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STIMULATION OF MONOVALENT CATION FLUXES BY ELECTRON DONORS IN THE HUMAN RED CELL MEMBRANE

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Summary

When human red cells are incubated at 37°C with the artificial electron donor system ascorbate + phenazine methosulphate the fluxes of Rb^+ (K^+) through the cell membrane are increased. The effect of this donor system is much stronger in energy-depleted than in normal cells. The same effects are produced by HS-glutathione, NADH or NADPH loaded into resealed ghosts, but these electron donors were ineffective when added to the incubation medium. The Rb^+ (K^+) fluxes induced by electron donors resemble closely those induced by an increase of intracellular Ca^{2+} (Gardos effect). The electron donors require the presence of intracellular Ca^{2+} to be effective, but at levels that do not stimulate by themselves the fluxes of K^+. Flavoenzyme inhibitors (atebrin and chlorpromazine), oligomycin and quinine prevented the effects of both electron donors and Ca^{2+} alone; antimycin, uncouplers and ethacrynic acid inhibited them partially; ouabain, furosemide, and rotenone had no effect.

The results could be explained if the effect of electron donors is to bring about a change in the redox state of some membrane component(s) that makes intracellular Ca^{2+} more effective to elicit rapid K^+ movements. Plasma membrane oxidoreductase activities could be engaged in this change.

Redox functions in the plasma membrane of animal cells have received considerable interest in the last few years. Oxidorectuase activities have been found in the plasma membrane of several cell lines [1] and their possible participa-

Abbreviations: asc-PMS, 20 mM ascorbate + 0.1 mM phenazine methosulphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid; HEDTA, *N*-hydroxyethylethylenediamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

tion in the regulation of adenylate cyclase activity [2,3] or the energization of active transport [4] have been suggested. Several NADH-dehydrogenase and diaphorase activities have been described in red cell membranes [5,6], in some instances associated to preparations of the $(Na^+ + K^+)$-ATPase [7]. On the other hand, the mature mammalian erythrocyte has the unique feature of lacking mitochondria, and hence any oxidoreductase associated to it.

As an initial approach to test whether these redox systems of the plasma membrane bear some relationship with transport processes, we have studied the effects of electron donors on the fluxes of Rb^+ (K^+) and Na^+ in intact red cells and resealed ghosts.

Material and Methods

Freshly drawn human blood was collected using heparin as the anticoagulant. The blood was centrifuged at 3000 rev./min for 5 min, the plasma and buffy coat were removed, and the red cells washed 3—4 times by centrifugation in a solution of 0.15 M NaCl.

Experiments with intact erythrocytes

(i) Depleted cells with low ATP levels were prepared by incubation of the cells (hematocrit 10%) for up to 30 h in the substrate-free medium, or for up to 8 h in the presence of either 1 mM sodium iodoacetate, 18.2 mM sodium arsenate (replacing phosphate) or 5 mM 2-deoxy-D-glucose. (ii) Normal non-depleted cells were generally used after a previous incubation of 4 to 6 h in medium containing 10 mM glucose. The intracellular concentrations of Na^+ and K^+ were only moderately modified by the ATP-depletion treatments used in this study. For example, in a set of 4 experiments, the final levels of Na^+ and K^+ in cells treated with 1 mM iodoacetate during 6 h were (mean ± S.D.) 27 ± 3 and 134 ± 2 mmol/kg intracellular water, respectively. In cells of the same batch incubated in parallel with glucose these levels were 20 ± 2 and 159 ± 4. After the incubation period with or without the inhibitors, the cells were washed 3 times with ice-cold 0.15 M NaCl, resuspended in the same solution at a hematocrit of 50% and used immediately for the uptake experiments.

