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Reversal of the Calcium Pump in Human Red Cells

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Summary. Human red cells containing low ATP and high P_i concentrations were suspended in media with and without 2 mM Ca^{2+}, and the incorporation of $(^{32}P)P_i$ into ATP was measured. There was some incorporation whatever the medium, but in every experiment there was an extra incorporation when the cells were in the Ca^{2+}-containing medium. This extra incorporation was abolished by the ionophore A23187, which collapses the Ca^{2+} concentration gradient across the membranes, or by $LaCl_3$, which blocks the Ca^{2+} pump. Starved and phosphate-loaded cells also show an uptake of Ca^{2+} which is not apparent in fresh cells. Results are consistent with the idea that Ca^{2+}-dependent incorporation of P_i into ATP is catalyzed by the Ca^{2+} pump using energy derived from the Ca^{2+} concentration gradient.

It is now accepted that the very low intracellular concentration of Ca^{2+} in human red cells is mantained by the operation of a pump that couples the hydrolysis of ATP to the outward movement of Ca^{2+} (*see* Schatzmann, 1975). Since there is no experimental evidence that the transport of Ca^{2+} is linked to the transport of other ions (Schatzmann & Vincenzi, 1969), the overall transport reaction can be written as follows:

$$nCa_i^{2+} + ATP \rightleftharpoons nCa_o^{2+} + ADP + P_i \qquad (1)$$

The value of n has been reported to be either one (Schatzmann, 1973) or two (Quist & Roufogalis, 1975; Ferreira & Lew, 1976; Sarkadi *et al.*, 1977). Equation (1) predicts that, when the Gibbs energy change necessary for the extrusion of n calcium ions is higher than that of the hydrolysis of ATP, the reaction will proceed from right to left with the synthesis of ATP catalyzed by the Ca^{2+} pump using energy derived from the calcium concentration gradient across the membrane. Reversal of ATP-driven cation pumps has been demonstrated in the Na^+ pump of red cells (Garrahan & Glynn, 1967; Glynn & Lew, 1970) and in the Ca^{2+} pump of sarcoplasmic reticulum (Hasselbach & Makinose, 1972). Fer-

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reira and Lew (1975) reported a very low ^{32}P incorporation into ATP in an attempt to demonstrate the reversal of the Ca^{2+} pump in resealed ghosts.

We have already shown that the first elementary step of ATP hydrolysis by the Ca^{2+} pump of red cells is reversible (Rega & Garrahan, 1978). In this paper we report experiments to demonstrate the reversibility of the overall reaction of the Ca^{2+} pump in human red cells.

Materials and Methods

Preparation of the Cells

Human red cells from freshly drawn heparinized blood were washed three times at room temperature with 10 vol of a solution containing (mM): NaCl, 150; $MgCl_2$, 1; ethylene-glycol-1-bis (β-aminoethyl ether) N.N'-tetra-acetic acid (EGTA), 0.1; TrisHCl (pH 7.4 at 37 °C), 10. The cells were allowed to stand for 2 min between washes and then were suspended in enough of the wash solution to give a hematocrit of 20%. The cell suspension was incubated at 37 °C for 6 hr, after which it received enough of a 55 mM solution of iodoacetamide in the wash solution to give a final concentration of 5 mM iodoacetamide. The cells were then incubated for an additional hour. In some experiments depletion of the cells was accomplished including 50 mM deoxyglucose during the incubation with iodoacetamide and omitting the six hour long preincubation. After incubation with iodoacetamide the cells were loaded with orthophosphate (Glynn and Lew, 1970) washing them with a solution of one volume of 100 mM KH_2PO_4:K_2HPO_4 (pH 7.4) buffer and two volumes of 75 mM citric acid:potassium citrate (pH 7.4) buffer. After this the cells were incubated for 10 min at 37 °C in five volumes of the same phosphatecitrate buffer labelled with (^{32}P) orthophosphate (specific activity 0.1 mCi/mmol).

