J. Membrane Biol. 8, 237–258 (1972)

ATP-Independent Calcium Net Movements in Human Red Cell Ghosts

H. Porzig

Pharmakologisches Institut, Universität Bern, Switzerland

Received 11 January 1972

Summary. Net Ca movements in metabolically depleted red cell ghosts were measured under the influence of different inwardly and outwardly directed Ca concentration gradients. Four variants of potassium-rich ghosts were prepared by reversal of osmotic hemolysis: (1) OCa-ghosts (hemolyzing medium (HM) contained no Ca); (2) OCa-EGTA-ghosts (HM contained 2 to 4 mM EGTA[1] and no Ca); (3) Ca-ghosts (HM contained 0.1 to 4.0 mM Ca); and (4) Ca-EGTA-ghosts [HM contained Ca and EGTA in various proportions ([Ca] < [EGTA])]. Ca uptake in OCa- and OCa-EGTA-ghosts at any particular extracellular concentration ($[Ca]_0$) within 60 min reached a steady state when cellular Ca and $[Ca]_0$ were still far from equilibration. About half of the total Ca taken up by OCa-EGTA-ghosts penetrated into the cell interior whereas the other half seemed to be bound to membrane sites. The amount of cellular Ca uptake (i.e., uptake into the cell interior as well as binding to the membrane) decreased with increasing internal concentration of ionized Ca. With 10^{-6} M internal free Ca, the uptake was nearly completely inhibited. In OCa-EGTA-ghosts the amount of Ca taken up into the intracellular space was markedly increased by the addition of mersalyl (0.05 mM) to the external medium. Ca outflow under outward gradients was larger than inflow under inward concentration gradients of similar magnitude and no steady state was reached within 180 min. The incorporation of more than 5 mM Ca into red cell ghosts led to a general increase in membrane permeability and finally to hemolysis of the ghosts. Lanthanum was found to inhibit Ca uptake in OCa- and OCa-EGTA-ghosts. The experimental results suggest that membrane-bound Ca regulates the Ca permeability of the red cell membrane. In the absence of ATP the uptake of Ca into red cell ghosts in contrast to outflow is a self-limiting process. This mechanism, in addition to active outward Ca pumping, may help to keep the free internal Ca in red cells at a low level.

Recent work on calcium transfer in human erythrocytes has been focused predominantly on the active, ATP-dependent calcium extrusion which enables the red cell to maintain a very low intracellular calcium concentration against a considerable electrochemical gradient (Schatzmann & Vincenzi, 1969; Olson & Cazort, 1969; Lee & Shin, 1969; Cha, Shin & Lee, 1971). This ability of the red cell to keep its internal calcium at a low level

1 EGTA = ethyleneglycol-bis-(β-aminoethylether)-N,N′-tetraacetic acid.

has important functional implications since intracellular calcium inhibits Na, K-ATPase (Dunham & Glynn, 1961; Epstein & Whittam, 1966) and increases the passive permeability of the membrane to sodium and potassium (Lew, 1970; Romero & Whittam, 1971).

The calcium efflux from red cell ghosts does not follow diffusion kinetics in the absence of ATP. When Ca^{45} was used as a tracer, results suggestive of a carrier-mediated Ca-Ca exchange diffusion were obtained (Porzig, 1970).

Ca influx in human red cells has not been studied thoroughly. However, it appears that inactivation of the "calcium pump" in intact cells is not sufficient to allow an appreciable net inward movement of extracellular Ca. When metabolically depleted cells are stored in the cold for prolonged periods of time, equilibrium between the calcium in the cells and in the medium is not reached even when extracellular Ca is increased to 10 mM/liter (Schatzmann & Vincenzi, 1969; Romero & Whittam, 1971).

The present study was designed to assess whether: (1) the ATP-dependent transport system is the only factor responsible for the inequality in Ca inflow and outflow in red cells; and (2) Ca binding to ghost membranes interferes with net Ca transfer.

These studies suggest that the membrane-bound Ca controls the net transfer of Ca in both inward and outward directions and may confer some rectifier properties to the membrane so that outflow appears to be favored over inflow. The size of the membrane compartment as an additional compartment for Ca uptake varies considerably under different experimental conditions.

Materials and Methods

Human erythrocytes were supplied by the central laboratory of the Swiss Red Cross Blood Transfusion Service in Bern and were stored at 4 °C for 2 to 6 days prior to use. Usually the cells were incubated for 13 to 17 hr at 37 °C in sterile isotonic NaCl solution without substrate to deplete the energy stores. The cells were then washed three times in 166 mM NaCl solution and ghosts rich in KCl were prepared following the method of Hoffman, Tosteson and Whittam (1960) as modified by Passow (1969). A detailed description is given by Porzig (1970). Washed ghosts were resuspended in appropriate media at 37 °C either directly or after the pretreatments indicated below. The uptake or loss of Ca and Mg under various conditions was then usually followed during a 3-hr incubation period. The standard incubation medium contained 140 mM KCl and 20 mM Tris buffer. The pH of all incubation media was adjusted to 7.4 at room temperature (corresponding to pH 7.2 at 37 °C and pH 7.7 at 1 °C). Potassium was used as basic cation for all incubation media to have the same cation as major osmotic constituent on both sides of the membrane. Control experiments showed that volume changes of ghosts caused by asymmetry in cation fluxes during incubation could thus be avoided. In these experiments, the volume change of ghosts in different media was monitored continuously for 3 hr with a light-scattering method devised by Fuhrmann, Liggenstorfer and Wilbrandt (1971).

In some of the uptake experiments the ghosts were not washed prior to incubation but Ca was added directly to the suspension at the end of the equilibration period (60 min at 37 °C) which always followed the reversal of hemolysis by addition of KCl. There was no significant difference in Ca uptake when Ca was added to washed ghosts suspended in the standard incubation medium.

Erythrocyte ghosts into which a known amount of Ca or EGTA or both had been incorporated during osmotic hemolysis are referred to as "Ca-ghosts", "OCa-EGTA-ghosts" and "Ca-EGTA-ghosts", respectively. "OCa-ghosts" are those without the addition of any of these substances. The cellular Ca concentration ($[Ca]_i$) was usually smaller than the Ca concentration in the hemolyzing fluid ($[Ca]_{hem}$). The ratio between $[Ca]_i$ and $[Ca]_{hem}$ dropped from about 1 to about 0.6 when $[Ca]_{hem}$ was raised from 0.1 to 4.0 mM.

On the basis of measurements reported by Weed, LaCelle and Merrill (1969) the ATP content of ghosts prepared from starved red cells as outlined above was estimated to be less than 10 μM/liter ghosts. In the present experiments no measurable amount of ATP (evaluated according to Lamprecht & Trautschold, 1962) was found in the ghosts. However, this result is not very conclusive because the actual detection limit for ATP in this assay was calculated to be higher than 10 μM/liter ghosts.

The hematocrit of the ghost suspensions was carefully controlled by a micro-hematocrit method using commercially available Van Allen hematocrit tubes because it seemed useful to express all cellular concentrations as mM/liter packed ghosts. The Van Allen tubes were filled with a constant volume (0.1 or 0.2 ml) of the ghost suspension by means of an automatic, fixed-volume-micropipette (Eppendorf) and were centrifuged for 40 min at $6{,}000 \times g$. In some experiments standard microhematocrit tubes and an ECCO hematocrit centrifuge were used (15 min at $19{,}000 \times g$) for a comparison of both methods. Only the standard microhematocrit method was applicable to measure the hematocrit in cell suspensions from La-containing media. The adhesiveness of the cells was increased to such an extent by La that they formed an occlusive plug at the lower part of the bulb of the Van Allen tubes. In most experiments the actual hematocrit values in the incubation suspension varied between 4.5 and 9%.

Samples for analysis of cellular Ca content were prepared by a modified method of Passow (1969): 5-ml samples of the experimental ghost suspension were taken at suitable intervals and washed twice at 2 °C in a large volume of buffered isotonic KCl or choline solution containing 10 mM EDTA (ethylenediamine tetraacetic acid). In the later experiments KCl was preferred over choline because the commercial choline was contaminated with up to 0.15 mM Ca/mole choline. The final sediment was hemolyzed overnight in doubly distilled water and filled up to the desired volume with doubly distilled water.