Uptake experiments were started by adding 0.3 ml of the 50% cell suspension to 3 ml of medium at 37°C containing $^{86}Rb^+$ ($2—5 \cdot 10^5$ cpm/ml) or $^{22}Na^+$ ($2 \cdot 10^6$ cpm/ml). The uptake period was terminated by mixing the contents of the incubation flasks with 3 volumes of ice-cold 0.15 M choline chloride. The cells were sedimented by centrifugation in the cold at 3000 rev./min for 5 min and washed twice with 10-ml aliquots of ice-cold choline chloride. The supernatant was aspirated and the residual fluid removed as much as possible form the cell pellet by touching it with pointed strips of filter paper. The pellets were weighed and extracted with 1 ml of 0.6 N perchloric acid or 6% trichloroacetic acid. $^{86}Rb^+$ or $^{22}Na^+$ uptake were estimated from the radioactivity present in the cell pellets. $^{86}Rb^+$ uptake was expressed either as the distribution ratio (cpm per ml of intracellular water/cpm per ml of medium) or as the inward rate constant (distribution ratio/incubation time). Since Rb^+ and K^+ behave similarly with regard to transport [8—12], the equivalent K^+ uptake can be estimated as the product inward rate constant × extracellular K^+ concentra-

tion. Na^+ uptake was expressed in mmol/kg intracellular water per h (inward rate constant × extracellular Na^+ concentration).

For efflux experiments, the cells were first incubated (hematocrit 25%) during 6 h at 37°C in medium containing $^{86}Rb^+$ ($2 \cdot 10^5$ cpm/ml) or $^{22}Na^+$ ($4 \cdot 10^6$ cpm/ml) and either 1 mM iodoacetate (depleted cells) or 10 mM glucose (normal cells). After this step, the cells were washed 4 times with ice-cold 0.15 M choline chloride and resuspended in the same solution to a hematocrit of 50%. Aliquots of this suspension were diluted with standard medium (hematocrit 4%) and incubated at 37°C for 5 or 15 min. When $^{86}Rb^+$ exodus was studied, the incubation period was terminated and the flask contents processed as described for the uptake experiments. The exodus of $^{86}Rb^+$ was estimated from the difference between the radioactivity present in the cells at the beginning and at the end of the incubation period, and was expressed as the per cent of $^{86}Rb^+$ remaining in the cells. $^{22}Na^+$ exodus was estimated from the difference between the radioactivity present in the medium at the beginning and at the end of the incubation. In this case the incubation was terminated by 2 min centrifugation of the flask contents at 12 000 × *g* in the cold. Cell Na^+ concentration was determined in the zero-time samples in order to calculate their specific activities, and Na^+ efflux was expressed as mmol/kg intracellular water per h. Total water in the cell pellets was determined from the loss of weight after 12 h at 100°C. The extracellular space was estimated as the sucrose space.

Experiments with resealed ghosts

Resealed ghosts were prepared either from normal cells or from depleted cells by the method of Bodemann and Passow [13]. Hemolysis was accomplished by rapid mixing of 1 vol. of a 50% cell suspension in 0.17 M Tris-HCl, pH 7.45, with 10 vols. of a 3.6 mM $MgSO_4$ solution. After 5 min, 0.5 vol. of a 1.6 M NaCl/1.6 M KCl solution was added (final concentration of Na^+ and K^+, 70 mM), mixed quickly, and allowed to stand for 10 min. All these steps were carried out at 4°C. Resealing was accomplished by a 30 min incubation at 37°C. For loading of the ghosts with different substances, these were dissolved in the hemolyzing 3.6 mM $MgSO_4$ solution. Fixed concentrations of free calcium were obtained by the use of mixtures containing $CaCl_2$ and several chelators. Concentrations of free calcium were calculated using the following values for the apparent association constants for calcium corrected for pH 7.4, the ionic strength and the Mg^{2+} content of the medium [14–16]: Ethyleneglycol-bis-(β-amino-ethyl ether) *N,N'*-tetraacetic acid (EGTA), $6.5 \cdot 10^6$; *N*-hydroxyethyl-ethylenediamine (HEDTA), $6.8 \cdot 10^4$. From now on, free calcium will be refered to as Ca^{2+} and total calcium (free + bound) as Ca.