Incubation Procedure

The starved and (^{32}P)P_i-loaded cells were washed with 20 vol of a solution having the same composition as the NaCl solution used for the initial wash of the cells and separated into two portions. One portion was suspended in a solution containing (mM): NaCl, 150; $MgCl_2$, 1; $CaCl_2$, 2.1; EGTA, 0.1; TrisHCl (pH 7.4 at 37 °C), 10; ouabain, 0.05; and the other in a solution of the same composition except that $CaCl_2$ was omitted. The suspensions (30 ml, 10% hematocrit) were incubated at 37 °C. After 10 min the tubes were cooled and the cells were separated by centrifugation. The packed cells were hemolyzed with 10 vol of distilled water. The amount of cells was calculated measuring the concentration of hemoglobin in the hemolyzate. The hemolyzate was deproteinized with perchloric acid (final concentration 3% vol/vol) and the perchloric acid was precipitated with KOH. The precipitated material was removed by centrifugation. A small aliquot of the clear supernatant was set aside to determine orthophosphate by the procedure of Fiske and Subbarow (1925) and total radioactivity, and the rest was used for measuring the amount of radiactive P_i and ATP.

Separation of Labeled Material

This was performed on small columns of Dowex 1 (Cl) resin (200–400 mesh, $\times 8$) 5 cm long and 0.4 cm in diameter, following the procedure already described (Garrahan

& Glynn, 1967). The amount of clear supernatant run onto each column came from 1 ml of cells. In most of the experiments ADP, AMP, and P_i were eluted together with a solution of 20 mM NH_4Cl in 0.02 N HCl, and all the ATP was collected by elution with 0.25 N HCl. Nucleotides were estimated from their absorbance at 260 nm. For each experimental condition the incorporation of (^{32}P) into ATP was calculated from the product of the relative specific activity of ATP and the intracellular concentration of the nucleotide.

Measurements of Ca^{2+} Uptake

5 ml of a cell suspension (10% hematocrit) in (mM): NaCl, 150; $MgCl_2$, 1; EGTA, 0.1; (^{45}Ca)$CaCl_2$ (specific activity 2 mCi/mol), 2.1; Tris HCl (pH 7.4 at 37 °C), 10; ouabain, 0.05; were measured into a series of glass tubes immersed in an ice-water bath. The experiment was initiated transfering the tubes to a water bath at 37 °C. One-half of the tubes were incubated for 5 min and the rest for 15 min. After incubation the tubes were returned to the ice-water bath and the cells were washed three times with an ice-cold solution having the composition of the solution used for incubation but devoid of radioactivity, followed by one wash in an ice-cold solution containing (mM): NaCl, 150; $MgCl_2$, 1; EGTA, 1; TrisHCl (pH 7.4 at 37 °C), 10. The washed cells were hemolyzed in 10 vol of 1 mM EGTA. The amount of cells was estimated by measuring the concentration of hemoglobin in an aliquot of the hemolizate. The rest of the hemolyzate was deproteinized with perchloric acid (3% vol/vol). After neutralization with KOH, the precipitated material was removed by centrifugation and the clear supernatant used for radioactivity counting. The Ca^{2+} uptake was calculated from the difference in radioactivity in the cells incubated 5 and 15 min at 37 °C.

Measurement of Ca^{2+}-ATPase Activity

This was performed in isolated membranes (Garrahan, Pouchan & Rega, 1969) following the procedure already described (Richards, Rega & Garrahan, 1977). Protein was measured by the procedure of Lowry *et al.* (1951).

Measurement of Cell Volume

Cell volume was estimated by measuring the concentration of hemoglobin in the cells (Garay & Garrahan, 1973). Hemoglobin was measured as oxyhemoglobin by its absorbance at 541 nm. The absorbance of packed cells was taken as 284.

Sources of Materials

(^{32}P)orthophosphate and (^{45}Ca)$CaCl_2$ were obtained from Comisión Nacional de Energía Atómica (Argentina) Ionophore A23187 was a kind gift of Dr. Robert L. Hamill from Lilly Research Laboratories. All salts were reagent grade.

