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Calcium Efflux from Human Erythrocyte Ghosts

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Summary. The passive Ca efflux from human red cell ghosts was studied in media of differing ion compositions and compared to the ATP-dependent Ca efflux. Cells were loaded with ^{45}Ca during reversible hemolysis, and the loss of radioactivity into the non-radioactive incubation medium was measured, usually for 3 hr at 37 °C. Analysis of the efflux curves revealed that ^{45}Ca efflux followed the kinetics of a simple two-compartment system. In the concentration range between 0 and 1 mM Ca in the external solution ($[Ca^{++}]_o$), the rate constant of passive Ca efflux (k min^{-1}, fraction of ^{45}Ca lost per minute into the medium) increased from 0.00732 to 0.0150 min^{-1}. There was no further increase at higher $[Ca^{++}]_o$. The relation between the rate constant of Ca efflux and $[Ca^{++}]_o$ is thus characterized by saturation kinetics. The passive transfer system for Ca could also be activated by Sr. The alkali metal ions Na, K and Li did not seem to have any significant influence on passive Ca transfer. The passive Ca efflux was slightly inhibited by Mg and strongly inhibited by Pb. Under most experimental conditions, a fraction of 15 to 50% of the intracellular Ca seemed to be "inexchangeable". The inexchangeable fraction decreased with increasing $[Ca^{++}]_o$ and increased with increasing $[Ca^{++}]_i$. It was not influenced by alkali metal ions, CN or Pb, but it could be completely removed from the cells by the addition of 0.1 mM Mersalyl to the incubation medium or by hemolysis with addition of a detergent. The active ATP-dependent Ca transport differed characteristically from passive transfer; the rate constant decreased with increasing $[Ca^{++}]_o$, and the inexchangeable Ca fraction increased with increasing $[Ca^{++}]_o$. The experimental results suggest that there exists a carrier-mediated Ca–Ca exchange diffusion in the erythrocyte membrane and that only a fraction of the ghost cell population participates in the Ca exchange diffusion.

Erythrocytes, like nerve and muscle fibers, maintain a low intracellular Ca concentration in spite of the existence of a considerable electrochemical gradient (*cf.* Harrison & Long, 1968). Since the erythrocyte membrane was shown not to be impermeable to Ca (*cf.* Ponder, 1953; Rummel, Seifen & Baldauf, 1962; Passow, 1963), an active transport of Ca in the outward direction may be postulated. As first shown by Schatzmann (1966, 1967) and by Schatzmann and Vincenzi (1969), human red cells indeed seem to possess an active ATP-dependent Ca transport mechanism. However, in

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mammalian heart muscle cells and the squid axon, which are even more permeable to Ca than red cells, active Ca extrusion has not been demonstrated. In these tissues, a passive transfer system could be characterized which effects a Ca–Na and a Ca–Ca exchange. Ca can be replaced by Sr (Reuter & Seitz, 1968; Blaustein & Hodgkin, 1969; Baker, Blaustein, Hodgkin & Steinhardt, 1969; Glitsch, 1969). It therefore seemed worthwhile to study whether the passive Ca movements in human erythrocyte ghosts were also due to a specific transfer system or merely to simple diffusion. Furthermore, the possible relations between active and passive Ca efflux were investigated.

The experimental results lead to the conclusions that in addition to active Ca transport, there exists a distinct carrier-mediated Ca–Ca exchange diffusion in the erythrocyte membrane and that Na does not compete with Ca for the carrier binding sites. The ghost population was kinetically inhomogeneous. Only a fraction of the cells participated in the Ca exchange diffusion.

Materials and Methods

Human erythrocytes were obtained from fresh citrated blood of healthy donors (20 ml of 3.6% citrate solution per 100 ml blood). The cells were washed three to five times at room temperature in about four volumes of isotonic NaCl solution (161 mM NaCl, 5 mM Tris buffer, pH 7.4). Finally, a 50% suspension of washed cells in isotonic Tris solution (166 mM Tris buffer, pH 7.4) was prepared.

$CaCl_2$, ^{45}Ca, Na–ATP or other substances were incorporated into the cells during osmotic hemolysis (Szekely, Mányai & Straub, 1954). The specific method employed was that of Hoffman, Tosteson and Whittam (1960), as modified by Lepke and Passow (1968) and Passow (1969). The 50% cell suspension in Tris buffer was mixed at 0 °C with a 10-fold volume of hemolyzing fluid. In most experiments, the hemolyzing fluid contained 4 mM $MgCl_2$ and the substances (except K, Na and Li) to be incorporated into the ghosts, including ^{45}Ca at a concentration of 160 to 200 μc/liter. Then, 5 to 10 min later, isotonicity was restored by the addition of 3.32 M KCl, NaCl or LiCl. In all experiments designed to measure the passive Ca efflux, the cells were preincubated for 60 min at 37 °C in this medium. The suspension was then centrifuged (6 min, 30,000 × *g*, refrigerated centrifuge), the supernatant was discarded and the cells were washed twice with a 10-fold volume of an ice-cold Ca-free solution (140 mM NaCl, 5 mM KCl, 20 mM Tris buffer, pH 7.4) and once with the same medium as that used for measuring the Ca exchange. This latter medium had the same composition as the Ca-free washing solution, except that $CaCl_2$, $MgCl_2$ or other substances were added as required. If the concentration of the added substance exceeded 4 mM, the external $[Na^+]$ was correspondingly reduced in order to maintain isotonicity.

