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TRANSPORT PARAMETERS AND STOICHIOMETRY OF ACTIVE CALCIUM ION EXTRUSION IN INTACT HUMAN RED CELLS

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Summary

Ca^{2+}-transport and its energy consumption were studied in intact human red cells loaded with Ca^{2+} by the aid of the ionophore A23187.

After the complete elimination of the ionophore the passive Ca^{2+}-permeability of the membrane returned to its normal low value, except when the intracellular Ca^{2+}-concentration was higher than 3 mM or the ATP level fell below 100 μM. Within these limits the rate of Ca^{2+}-extrusion was independent of the cellular ATP content but was greatly enhanced by increasing $[Ca^{2+}]_i$ and reached a plateau at about 1 mM intracellular Ca^{2+}-concentration. The maximum rate of Ca^{2+}-efflux was about 85 μmol/l of cells per min at 37° C, pH 7.4. The activation energy of active Ca^{2+}-extrusion was found to be 15 200 cal/mol, and the optimum pH in the suspension was 7.7.

Ca^{2+}-efflux was not connected with the counter-transport of cations.

The Ca^{2+}-pump was not affected by ouabain or oligomycin and only partial inhibition could be achieved by the SH-reagents: ethacrynic acid, *N*-ethylmaleimide and *p*-chloromercuribenzoate or with propranolol and ruthenium red. An 80 to 95% inhibition of the active Ca^{2+}-extrusion was brought about by 50—250 μM lanthanum, which in the above concentrations caused no aggregation or haemolysis. The inhibition of the Ca^{2+}-pump by lanthanum was found to be reversible, the site of inhibition being at the external surface of the cell membrane.

To examine the energy consumption of the Ca^{2+}-extrusion, ATPase activity was assessed by measuring inorganic phosphate liberation in Ca^{2+}-loaded red cells the metabolism of which was inhibited by iodoacetamide + Na^+-tetrathionate. Ca^{2+}-activated ATPase activity connected with the Ca^{2+}-pump was distinguished from other Ca^{2+}-ATPases by using the non-penetrating inhibitor, lanthanum. The molar ratio of Ca^{2+}-transported per ATP split was found to be 2 : 1.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetate.

Introduction

The active Ca^{2+}-transport of human red cells has so far been studied in Ca^{2+}-loaded resealed ghosts [1—8], in metabolically depleted [9] and in drug-treated [7,10] cells, or approached by computations based on data obtained with red cells in the presence of the Ca^{2+}-ionophore A23187 [11]. As reported recently [12] we succeeded in loading fresh red cells with Ca^{2+} by the aid of compound A23187 and subsequently eliminating the ionophore from the cell membrane completely. Thus it became possible to study Ca^{2+}-transport in a practically homogeneous population of Ca^{2+}-loaded intact red cells.

It is generally accepted that Ca^{2+}-Mg^{2+}-activated ATPase of the red cell membrane plays a fundamental role in the active Ca^{2+}-extrusion (see ref. 8). However, the amount of ATP used for active Ca^{2+}-transport has not yet been firmly established. In resealed ghosts Schatzmann and Vincenzi [2] and Schatzmann [7] estimated a Ca^{2+}/ATP molar ratio of 1 : 1, whereas Quist and Roufogalis [13] found a ratio of 2 : 1.

In the present paper experiments obtained with Ca^{2+}-loaded intact human red cells and aimed at the elucidiation of the transport characteristics and stoichiometry of the active Ca^{2+}-extrusion are described.

Materials and Methods

Washed red cells from freshly drawn heparinized human blood were used.

Chemicals: ouabain, iodoacetamide, Na^+-tetrathionate, *N*-ethyl-maleimide, *p*-chloromercuribenzoate, ruthenium red (products of Fluka A.G., Buchs), ethyleneglycol-bis-(β-aminoethylether-*N*,*N*′-tetraacetate (EGTA), oligomycin and ethacrynic acid (SERVA Feinbiochemica, Heidelberg), chlorobutanol (Richter, Budapest, propranolol (Sigma Chemical Company, St. Louis, Mo.) and $LaCl_3$ (Reanal, Budapest) were of reagent grade. A23187 was kindly provided by Ely-Lilly and Co. (Indianapolis, Indiana) and nigericin by Prof. I. Horvath. The chloride salts of lanthanides (Pr, Nd, Sm, Eu, Gd, Ho, Yb, Lu) were gifts of the Central Research Institute of Physics, Budapest, Hungary.

Na^+ and K^+ were determined with an EEL flame photometer, whereas Mg^{2+} and Ca^{2+} were measured by an atomic absorption spectrometer (Unicam SP 90) in trichloroacetic acid extracts.

Cellular ATP was determined by the luciferine-luciferase method of Stanley and Williams [14] by using the enzyme-preparation of SERVA Feinbiochemica. 2,3-diphosphoglycerate was measured by the aid of the Calbiochem 2,3-diphosphoglycerate kit. Haemoglobin was assessed by the cyanomethaemoglobin method, haematocrit by the microhaematocrit tube technique.

^{45}Ca (2.5 Ci/g) radioactivity was counted in an Intertechnique SL-30 liquid scintillation spectrometer or, in the presence of coloured compounds, in a Frieseke-Hoepfner gas-flow GM counter. For ^{45}Ca countings made with less than 0.2 ml of trichloroacetic acid extract in 10 ml scintillation liquid (Liquid Scintillator, Nuclear E.L. in toluene mixed with equal volume of absolute ethanol) no correction for quenching was needed. ^{22}Na (188 Ci/g) and ^{140}La (9.5 Ci/g) activities were measured in a Beckman Biogamma spectrometer.