Ca and Mg were estimated in these samples by atomic absorption photometry with an Instrumentation Laboratory 353 absorption/emission flame photometer. To avoid phosphate interference in Ca measurements, La was added to samples and standards to make up a 1% solution. Deproteinization of the samples previous to Ca measurements proved to be unnecessary since no significant difference in Ca concentration measured before and after deproteinization could be detected. The calcium standards were prepared from either $CaCl_2$ or from $CaCO_3$. The $CaCl_2$ content in the main stock solution prepared from $CaCl_2 \cdot 2H_2O$ was controlled by chloride titration with $AgNO_3$ using potassium bichromate as indicator. The calibration curves prepared from $CaCO_3$ stock solution were identical with those made up from $CaCl_2$ stock solution.

Control measurements included calcium estimation in the supernatant at the beginning and at the end of each experiment, in the hemolyzing medium and in the different solutions used for any particular experiment.

The Ca^{++} concentration in the Ca-EGTA-buffers was calculated using a pK′ (log of apparent formation constant) of 7.02[2] (for pH 7.2 at 37 °C and an ionic strength of 0.16 M). The dissociation curve was plotted with the Ca/EGTA ratio on a linear scale (ordinate) and the Ca^{++} concentration on a log scale (abscissa). The Ca^{++} concentration could thus be read directly for all Ca/EGTA ratios (*see* Portzehl, Caldwell & Rüegg, 1964). No correction has been applied for the small amount of Mg (2.5 mM) present in the cells.

Whenever commercially available, chemicals to prepare solutions were of analytical grade. Mersalyl (Salyrgan®) was a gift of the Farbwerke Hoechst, Frankfurt/M-Hoechst, Germany. All concentrations in the solutions are given as mM/liter.

Results

The Influence of External Ca on Calcium Inward Movement

Potassium-rich red cell ghosts were prepared either in the presence or in the absence of 1 mM Ca in the hemolyzing medium. This treatment resulted in ghosts with a cellular Ca content of 0.064 ± 0.008 and 0.747 ± 0.032 mM/liter packed cells, respectively.

Ca-ghosts differed characteristically from OCa-ghosts and OCa-EGTA-ghosts in that they retained more hemoglobin, had a smaller volume and a higher viscosity. These features have been described earlier (Dodge, Mitchell & Hanahan, 1963; Weed *et al.*, 1969; Duchon & Collier, 1971; Palek, Curby & Lionetti, 1971) and will not be discussed further in the present paper.

The cells were incubated for 180 min at 37 °C in isotonic, buffered KCl solutions containing 0.1 to 12 mM Ca to study the calcium uptake under a variety of inward concentration gradients. Fig. 1 shows an experiment of this kind. The cellular calcium did not increase linearly with time and the "steady state" reached within 180 min was far from equilibrium. This level is certainly not a true steady state and the expression in the context of the present paper should only be taken to indicate a condition where the rate of Ca uptake becomes very small. In the presence of 4.83 mM extracellular Ca, the Ca-free ghosts took up more than twice as much Ca as Ca-ghosts. This difference in Ca uptake was present over the entire range of inward gradients. The two straight lines of Fig. 2 are regression lines relating uptake and extracellular Ca concentration ($[Ca]_0$) in 14 similar experiments. A linear regression seemed an adequate way of presenting these results because the uptake did not show saturation within the given concentration range. Even though the scatter of the data and the small number of experiments with $[Ca]_0$ above 7 mM (3) do not allow a firm

2 Personal communication of Prof. Anderegg, ETH Zürich, to Dr. P. Bally, Dept. of Pharmacology, University of Bern.

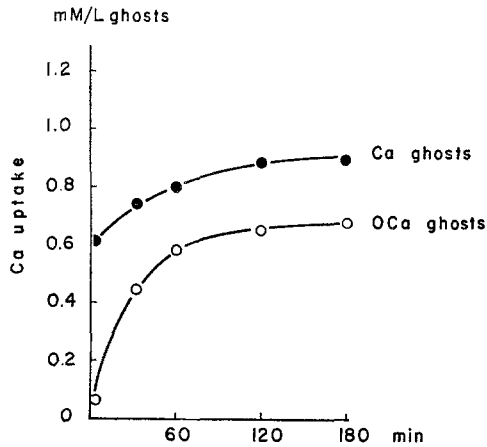

Fig. 1. Time course of Ca net uptake into erythrocyte ghosts. Starved red cells were hemolyzed in a solution containing 4 mM $MgCl_2$ and 8 mM Tris-Cl (OCa-ghosts, o——o) or 4 mM $MgCl_2$, 8 mM Tris-Cl and 1 mM $CaCl_2$ (Ca-ghosts, •——•). Reversal of hemolysis with KCl. After 60 min of equilibration at 37 °C the experiment was started by adding 5 mM $CaCl_2$ to each ghost suspension. The Ca concentration in the ghosts (ordinate, mM Ca/liter packed ghosts) is plotted versus time (abscissa, minutes). Hematocrit, 10.3% (OCa-ghosts) and 7.7% (Ca-ghosts). Single experiment

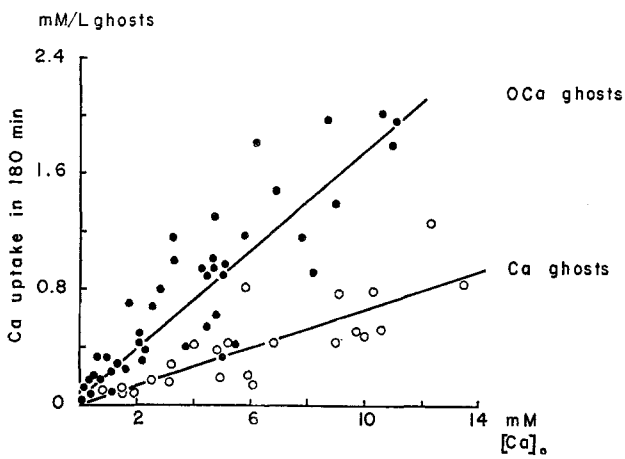

Fig. 2. Relation between external Ca concentration ($[Ca]_0$) and Ca uptake into ghosts. Method as in Fig. 1. Ca uptake in 180 min (ordinate, mM/liter packed ghosts) is plotted against the Ca concentration in the medium (abscissa, mM/liter); i.e., the mean value of the Ca concentration in the supernatent at the beginning and the end of each experiment. The filled circles represent single estimates of Ca uptake into OCa-ghosts from 13 experiments; the open circles give single estimates for uptake into Ca-ghosts from 6 experiments. The straight lines represent regression lines calculated from these data. The difference between the two regression coefficients is highly significant ($p < 0.0001$)

conclusion for high concentrations (7 to 12 mM $[Ca]_0$), the deviation from linearity is certainly small. The total amount of Ca taken up by Ca-free ghosts at 7 to 12 mM/liter $[Ca]_0$ was approximately equal to the sum of uptake and initial content of identically treated 0.75 mM Ca-ghosts.

The Dependence of Calcium Uptake on $[Ca]_i$

The following experiments were designed to determine why Ca uptake reached a steady state even when an inward gradient persisted and even though the calcium pump was inactivated by starving the cells prior to hemolysis. The difference in Ca entry between Ca-free and Ca-containing ghosts suggested that the inward movement of Ca from the external medium was influenced by the intracellular concentration. Therefore, the Ca uptake of ghosts containing 0.1 to 3.0 mM Ca/liter cells was measured. In Fig. 3, the Ca uptake during an incubation of 180 min in the standard medium containing 10 mM Ca is plotted against the total cellular Ca at the beginning of the experiment. The figure shows that Ca uptake decreased markedly as the initial cellular Ca was increased from 0.05 to 0.09 mM. This was followed by a plateau where an increase in cellular Ca from 0.1 to 1.0 mM/liter cells had almost no influence on Ca uptake. The Ca uptake decreased again when cellular Ca increased from 1 to 2 mM and was completely inhibited with Ca concentrations above 2 mM/liter cells. The values in Fig. 3 represent mean values of two similar experiments. Two other experiments in which a lower $[Ca]_0$ had been used showed the same relationship between $[Ca]_i$ and uptake even though the absolute values of Ca uptake were smaller.