The sediment was resuspended in an equal volume of the final incubation medium. A 0.5-ml sample of this suspension was taken to determine the wet and dry weights. The experiment was started by adding to each 50 ml of prewarmed (37 °C) inactive incubation medium, 3 to 5 ml of the cold ghost suspension. This led to a temperature drop of about 2 to 3 °C; the initial temperature of 37 °C was attained 5 to 8 min later. The amount of cells in the medium was 2 to 6 mg dry weight/ml solution which roughly corresponds

to a hematocrit of 1.5 to 3%. Measurement of ATP-dependent Ca efflux was performed after hemolysis in the presence of 2 mM Tris-buffered disodium-ATP (Boehringer, Mannheim, Germany) and subsequent reversal of hemolysis with 3.32 M KCl solution. There was no preincubation period preceding the start of the experiment. After being washed once, the ghosts were simply suspended in 50 ml of the appropriate medium at 25 °C.

One-ml samples of supernatant were taken at suitable intervals to measure ^{45}Ca activity. At the beginning and the end of the experiment, additional samples were taken to estimate the degree of hemolysis. At the end of the incubation period, extra samples were drawn to estimate total hemoglobin concentration and total ^{45}Ca activity.

To measure ^{45}Ca activity, samples were evaporated to dryness at 95 °C and subsequently digested by a 1:1 mixture of 65% HNO_3/60% $HClO_4$ on an electrically heated aluminium block (Hattingberg, Klaus, Lüllmann & Zepf, 1966). After evaporation to dryness, the residue was dissolved in 1 ml 0.1 M HCl, and 0.25 ml was then transferred into 10 ml of liquid scintillation fluid (Toluene/methanol, 1:1, containing 0.025 g/liter POPOP, 2.5 g/liter PPO and 0.0555 g/liter $CaCl_2$) [1] and counted in a Packard liquid scintillation counter.

In some experiments the samples taken from the supernatant were dried but not digested in $HNO_3/HClO_4$ before dissolving in 0.25 ml of 0.1 M HCl. Under these conditions quenching could be neglected, provided that hemolysis did not exceed 2%.

Hemoglobin was estimated photometrically in the suspension and in the supernatant at 410 nm (Soret band). In the experiments presented below, hemolysis never exceeded 3%. To avoid Ca adsorption to glass walls, incubation and centrifugation were done in plastic vessels.

The efflux of ^{45}Ca was calculated from the increase of ^{45}Ca activity in the supernatant and the known total activity in the incubation mixture. Single exponential curves could be fitted to the data by the method of the least squares. The rate constant (i.e., fraction of total ^{45}Ca activity in the cells lost per minute) was calculated by a digital computer.[2] Wherever possible, the mean $\pm$ standard deviation is given.

Results

Passive Ca efflux was determined as a function of the $[Ca^{++}]$ on the outside ($[Ca^{++}]_o$) and inside ($[Ca^{++}]_i$) of the red cell ghost membrane.

Fig. 1 shows one of twelve experiments, where ^{45}Ca efflux from erythrocyte ghosts containing 1 mM $CaCl_2$ was measured in media with $[Ca^{++}]$ between 0 and 4 mM. The plotted curves represent single exponential curves fitted to the experimental points by the method of the least squares. Thus, ^{45}Ca efflux follows the kinetics of a simple two-compartment system. However, the individual curves asymptotically approach end points which are far from an equilibrium distribution of the ^{45}Ca between cells and medium.

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1 In glass vials, at low $[Ca^{++}]$ the counting efficiency depends on the concentration of nonradioactive Ca. The addition of an excess of nonradioactive Ca to the scintillation fluid guarantees a constant and maximal efficiency (Fuchs, II. Dept. of Physiology, Homburg/Saar, *personal communication*, 1969).