Results

Effects of Iodoacetamide on Ca^{2+}-Dependent ATPase

One of the main difficulties in studying the incorporation of ^{32}P from P_i into ATP in red cells is the very quick ATP-P_i exchange catalyzed by

the glycolytic enzymes phosphoglycerate kinase and triosephosphate deshydrogenase. This exchange can be blocked to a large extent by 5 mM iodoacetamide acting on triosephosphate deshydrogenase (Garrahan & Glynn, 1967). It is known that 5 mM iodoacetamide has little effect on the Na^+ pump in red cells (Garrahan & Glynn, 1967). However, since the Ca^{2+} pump in red cells is more sensitive to sulfhydryl blocking agents than the Na^+ pump (Richards *et al.*, 1977), it was necessary to test the effect of iodoacetamide on the Ca^{2+}-ATPase. This was accomplished measuring Ca^{2+}-ATPase activity of fragmented membranes preincubated in media with and without 5 mM iodoacetamide. Results in Table 1 show that, although iodoacetamide partially inhibits Ca^{2+}-ATPase, there remains a substantial amount of activity after treatment with the reagent. After this finding all P_i incorporation experiments were done on cells which had been treated with 5 mM iodoacetamide for 1 hr. Control experiments (not shown) demonstrated that starvation and phosphate loading did not affect Ca^{2+}-ATPase activity in iodoacetamide-treated cells.

Effects of External Ca^{2+} on the Incorporation of (^{32}P)P_i into ATP

Table 2 shows the results of nine independent experiments in which the incorporation of P_i into ATP in cells containing low ATP and high P_i concentrations was measured in media with and without 2 mM Ca^{2+}. Results show that in the cells suspended in the medium without Ca^{2+} there is labeling of ATP. This labeling is attributable to the residual activity of glycolytic enzymes (*see* Garrahan & Glynn, 1967). In all the experiments the specific activity of ATP was higher in the cells incubated in the Ca^{2+} medium, indicating that the fraction of ATP that has exchanged its phosphate with inorganic phosphate is larger in the cells incubated in the Ca^{2+} medium. Control experiments (not shown) demonstrated that external Ca^{2+} did not significantly affect the cell volume. This, together with the fact that external Ca^{2+} did not modify the concentration of intracellular P_i (Table 2), indicates that the Ca^{2+}-dependent increase in specific activity has to be attributed to a higher rate of incorporation of P_i into ATP rather than to changes in the concentration of enzymes and intermediates involved in glycolytic ATP-P_i exchange. Results in Table 2 also show that there is a small but significant decrease in the amount of ATP present in the cells incubated in the Ca^{2+} medium.

Table 3 shows the results of an experiment in which the effect of Ca^{2+} on the incorporation of (^{32}P)P_i into ATP was measured in the

Table 1. The effects of iodoacetamide on Ca^{2+}-dependent ATPase from red cell membranes

Pretreatment	Ca^{2+}-dependent ATPase (μmol P_i/mg protein · hr)
60 min in 15 mM Tris HCl	0.412 ± 0.011
60 min in 15 mM Tris HCl + 5 mM IAA	0.244 ± 0.019

Isolated membranes were suspended in 15 mM Tris HCl (pH 7.8 at 37 °C) to give a concentration of 3 mg protein/ml and incubated at 37 °C. After incubation the membrane suspension was diluted in 2 vol of the final incubation media for ATPase assay. Determinations were performed by triplicate. Results shown are the mean ± SEM.

Table 2. The intracellular concentration of P_i and ATP and the incorporation of $(^{32}P)P_i$ into ATP in starved and phosphate loaded red cells incubated in media with and without 2 mM Ca^{2+}

	(P_i)	(ATP)	Relative specific activity	$(^{32}P)P_i$ incorporated into ATP (μmol/ liter cells hr)
	$\left(\frac{\text{mmol}}{1 \text{ cell } H_2O}\right)$		$\left(\frac{\text{cpm/mol ATP}}{\text{cpm/mol } P_i}\right)$	
Cells in 0 mM Ca^{2+}	22.4	0.147	0.079	33.6
Cells in 2 mM Ca^{2+}	22.3	0.117	0.152	48.0
Difference between the mean values of cells in 0 mM Ca^{2+} and of cells in 2 mM Ca^{2+}	−0.1	−0.030	0.076	14.4
Standard error of the difference	±0.74	±0.006	±0.014	±1.6
P values	>0.9	<0.01	<0.001	<0.001