Freshly prepared resealed ghosts were sedimented by centrifugation in the cold (10 min at 35 000 × *g*), washed 3 times with 20 volumes of ice-cold choline chloride, and resuspended in the same solution to about 50%. Initial intracellular water content per ml of this ghost suspension was calculated from its contents of Na^+ and K^+ under the assumption that the intracellular concentrations of Na^+ and K^+ in the ghosts were 70 mM. This was routinely checked by microhematocrit determinations.

For the uptake experiments, 0.1 ml of the ice cold 50% ghosts suspension

were mixed with 0.4 ml of ice-cold incubation medium containing $^{86}Rb^+$ in a 1.5 ml Eppendorf tube. The tubes were immediately transferred to a water bath at 37°C and incubated during 5 min with continous shaking. The incubation period was terminated by 5 min centrifugation at 13 000 × *g* in the cold. The pellets were washed twice with 1-ml aliquots of 0.15 M choline chloride, and extracted with 0.5 ml of 6% trichloroacetic acid. The concentrations of $^{86}Rb^+$, Na^+ and K^+ in the ghosts were calculated with reference to their initial intracellular water. $^{86}Rb^+$ uptake was expressed as the inward rate constant, calculated as described above for the intact cells.

Incubation media

For the intact cells, the standard incubation medium was a modified Krebs-Ringer-phosphate solution containing (mM): NaCl, 118.7; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 0.5; sodium phosphate buffer, 17, pH 7.4. In most experiments, equiosmolar HEPES/NaOH buffer replaced phosphate, similar results being obtained with both buffers. For the experiments with resealed ghosts, the incubation medium had the following composition (mM): NaCl, 130; KCl, 6; $MgCl_2$, 1; HEPES/NaOH buffer, 20, pH 7.4. Sodium ascorbate and phenazine methosulphate were added to the incubation medium from freshly prepared concentrated solutions to give final concentrations of 20 and 0.1 mM, respectively, and replaced an equimolar amount of NaCl. Other additions to the medium are specified in the legends of tables and figures. When media with different concentrations of Na^+ or K^+ were used, choline replaced these cations to keep the osmolarity of the medium at 305 ± 5 mosM.

Analytical procedures

Radioactivity was measured by liquid scintillation counting, using a toluene-ethanol counting solution [17]. Na^+ and K^+ were determined by flame photometry, ATP by a fluorometric procedure [18], and sucrose by a spectrophotometric procedure [19]. Hemoglobin was determined by measuring the absorbance at 415 or 541 nm. None of the electron donors produced measurable hemolysis during the incubation periods used in this study. On addition of ascorbate + phenazine methosulphate (asc-PMS), the cell suspension darkened within a few seconds. This phenomenon was probably due to the formation of methemoglobin by H_2O_2 produced by spontaneous oxidation of reduced PMS. It appeared also with hemolysates and was not prevented by any of the inhibitors used in this work, which otherwise prevented the effect of asc-PMS on transport.

Chemicals

$^{86}RbCl$ and $^{22}NaCl$ were purchased from The Radiochemical Center, Amersham. Ethacrynic acid, furosemide and chlorpromazine were gifts from Merck Sharp & Dhome, Hoechst Iberica and Rhodia Iberica, respectively. Other chemicals were obtained either from Sigma London Chem. Co. Ltd. or from E. Merck, Darmstadt.

Results

Effects of electron donors on the uptake of $^{86}Rb^+$ by the intact erythrocyte. Dependence on the cellular level of ATP

In a series of preliminary experiments, it was found that asc-PMS stimulated the uptake of $^{86}Rb^+$ by the cells. In normal cells this effect was not constant (appearing in 6 out of 11 experiments), and the magnitude of the stimulation in short (2—5 min) incubation periods ranged between 1.4 and 7-fold of the control values. In ATP-depleted cells the stimulation of $^{86}Rb^+$ uptake appeared consistently in all the experiments, the effect being much stronger than in normal cells and presenting a definite time course. Fig. 1 shows the time course of the stimulation of $^{86}Rb^+$ uptake by asc-PMS in a representative experiment with depleted cells. The distribution ratios reached were higher than 1 in incubation periods of 5 to 20 min. The effect of asc-PMS was stronger in short incubation periods, the uptake of $^{86}Rb^+$ remaining reasonably linear during the first 5 min. This incubation period was chosen for the study of the initial rate of uptake in subsequent experiments. PMS alone had no significant effects (Fig. 1).