This curve resembles a titration curve and suggests that the ionized intracellular ("free") Ca concentration ($[Ca^{++}]_i$) is the most important determinant of Ca entry and not the total cellular Ca. I therefore attempted to evaluate the relationship between $[Ca]_0$, overall cellular Ca content, Ca uptake and free intracellular Ca concentration.

Ca-free ghosts containing 1.2 to 2.4 mM/liter EGTA were prepared in order to maintain a low level of free intracellular Ca. Control experiments established that only $13.9 \pm 3.5\%$ of the EGTA left the cells during the experiment (*compare* Table 1). The uptake was measured as above in the presence of 0.1 to 12 mM/liter $[Ca]_0$. If $[Ca]_0$ directly controlled the level of free intracellular Ca and if a considerable part of the total Ca taken up from the medium could reach the intracellular space, then the inflow of Ca in EGTA-cells should increase markedly and reach a much higher level. Most of the EGTA would have to be converted into Ca-EGTA before a substantial increase in the free intracellular Ca could occur.

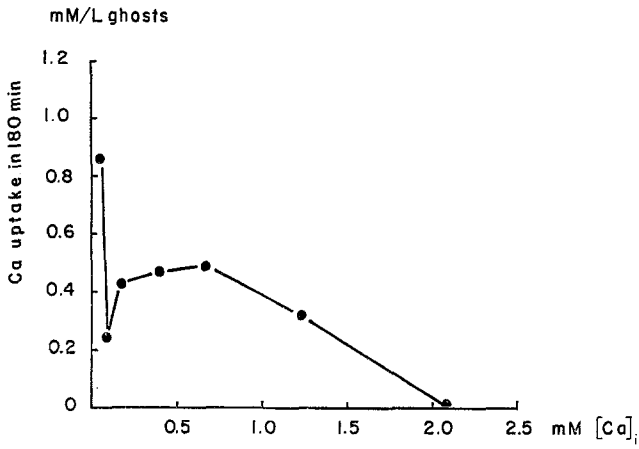

Fig. 3. Influence of cellular Ca content (abscissa) on steady state (=180 min) Ca uptake (ordinate). Starved red cells were hemolyzed in solutions containing 4 mM $MgCl_2$, 8 mM Tris-Cl and different Ca concentrations (0; 0.1; 0.25; 0.5; 1; 2; 4 mM). Reversal of hemolysis with KCl. After 60 min of equilibration at 37 °C the ghosts were washed 3 times in Ca-free solution (140 mM KCl, 20 mM Tris-Cl, pH 7.4) and then incubated at 37 °C in a solution containing 140 mM KCl, 20 mM Tris-Cl, 10 mM $CaCl_2$ (pH 7.4). The cellular content given on the abscissa refers to the values measured in the washed ghosts prior to incubation. Hematocrit, 5.3%. The figure presents mean values of two similar experiments

The curve in Fig. 4(*b*) relates $[Ca]_0$ and the steady state value of overall cellular Ca uptake. Fig. 4(*a*) shows the uptake versus time curves for various external concentrations in a single experiment. The total amount of Ca taken up by these cells at any particular concentration was larger than in cells without EGTA but much less than anticipated (*compare* Fig. 4(*b*) and Fig. 2). Only with the highest $[Ca]_0$ (12 mM) did the total uptake slightly exceed the intracellular EGTA concentration (about 2.4 mM). This indicates that even if all the Ca had penetrated into the intracellular space, the concentration of free Ca would have remained as low as 10^{-4} M. Varying the EGTA concentration from 1.2 to 2.4 mM/liter cells had no influence on the absolute amount of Ca taken up. However, the intracellular EGTA concentration in these experiments is subjected to some uncertainty since it could not be measured directly. It was assumed that the concentration ratio between EGTA in the hemolyzing fluid and EGTA in the resulting ghosts was similar to the corresponding ratio for Ca-EGTA measured in parallel experiments (*see below*). Although the persistantly low free $[Ca^{++}]_i$ in OCa-EGTA-ghosts ensured a large concentration gradient, the uptake of Ca was only slightly increased. This observation, together with the fact that the steady state concentration was reached within approximately 60 min,

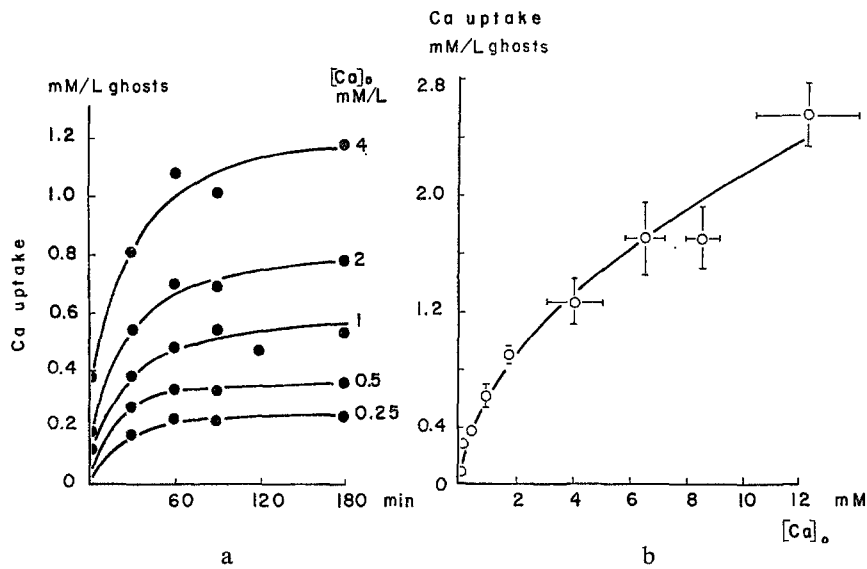

Fig. 4. (*a*) Time course of Ca uptake into OCa-EGTA-ghosts. Starved red cells were hemolyzed in a solution containing 4 mM $MgCl_2$, 8 mM Tris-Cl, 4 mM EGTA. Reversal of hemolysis with KCl. Further procedure as in Fig. 3. The five curves correspond to the uptake in mM/liter packed ghosts (ordinate) measured at 37 °C in presence of 5 different Ca concentrations (0.25; 0.5; 1; 2; 4 mM/liter as indicated on the figure) in the standard incubation medium (140 mM KCl, 20 mM Tris-Cl, pH 7.4). Single experiment. Hematocrit, 6.2%. (*b*) Dependence of Ca uptake into OCa-EGTA-ghosts on $[Ca]_0$. Procedure as in (*a*). The steady state Ca uptake as measured at the end of a 120- or 180-min incubation period at 37 °C (ordinate) is plotted against the Ca concentration in the medium. Hematocrit 5.5 to 8.5%. Vertical bars: ± SE; horizontal bars: range of external Ca concentrations; no bar: SE or range smaller than the dimensions of the symbol. Nine experiments. Each point represents 3 to 5 estimations

suggests that the free intracellular Ca is not directly related to $[Ca]_0$ but contributes to the control of overall cellular uptake.

To substantiate this point, the Ca^{++} concentration in ghosts was varied from 10^{-8} to 10^{-6} M using EGTA. As compared to EGTA-free OCa-ghosts, the incorporation of EGTA or Ca-EGTA with Ca/EGTA ratios less than one did not induce any additional loss of magnesium or hemoglobin from the cells. This was interpreted as indicating that EGTA did not alter the permeability of the ghosts membrane. Fig. 5 illustrates a representative experiment. A constant amount of EGTA and different amounts of Ca were incorporated into erythrocyte ghosts to establish Ca/EGTA ratios ranging from 0.002 to 0.9 (corresponding to 10^{-8} to 10^{-6} M Ca^{++}). The assumption was that this Ca/EGTA concentration ratio was identical in the ghosts and the hemolyzing medium. The total amount of cellular Ca at the beginning of the experiment was taken as being equal to the cellular Ca-EGTA content.