Loading of human red cells with Ca^{2+} was carried out as described earlier by

Sarkadi et al. [12]. Intracellular Ca^{2+}-concentration was calculated from tracer specific activities and the original cell $[Ca^{2+}]$ was neglected. The simultaneous loading of cells with ^{22}Na and Ca^{2+} was carried out by combining the alkalication and Ca^{2+}-loading methods [12] as follows. Packed red cells were incubated in isosmotic choline chloride containing 2 μM A23187 and 5 μM nigericin, ^{22}Na or ^{45}Ca and non-radioactive Ca^{2+}. After 2 min of incubation at 37°C, the cells were washed once in ice-cold isosmotic choline chloride containing 0.5% human albumin and subsequently twice with ice-cold isosmotic KCl + 0.5% albumin solution.

Red cells were ATP-depleted either by incubating them with 2 mM iodoacetamide + 10 mM inosine in a high-K^+ (120 mM) medium at 37°C, pH 7.4 for 30—60 min or by the method of Szász et al. [15]. In the latter case cells were incubated for 4 h at 37°C with 2 mM iodoacetamide + 15 mM Na^+-bisulphite for 2,3-diphosphoglycerate depletion and then for another 2 h with 2 mM iodoacetamide + 10 mM inosine for ATP-depletion.

Tracer efflux and influx were followed as described in our previous paper [12].

ATPase activity in the red cells was estimated according to the principle of Whittam and Ager [16] as applied in the technique of Szász [17]. Inorganic phosphate liberation was measured in glucose-depleted cells treated with 2 mM iodoacetamide + 2 mM Na^+-tetrathionate.

Inorganic phosphate (P_i) was determined by the sensitive and simple method of Murphy and Riley [18], which is based on the complex formation of phosphate with potassium-antimonyl-tartarate in acidic ammonium molybdate. After reduction the complex has a stable blue colour with an absorption maximum at 880 nm. In the method simplified by us two reagents were used: reagent (A) contained 1% ammonium molybdate and 0.014% potassium-antimonyl-tartarate in 2.5 N H_2SO_4, whereas reagent (B) was 1% ascorbic acid. In the assay procedure 0.1—4.0 ml of trichloroacetic acid or perchloric acid extracts of red cells (containing 0.5—10.0 μg of P_i) were pipetted into calibrated 10.0 ml test tubes. Then 2.0 ml of reagent (A), 1.0 ml of freshly prepared reagent (B) and distilled water to a final volume of 10.0 ml were added. After 10 min at room temperature the absorbance of the samples was read at 880 nm. Readings were made within 30 min to avoid error due to ATP breakdown in the reagent mixture (less than 1% of ATP was broken down in the first 30 min).

Results

1. Characterization of the Ca^{2+}-loaded red cells

As reported earlier [12] there was no significant change, relative to untreated cells, in the ATP, 2,3-diphosphoglycerate, Na^+ and K^+ content of fresh red cells loaded with 0.5—2.0 mM Ca^{2+} by the A23187 ionophore method. Their Mg^{2+}-content (2.52 ± 0.46 mmol/l of cells; $n = 6$, ± S.D.) was not significantly different from that of untreated cells (2.62 ± 0.42 mmol/l of cells; $n = 6$) either.

Whereas Ca^{2+}-influx into Ca^{2+}-loaded intact red cells is immeasurably slow [12], these cells are highly permeable to potassium [19]. We examined the

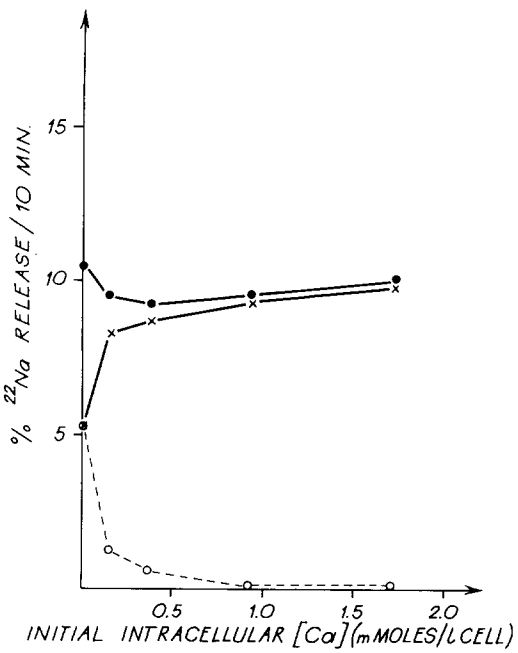

Fig. 1. Effect of intracellular Ca^{2+}-concentration on ^{22}Na-efflux from intact human red cells. Incubation medium: 120 mM KCl, 15 mM NaCl, 2.0 mM $CaCl_2$, 15 mM Tris · HCl (pH 7.4). Haematocrit 5%; temperature 37°C. •, total ^{22}Na-efflux; X, ouabain-insensitive ^{22}Na-efflux; ○, ouabain-sensitive ^{22}Na-efflux. (Ouabain-insensitive ^{22}Na-efflux was measured in the presence of 10^{-4} M ouabain).

selectivity of this permeability change and the effect of intracellular Ca^{2+}-concentration on the active Na^+-extrusion. As demonstrated in Fig. 1, ouabain-insensitive Na^+-efflux was only slightly enhanced by increasing $[Ca^{2+}]_i$, whereas ouabain-sensitive Na^+-efflux was completely inhibited at intracellular Ca^{2+}-concentration higher than 1 mM.