Fig. 2 shows the effect of asc-PMS in cells containing different levels of ATP. When cell ATP was higher than 0.5 mM the stimulation of $^{86}Rb^+$ uptake by asc-PMS was relatively small, but it increased strikingly when ATP was under 0.3 mM, the uptake of $^{86}Rb^+$ reaching values near 100 times the control values at ATP levels close to zero. The magnitude of this stimulation was largely independent of the type of treatment used to lower the cellular ATP.

The effect of asc-PMS was reversible. If depleted cells treated with the electron donor for 5 min were washed once and resuspended in fresh medium containing no asc-PMS, the uptake of $^{86}Rb^+$ returned to the values found in non-treated cells.

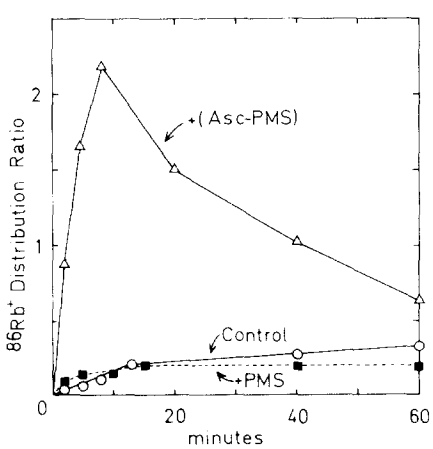


Fig. 1. Time course of the effect of asc-PMS on the uptake of $^{86}Rb^+$ by iodoacetate-treated (6 h) depleted cells.

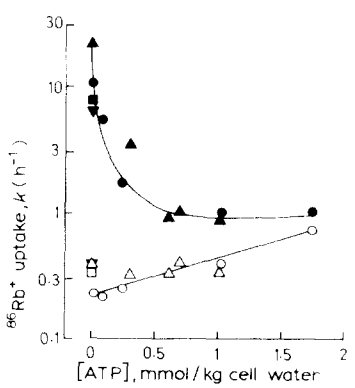


Fig. 2. Effect of the cellular level of ATP on the uptake of $^{86}Rb^+$ induced by asc-PMS. Cells were first incubated during different periods in medium containing no substrate (circles), 1 mM iodoacetate (triangles), 17 mM aresenate (squares) or 5 mM 2-deoxy-D-glucose (inverted triangles). Uptake of ^{86}Rb was studied during 5 min in medium with (solid symbols) or without (open symbols) asc-PMS, and is expressed as the inward rate constant (h^{-1}). Note the logarithmic scale on the ordinate axis.

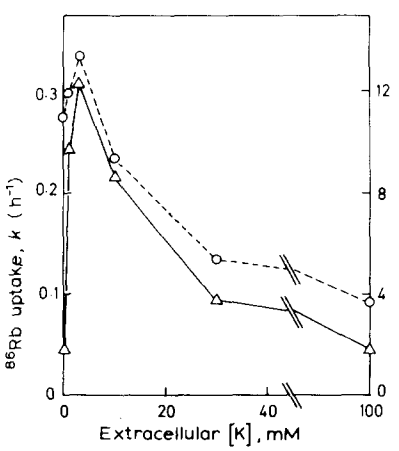
Other reducing agents tested (10 mM dithiothreitol, 20 mM ascorbate, either alone or together with 0.2 mM TMPD, 10 mM sodium bisulphite) produced much smaller or no effects on ^{86}Rb uptake during incubation periods of up to 30 min.