2 The calculations were performed on the computer of the "Deutsches Rechenzentrum", Darmstadt, Germany.

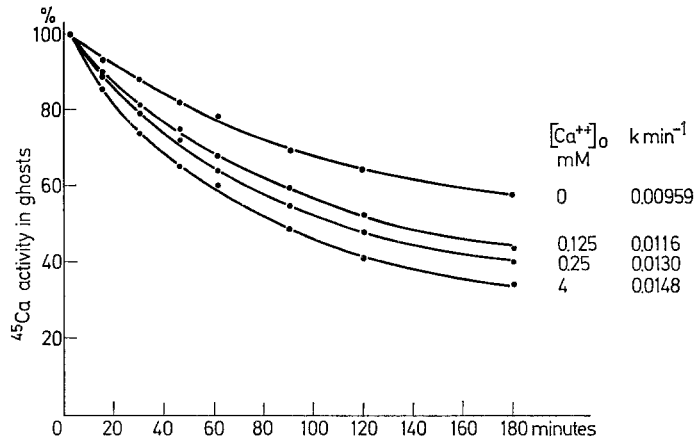


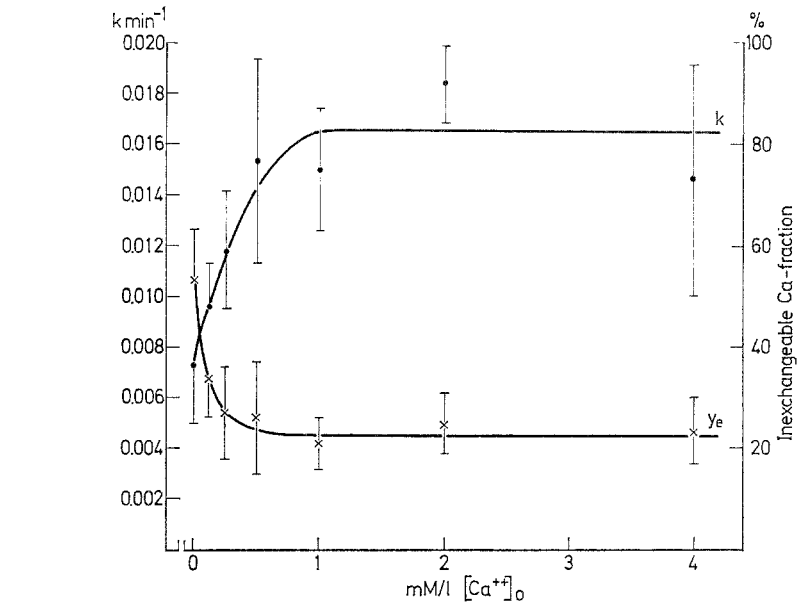
Fig. 1. Time course of ^{45}Ca efflux from human erythrocyte ghosts. Red cells were hemolyzed in a solution containing 8 mM Tris–Cl, 4 mM $MgCl_2$, 1 mM $CaCl_2$ and 160 to 200 µc/liter ^{45}Ca. Reversal of hemolysis in presence of KCl. Abscissa: efflux time in minutes. Ordinate: ^{45}Ca activity in the ghosts in percent of initial value. The four curves correspond to the results obtained with four different $[Ca^{++}]_o$ (0, 0.125, 0.25 and 4 mM) in the incubation medium (140 mM NaCl; 5 mM KCl; 20 mM Tris buffer, pH 7.4). Temperature of incubation: 37 °C. The rate constant k (min^{-1}) is calculated for each of the exponential curves. The experimental points are fitted by these exponentials by the method of the least squares. Volume ratio cells/medium in this and subsequent figures $\approx 1:50$

The calculated end points are inversely related to the $[Ca^{++}]$ in the medium and to the rate constants (k). The calculated fraction of the intracellular ^{45}Ca which remains in the cells at $t = \infty$ will, hereafter, be referred to as "inexchangeable".

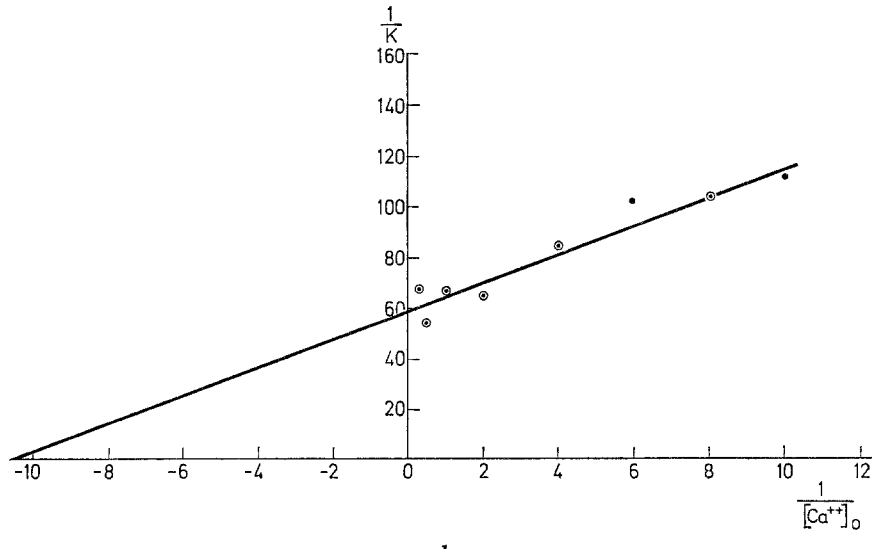
It seems unlikely that the inexchangeable intracellular Ca, as calculated for $t = \infty$, does not exchange at all with the medium. Therefore, an attempt was made to determine the rate of Ca turnover of the "inexchangeable" fraction in long-term experiments. However, even after 16-hr incubation at 37 °C, there was no equal distribution of ^{45}Ca across the membrane. The intracellular Ca concentration finally reached was never more than 5 to 6% below the calculated asymptotic value.

Where is the bound Ca located? It seemed conceivable that the inexchangeable fraction is firmly bound to the cell membrane. This possibility could be excluded by the finding that hemolyzing the cells by the addition of a detergent led to an equal distribution of Ca between cells and medium. In addition it could be shown that Mersalyl (Salyrgan^R [3]) may induce a

3 I am grateful to the Farbwerke Hoechst, Frankfurt/Main-Hoechst, for a gift of the substance.



a



b

Fig. 2. a) Effect of the $[Ca^{++}]_o$ on the rate constants of Ca efflux (k min^{-1}; left ordinate; •——•) and on the inexchangeable Ca (y_e; in percent of initial Ca content in the ghosts; right ordinate; ×——×). Same ghost preparation as in Fig. 1. Abscissa: $[Ca^{++}]_o$ in mM. Solutions and temperature as in Fig. 1. The points represent mean values of 4 to 22 experimental determinations. Vertical bars = ± SD. b) Lineweaver-Burk plot of the reciprocals of $[Ca^{++}]_o$ (abscissa) vs. $1/k$ (ordinate). The values were taken from Fig. 2a. The dissociation constant, K_m, is 0.097 mM $[Ca^{++}]_o$. ⊙ mean values; • single values

100% Ca loss from the cells without causing a significant degree of hemolysis. Finally, the addition of a complexing agent, ethylenediaminetetraacetate (EDTA), 0.5 to 5 mM, to a Ca-free solution did not remove any Ca from the cells in addition to that leaving the cells spontaneously.

In Fig. 2a, the k's of Ca efflux and the fraction of inexchangeable Ca derived from experiments similar to that presented in Fig. 1 are plotted as functions of the $[Ca^{++}]_o$.