Ca^{2+}-loaded red cells undergo a reversible disk-sphere transformation as intracellular Ca^{2+}-concentration changes [12]. Since these shape-changes do not alter the cell volume [20,15], they could be neglected in the flux-calculations.

2. Effects of cellular Ca^{2+}- and ATP-concentration on the Ca^{2+}-transport

The rates of Ca^{2+}-efflux and influx in Ca^{2+}-loaded red cells were studied simultaneously. Fig. 2 shows the Ca^{2+}-efflux rate measured in red cells loaded with 10—5000 μM Ca^{2+}. The efflux rate increased linearly with increasing $[Ca^{2+}]_i$ up to about 1 mM and reached a plateau at higher values. The maximum Ca^{2+}-efflux rate was found to be 86.2 ± 5.6 μmol/l of cells per min at 37°C, pH 7.4 (± S.D. values, n = 24), and was constant for 20 min without the addition of glycolytic substrates. Within this period red cells with initial $[Ca^{2+}]_i$ of about 1 mM completely eliminated excess Ca^{2+} reaching a final intracellular Ca^{2+}-concentration less than 10 μM.

The rate of Ca^{2+}-efflux was practically unaffected by 2 mM Ca^{2+}, EGTA or EDTA in the incubation medium up to an intracellular Ca^{2+}-concentration of 3 mM. At higher $[Ca^{2+}]_i$ the efflux was stimulated by EGTA or EDTA and inhibited by Ca^{2+} in the medium.

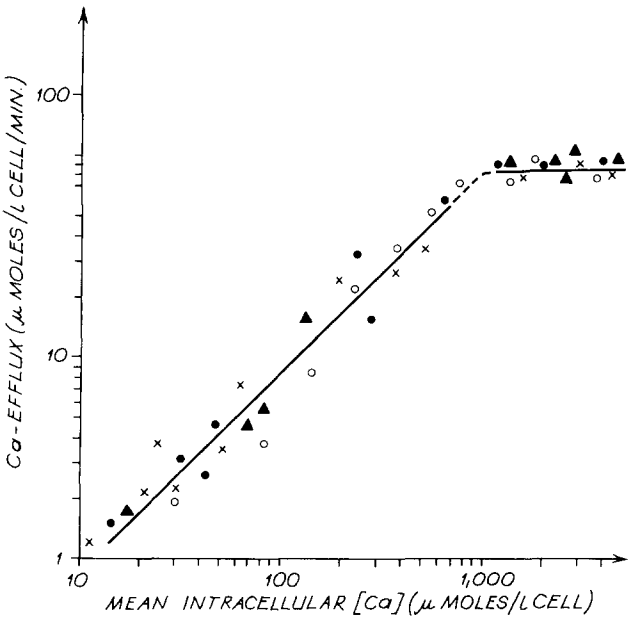

Fig. 2. Effect of intracellular Ca^{2+}-concentration on the rate of Ca^{2+}-efflux from intact human red cells. For incubation medium and conditions see the legend of Fig. 1. Data of four separate experiments (×, ●, ▲, ○).

Ca^{2+}-efflux rate did not change if cellular ATP was reduced by iodoacetamide plus inosine treatment to about 100 μM. At lower ATP-levels Ca^{2+}-efflux rate declined and became dependent on extracellular EGTA and Ca^{2+}, insofar as it was increased by EGTA and decreased by Ca^{2+}. These effects were even more pronounced in completely phosphate ester depleted, Ca^{2+}-loaded red cells (cell ATP < 1 μM).

The Ca^{2+}-influx into Ca^{2+}-loaded fresh red cells was negligible (below 1 μmol/l of cells per min) up to 3 mM $[Ca^{2+}]_i$. Above this level Ca^{2+}-influx rate increased and a slight haemolysis occurred. Again the increase in Ca^{2+}-influx rate was much more pronounced in exhaustively phosphate-ester depleted Ca^{2+}-loaded cells. The above results indicate that the passive Ca^{2+}-fluxes increase if intracellular Ca^{2+}-concentration is higher than 3 mM and cellular ATP level is lower than 100 μM (see also ref. 19). Therefore, active Ca^{2+}-extrusion was studied in cells with initial $[Ca^{2+}]_i$ not higher than 2.9 mM and flux was measured for periods within which ATP content did not decrease below 100 μM.