Effects of extracellular K^+ on the stimulation of $^{86}Rb^+$ uptake induced by asc-PMS

The aim of these experiments was to investigate the type of transport mechanism involved in the uptake of $^{86}Rb^+$ induced by asc-PMS. The results of a representative experiment with depleted cells are presented in Fig. 3. External K^+ up to 3 mM increased the uptake of $^{86}Rb^+$. However, above that concentration extracellular K^+ decreased progressively the rate of uptake of $^{86}Rb^+$, suggesting competition of both cations for transport. The kinetics of the effects of extracellular K^+ on the uptake of $^{86}Rb^+$ by depleted cells not treated with asc-PMS was very similar, although the uptake was about 40 times slower.

Effects of asc-PMS on the exodus of $^{86}Rb^+$. Dependence on external K

The treatment with asc-PMS increased the efflux of $^{86}Rb^+$ from depleted cells, this effect being activated by extracellular K^+ (Fig. 4). The stimulation by external K^+ presented saturation kinetics, and was half-maximal at about 1 mM K^+. With external K^+ concentrations of 5 mM or higher the rate constant for $^{86}Rb^+$ efflux was increased by at least one order of magnitude on treatment with asc-PMS. Therefore, it seems that the stimulation of $^{86}Rb^+$ uptake by asc-PMS is largely balanced by a parallel, K^+-stimulated, efflux increase, the increase of the inward and the outward rate constants being of the same order



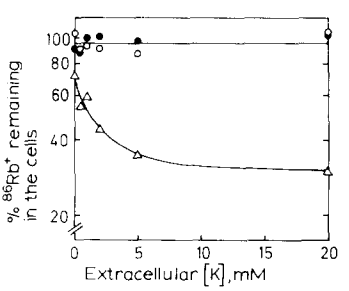


Fig. 3. Effect of extracellular K^+ on the uptake of $^{86}Rb^+$ by iodoacetate-treated depleted cells with or without asc-PMS. Uptake of $^{86}Rb^+$ was studied during a 5-min period in medium with (△, scale at right) or without (○, scale at left) asc-PMS and different K^+ concentrations. It is expressed as the inward rate constant (h^{-1}). The concentration of Na^+ in the medium was always 40 mM.

Fig. 4. Effects of external K^+ on the exodus of $^{86}Rb^+$ from iodoacetate-treated depleted cells. The per cent of $^{86}Rb^+$ remaining in the cells after a 15-min incubation period was measured. Medium contained different K^+ concentrations and either no additions (circles), asc-PMS (triangles) or asc-PMS + 1 mM atebrin (closed circles). The concentration of Na^+ in the medium was always 40 mM. Note the logarithmic scale on the ordinate axis.

of magnitude. Hence, the net results of the treatment with asc-PMS in standard medium containing 6 mM K^+ should be a net loss of cellular K^+, since its intracellular concentration is about 20 times the extracellular one. This prediction was confirmed: in depleted cells incubated for 15 min with asc-PMS the levels of Na^+ and K^+ were (mean ± S.D. of 4 experiments) 32 ± 3 and 105 ± 7 mmol/kg intracellular water, respectively. In cells incubated in parallel without asc-PMS these levels were 29 ± 4 and 127 ± 3. As can be seen, the cells' levels of Na^+ were modified little by asc-PMS.

Effects of asc-PMS on the transport of Na

The effects of asc-PMS on the unidirectional fluxes of Na are shown in Table I. These effects were similar in normal and in depleted cells. The influx of Na^+ was stimulated by 80—90% on treatment with asc-PMS, while the efflux was not significantly modified.

Calcium-dependence of the effects of asc-PMS on the uptake of $^{86}Rb^+$

Several characteristics of the cation fluxes elicited by asc-PMS are reminiscent of those of the fluxes of K^+ elicited by an increase of the intracellular Ca^{2+} concentration, as discussed below. Therefore, experiments were carried out to study the possible dependence on Ca^{2+} of the effects of asc-PMS.