The k increases rapidly in the concentration range between 0 and 0.5 mM $[Ca^{++}]_o$. However, k does not show a significant further increase when the $[Ca^{++}]_o$ is raised in the range between 1 and 120 mM.

This type of relationship between the $[Ca^{++}]_o$ and the k of the passive Ca efflux from the ghosts characterizes saturation kinetics and thus suggests that a carrier system participates in the passive Ca efflux. At the saturation level, in the presence of 1 mM $[Ca^{++}]_o$, the ghosts lose the incorporated ^{45}Ca about twice as fast as in absence of external Ca (k values 0.0154 ± 0.0024 and 0.00736 ± 0.00247, respectively). The inexchangeable Ca fraction decreases with increasing $[Ca^{++}]_o$. In a Ca-free medium, it amounts to $53.5 \pm 9.7\%$, whereas in the presence of 1 mM Ca in the incubation medium, only $21.4 \pm 5.4\%$ of the incorporated ^{45}Ca is "inexchangeable". As may be calculated, leakage of Ca from the cells causes an increase in total $[Ca^{++}]_o$ of no more than 0.01 to 0.02 mM after 3 hr and thus can be neglected during the course of an experiment.

To test if Michaelis-Menton kinetics may be applied to the described saturation kinetics, the reciprocals of the mean values of Fig. 2a were plotted vs. the reciprocals of $[Ca^{++}]_o$, using the method of Lineweaver and Burk (Fig. 2b). A straight line can be drawn through the experimental points. Half saturation (K_m) seems to be reached with about 0.1 mM $[Ca^{++}]_o$.

The results of experiments with constant $[Ca^{++}]_o$ (1 mM) and varying levels of $[Ca^{++}]_i$ (0 to 4 mM) are shown in Fig. 3. When hemolyzed in the absence of nonradioactive Ca but in the presence of 4 mM $MgCl_2$ and 200 μc/liter ^{45}Ca, the ghosts lost more than 90% of the incorporated ^{45}Ca during the washing procedure which preceded the final incubation. Nevertheless, these ghosts maintained a low permeability to K and Na. Thus, rate constants for Ca efflux were measured only in the range between 0.25 and 4 mM $[Ca^{++}]_i$.

In these experiments, $[Ca^{++}]_i$ refers to the concentration in the hemolyzing fluid which may differ considerably from the level of the intracellular Ca at the beginning of the experiments (Schatzmann, 1966; Schatzmann &

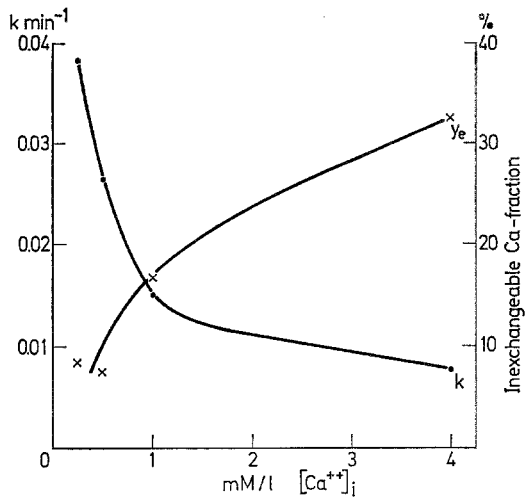


Fig. 3. Effect of the $[Ca^{++}]_i$ (abscissa, mM) on the k of Ca efflux (k min^{-1}; left ordinate), and on the inexchangeable fraction (y_e; in percent of the initial Ca content as in Fig. 2a; right ordinate). Composition of the external medium: 140 mM NaCl; 5 mM KCl; 1 mM $CaCl_2$; 20 mM Tris buffer, pH 7.4. Temperature: 37 °C. $[Ca^{++}]_i$ refers to the $[Ca^{++}]$ of the hemolyzing fluid which contained, additionally, 4 mM $MgCl_2$ and 8 mM Tris-Cl. Reversal of hemolysis in presence of KCl. The figure gives the results of one of four similar experiments

Vincenzi, 1969). Moreover, the ratio between $[Ca^{++}]_i$ and the $[Ca^{++}]$ in the hemolyzing fluid is possibly not constant over the concentration range used in the present experiments. Thus the observed relationship between k and $[Ca^{++}]_i$ could be subject to some error.

At the highest $[Ca^{++}]_i$, the k is reduced to 1/6 of that at the lowest concentration (0.0373 min^{-1} at 0.25 mM Ca^{++} and 0.00604 min^{-1} at 4 mM), whereas the ^{45}Ca efflux rose by a factor of 2.6 (0.00927 mmole/min at 0.25 mM and 0.0242 mmole/min at 4 mM). An unchanged k would have indicated a direct proportionality between $[Ca^{++}]_i$ and Ca efflux. The inexchangeable Ca fraction increased with increasing intracellular $[Ca^{++}]$ (9.9% at 0.25 mM and 26.2% at 4 mM). In the absence of nonradioactive Ca inside the ghosts, no inexchangeable ^{45}Ca could be measured. The described behavior of k and the inexchangeable Ca suggests either that the diffusional resistance of the membrane for Ca depends on the intracellular $[Ca^{++}]$ or that a saturatable transfer system participates in passive Ca efflux. Both the decrease in k with increasing $[Ca^{++}]_i$ and the increase in k with increasing $[Ca^{++}]_o$ are in agreement with the notion of a saturable Ca exchange system. However, a definite choice between the two possibilities could not be made on basis of the present information.