3. Effect of the incubation conditions on the active Ca^{2+}-transport

Owing to the high K^+-permeability of the membrane in Ca^{2+}-loaded human red cells K^+ had to be included in the incubation medium to avoid the considerable shrinkage of cells during the Ca^{2+}-flux measurements. Nevertheless, the initial rate of Ca^{2+}-efflux, measured over a 5-min interval, was not altered by the absence of external K^+. The external Na^+ could be replaced by choline without any effect on the rate of Ca^{2+}-efflux. EGTA or EDTA (2 mM) in the medium, (which practically chelated all divalent cations) and the addition of

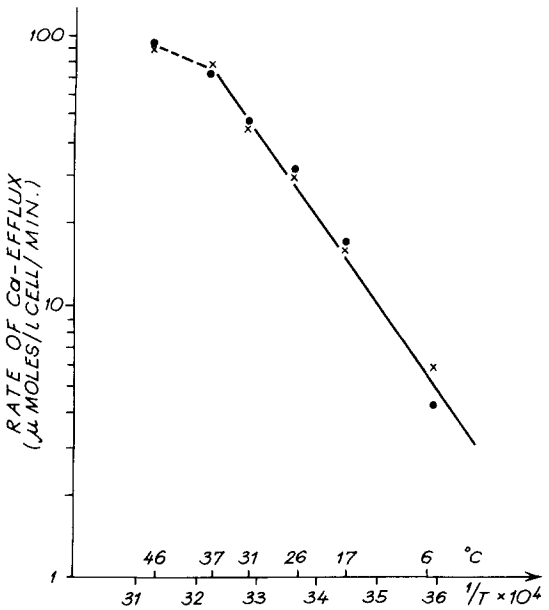

Fig. 3. Temperature-dependence of active Ca^{2+}-extrusion. Data of a representative experiment. Samples were taken at 5 (X) and 10 min (•). For incubation medium see the legend of Fig. 1. Haematocrit, 5%; initial intracellular [Ca^{2+}] 2.2 mM.

calcium, strontium, barium, magnesium or manganese ions (up to 2 mM) did not affect active Ca^{2+}-extrusion.

On examining the effect of temperature on the rate of Ca^{2+}-efflux the Arrhenius plot gave a straight line between 6 and 37° C, whereas above 37° C the slope of the curve declined (Fig. 3). The activation energy of active Ca^{2+}-extrusion estimated from the linear part of the plot was 15 200 cal/mol (average of 5 experiments). The rate of Ca^{2+}-influx was not increased at any temperature examined but slightly at 46° C.

If the pH of the suspension was reduced from pH 7.4 to 6.8 the rate of Ca^{2+}-efflux diminished by an average of 25%. Conversely, if the pH value was 7.7, Ca^{2+}-efflux rate increased by 20% and reached its maximum value. At higher pH values the Ca^{2+}-efflux rate declined rapidly. The rate of Ca^{2+}-influx was invariably low between pH 6.8 and 8.0.

4. Effects of drugs on the active Ca^{2+}-transport

Ouabain (10^{-4} M) and oligomycin (10 μg/ml) did not inhibit Ca^{2+}-efflux. Whereas iodoacetamide and Na^{+}-tetrathionate up to 3 mM had no effect on this process, 1 mM ethacrynic acid, *N*-ethyl-maleimide or *p*-chloromercuribenzoate reduced Ca^{2+}-efflux by 25—30%. At higher *p*-chloromercuribenzoate and *N*-ethyl-maleimide concentrations passive Ca^{2+}-permeability substantially increased. Propranolol (2—5 mM) caused a partial inhibition of active Ca^{2+}-extrusion (see also ref. 19). Ruthenium red inhibited Ca^{2+}-efflux to 60% at 1 mM concentration.

Lanthanum and lanthanides proved to be the most potent inhibitors of the

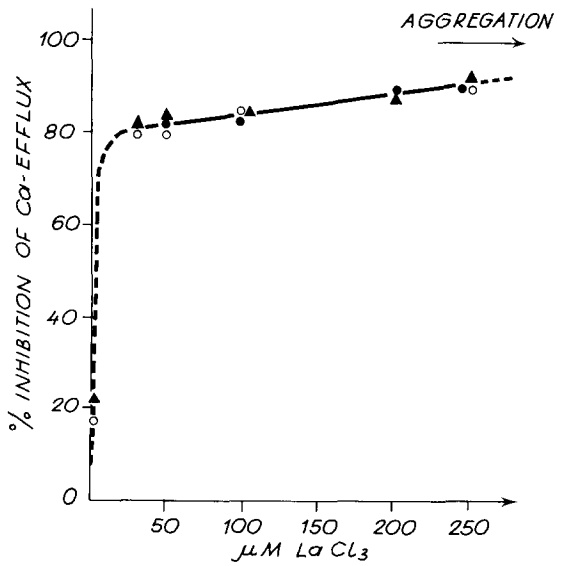

Fig. 4. Inhibition of Ca^{2+}-efflux from Ca^{2+}-loaded intact red cells by lanthanum. For incubation medium and conditions see the legend of Fig. 1. Data of three separate experiments (●, ○, ▲); 10-min incubation periods. Initial intracellular [Ca^{2+}] 1.5—2.5 mM.