Table II shows the results of two sets of experiments with intact cells and resealed ghosts. The results with intact cells showed that the effect of asc-PMS is Ca^{2+}-dependent, since it was completely prevented in Ca^{2+}-free medium provided that the cells had been previously depleted of Ca^{2+} (— — row). The results with intact cells did not clearly show, however, at which side of the membrane Ca^{2+} is needed. The answer to this question was provided by the results with resealed ghosts. As shown in Table II, asc-PMS increased the uptake of $^{86}Rb^+$ by the ghosts only when Ca^{2+} was present at the intracellular side. In thise case, the presence of intracellular Ca^{2+} in the μM range used already increased the uptake of $^{86}Rb^+$, but the treatment with asc-PMS produced an additional stimulation. Since the results with resealed ghosts show that Ca^{2+} is needed only at the intracellular side, the results in the two last entries with intact cells in Table II suggest that significant movements of Ca^{2+} across the cell membrane take place during the incubation period.

In another set of experiments, the effects of asc-PMS were studied in ghosts containing a broad range of buffered Ca^{2+} concentrations (Fig. 5). In the

TABLE I

EFFECTS OF asc-PMS ON THE TRANSPORT OF Na^+ BY NORMAL AND DEPLETED CELLS

Cells were first incubated for 6 h with either 10 mM glucose (normal) or 1 mM iodoacetate (depleted). The fluxes of Na^+ were studied during an additional 5 min incubation period. Data are means ± S.D. of 3 experiments and are expressed as mmol/kg intracellular water per h.

Cell type	Influx		Efflux	
	Control	Asc-PMS	Control	Asc-PMS
Normal	5.5 ± 0.2	10.3 ± 0.6	6.0 ± 0.1	5.7 ± 0.6
Depleted	4.8 ± 0.6	8.7 ± 0.7	2.1 ± 0.1	2.0 ± 0.3

TABLE II

CALCIUM-DEPENDENCE OF THE EFFECT OF asc-PMS ON THE UPTAKE OF $^{86}Rb^+$

Intact cells were preincubated for 6 h in medium containing 1.8 mM iodoacetate and either 0.5 mM $CaCl_2$ or 5 mM EGTA. Then the cells were incubated for 5 min in medium containing ^{86}Rb with 0.5 mM $CaCl_2$ or without Ca^{2+} (5 mM EGTA). Resealed ghosts were prepared to contain no Ca^{2+} (3 mM EGTA) or 1.4 μM Ca^{2+} (3 mM EGTA + 2.7 mM $CaCl_2$), and were incubated for 5 min in medium containing $^{86}Rb^+$ with 1.4 μM Ca^{2+} or without Ca^{2+} (3 mM EGTA). $^{86}Rb^+$ uptake is expressed as the inward rate constant (mean ± S.D. of 3 experiments).

Preparation	Presence of Ca^{2+}		Uptake of ^{86}Rb	
			Without asc-PMS	With asc-PMS
Intact cells	Preincubation	Incubation		
	+	+	0.36 ± 0.01	8.39 ± 0.26
	—	—	0.31 ± 0.02	0.32 ± 0.04
	+	—	0.38 ± 0.02	1.97 ± 9,94
	—	+	0.30 ± 0.01	7.87 ± 0.95
Resealed ghosts	Intracellular	Extracellular		
	—	+	2.59 ± 0.05	1.82 ± 0.13
	+	+	6.01 ± 0.28	10.83 ± 0.47
	+	—	5.96 ± 0.22	9.57 ± 0.36

absence of asc-PMS, the uptake of $^{86}Rb^+$ increased sharply when the concentration of internal Ca^{2+} was above 10^{-6} M. In the presence of asc-PMS the stimulation of the uptake of $^{86}Rb^+$ appeared at lower Ca^{2+} levels (above $2 \cdot 10^{-7}$ M), and this stimulation was greater than with Ca^{2+} alone even at concentrations at which the effect of Ca^{2+} alone was already maximal (Fig. 5, left). The stimulation of $^{86}Rb^+$ uptake by Ca^{2+} or by asc-PMS was associated with a large net loss of K^+ from the ghosts, the effect seen with Ca^{2+} alone being increased by the presence of asc-PMS (Fig. 5, right). The intracellular Na^+ concentrations were not significantly modified.