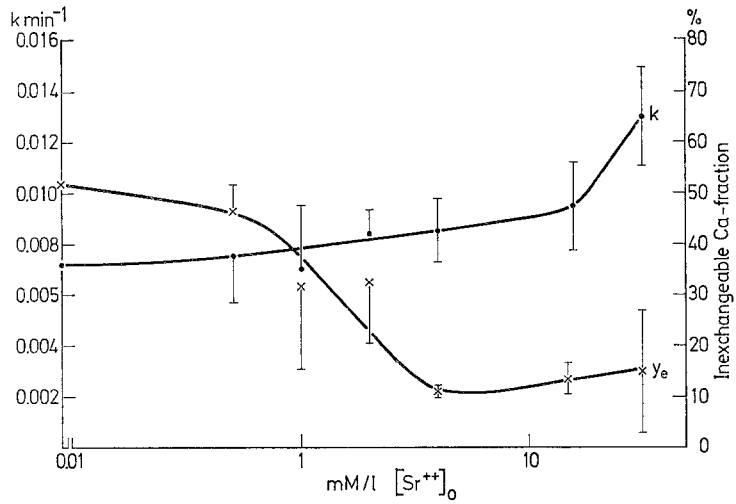


Fig. 4. Effect of the $[Sr^{++}]_o$ on the k of Ca efflux (k min^{-1}; left ordinate; •——•) and on the inexchangeable Ca (y_e; in percent of the initial Ca content; right ordinate; ×——×). Ghost preparation as in Fig. 1. Abscissa: $[Sr^{++}]_o$ in mM, logarithmic scale. Incubation media: 0.5 to 4 mM $SrCl_2$ was added to a solution containing 140 mM NaCl, 5 mM KCl and 20 mM Tris buffer, pH 7.4; temperature 37 °C. Higher concentration of Sr were achieved by replacing an equivalent amount of NaCl by $SrCl_2$. The values obtained in the absence of Ca or Sr in the medium were taken from Fig. 2a. Plotted are the results of six experiments; each point represents three determinations. Vertical bars = ± SD

Replacement of Ca by Sr

In another set of experiments, it was studied if Sr can substitute for Ca in the hypothetical transport system (Fig. 4). If applied to the outside, Sr is much less effective than Ca in activating Ca efflux. The increase in Ca efflux, evoked by 32 mM Sr^{++} in the medium, is similar to the increase produced by 0.25 mM Ca^{++}. The respective k values were 0.013 and 0.012 min^{-1}. There is a more distinct influence of Sr on the diminution of the inexchangeable Ca fraction. In the concentration range of 1 to 4 mM Sr^{++} in the medium, the fraction of inexchangeable Ca was reduced by about 60%, although the k for Ca efflux was barely affected.

Analogous results are obtained when nonradioactive Ca in the interior of the cells was replaced by Sr. At a $[Ca^{++}]_i$ of 1 mM the k of the Ca efflux was reduced to 0.0154 min^{-1}, whereas the same $[Sr^{++}]$ inside the cells decreased the Ca efflux only to 0.063 min^{-1}.

The Influence of Mg on Ca Efflux

Over the limited concentration range from 0 to 4 mM, intracellular Mg does not affect the rate of ^{45}Ca efflux in either the absence or presence of

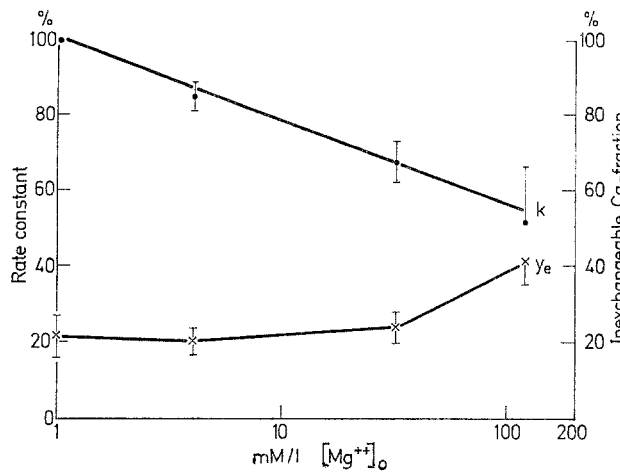


Fig. 5. Effect of the $[Mg^{++}]_o$ on the relative k of Ca efflux (k, •——•) and on the inexchangeable Ca (y_e, ×——×). Ghost preparation as in Fig. 1. Abscissa: $[Mg^{++}]_o$ in mM, logarithmic scale. Left ordinate: relative k's in percent of the value obtained in absence of Mg in the external solution. Right ordinate: inexchangeable Ca in percent of the initial Ca content. Incubation media: up to 4 mM $MgCl_2$ was added to a solution containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 20 mM Tris buffer, pH 7.4; temperature 37 °C. Higher concentrations of Mg were achieved by replacing an equivalent amount of NaCl by $MgCl_2$. Plotted are the results of five experiments, the points represent three to four determinations. Vertical bars = ± SD

intracellular nonradioactive Ca. Increasing the $[Mg^{++}]_o$ results in a slight inhibition of Ca efflux (Fig. 5). In the experiments depicted in Fig. 5, where the $[Ca^{++}]_o$ amounted to 1 mM, the k was reduced by about 48% with 120 mM $MgCl_2$ in the external solution.

The Effect of CN and Pb on Passive Ca Efflux

In the search for agents which are capable of influencing the amount of inexchangeable Ca, we studied the effects of KCN and Pb. There is some evidence that KCN may release Ca from binding sites in the squid axon (Blaustein & Hodgkin, 1969). In our experiments, 2 to 4 mM KCN was added to the standard incubation medium containing 0 or 1 mM $CaCl_2$. However, these concentrations have an effect neither on the k nor on the fraction of inexchangeable Ca.