Paired determinations of the effects of external Ca^{2+} were performed in nine independent experiments. The concentrations of P_i and ATP were calculated assuming that the water content of the cells was 0.7 (vol/vol) (Savitz, Sidel & Solomon, 1964). To allow paired comparisons of the effects of Ca^{2+}, P_i incorporation into ATP was estimated in each of the nine experiments from the product of the relative specific activity of ATP and the intracellular concentration of ATP. The mean value of the incorporation calculated in this way will differ from the product of the mean relative specific activity and the mean ATP concentration. In three experiments the concentration of ADP was also measured and found to be 0.233 ± 0.043 mmol/liter cell H_2O. Ca^{2+}-dependent incorporation of P_i into ADP was about 5% of the Ca^{2+}-dependent incorporation into ATP. The concentrations of ATP and P_i were measured at the end of the incubation. Control experiments (not shown) demonstrated that phosphate-loaded cells lose P_i with a half-time of 30 min. Therefore, the measured P_i concentration is about 80% the initial concentration of P_i. For this reason the concentration of P_i in the initially P_i-free incubation media must have been about 0.5 mM at the end of the experiment. This amount of P_i will reduce in about 0.12 mM the concentration of free Ca^{2+} in the external media. *P* values were determined using Student's *t* test for paired samples (not significant $P = 0.05$). All solutions contained 0.05 mM ouabain to avoid P_i incorporation by reversal of the Na pump (Garrahan & Glynn, 1967).

Table 3. The effects of the ionophore A23187 and of La^{3+} on the incorporation of $(^{32}P)P_i$ into ATP in starved and phosphate-loaded cells

Conditions	Relative specific activity $\left(\frac{\text{cpm/mol ATP}}{\text{cpm/mol } P_i}\right)$
EGTA	0.175
Ca^{2+}	0.322
EGTA + 5 μM A23187	0.130
Ca^{2+} + 5 μM A23187	0.147
EGTA + 100 μM $LaCl_3$	0.180
Ca^{2+} + 100 μM $LaCl_3$	0.162

The experimental procedure was identical to that of the experiments in Table 2.

presence of either the Ca^{2+} ionophore A23187 or $LaCl_3$. The ionophore abolishes in a few minutes the Ca^{2+} gradient across the cell membrane (Sarkadi, Szász & Gárdos, 1976). Results demonstrate that the Ca^{2+}-ionophores A23187 sharply decreases the Ca^{2+}-dependent incorporation of P_i into ATP. This suggests that Ca^{2+}-dependent incorporation requires the transmembrane Ca^{2+} gradient. The effect of A23187 allows us to discard the posibility that the Ca^{2+}-dependent incorporation of P_i into ATP is caused by an effect of Ca^{2+} on glycolysis, since if this were so there seems to be no reason for this effect to be absent in ionophore-treated cells. The experiment with $LaCl_3$ (Table 3) was performed to see whether Ca^{2+}-dependent P_i incorporation into ATP requires the functioning of the Ca^{2+} pump. Results show that the effect of Ca^{2+} is absent when the incubation medium contains 0.1 mM $LaCl_3$, concentration high enough to fully block the Ca^{2+} pump (Quist & Roufogalis, 1975; Sarkady *et al.*, 1977).

Calcium Uptake

Table 4 summarizes the results of seven separate experiments in which the uptake of Ca^{2+} was measured in red cells submitted to an identical treatment to those used for the experiments in Table 2. Starved and phosphate-loaded cells take up calcium at a significant rate. This behavior contrasts with that of fresh cells which, under the same experimental conditions, show no detectable uptake of ^{45}Ca (Table 4), a fact which agrees with findings by others (Lew, 1971).