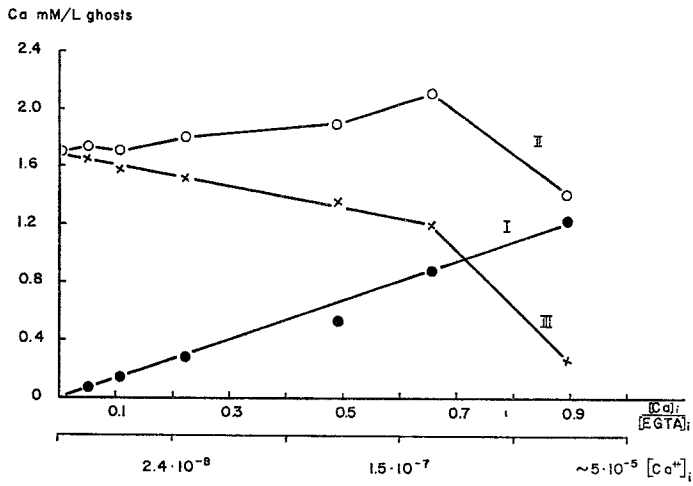

Fig. 5. Relation between intracellular ionized Ca concentration ($[Ca^{++}]_i$) and Ca uptake. Starved red cells were hemolyzed in solutions containing 4 mM $MgCl_2$, 10 to 15 mM Tris-Cl, 2 mM EGTA and increasing amounts of $CaCl_2$ in order to establish a range of Ca/EGTA ratios between 0.01 and 0.9 corresponding to 10^{-9} to 10^{-6} M $[Ca^{++}]_i$ (abscissa). Reversal of hemolysis with KCl. After the equilibration period (60 min at 37 °C), cells were washed 3 times in ice cold Ca-free solution (140 mM KCl, 20 mM Tris-Cl, pH 7.4). At the last wash samples were taken to measure the initial cellular Ca content, considered to be identical with cellular Ca-EGTA content (•——•, curve *I*). The cells were then incubated for 120 min at 37 °C in a Ca-containing medium (129 mM KCl, 18 mM Tris-Cl, 10 mM $CaCl_2$). At the end of 120 min the cells were washed twice in Ca-free solution (composition as above), twice in Ca-free EDTA-containing solution (130 mM KCl, 20 mM Tris-Cl, 10 mM EDTA, pH 7.5), and the cellular Ca content measured again (o——o, curve *II*). The difference between Ca content at the beginning (*I*) and at the end (*II*) of the incubation period (= uptake) is plotted as curve *III* (x-x). Hematocrit, 5.7 to 6.5%. One of 4 similar experiments

These ghosts were incubated for 120 min in the standard medium containing 10 mM Ca and the cellular Ca concentration was measured again. The difference between $[Ca]_i$ at the beginning and the end of the incubation period is plotted as "uptake" in Fig. 5.

An intracellular free calcium concentration of 10^{-6} M was associated already with a reduction in Ca influx of 30%. By increasing $[Ca^{++}]_i$ further, the uptake was completely inhibited. The sharp decrease in Ca entry at a Ca/EGTA ratio above 0.7 probably reflects a steep increase in $[Ca^{++}]_i$ due to the poor buffering capacity of the system when most of the EGTA is occupied by Ca. Therefore, the Ca^{++} concentration associated with a complete inhibition of uptake could not be evaluated accurately by this method. Since the internal Ca^{++} concentration is closely releated to Ca uptake it is perhaps justified to take the amount of uptake as an indicator of $[Ca^{++}]_i$.

Hence, comparing Fig. 3 to 5 it seems that Ca-ghosts were able to "buffer" the Ca concentration internally so that the free concentration was kept below 10^{-5} M even though the overall cellular concentration ranged between 0.1 and 1.0 mM.

The Effect of Mersalyl on Ca Uptake

The experiments reported above suggest that a large part of the total Ca taken up into OCa-ghosts and OCa-EGTA-ghosts was bound to the ghost membrane and did not penetrate into the intracellular space. To confirm this point I used mersalyl (Salyrgan ®), a SH-group blocking organic mercury compound, as a means of increasing membrane permeability and decreasing the binding capacity of the membrane for calcium (Porzig, 1970). EGTA-containing OCa-ghosts were prepared and incubated in the presence and absence of 0.1 or 0.05 mM mersalyl. The external medium contained 1 mM Ca (Fig. 6). The mersalyl-treated cells, after a latency period of 60 min where the Ca uptake did not differ from the uptake into untreated cells, took up five times as much Ca as control cells during the subsequent 120 min. At the end of 3 hr this additional uptake caused a cellular Ca content which was higher than the Ca concentration in the incubation medium. This effect must be attributed to the intracellular complexing of Ca by EGTA. Thus,

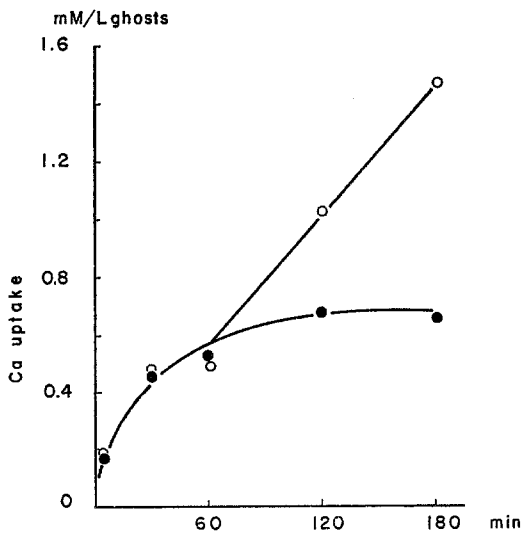

Fig. 6. Time course of Ca uptake into OCa-EGTA-ghosts in the presence (o——o) and in the absence (•——•) of 0.1 mM/liter mersalyl. Method as described in Fig. 4(*a*). Composition of the incubation medium: 140 mM KCl, 20 mM Tris-Cl, 1 mM $CaCl_2$, pH 7.4. Hematocrit, 7.3 % (without mersalyl), 8.1 % (with mersalyl). One of 3 similar experiments

a change in membrane permeability, such as that resulting from treatment with mersalyl, appeared to be a prerequisite for a direct influence of $[Ca]_o$ on the Ca uptake into the intracellular space.

The Membrane-Bound Fraction of Ca Uptake

If the membrane and the intracellular space can be considered as two different compartments for Ca uptake, the kinetics of the calcium movements into and out of these compartments should be separable. This was tested by taking advantage of the previous finding that intracellular Ca was largely immobilized if complexed to EGTA. With respect to Ca outflow the three-compartment system (intracellular space – membrane – extracellular medium) could thus be converted into a two-compartment system (membrane – extracellular space). Calcium taken up intracellularly by OCa-EGTA-ghosts during an incubation period in Ca-containing medium would contribute little to Ca outflow during a subsequent incubation of these cells in Ca-free medium. If, under these conditions, an appreciable Ca outflow were observed it would have to be mainly a result of Ca release from membrane sites. Fig. 7 shows a pertinent experiment. Ca outflow from Ca-

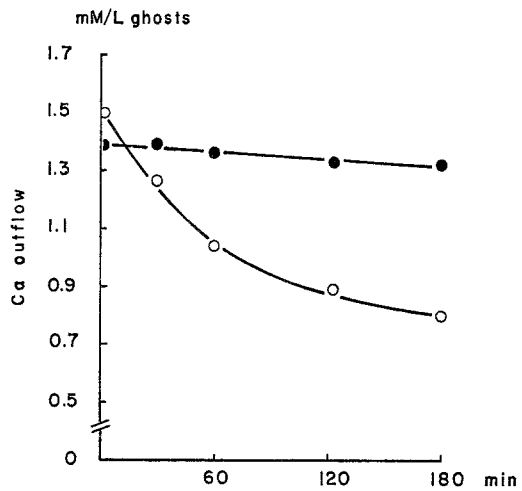

Fig. 7. Time course of Ca outflow from Ca-EGTA-ghosts (•——•) and from Ca-loaded OCa-EGTA-ghosts (o——o). Ca-EGTA-ghosts were prepared as in Fig. 5. Composition of hemolyzing fluid: 4 mM $MgCl_2$, 8 mM Tris-Cl, 4 mM EGTA, 2 mM $CaCl_2$. The cells were incubated at 37 °C in Ca-free solution (140 mM KCl, 20 mM Tris-Cl, pH 7.4). OCa-EGTA-ghosts were prepared as in Fig. 4(*a*). They were loaded with Ca for 60 min at 37 °C in a Ca-containing medium (140 mM KCl, 20 mM Tris-Cl, 10 mM $CaCl_2$, pH 7.4), washed twice in ice cold Ca-free solution (composition as above) and incubated again at 37 °C in the same Ca-free solution to measure the Ca outflow. Hematocrit, 8% (Ca-EGTA-ghosts), 8.7% (OCa-EGTA-ghosts). One of 7 similar experiments