In red cells and red cell ghosts, Pb induces a dramatic increase in passive K permeability without a concomitant change in Na permeability (Lindemann & Passow, 1960). Pb also strongly reduces the k for Ca efflux (Fig. 6). In the presence of 0.1 mM lead acetate, there was a more than 70% decrease

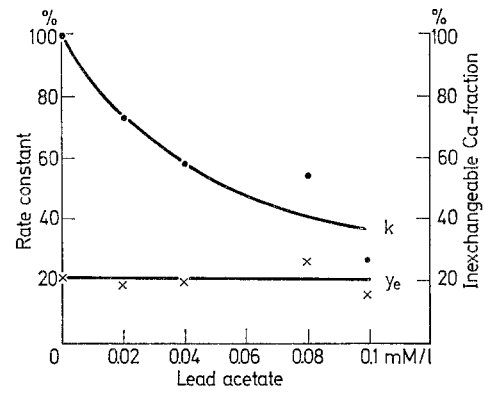


Fig. 6. Action of Pb on the relative k of Ca efflux (k, •——•) and on the inexchangeable Ca (y_e, ×——×). Ghost preparation as in Fig. 1. Abscissa: concentration of lead acetate in the external solution in mM/liter. Left ordinate: k in percent of the value measured in the absence of Pb. Right ordinate: inexchangeable Ca in percent of the initial Ca content. Incubation medium: lead acetate was added to a solution containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 20 mM Tris buffer, pH 7.4; temperature 37 °C. Plotted are the results of one of five similar experiments

in k (0.0044 min^{-1} in the presence of Pb as compared to 0.0154 min^{-1} in the absence of Pb). As in the case of Mg inhibition of Ca efflux, Ca efflux is inhibited by Pb in the presence and absence of external Ca. The size of the inexchangeable Ca fraction, however, remains unchanged in the presence of Pb.

The Influence of Alkali Metal Ions on Ca Efflux

It was interesting to study if Na competed with Ca for transport sites of the Ca-activated fraction of total Ca efflux on either side of the membrane as they probably do in the squid axon and in mammalian heart muscle. Such investigation would require constant levels of intra- and extracellular Na and K throughout the duration of the experiments. The recovery of the low permeability to K and Na is normally achieved in ghosts by preincubation at 37 °C. Erythrocytes, hemolyzed and resealed in presence of 1 to 4 mM $CaCl_2$, however, remained leaky for K and Na, thus behaving similarly to Pb-poisoned cells. The same observation was made by Hoffman (1962) and by Lepke and Passow (1966). In order to keep conditions constant, hemolyzed cells were resealed with NaCl, KCl or LiCl and then incubated in the corresponding isotonic NaCl, KCl or LiCl solution. In this manner the composition of the media on both sides of the membrane was nearly identical. This treatment, of course, made it im-

Table. *Influence of some monovalent cations on the ^{45}Ca efflux from erythrocyte ghosts*[a]

$[Ca^{++}]_o$	k min^{-1} [b]				
	Control[c]	Na[d]	K[e]	Li[f]	Cholin[g]
0 mM	0.00736 (22)	0.00888 (4)	0.00902 (2)	0.00828 (2)	0.00669 (4)
1 mM	0.0150 (15)	0.0159 (3)	0.0159 (2)	0.0151 (2)	0.0149 (3)

[a] In all experiments, cells were hemolyzed in presence of 4 mM $MgCl_2$, 1 mM $CaCl_2$ and 8 mM Tris Cl. Isotonicity during reconstitution was achieved by appropriate volumes of a 3.32 M KCl, LiCl or NaCl solution.

[b] Mean values of the rate constants given. Number of experiments in parentheses.

[c] Reconstituted with KCl, incubated in NaCl. Incubation media: 141 mM NaCl, 5 mM KCl, 20 mM Tris buffer, pH 7.4.

[d] Reconstituted with NaCl, incubated in NaCl. Incubation media: 146 mM NaCl, 20 mM Tris buffer, pH 7.4.

[e] Reconstituted with KCl, incubated in KCl. Incubation media: 146 mM KCl, 20 mM Tris buffer, pH 7.4.

[f] Reconstituted with LiCl, incubated in LiCl. Incubation media: 146 mM LiCl, 20 mM Tris buffer, pH 7.4.

[g] Reconstituted with KCl, incubated in cholinchloride. Incubation media: 146 mM cholinchloride, 20 mM Tris buffer, pH 7.4.

possible to decide between effects on the outside or the inside of the membrane. Na or K or Li had no demonstrable influence on the Ca transport system (Table).

Comparison Between Passive and ATP-Dependent Ca Efflux

Rate constants of ^{45}Ca efflux were measured in the presence and absence of intracellular ATP in order to compare the passive Ca movements described above and the active Ca transport that has been demonstrated by Schatzmann and Vincenzi (1969).

The experimental procedure involving the use of ^{45}Ca was not suited to detect transport against a concentration gradient. Nevertheless, there exist pronounced differences between the kinetics of ATP-induced and ATP-independent Ca movements which, on the basis of Schatzmann's work, seem to justify the term "active transport" for the ATP-dependent ^{45}Ca extrusion.