Ca^{2+}-pump. As shown in Fig. 4, 250 μM lanthanum inhibited Ca^{2+}-extrusion to about 95%, and a lanthanum concentration as low as 50 μM already caused an 80% inhibition. Lanthanides Ho, Pr, Gd, Sm, etc., at a concentration of 250 μM were found to be as effective inhibitors of the process as lanthanum but the inhibition declined more rapidly toward lower concentrations. Above 280—

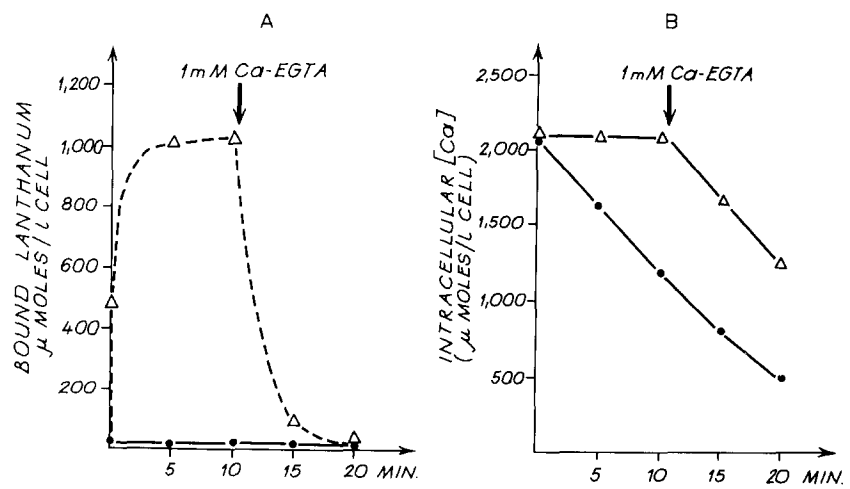

Fig. 5. Binding of lanthanum to the red cells (A) and inhibition of Ca^{2+}-efflux by lanthanum (B). For incubation medium see the legend of Fig. 1. Haematocrit, 12%; temperature, 37°C. Data of a representative experiment. Initial intracellular [Ca^{2+}] 2.1 mM ●, 0.2 mM $LaCl_3$ (in (A) supplemented with 5 μCi of $^{140}LaCl_3$) plus 1 mM Ca-EGTA; △, 0.2 mM $LaCl_3$. (In (A) supplemented with 5 μCi of $^{140}LaCl_3$).

300 μM all the lanthanides tested caused the aggregation of red cells and an increased rate of haemolysis.

The inhibition of Ca^{2+}-efflux caused by lanthanum developed immediately after adding this trivalent cation to the red cell suspension and could be reversed by adding Ca^{2+}-EGTA (Fig. 5B). (Addition of Na^{+}-EGTA caused a marked haemolysis of lanthanum-treated cells). The binding of lanthanum to the red cells was examined by the aid of radioactive ^{140}La isotope (Fig. 5A). The binding process was very rapid within the temperature range 6—37° C and after the addition of Ca^{2+}-EGTA to the medium a rapid release of lanthanum from the cells was detected.

It is to be mentioned here that increased Ca^{2+}-influx at high intracellular Ca^{2+}-concentrations (>3 mM) or at low cellular ATP levels (<100 μM) could also be inhibited by 250 μM lanthanum to about 40—50%.

5. ATPase activity in Ca^{2+}-loaded red cells. Relation of ATP consumption to Ca^{2+}-extrusion

For estimating ATPase activity the P_i liberation in red cells loaded with different amounts of calcium was measured in the presence of 2 mM iodoacetamide plus 2 mM Na^{+}-tetrathionate. As mentioned before these drugs up to 3 mM concentration had no effect on active Ca^{2+}-extrusion or on passive Ca^{2+}-fluxes. In the experiments presented in Fig. 6, the effects of 10^{-4} M ouabain and 250 μM $LaCl_3$ were studied on the rate of P_i liberation. In normal, fresh red cells the amount of inorganic phosphate liberated in the presence of metabolic inhibitors was found to be 37.5 ± 8.2 μmol/l of cells per min (n = 5; ± S.D. values), whereas in the presence of ouabain this value decreased to 22.2 ± 4.5 μmol/l of cells per min. In these control red cells lanthanum caused only a slight (non-significant) decrease in P_i liberation. Similarly, P_i liberation was not altered if cells were pretreated with A23187 as in the Ca^{2+}-loading proce-

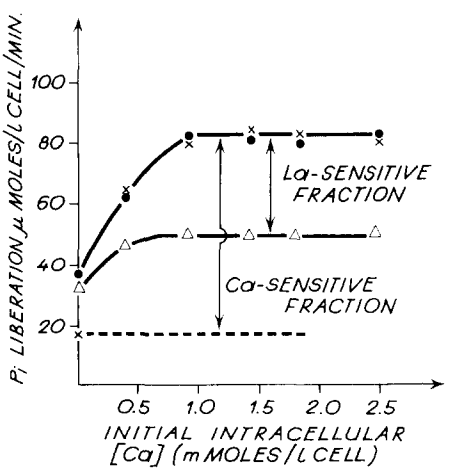

Fig. 6. Effect of intracellular Ca^{2+}-concentration on the rate of P_i liberation in intact red cells in the presence of 2 mM iodoacetamide plus 2 mM Na^{+}-tetrathionate. For incubation medium see the legend of Fig. 1. Haematocrit, 10%; temperature, 37°C. Data of a representative experiment. •, control; ×, 10^{-4} M ouabain; △, 250 μM $LaCl_3$.