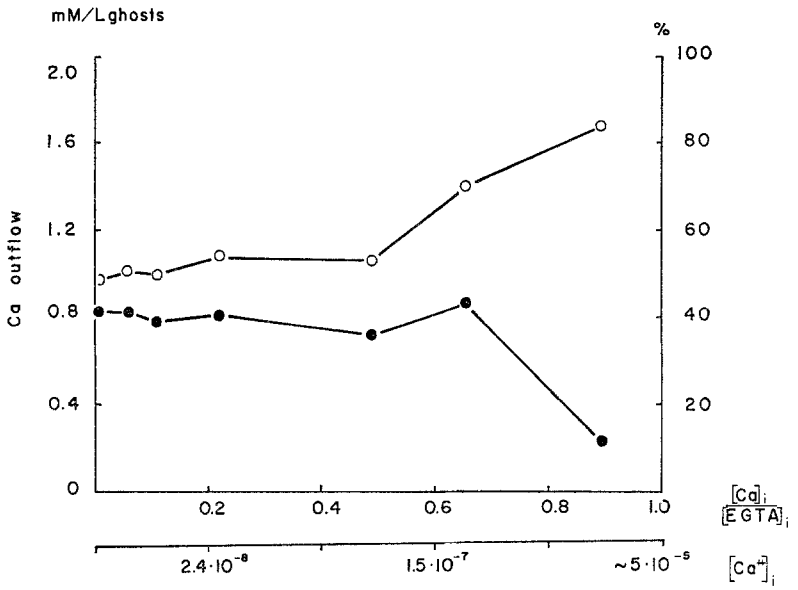

Fig. 8. Effect of $[Ca^{++}]_i$ on Ca loss from Ca-loaded Ca-EGTA-ghosts. Abscissa: intracellular Ca/EGTA ratio and corresponding $[Ca^{++}]_i$. The Ca-loaded cells from the experiment in Fig. 5 were washed twice in a Ca-free medium (140 mM KCl, 20 mM Tris-Cl, pH 7.4) and incubated in the same medium at 37 °C for 180 min. The lower curve (•——•) gives the total amount of Ca having left the cells at the end of 180 min in mM/liter packed ghosts (right ordinate). The upper curve (o——o) expresses the Ca loss in 180 min as per cent of the preceding uptake (left ordinate), (*compare* Fig. 5). Hematocrit, 5.7 to 6.5%. One of 5 similar experiments

EGTA-ghosts is compared to Ca outflow from Ca-loaded OCa-EGTA-ghosts. Ca-EGTA-cells lost 5% of the incorporated Ca whereas the Ca-loaded OCa-EGTA-cells lost 45% of their Ca in 3 hr. The rate of Ca loss in the two cases differed markedly. The rate constant (i.e., the fraction of total Ca lost per minute into the external medium) was 0.00033 and 0.0147 min^{-1}, respectively. The magnesium content of these cells did not diminish more than 10% indicating that a general increase in membrane permeability was not responsible for the observed Ca outflow. These results confirmed the view that the membrane compartment contained a large fraction of the total amount of Ca taken up by OCa- and OCa-EGTA-ghosts in a Ca-containing medium.

In Fig. 8, the Ca loss from Ca-loaded OCa-EGTA-cells is plotted against the free intracellular Ca concentration. The lower curve demonstrates that the absolute amount of Ca released by preloaded cells remained fairly constant when $[Ca^{++}]_i$ increased from 10^{-9} to 4×10^{-7} M and then decreased to 1/4 of the original value as $[Ca^{++}]_i$ was raised further to about 10^{-6} M. As

pointed out in the context of the uptake curve of Fig. 5, here again the sharp downward deflection of the curve reflects probably the unsatisfactory buffering capacity of EGTA in this concentration range. The small absolute amount leaving the cells in the presence of the highest Ca-EGTA concentration shows that the intracellulary complexed Ca contributed little to the total loss. Plotting the Ca outflow in per cent of the preceding uptake (upper curve in Fig. 8) shows an increase in the outflow from 55 to 85% when $[Ca^{++}]_i$ was raised from 10^{-7} to 10^{-6} M. This finding reflects the fact that total cellular uptake started to decrease when the outflow was still unchanged (compare with Fig. 5). Furthermore, with $[Ca^{++}]_i$ above 2×10^{-7} M the reduction in uptake was even greater than the outflow. Since Ca outflow from Ca-loaded OCa-EGTA-ghosts was unchanged in the range between 10^{-9} to 2×10^{-7} M it is reasonable to assume that the amount of membrane-bound Ca was also unchanged. Therefore, the reduction in uptake at these concentrations suggests a decrease in the ability of Ca to penetrate into the intracellular space.

The fraction of total Ca uptake which is caused by membrane binding is probably larger than the amount released by Ca-loaded OCa-EGTA-ghosts into a Ca-free medium. In the steady state, where the rate of Ca loss from these cells is equal to the rate at which Ca moves out of Ca-EGTA-cells, some Ca will still be fixed by membrane binding sites. Therefore, experiments like the one in Fig. 7 were carried out with 0.05 mM mersalyl added to the outflow medium (Table 1). Fig. 9 illustrates the results of such an experiment. Mersalyl-treated Ca-EGTA-cells lost 19.1 ± 3.3% of the incorporated Ca in 180 min, Ca-loaded OCa-EGTA-cells lost 62.5 ± 6.4% of the cellular Ca in the presence and 44.9 ± 2.4% in the absence of mersalyl. Since the same mersalyl treatment induced an almost complete release of cellular Ca in normal Ca-ghosts without EGTA these figures suggest that at least 37% of cellular Ca has been bound to intracellular EGTA. This estimate is certainly too low because no correction has been made for the Ca loss from Ca-EGTA. Since Ca-EGTA-ghosts lost 19% of the cellular Ca, this correction can be assumed to be about 8%. Therefore, about 45% of total Ca taken up appears to be located intracellularly and 55% appears to be located in the membrane.

The membrane-bound Ca fraction as characterized in the preceding section could be demonstrated only in intact ghosts suspended in isotonic media. Therefore, it is not directly comparable to the amount of Ca bound to hemoglobin-free erythrocyte membranes which are freely accessible to ions on the internal and external face. This can be inferred from experiments where Ca-EGTA-ghosts and Ca-loaded OCa-EGTA-ghosts from the same incuba-

Table 1. Effect of mersalyl on Ca outflow from different ghost preparations[a]

Ghost preparation	Standard medium			Standard medium + 0.05 mM/liter mersalyl		
	$[Ca]_i$ initial mM/liter	Outflow in 180 min mM/liter	Outflow in % of initial $[Ca]_i$	$[Ca]_i$ initial mM/liter	Outflow in 180 min mM/liter	Outflow in % of initial $[Ca]_i$
(I) CaEGTA-ghosts	0.591 ± 0.038 (10)	0.075 ± 0.014 (10)	13.9 ± 3.5 (10)	0.686 ± 0.031 (5)	0.130 ± 0.021 (5)	19.1 ± 3.3 (5)
(II) Ca-loaded OCa-EGTA-ghosts	1.282 ± 0.088 (10)	0.588 ± 0.054 (10)	44.9 ± 2.4 (10)	0.989 ± 0.063 (5)	0.603 ± 0.029 (5)	62.5 ± 6.4 (5)
(III) Ca-ghosts	0.705 ± 0.045 (9)	0.238 ± 0.025 (9)	35.1 ± 4.4 (9)	0.843 ± 0.065 (4)	0.733 ± 0.050 (4)	87.3 ± 2.0 (4)
(IV) Ca-loaded OCa-ghosts	0.861 ± 0.122 (4)	0.542 ± 0.064 (4)	63.6 ± 2.8 (4)	0.731 ± 0.036 (3)	0.653 ± 0.037 (3)	89.4 ± 0.9 (3)