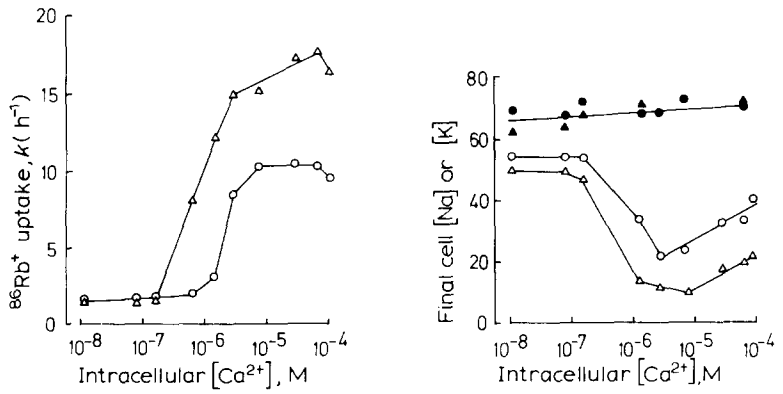


Fig. 5. Effects of asc-PMS on the uptake of $^{86}Rb^+$ (left) or the contents of Na^+ and K^+ (right) of ghosts containing different Ca^{2+} concentrations. Resealed ghosts were prepared from iodoacetate-treated depleted cells to contain 70 mM KCl, 70 mM NaCl and the indicated concentration of Ca^{2+} by the use of Ca^{2+} buffers prepared with EGTA or HEDTA and $CaCl_2$. The uptake of $^{86}Rb^+$ was studied during 5 min at 37°C in medium containing the same concentration of Ca^{2+}, with (triangles) or without (circles) asc-PMS. $^{86}Rb^+$ uptake is expressed as the inward rate constant (h^{-1}). Cell Na^+ and K^+ were determined at the end of the 5 min incubation period and are expressed as mmol/kg initial cell water. Open symbols refer to K^+ and closed symbols to Na^+.

Effects of NADH, NADPH, HS-glutathione and other electron donors on the uptake of $^{86}Rb^+$ by resealed ghosts

Loading of the ghosts with 2 mM of either NADH, NADPH or HS-glutathione increased significantly the uptake of $^{86}Rb^+$ when the intracellular Ca^{2+} concentration was in the μM range (Table III, Expt. 1). For comparison, the effect of asc-PMS added to the incubation medium is also shown. The effect of electron donors can not be attributed to an increase of the proportion of ghosts which reseal in the presence of these agents, since: (i) asc-PMS was not present during the resealing step, and (ii) the uptake rates are refered to the initial intracellular water (see Methods) so that changes in the proportion of resealed ghosts should not affect the calculated values. In ghosts prepared without Ca^{2+} (containing 3 mM EGTA) none of these electron donors increased the uptake of ^{86}Rb. Neither NADH, NADPH or HS-glutathione were effective when added to the extracellular medium.

Table III (expt. 2) shows that ascorbate either alone or together with TMPD, or dithiothreitol also stimulated the uptake of $^{86}Rb^+$ when incorporated into the ghosts. The presence of TMPD did not increased the effect of ascorbate alone. The stimulation of $^{86}Rb^+$ uptake was always associated with a parallel net loss of K^+ from the ghosts with no significant modifications of the Na^+ contents.