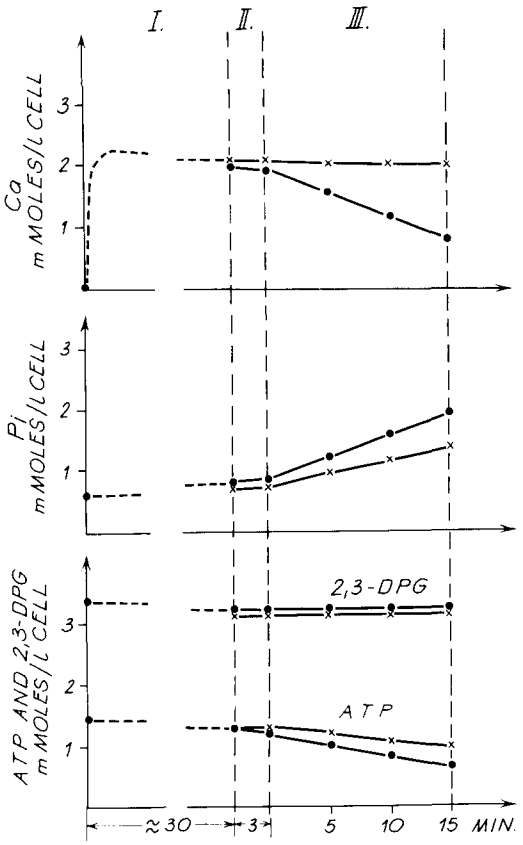

Fig. 7. Ca^{2+}-efflux, P_i liberation and changes in ATP and 2,3-diphosphoglycerate (2,3-DPG) levels in Ca^{2+}-loaded intact red cells in the presence of 2 mM iodoacetamide plus 2 mM Na^+-tetrathionate. For incubation medium see the legend of Fig. 1. Haematocrit, 11%. Data of a representative experiment. I, Ca^{2+}-loading period (0°C); II, preincubation with iodoacetamide plus Na^+-tetrathionate (25°C); III, assay period (37°C). •, control; X, 250 μM $LaCl_3$.

dure but in the presence of 0.1 mM EGTA. On raising $[Ca^{2+}]_i$ the rate of P_i liberation increased significantly. At $[Ca^{2+}]_i$ higher than 0.5 mM the ouabain-sensitivity of P_i production was lost but lanthanum decreased the rate of P_i liberation to about 50%. Both La^{3+}-sensitive and La^{3+}-insensitive ATPase activities were hardly affected by a further rise of $[Ca^{2+}]_i$ in the range of 1.0—3.0 mM.

For determining Ca^{2+}/ATP stoichiometry red cells loaded with Ca^{2+} from 1.5 to 2.9 mmol/l of cells were used. The changes in cellular ATP and 2,3-diphosphoglycerate concentrations were measured parallel with the determination of Ca^{2+}-efflux and P_i liberation. The results of a typical experiment (Table I, No. 3) are shown in Fig. 7.

After the Ca^{2+}-loading procedure (phase I in Fig. 7) the red cells were incubated for 3 min at 25°C (phase II) to develop a complete metabolic inhibition by the added iodoacetamide plus Na^+-tetrathionate. In the following 15-min period (phase III, at 37°C) Ca^{2+} was extruded at a constant rate in the control

TABLE I

Ca^{2+}-EXTRUSION AND P_i LIBERATION IN Ca^{2+}-LOADED INTACT RED CELLS IN THE PRESENCE OF 2 mM IODOACETAMIDE PLUS 2 mM Na^+-TETRATHIONATE

For incubation medium and conditions see the legend of Fig. 7.

No.	Initial $[Ca^{2+}]_i$ (mmol/l cells)	Ca^{2+}-extrusion (μmol/l of cells per min)			P_i liberation (μmol/l of cells per min)			La^{3+}-sensitive Ca^{2+}/ATP ratio
		Control	+$LaCl_3$ *	La^{3+}-sensitive	Control	+$LaCl_3$ *	La^{3+}-sensitive	
1	2.86	80.1	7.8	72.3	85.1	53.0	32.1	2.25
2	2.54	103.9	8.8	95.1	85.5	35.5	50.0	1.90
3	2.12	92.9	6.9	86.0	82.4	37.9	44.5	1.93
4	2.65	88.8	5.9	82.9	77.5	32.4	45.1	1.84
5	1.80	83.0	8.2	74.8	92.5	54.6	37.9	1.97
6	1.96	72.2	9.1	63.1	72.1	37.5	34.6	1.82
7	2.14	107.1	6.8	100.3	86.2	32.4	53.8	1.86
8	2.42	65.4	4.2	61.2	70.4	41.8	28.6	2.14
Mean	2.31	86.7	7.2	79.5	81.5	40.7	40.8	1.96
± S.D.		14.5	1.6	14.2	7.6	8.7	8.9	0.15

* 250 μM of $LaCl_3$.