[a] Ca-EGTA-ghosts (I) were prepared as decribed in Fig. 5. Composition of the hemolyzing fluid: 4 mM $MgCl_2$, 8 to 10 mM Tris-Cl, 2 to 4 mM EGTA, 1 mM $CaCl_2$. Reversal of hemolysis with KCl. Incubation in presence or absence of mersalyl for 180 min in a Ca-free medium at 37 °C (140 mM KCl, 20 mM Tris-Cl, pH 7.4 = standard medium). OCa-EGTA-ghosts (II) were prepared as described in Fig. 4(*a*). The cells were loaded with Ca during incubation in Ca-containing medium (140 mM KCl, 20 mM Tris-Cl, 10 mM $CaCl_2$ (in 4 experiments, 5 mM $CaCl_2$), pH 7.4, washed twice in the standard medium and incubated again in the same medium with or without mersalyl for 180 min at 37 °C. Ca-ghosts (III) were prepared as described in Fig. 1. At the end of the equilibration period, the ghosts were washed 3 times in the standard medium and incubated in the same medium with or without mersalyl at 37 °C for 180 min. OCa-ghosts (IV) were prepared as described in Fig. 1. At the end of the equilibration period, the cells were washed 3 times in the standard medium. The further procedure for loading the ghosts with Ca and for the final incubation was identical to the one described for OCa-EGTA-ghosts. The numbers give mean values ± SE (number of experiments in parentheses).

tion suspension were washed either with isotonic KCl solution containing 10 mM EGTA or with distilled water. In both types of ghosts the hypotonic wash removed about 95% of the total cellular Ca. A similar observation was made on OCa-ghosts which did not seal after reversal of hemolysis and therefore lost most of their hemoglobin during the course of the experiment. The uptake of Ca in these hemolytic ghosts was only about 20% of the uptake in normal low-permeability-ghosts. Moreover, hemolytic ghosts lost nearly all of the Ca taken up in a Ca-containing solution during a subsequent incubation in Ca-free medium.

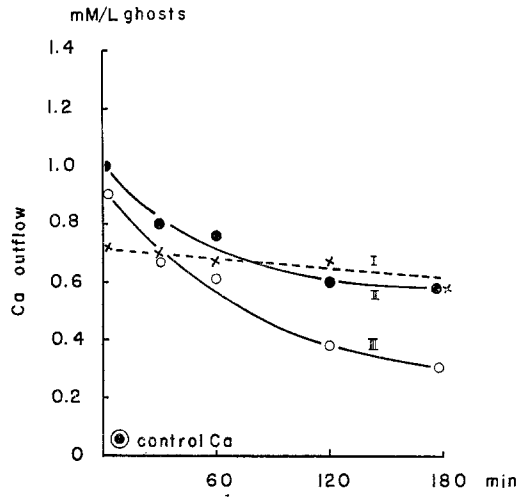

Fig. 9. Time course of Ca outflow from Ca-loaded OCa-EGTA-ghosts in the presence and in the absence of mersalyl. Same procedure as described in Fig. 7. OCa-EGTA-ghosts previously loaded with Ca for 120 min at 37 °C in a solution containing 140 mM KCl, 20 mM Tris-Cl, 5 mM $CaCl_2$, pH 7.4 were incubated in Ca-free medium (140 mM KCl, 20 mM Tris-Cl, pH 7.4 with (*III*, o——o) or without (*II*, •——•) 0.05 mM mersalyl. Ca outflow from Ca-EGTA-ghosts under the same conditions is shown as a control (*I*, ×——×). The point at the bottom of the figure marked as "control" indicates the cellular Ca content in OCa-EGTA-ghosts prior to loading. Hematocrit, 5.1 to 5.7%. One of 3 similar experiments

Ca-free ghosts had a much higher Ca binding capacity than intact starved erythrocytes. Starved red cells when incubated in the standard medium containing 0.5 to 10 mM Ca took up a maximum of 0.1 mM/liter packed cells with the highest concentration used. No uptake was measurable with smaller concentrations.

Efflux of Ca Incorporated into Red Cell Ghosts by Reversible Osmotic Hemolysis

In Fig. 10(*a*), the outflow of Ca into a Ca-free medium is plotted against the Ca concentration in the cells at the start of the experiment (upper curve) and compared to Ca inflow into OCa-ghosts in the presence of the same but inwardly directed Ca concentration gradients (lower curve). A nearly linear relationship between outflow and $[Ca]_i$ was obtained for internal concentrations ranging from 0.1 to 2.7 mM/liter packed cells. As $[Ca]_i$ was raised above 3 mM, the ghosts (after hemolysis and restoration of isotonicity) resealed only incompletely to Ca, Mg and hemoglobin. Subsequent washings

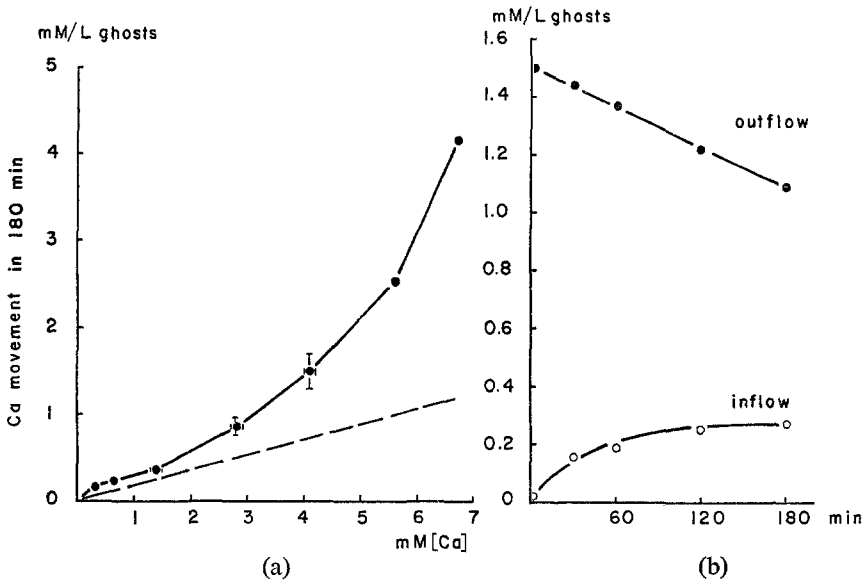

Fig. 10. (*a*) Effect of $[Ca]_i$ on Ca outflow from Ca-ghosts. The total Ca loss in 180 min into a Ca-free medium (ordinate) is plotted versus the initial cellular Ca concentration (abscissa). Starved red cells were hemolyzed in solutions containing 4 mM $MgCl_2$, 8 mM Tris-Cl, and 0.1 to 15 mM $CaCl_2$. Reversal of hemolysis with KCl. Further procedure as in Fig. 3. Composition of the incubation medium: 140 mM KCl, 20 mM Tris-Cl, pH 7.4. The points are mean values of 5 to 10 estimations. Vertical bars = ± SE; horizontal bars = ± SE of the range of Ca concentrations. The two points for the highest $[Ca]_i$ are single values. At low concentrations, the SE was smaller than the dimension of the symbols. Hematocrit, 5.5 to 7.5%. Data from 10 experiments. For comparison, the regression line for Ca uptake into OCa-ghosts, taken from Fig. 2, is also shown (dotted line). (*b*) Comparison between time course of Ca inflow into OCa-ghosts and Ca outflow from Ca-ghosts into a Ca-free medium. Preparation of ghosts as in Fig. 1. The initial Ca content of OCa-ghosts was 0.023, of Ca-ghosts 1.504 mM/liter packed ghosts. The external medium contained 140 mM KCl, 20 mM Tris-Cl and either none or 1.6 mM Ca (pH 7.4). Hematocrit, 6.7 (Ca-ghosts) and 7.4% (OCa-ghosts). One of two similar experiments (with a total of 8 parallel estimations of inflow and outflow)

in ice cold isotonic Ca-free solution itself removed 30 to 50% of the incorporated Ca. The same procedure did not change $[Ca]_i$ in cells with an initial content of 0.1 to 3.0 mM Ca per liter packed cells. Therefore, only cells with $[Ca]_i$ in this range (corresponding to Ca concentrations in the hemolyzing medium of 0.1 to 4.0 mM) have been used in the inflow experiments.