Table 4. The uptake of Ca^{2+} by fresh and by starved and phosphate-loaded red cells

	Ca^{2+}-uptake (μmol/liter cells hr)
Fresh cells	-0.25 ± 0.52 $(n=3)$
Starved cells	22.27 ± 1.90 $(n=7)$

Starved and phosphate-locaded cells were treated as those in Table 2. Figures are the mean ± SEM. The number of experiments is given in parentheses.

Discussion

Results presented in this paper demonstrate that external Ca^{2+} increases the rate of incorporation of P_i into ATP in partially starved and phosphate loaded red cells. The effect of external Ca^{2+} on the incorporation disappears either when the Ca^{2+} concentration gradient across the membrane is abolished with A23187 or when the Ca^{2+} pump is blocked with La^{3+}. Results are consistent with the idea that Ca^{2+}-dependent incorporation of P_i into ATP is catalyzed by the Ca^{2+} pump using energy derived from the Ca^{2+} gradient.

The overall Gibbs energy change (ΔG_t) for active Ca^{2+} transport coupled to the hydrolysis of ATP in red cells [Eq. (1)] can be calculated as

$$\Delta G_t = \Delta G^{\circ\prime} + RT \ln (\mathrm{ATP})_i/(\mathrm{ADP})_i(\mathrm{P_i})_i + nRT \ln (\mathrm{Ca^{2+}})_o/(\mathrm{Ca^{2+}})_i + nZFE \qquad (2)$$

where $\Delta G^{\circ\prime} = -30.5$ kJ/mol (Lehninger, 1975), n is the number of Ca^{2+} ions transported per molecule of ATP hydrolyzed, E is the membrane potential (external minus internal) and the subscripts i and o mean intracellular and extracellular respectively. R, T and F have their usual meanings.

The application of Eq. (2) to Ca^{2+} transport in red cells is hampered by two uncertainties; (i) no precise values for $(Ca^{2+})_i$ is yet available and, (ii) as it was mentioned in the introduction, the value of n is still a matter of dispute.

In Fig. 1 the value of ΔG_t at constant (2×10^{-3} M) external Ca^{2+} concentration is plotted as a function of internal Ca^{2+} concentration. 10^{-7} M was taken as a lower limit for internal free Ca^{2+} in fresh cells (Simons, 1976). In the case of starved and phosphate-loaded cells data in Table 4 show that, if all the Ca^{2+} that enters the cells remains in