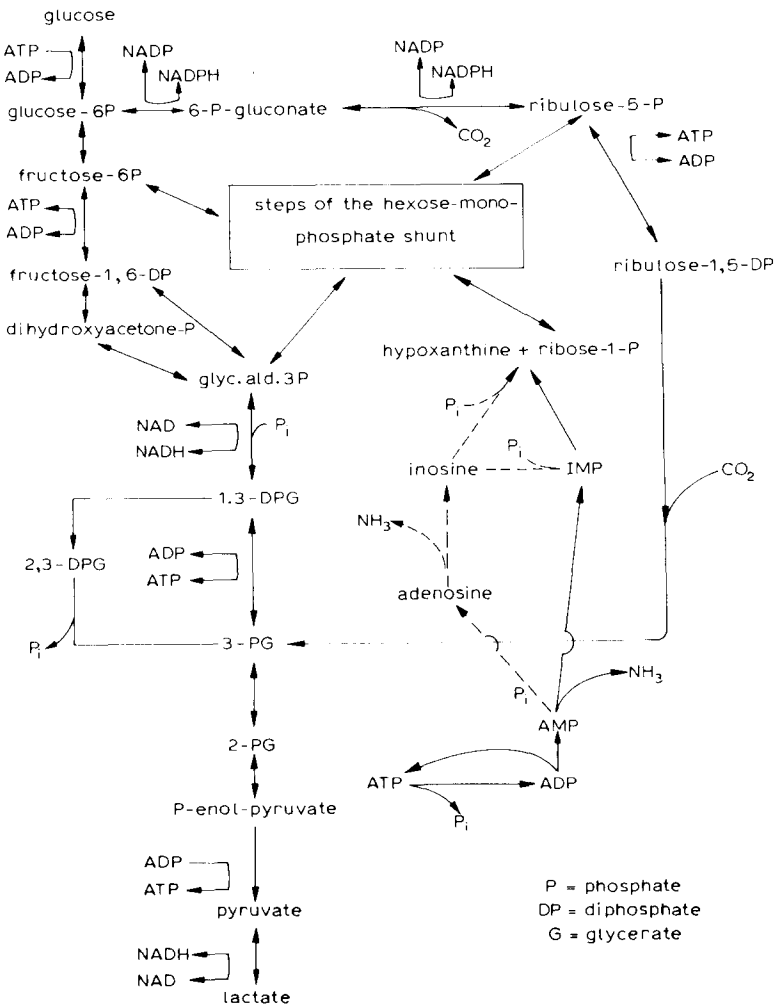

Fig. 8. The scheme of carbohydrate and adenine nucleotide metabolism in human red cells.

cells, whereas Ca^{2+}-efflux was almost completely inhibited in the presence of lanthanum. ATP splitting was reflected by the liberation of P_i in the cells. Its rate was reduced by lanthanum to about half of the control. ATP loss measured directly was also lanthanum-sensitive, while on the other hand, 2,3-diphosphoglycerate concentration did not decrease in the cells during the whole procedure.

The data for Ca^{2+}-transport and P_i liberation obtained in 8 separate experiments under the above conditions are summarized in Table I. The mean value for lanthanum-sensitive Ca^{2+}-efflux/P_i liberation was found to be 1.96 ± 0.15 (n = 8, ± S.D.), which does not differ from 2 within the limits of error. The ratio of the La^{3+}-sensitive fractions of Ca^{2+}-efflux and ATP loss measured directly was 3.24 ± 0.46 (n = 5).

Discussion

The advantages of using intact red cells for studying the functional characteristics of the Ca^{2+}-pump are obvious: the intracellular milieu is unchanged and the cell population possessing intact membranes is homogeneous. In the following the data obtained with this preparation will be compared with findings on resealed ghosts, energy-depleted cells and haemoglobin-free membranes.

Contradictory results have been published concerning the Ca^{2+}-activation of the pump-system. The experiments of Schatzmann and Rossi [3] on Ca^{2+}-loaded resealed ghosts and those of Romero and Whittam [9] on red cells treated with Ca^{2+} during starvation followed by the regeneration of ATP indicated that the intracellular Ca^{2+}-concentration that saturates the Ca^{2+}-pump is in the millimolar range. In contrast, Schatzmann [7] in ghosts loaded with Ca^{2+}-EGTA buffer found this value to be lower than 10^{-5} M. The Ca^{2+}-concentration resulting in maximum activation of the Ca^{2+}-Mg^{2+}-ATPase of isolated membranes varied from 300—500 μM [21] and 500—700 μM [3] to as low as 10—25 μM [7,22,23], depending on the method of membrane preparation. Recently Quist and Roufogalis [24] claimed that "low-affinity" membrane Ca^{2+}-Mg^{2+}-ATPase is not an "artifact", and that indeed this type of ATP splitting enzymes seems to play the basic role in the active Ca^{2+}-transport.

In intact red cells we found the lowest $[Ca^{2+}]_i$ eliciting maximum activity in Ca^{2+}-extrusion to be about 1 mM. In view of the data that over a wide range of Ca^{2+}-concentrations about 50% of intracellular Ca^{2+} is in the ionized form [7,11] our results are in good agreement with the assumption that the "low affinity" Ca^{2+}-Mg^{2+}-ATPase is involved in the active Ca^{2+}-transport.

The capacity of Ca^{2+}-extrusion in red cells seems to exceed greatly the physiological demands. The maximum efflux rate is about 85 μmol/l of cells per min, whereas the rate of Ca^{2+}-influx at 2—4 mM external $[Ca^{2+}]$ is well below 1 μmol/l of cells per min [6,19,25], and becomes increased only in certain pathological conditions [26,27]. The Ca^{2+}-leakage found in our cell preparation at high $[Ca^{2+}]_i$ and in the case of exhaustive ATP-depletion can be taken as a sign of membrane-demage.

The activation energy of the Ca^{2+}-pump in intact red cells was found to be 15 200 cal/mol, which is only slightly higher than the values obtained for resealed ghosts by Lee and Shin [4] or for isolated membranes by Quist and Roufogalis [24]. In membrane-preparations the pH optimum of Ca^{2+}-Mg^{2+}-ATPase was reported to be 7.0—7.2 [3,8]. In intact cells, in accordance with data on resealed ghosts [3] we found that Ca^{2+}-extrusion has a higher pH optimum value in the suspension, centering here about pH 7.7.

