement of the Ca\(^{2+}\)-sensitive K\(^{+}\) channel in the maintenance of the steady state level of intracellular Ca\(^{2+}\), and, more in general, the concept that the transmembrane K\(^{+}\) gradient is an important ingredient in determining the balance of Ca\(^{2+}\) between cells and medium. The experiment with DIDS-treated red blood cells, in which the Ca\(^{2+}\)-sensitive K\(^{+}\) channel is blocked (Fig. 10C), offer additional support for this suggestion.

The role of the K\(^{+}\)-dependent Ca\(^{2+}\) influx system, and of the Ca\(^{2+}\)-activated K\(^{+}\) channel as regulators of the Ca\(^{2+}\) homeostasis is probably not dramatically important in red blood cells where the cycling of Ca\(^{2+}\) occurs at a slow rate. However, cells with high Ca\(^{2+}\)-cycling rates could effectively modulate large fluctuations in intracellular Ca\(^{2+}\) without the significant losses of K\(^{+}\) observed in red blood cells. The main role of the Ca\(^{2+}\)-sensitive K\(^{+}\) channel might just be to defend the intracellular milieu from uncontrolled Ca\(^{2+}\) increases.

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