I first examined if the ghosts used in our experiments were sufficiently free from energy-supplying enzyme systems. To do this, the ^{45}Ca efflux was measured in the presence and absence of a substrate, 10 mM adenosine, in the standard incubation medium. Moreover, in parallel experiments, ghosts that were prepared from fresh erythrocytes were compared with ghosts from erythrocytes preincubated for 12 to 15 hr at 37 °C in a substrate-free isotonic

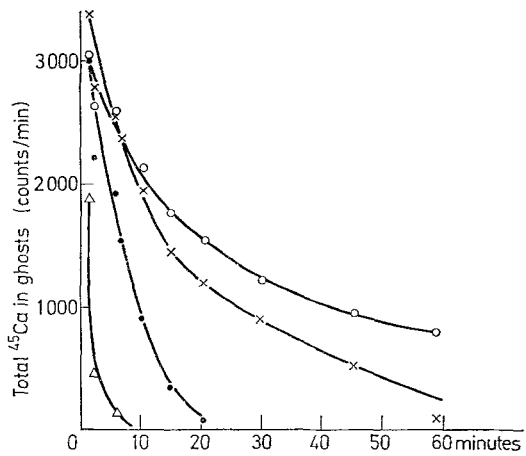


Fig. 7. The effect of intracellular ATP on Ca efflux at different extracellular $[Ca^{++}]$. Hemolysis in presence of 2 mM Tris-buffered disodium ATP, 4 mM $MgCl_2$ and 1 mM $CaCl_2$. Reversal of hemolysis in presence of KCl. Abscissa: efflux time in minutes. Ordinate: ^{45}Ca content of the ghosts suspended in 1 ml of the incubation medium in counts/min. Incubation media with 0 mM (▵——▵), 0.5 mM (•——•), 1 mM (×——×), and 2 mM (○——○) $CaCl_2$ added to a solution containing 140 mM NaCl, 5 mM KCl, 20 mM Tris buffer, pH 7.4; temperature 25 °C. The k's for 0, 0.5, 1, and 2 mM $[Ca^{++}]_o$ were 1.295, 0.0882, 0.0560, 0.0505 min^{-1}, respectively. Volume ratio of cells/medium ≈ 2:50. Plotted are the results of one of four similar experiments

NaCl medium. In none of these experiments was the rate of Ca efflux significantly different from that observed in untreated ghosts.

During the first hour, like the passive efflux, ATP-dependent efflux could be described by a single exponential, although the considerable scatter of the experimental points does not exclude the possibility that other functions fit the data equally well. In spite of this uncertainty, k's were determined as described above for passive Ca movements.

In contrast to passive Ca efflux, the k of ^{45}Ca efflux from ghosts loaded with 2 mM ATP decreased with increasing $[Ca^{++}]_o$ (Fig. 7). At 0 mM $[Ca^{++}]_o$, 1 mM $[Ca^{++}]_i$ and 25 °C, the mean value of k was 1.10 min^{-1} compared to 0.0797 min^{-1} with 1 mM $[Ca^{++}]$ in the medium. $[Ca^{++}]_o$ from 1 to 16 mM induced only a slight further reduction of k (Fig. 8).

In the presence of intracellular ATP and with a $[Ca^{++}]_o$ exceeding 1 mM, the calculated $[Ca^{++}]$ in the cells at $t = \infty$ increases with increasing $[Ca^{++}]_o$ (Figs. 7 & 8), whereas in passive efflux an inverse relationship has been observed. In isotonic $CaCl_2$ solution, 60 to 75% of the incorporated ^{45}Ca remains inside the cells at the end of active Ca extrusion. At 25 °C [4],

4 At 37 °C and a $[Ca^{++}]$ of less than 2 mM in the medium, the rate of Ca^{++} exit is too rapid to be followed by the methods employed in this study.

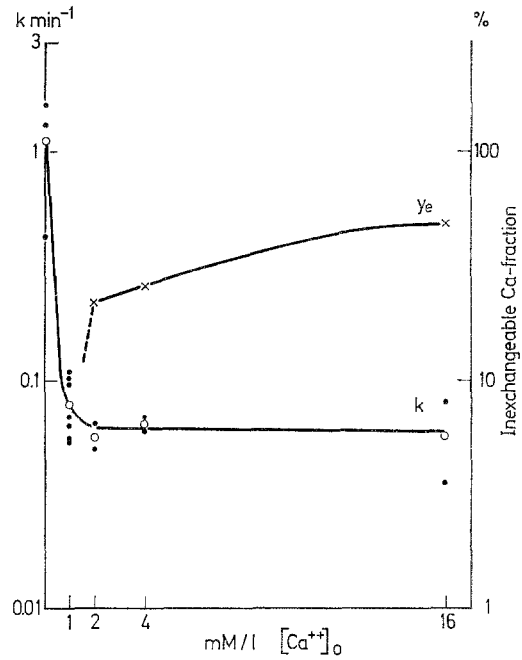


Fig. 8. Effects of the $[Ca^{++}]_o$ (abscissa, mM) on the k's of ATP-dependent Ca efflux (k min^{-1}, o——o) and on the inexchangeable Ca (y_e, ×——×). Ghost preparation as in Fig. 7. Left ordinate: k, logarithmic scale. Right ordinate: inexchangeable Ca in percent of the initial Ca content, logarithmic scale. Incubation media: up to 4 mM $CaCl_2$ was added to a solution containing 140 mM NaCl, 5 mM KCl, and 20 mM Tris buffer, pH 7.4. Higher concentrations of Ca were achieved by replacing an equivalent amount of NaCl by $CaCl_2$. Plotted are the results of eight experiments. Each point represents a single estimation; circles indicate mean values. In the y_e curve, crosses represent mean values. No inexchangeable Ca fraction was detectable in the range between 0 and 1 mM $[Ca^{++}]_o$. Volume ratio cells/medium ≈2:50

ATP-loaded ghosts expel all their ^{45}Ca at a rate which depends on the $[Ca^{++}]$ in the medium. At 0 mM $[Ca^{++}]_o$, Ca extrusion is complete after 10 min; at 1 mM $[Ca^{++}]_o$, it is complete after 50 min.