Our observations about the effect of intracellular $[Ca^{2+}]$ on the active Na^+-extrusion in intact red cells are in good agreement with the data concerning the Ca^{2+}-inhibition of K^+-Na^+-activated ATPase of isolated red cell membranes [8,21]. We could also confirm the suggestions derived from experiments with resealed ghosts that there is no counter-transport of alkali or alkali earth cations related to active Ca^{2+}-extrusion [8].

In Ca^{2+}-loaded intact red cells the effects of Ca^{2+}-transport inhibitors, especially of lanthanum, could be reliably studied. The penetration of La^{3+} into the cells could be excluded by measuring La^{3+}-binding and the inhibition

of Ca^{2+}-transport simultaneously. The rapid binding of lanthanum to the cell membrane and the La^{3+}-detachment caused by non-penetrating chelators manifested themselves in the instantaneous and reversible inhibition of the Ca^{2+}-pumping. Lanthanum does not inhibit the Na^{+}-K^{+} pump of red cells [12], in Ca^{2+}-loaded red cells it does not affect passive K^{+}-transport and shape changes [19], but hinders passive Ca^{2+}-movements caused by high $[Ca^{2+}]_i$ or low cellular ATP level. If passive Ca^{2+}-fluxes are negligible La^{3+} can be safely used for studying the energy-requirement of the Ca^{2+}-pump.

The crucial point of determining the ATP consumption related to active Ca^{2+}-transport is the reliability of the method applied. An assay procedure for ATPase in intact red cells has been reported by Whittam and Ager [16]. The principle of this method is that in human red cells with intact metabolism the P_i liberated from ATP can disappear practically only via the glyceraldehyde-3-phosphate dehydrogenase enzyme reaction. Since this P_i incorporation results in equimolar lactate production, under controlled conditions ATPase activity is given by the sum of P_i liberation and lactate production. However, the prerequisites of this method are not fulfilled if Ca^{2+}-loaded red cells are used, since several glycolytic enzymes (especially kinases) are inhibited by Ca^{2+} [28]. Nevertheless, ATPase activity can be determined in these cells by the method of Szász [17]. In this technique the incorporation of liberated P_i into the glycolytic intermediates is prevented by inhibiting glyceraldehyde-3-phosphate dehydrogenase by iodoacetamide. Under such conditions 2,3-diphosphoglycerate starts to break down producing P_i. This process, in turn, can be blocked completely by Na^{+}-tetrathionate, an inhibitor of the 2,3-diphosphoglycerate phosphatase [29]. In order to prevent P_i liberation by phosphatases from the glycolytic intermediates accumulated above the glyceraldehyde-3-phosphate dehydrogenase step the cells are previously glucose-depleted by repeated washings. As it is shown by the scheme of human red cell metabolism (Fig. 8), after these treatments the amount of P_i liberated equals the amount of ATP split by ATPases, since in human red cells the end-products of adenine-nucleotide metabolism are IMP and/or hypoxanthine plus ribose-phosphate, formed without P_i liberation [30,31].

In the experiments based on the above method endogeneous ATP is the substrate of the ATPases. During the incubation ATP is partially regenerated by the active adenylate kinase enzyme and to a lesser extent via the Lionetti-shunt [32], which produces 3-phospho-glycerate from the metabolites of the hexose-monophosphate shunt. As under these condtions, in normal red cells the Na^{+} and K^{+} activation and the ouabain-sensitivity of P_i liberation are the same as in the Whittam-Ager procedure [17] and, furthermore, since neither iodoacetamide nor tetrathionate, in the concentrations applied, has any effect on the Ca^{2+}-fluxes, the method is suitable for the determination of ATPase activity connected with Ca^{2+}-transport. On the other hand, by the aid of lanthanum, which blocks active Ca^{2+}-extrusion completely without entering the intact red cells, ATP splitting related to active Ca^{2+}-transport can be separated from other Ca^{2+}-ATPase activities.

By loading the red cells with Ca^{2+} a significant increase in both total and La^{3+}-sensitive ATPase activities can be observed reaching plateaus at $[Ca^{2+}]_i$ about 1.0 mM. Irrespective of the source of the lanthanum-insensitive, Ca^{2+}-

activated ATPase activity for the determination of the Ca^{2+}/ATP stoichiometry of the pump, cells containing Ca^{2+} at a level saturating both La^{3+}-sensitive and insensitive ATPases had to be used. The lack of ouabain-sensitivity of ATP splitting in Ca^{2+}-loaded cells reflects the inhibition of the Na^+-K^+ pump by intracellular Ca^{2+}.

The Ca^{2+}/ATP molar ratio for active Ca^{2+}-extrusion is 2 : 1, as calculated from the present experiments. Schatzmann [7] by measuring Ca^{2+}-efflux and total Ca^{2+}-activated ATPase in resealed ghosts found a molar ratio of 1 : 1. However, in his experiments the Ca^{2+}-ATPase activity not related to the Ca^{2+}-pump was not separated from the transport ATPase. On the other hand, Quist and Roufogalis [13] have got a ratio similar to ours by comparing lanthanum-sensitive Ca^{2+}-transport and ATP splitting in resealed ghosts. From experiments on red cells treated with A23187 plus Ca^{2+}, Ferreira and Lew [11] also concluded that Ca^{2+}-transport system has two equivalent binding sites for Ca^{2+}.

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