Apparently, high Ca concentration (above 5 mM) exerted a damaging effect on membrane integrity only during the preparation of ghosts when the medium was hypotonic. The same Ca concentrations when applied in an isotonic medium after reconstitution of the ghosts had no deleterious effect on the membrane. The insensitivity of the ghost membrane to external Ca may be inferred from Fig. 2. The internal Ca of reconstituted ghosts could

be raised by simply suspending Ca containing K-rich cells in buffered isotonic NaCl solution. Ca-ghosts are not sealed for K (Hoffman, 1962). In a Na medium they lost K rapidly and gained Na slowly. This resulted in a volume decrease of approximately 70%. Since Ca moved out much slower than K, $[Ca]_i$ was raised nearly threefold. A $[Ca]_i$ of 8 mM could easily be obtained by this method. The rate and the amount of Ca loss from the shrunken cells was, however, independent of the volume and hence, under these conditions, independent of the internal Ca concentration.

The following data on Ca outflow were obtained from experiments with 0.1 to 3.0 mM Ca-ghosts. In Fig. 10(*b*), the time course of Ca outward movement into a Ca-free solution and the inward movement into OCa-ghosts in the presence of approximately equal concentration gradients in the two directions (1.5 mM) are compared. In general, there was no significant difference between the amount of uptake into OCa-ghosts and the amount of loss from Ca-ghosts into a Ca-free medium during the first 60 min. However, two important differences between inward and outward movements were observed: (1) The inflow rate leveled off within 120 min whereas in most experiments the outflow versus time curve deviated only slightly from a straight line. (2) The outflows into Ca-free and Ca-containing media did not differ, provided the concentration difference was kept constant, whereas the inflow was significantly reduced as the $[Ca]_i$ was increased even though the absolute gradient remained unchanged.

Influence of Lanthanum on Calcium Movements

La is known to have a strong affinity to Ca binding sites in biological tissues (*cf.* Williams, 1970). In squid giant axon and in smooth muscle La is reported to inhibit Ca fluxes across the cell membranes (*cf.* Van Breemen & De Weer, 1970). Therefore, it seemed useful to evaluate the effect of extracellular La on Ca movements in red cell membranes. Ca-ghosts or Ca-EGTA-ghosts were prepared and subsequently suspended in the standard medium with 0.5 or 0.25 mM/liter La added. In Ca-ghosts and in Ca-free ghosts loaded with Ca in a preceding incubation, La seemed to inhibit the Ca outward movement during the first hour of incubation and to enhance it in the second and third hour. This latter effect, however, was obscured by hemolysis which occurred simultaneously and indicated a nonspecific effect on membrane permeability. La did not influence the slow rate of Ca outflow from Ca-EGTA-ghosts. The Ca uptake into OCa- or OCa-EGTA-ghosts was completely inhibited if La (0.5 to 2.0 mM/liter) and Ca (1 to 10 mM/liter) were present together in the external medium.

It was not possible to test the effect of the incorporation of La into red cell ghosts on Ca movements. One mM La in the hemolyzing medium prevented the resealing of the ghost cells after reconstitution of isotonicity. White, nearly hemoglobin-free membranes could be obtained in a single hemolyzing step. These membranes did not contain a measurable amount of Ca nor did they take up Ca from a La-free, Ca-containing medium.

Discussion

The work of Romero and Whittam (1971) as well as that of Schatzmann and Vincenzi (1969) has indicated that the inhibition of the Ca-pump is not sufficient to raise Ca uptake into human erythrocytes substantially. Romero and Wittam combined inhibition of the active transport by metabolic depletion of the cells and increase in passive permeability for Ca by raising the pH to 7.5 to 7.6. Only a combination of both conditions resulted in a considerable increase of Ca uptake.

The results of the present experiments suggest that the Ca uptake into human erythrocyte ghosts is a self-limiting process. The binding of Ca to membrane sites prevents the penetration of external Ca into the cell interior. This mechanism would enable the cells to maintain a very low intracellular Ca concentration even in the absence of an active Ca outward transport. This hypothesis is based on the following findings: (1) The Ca uptake into OCa-EGTA-ghosts reached a "steady state" within 60 min even though a nearly constant, inwardly directed concentration gradient for Ca persisted [*see* Fig. 4(*a*)]; (2) No direct effect of $[Ca]_0$ on free $[Ca^{++}]_i$ was observed unless membrane permeability and binding capacity for Ca were altered chemically by mersalyl (*see* Fig. 6); (3) Raising $[Ca^{++}]_i$ from 10^{-9} to 10^{-6} M was associated with increasing inhibition of Ca uptake (*see* Fig. 5). The idea of membrane-bound Ca as a factor controlling Ca permeability fits into the general observation that this ion is important for the control of K^+ and Na^+ permeability in erythrocyte membranes as well (Ponder, 1953; Bolingbroke & Maizels, 1959; Lepke & Passow, 1960; Burt & Green, 1971; Romero & Whittam, 1971). An interesting feature of the present results is the dependence of the amount of membrane-bound Ca on the internal free Ca concentration. In ghosts prepared in the presence of less than 2×10^{-7} M Ca^{++}, more than 50% of the total Ca uptake was caused by membrane binding. On the other hand, an extracellular Ca concentration of more than 10^{-2} M was necessary to saturate the binding sites accessible

from the outside. If the intracellular ionized Ca was raised to 10^{-5} M, both the intracellular uptake and the uptake into the membrane were completely inhibited. Therefore, it seems justified to assume that at least two types of Ca binding sites exist in the ghost membrane. One type is available only in ghosts with an internal Ca^{++} concentration below 10^{-5} M. It binds relatively large amounts of Ca and equilibrates with $[Ca]_0$ (type "A"). The second type is present in all ghost preparations, limits the Ca uptake into the intracellular space, binds only a small amount of Ca, and is probably in equilibrium with $[Ca^{++}]_i$ (type "B"). It is important in this context that starved red cells, although comparable to OCa-ghosts with respect to their cellular Ca content (*cf.* Harrison & Long, 1968, own control measurements in connection with the present work), seemed to lack type "A" binding sites, since they did not take up any appreciable amount of Ca under comparable conditions (Rummel, Seifen & Baldauf, 1962; Long & Mouat, 1971; this paper, *see* p. 15). These observations suggest that type "A" binding sites are not accessible or do not exist under physiological conditions but are made available during the conversion of red cells into ghosts and by the concomitant changes in membrane structure.

The concept of Ca binding sites on the inside of the red cell membrane functionally different from those on the outside is supported by the findings of Palek *et al.* (1971). These authors observed volume shrinkage of ghost cells associated with changes in osmotic behavior and hence membrane permeability when Ca was incorporated into the cells during osmotic hemolysis. The lowest concentration required for this ATP-independent effect was 2×10^{-6} M ionic Ca. Extracellular Ca had no effect. The alteration in membrane physical properties described by Palek *et al.* (1971) may well reflect the same interaction of Ca with the inner surface of the membrane, which also causes the drop in Ca permeability.

A change in membrane structure may also be the reason for the difference in Ca binding to normal OCa-ghosts and to fragmented red blood cell membranes (white membranes). Prolonged treatment in solutions of low ionic strength required to prepare white membranes may introduce such structural change. Ca binding to type "A" sites was a rather slow process. It took at least 60 min to establish the equilibrium between membrane and solution. The bound Ca could not be removed from these sites by washing the cells in EDTA-containing isotonic solution but was largely removed by washing in hypotonic solution. In white erythrocyte membranes the equilibrium between membranes and medium is established within 10 min, EDTA reduces the binding markedly and diminishing the ionic strength increases the amount of membrane-bound Ca (Gent, Trounce & Walser, 1964;

Long & Mouat, 1971; Forstner & Manery, 1971). The conclusion seems reasonable that type "A" binding sites are located in the depth of the membrane, separated from the medium by diffusion barriers to Ca, whereas in high-permeability-ghosts the Ca binding sites are freely accessible to external Ca and EDTA. The absolute amount of Ca bound to OCa-EGTA-ghosts in media of normal ionic strength (0.166) was about 5 times higher than the amount reported by Long and Mouat (1971) as being bound to white membranes under comparable conditions (0.1 and 0.5 mM/liter packed cells, respectively). However, in media of low ionic strength red cell membranes have been found by the same authors to bind up to 1.2 mM Ca/liter in the presence of 2.5 mM external Ca. Comparable values have been reported recently by Forstner and Manery (1971). Apparently, under these conditions the binding capacity for Ca in white membranes is even higher than in OCa-EGTA-ghosts.