We did not succeed in inhibiting the ATP-dependent ^{45}Ca efflux with Mersalyl (Salyrgan^R). This SH-group blocking agent has been described as an unspecific inhibitor of the Ca^{++}–Mg^{++}–ATPase (Schatzmann, 1966; Wins & Schoffeniels, 1966). 0.5 mM Mersalyl at 25 °C was without any effect on the ^{45}Ca efflux from ATP-loaded ghosts. As mentioned above, at 37 °C, similar or even lower concentrations of Mersalyl drastically enhanced the membrane permeability of ATP-free cells. Thus, under our experimental conditions, an influence on passive permeability could have obscured an inhibition of active ^{45}Ca efflux.

Discussion

The values of the k's of passive ^{45}Ca efflux from human erythrocyte ghosts observed in the present experiments are higher than anticipated from previous measurements of ^{45}Ca permeability of intact erythrocytes (Rummel *et al.*, 1962; Passow, 1963; Schatzmann & Vincenzi, 1969). In an attempt to load intact erythrocytes with Ca, Schatzmann and Vincenzi (1969) found that the cells took up only very small quantities of ^{45}Ca during cold storage for periods up to 6 days. However, in contrast to the slow uptake, the rate of Ca loss from these cells was much faster and comparable to the rates observed in the present paper. The authors discuss the possibility that the high rate of Ca release was a consequence of some damage done to the membrane by rewarming the cold-stored cells. In the present experiments, the osmotic shock employed in preparing the ghosts may have acted as such an injury. The demonstration of a sizeable inexchangeable fraction of intracellular Ca (a minimum of 15 to 20% and a maximum of 60 to 70% of the total intracellular Ca) suggests that the ghost population is not homogeneous. Possibly some of the ghosts still preserved the original low Ca permeability that characterizes intact erythrocytes. In this fraction of the ghosts, Ca efflux could only be induced by chemical alteration of passive membrane permeability (Mersalyl, or hemolysis in presence of a detergent) or by stimulating the Ca pump.

The high passive Ca permeability of the rest of the ghosts apparently is not simply due to an irreversible rupture of the membrane during the hemolytic event. This can be inferred from the observation that passive Ca efflux could be promoted considerably by external Ca. At 0.5 to 1 mM $[Ca^{++}]_o$, this effect reaches a saturation level. This finding suggests that at least a large fraction of passive Ca efflux occurs through a carrier system. However, it is not certain if the fraction of total passive Ca efflux which is promoted by Ca in the external solution ("Ca-activated fraction") is identical in size with the carrier-mediated fraction of Ca efflux. This assumption would imply that the passive Ca efflux in absence of external Ca ("Ca-insensitive fraction") represents merely a "leak"-flux. The experimental results do not allow such a conclusion. On the contrary, the fact that both Ca-activated and Ca-insensitive fractions are inhibited similarly in the presence of Pb or Mg may indicate that the "true" leak-flux is considerably less than the Ca-insensitive fraction of total passive Ca efflux.

The inference that the described Ca transfer mechanism does not involve active transport is further supported by the finding that the kinetics of Ca exchange in ghosts prepared from fresh erythrocytes is similar

to that in erythrocytes which were preincubated in the absence of substrate for 13 to 15 hr at 37 °C.

The kinetics of the Ca-stimulated ^{45}Ca efflux from smooth muscle observed by van Breemen and van Breemen (1968) follows a pattern similar to that described in the present paper. These authors suggested, however, that their observations reflect a Ca exchange at binding sites on the cell surface rather than a carrier transport across the membrane. We first were tempted to interpret our results along similar lines by assuming that the exchangeable fraction is bound to the outer cell surface and that the inexchangeable fraction is located inside the ghosts. However, this hypothesis was rejected on the grounds that a destruction of the permeability barrier to Ca by Mersalyl or by a hemolytic detergent leads to a complete release not only of the inexchangeable Ca, but also of the exchangeable Ca.

An increase in the rate of Ca efflux induced by raising the $[Ca^{++}]_o$ is always associated with a decrease in the size of the inexchangeable Ca fraction. However, the rate constant does not always correlate with the size of the exchangeable fraction. Pb and, to a lesser extent, Mg were able to reduce the k without changing the inexchangeable Ca fraction. This suggests that the inexchangeable fraction is not an artifact resulting from the method of evaluating the flux measurements.

A competition between Ca and Na for the Ca carrier sites could not be demonstrated. Previous observations of the author (Porzig, 1969), which seemed to indicate such a competition, could not be confirmed. Thus, in contrast to other cells (Reuter & Seitz, 1968; Baker *et al.*, 1969), erythrocytes are probably unable to utilize the Na gradient for achieving a net Ca efflux against the electrochemical potential.

The characteristics of active Ca transport were found to be quite different from that of passive Ca movements. In contrast to passive Ca exchange, the rate of Ca extrusion against a concentration gradient in the presence of intracellular ATP decreased with increasing extracellular Ca. Moreover, the size of the inexchangeable fraction was augmented instead of being reduced by raising the $[Ca^{++}]$ in the medium. Nevertheless, the carrier participating in active and passive Ca transport through the membrane may not necessarily belong to different molecular species. There are a few observations which may suggest a common carrier for active and passive Ca transfer. Active as well as passive movements seemed saturated at about the same intracellular concentration of approximately 2 mM. Sr can substitute for Ca in active transport as well as in passive transfer (Schatzmann & Vincenzi, 1969; Olson & Cazort, 1969). Finally, neither

the active transport nor the passive transfer could be influenced by Na or K (Schatzmann & Vincenzi, 1969).

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