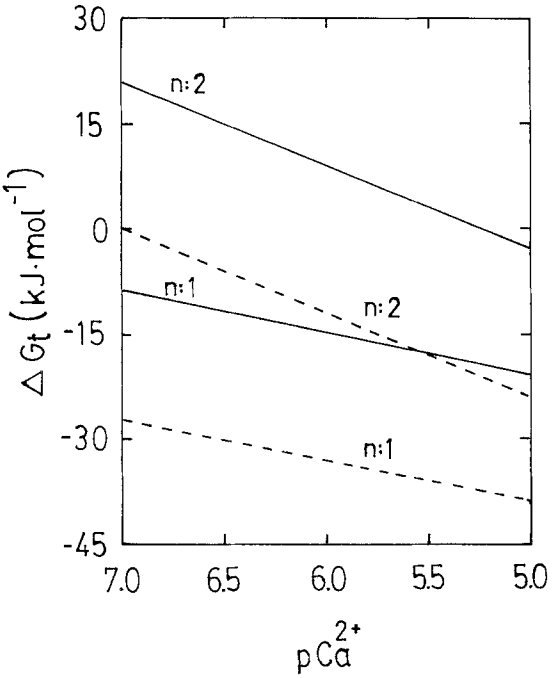

Fig. 1. The total Gibbs energy change of active Ca^{2+} transport as a function of internal Ca^{2+} concentration and at constant (2×10^{-3} M) external Ca^{2+} in fresh (----) and in starved and phosphate-loaded (——) red cells. ΔG_t was calculated using Eq. (2) for both $n = 1$ and $n = 2$. The following values were used: (ATP) $= 1.5 \times 10^{-3}$ M; (ADP) $= 0.32 \times 10^{-3}$ M; $P_i = 0.36 \times 10^{-3}$ M; $E = 0.01$ V, for fresh cells (Garrahan & Glynn, 1967) and: (ATP) $= 0.13 \times 10^{-3}$ M; (ADP) $= 0.23 \times 10^{-3}$ M; $P_i = 22.4 \times 10^{-3}$ M (*see* Table 2) for starved cells. In these cells, E was calculated to be 0.023 V by applying Nernst equation to the intra/extracellular chloride concentration ratio, assuming that 22.4×10^{-3} M intracellular P_i has displaced 44.8×10^{-3} M Cl^-. This calculation disregards the increase in the value of E that might result from the Ca^{2+}-dependent increase in the permeability to K^+ (*see* Simons, 1977)

solution, the intracellular concentration of Ca^{2+} will be 5×10^{-6} M ($pCa^{2+} = 5.3$) at the end of the incubation time. ΔG_t was calculated for both $n = 1$ and $n = 2$, taking the concentrations of ATP, ADP, and P_i of fresh cells and of cells whose content of nucleotides and of P_i is that of the cells used in the experiments reported in this paper. It can be seen that when $n = 1$, ΔG_t is negative for both fresh and starved cells in the whole range of $(Ca^{2+})_i$ represented. On the other hand, when $n = 2$, ΔG_t is positive up to 5.6×10^{-6} M Ca^{2+} ($pCa^{2+} = 5.25$) for cells with the composition of the starved cells, and zero or negative for fresh cells. Therefore if $n = 2$, Ca^{2+}-dependent incorporation of P_i into ATP in starved cells could represent net synthesis of ATP. If n were 1, net

hydrolysis rather than net synthesis of ATP is to be expected. However, it can be shown that the rate of the backwards component of the ATPase reaction will reach measurable levels in starved and phosphate-loaded cells since in these cells ΔG_t is 18 kJ/mol less negative than in fresh cells. A brief calculation may illustrate this point. Figure 1 shows that for $n=1$, at an intracellular Ca^{2+} concentration of 10^{-6} M, ΔG_t is -15 kJ/mol. It can be calculated that this value of ΔG_t corresponds to a reaction in which the ratio between backward to forward rates is 0.0296. If 14.3 μmol/liter cell·hr is taken as the backward rate of the ATPase reaction, the forward rate will be 480 μmol/liter cell hr. This value is not very different from 400 μmol/liter cells hr which is the rate for ATP hydrolysis that can be calculated for cells containing 10^{-4} M ATP and 10^{-6} M Ca^{2+} (Richards, Rega & Garrahan, 1978) and treated with 5 mM iodoacetamide.

It can therefore be concluded that for both $n=1$ and $n=2$ there are no thermodynamic restrictions against the idea that Ca^{2+}-dependent P_i incorporation into ATP in starved and phosphate-loaded cells is catalyzed by the Ca^{2+} pump using energy derived from the Ca^{2+} gradient.

On the basis of the results of this paper, it is difficult to decide whether Ca^{2+}-dependent P_i incorporation into ATP represents a net synthesis of ATP or ATP-P_i exchange during net hydrolysis of the nucleotide. The small, but significant, decrease in the concentration of ATP observed in cells incubated in media containing Ca^{2+} seems to favor the idea that there is net hydrolysis of ATP caused by activation of the Ca^{2+} pump. However, it cannot be discarded that intracellular Ca^{2+} may stimulate ATP hydrolysis unrelated to the Ca^{2+} pump or that, under these conditions, Ca^{2+} activates the Ca^{2+} pump without being transported.

The main argument for the idea that net synthesis of ATP is taking place during the incorporation of P_i into ATP lies in the fact that cells which show this phenomenon also show an uptake of Ca^{2+} which is not apparent in fresh cells. However, even if we accept that all the Ca^{2+} has entered the cells through the Ca^{2+} pump working in a reversed mode, it remains to be explained why under these conditions the ratio between Ca^{2+} uptake and P_i incorporation is 1.55 instead of 2.

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