The outward movement of Ca followed a different pattern from that described for Ca uptake in that the experiments provide no evidence for a self-limitation of Ca outflow. Ca-ghosts containing enough Ca to inhibit uptake will nevertheless lose a large part of the incorporated Ca at a constant rate into a Ca-free medium. This suggests that the pathways for Ca outward and inward movements are different. There is good evidence that an increase in intracellular Ca beyond 10^{-4} M raises the sodium and potassium permeability (Hoffman, 1962; Lew, 1970; Romero & Whittam, 1971). The present experiments show that Ca outward movement can also be enhanced by increasing $[Ca]_i$. However, the concentrations needed to affect Na^+ and K^+ permeability are smaller by at least an order of magnitude.

To summarize, these considerations point to a dual effect of Ca on membrane permeability in red cells. If applied to the outside, Ca will reduce the increased cation permeability in isotonic solutions of low ionic strength (Bolingbroke & Maizels, 1959; Wilbrandt & Schatzmann, 1960) or in butanol-containing isotonic salt solutions (Burt & Green, 1971). If applied to the inside in concentrations above 10^{-5} M Ca will increase cation permeability. It will abolish any selective permeability if applied in high concentrations to membranes in a medium of low ionic strength. With respect to cation permeability, Ca appears to be capable of introducing "rectifier properties" in erythrocyte membranes. The parallel changes in volume, shape, viscosity (Weed *et al.*, 1969; Palek *et al.*, 1971) and permeability of erythrocytes and ghosts are all associated with an increase in intracellular Ca and perhaps point to a causal relationship between conformational and permeability change in the red cell membrane.

Many thanks are due to Prof. Hildegard Portzehl for helpful information concerning the use of EGTA as calcium-buffer. The technical assistance of Miss M. Over, Mr. M. Staub and Mr. R. Binggeli is gratefully acknowledged.

This study was supported by the Schweizerischer Nationalfonds. Grant No. 3.91.69 to Prof. H. Reuter.

References

Bolingbroke, V., Maizels, M. 1959. Calcium ions and the permeability of human erythrocytes. *J. Physiol.* **149**:563.

Breemen, C. van, De Weer, P. 1970. Lanthanum inhibition of ^{45}Ca efflux from the squid giant axon. *Nature* **226**:760.

Burt, D.H., Green, J.W. 1971. The sodium permeability of butanol treated erythrocytes. The role of calcium. *Biochim. Biophys. Acta* **225**:46.

Cha, Y.N., Shin, B.C., Lee, K.S. 1971. Active uptake of Ca^{++} and Ca^{++}-activated Mg^{++} ATPase in red cell membrane fragments. *J. Gen. Physiol.* **57**:202.

Dodge, J.T., Mitchell, C., Hanahan, D.J. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**:119.

Duchon, G., Collier, H.B. 1971. Enzyme activities of human erythrocyte ghosts: Effects of various treatments. *J. Membrane Biol.* **6**:138.

Dunham, E.T., Glynn, I.M. 1961. Adenosinetriphosphatase activity and the active movements of alkali metal ions. *J. Physiol.* **156**:274.

Epstein, F.H., Whittam, R. 1966. The mode of inhibition by calcium of cell-membrane adenosine-triphosphatase activity. *Biochem. J.* **99**:232.

Forstner, J., Manery, J.F. 1971. Calcium binding by human erythrocyte membranes. *Biochem. J.* **124**:563.

Fuhrmann, G.F., Liggenstorfer, P., Wilbrandt, W. 1971. Änderung des Glukosetransports frischer menschlicher Erythrocyten bei längerer Inkubation. *Experientia* **27**:1428.

Gent, W.L.G., Trounce, J.R., Walser, M. 1964. The binding of calcium ion by the human erythrocyte membrane. *Arch. Biochim. Biophys.* **105**:582.

Harrison, D.G., Long, C. 1968. The calcium content of human erythrocytes. *J. Physiol.* **199**:367.

Hoffman, J.F. 1962. Cation transport and structure of the red cell plasma membrane. *Circulation* **26**:1201.

Hoffman, J.F., Tosteson, D.C., Whittam, R. 1960. Retention of potassium by human erythrocyte ghosts. *Nature* **185**:186.

Lamprecht, W., Trautschold, I. 1962. Adenosin-5′-triphosphat-Bestimmung mit Hexokinase und Zwischenferment. *In:* Methoden der enzymatischen Analyse. H.U. Bergmeyer, editor. p. 543. Verlag Chemie, Weinheim.

Lee, K.S., Shin, B.C. 1969. Studies on the active transport of calcium in human red cells. *J. Gen. Physiol.* **54**:713.

Lepke, S., Passow, H. 1960. Die Wirkung von Erdalkalimetallionen auf die Kationenpermeabilität fluoridvergifteter Erythrocyten. *Pflüg. Arch. Ges. Physiol.* **271**:473.

Lew, V.L. 1970. Effect of intracellular calcium on the potassium permeability of human red cells. *J. Physiol.* **206**:35P.

Long, C., Mouat, B. 1971. The binding of calcium ions by erythrocytes and "ghost"-cell membranes. *Biochem. J.* **123**:829.

Olson, E.J., Cazort, R.J. 1969. Active calcium and strontium transport in human erythrocyte ghosts. *J. Gen. Physiol.* **53**:311.

Palek, J., Curby, W.A., Lionetti, F.J. 1971. Effects of calcium and adenosine triphosphate on volume of human red cell ghosts. *Amer. J. Physiol.* **220**:19.

Passow, H. 1969. Ion permeability of erythrocyte ghosts. *In:* Laboratory Techniques in Membrane Biophysics. H. Passow and R. Stämpfli, editors. p. 21. Springer-Verlag, Berlin.

Ponder, E. 1953. Volume changes, ion exchanges and fragilities of human red cells in solutions of the chlorides of the alkaline earths. *J. Gen. Physiol.* **36**:767.

Portzehl, H., Caldwell, P.C., Rüegg, J.C. 1964. The dependence of contraction and relaxation of muscle fibres from the crab maia squinado on the internal concentration of free calcium ions. *Biochim. Biophys. Acta* **79**:581.

Porzig, H. 1970. Calcium efflux from human erythrocyte ghosts. *J. Membrane Biol.* **2**:324.

Romero, P.J., Whittam, R. 1971. The control by internal calcium of membrane permeability to sodium and potassium. *J. Physiol.* **214**:481.

Rummel, W., Seifen, W., Baldauf, J. 1962. Aufnahme und Abgabe von Calcium an Erythrocyten vom Menschen. *Arch. Exp. Pathol. Pharmakol.* **244**:172.

Schatzmann, H.J., Vincenzi, F.F. 1969. Calcium movements across the membrane of human red cells. *J. Physiol.* **201**:369.

Weed, R.I., LaCelle, P.L., Merrill, E.W. 1969. Metabolic dependence of red cell deformability. *J. Clin. Invest.* **48**:795.

Wilbrandt, W., Schatzmann, H.J. 1960. Changes in the passive cation permeability of erythrocytes in low electrolyte media. *In:* Regulation of the Inorganic Ion Content of Cells. Ciba Foundation Study Group No. 5.G. E.W. Wolstenholme and C.M. O'Connor, editors. p. 34. J. & A. Churchill Ltd., London.

Williams, R.J.P. 1970. The biochemistry of sodium, potassium, magnesium and calcium. *Quart. Rev. Chem.* **24**:331.