Effects of several inhibitors on the uptake of $^{86}Rb^+$ induced by Ca^{2+} and electron donors

Table IV shows the effect of several inhibitors on the stimulation of $^{86}Rb^+$ uptake elicited in intact cells by asc-PMS (Column A) or in resealed ghosts by either Ca^{2+} (Column B), HS-glutathione (Column C) or asc-PMS (Column D). In all the four cases the effect of each inhibitor was similar. The most potent inhibitors were quinine, atebrin, chlorpromazine and oligomycin, followed by

TABLE III

EFFECTS OF SEVERAL ELECTRON DONORS ON THE UPTAKE OF $^{86}Rb^+$ AND THE CONTENTS OF Na^+ AND K^+ OF RESEALED GHOSTS

Ghosts were prepared to contain the indicated electron donors and 1.4 μM Ca^{2+} (2.7 mM $CaCl_2$ + 3 mM EGTA). In Expt. 2 the electron donors and Ca^{2+} were added also to the incubation medium at the same concentrations. In the last row of each Expt., ghosts resealed without electron donors were incubated in medium containing asc-PMS. Incubation was carried out for 5 min at 37°C. $^{86}Rb^+$ uptake is expressed as the inward rate constant (h^{-1}), and Na^+ and K^+ as mmol/kg initial intracellular water. Each result is mean ± S.D. of 3 experiments.

Expt.	Electron donor	$^{86}Rb^+$ uptake	Na^+	K^+
1	None	0.59 ± 0.08		
	2 mM NADH	2.41 ± 0.17		
	2 mM NADPH	3.18 ± 0.27		
	2 mM HS-glutathione	2.28 ± 0.14		
	asc-PMS	9.19 ± 0.34		
2	None	1.90 ± 0.04	70 ± 2	34 ± 1
	20 mM ascorbate	3.34 ± 0.11	68 ± 2	23 ± 1
	20 mM ascorbate + 0.1 mM TMPD	3.50 ± 0.36	65 ± 5	20 ± 2
	5 mM dithiothreitol	2.45 ± 0.09	70 ± 5	31 ± 2
	asc-PMS	5.30 ± 0.42	73 ± 4	15 ± 1

TABLE IV

EFFECTS OF SEVERAL INHIBITORS ON THE UPTAKE OF $^{86}Rb^+$ INDUCED BY Ca^{2+} AND ELECTRON DONORS

The effects of inhibitors were studied in iodoacetate-treated depleted erythrocytes treated with asc-PMS (column A) or in resealed ghosts containing either 1.4 μM Ca^{2+} (2.7 mM $CaCl_2$ + 3 mM EGTA) (column B), 1.4 μM Ca^{2+} and 2 mM HS-glutathione (column C), or 1.4 μM Ca^{2+} and asc-PMS added to the incubation medium (column D). In the experiments of column A, the treatment of the intact cells with asc-PMS stimulated the uptake of $^{86}Rb^+$ by 20 to 66 times. The uptake of $^{86}Rb^+$ by ghosts containing 1.4 μM Ca^{2+} (column B) was 3 to 4 times that of ghosts without Ca^{2+} (3 mM EGTA). HS-glutathione and asc-PMS (columns C and D) produced an additional stimulation of 137% and 279%, respectively. $^{86}Rb^+$ uptake was studied during a 5 min incubation period with or without the inhibitor. Data are means (± S.D.) of 3—4 experiments and are expressed as per cent inhibition due to the presence of the inhibitor. The inhibitors were added to the medium just at the beginning of the $^{86}Rb^+$ uptake period except in several experiments in which they were added 15 min (a) or 60 min (b) before. (c) Dinitrophenol concentration was 2 mM in the experiment with intact cells and 1 mM in those with resealed ghosts.

Inhibitor	Percent inhibition			
	A	B	C	D
Atebrin, 1 mM	96 ± 4	76 ± 3	80 ± 2	88 ± 88
Chlorpromazine, 0.15 mM	94 ± 6 [a]	88 ± 1	73 ± 2	
Antimycin, 5 mg/l	28 ± 2 [a]	30 ± 1	52 ± 7	47 ± 9
Antimycin, 13 mg/l	55 ± 7 [a]			
Rotenone, 0.2 mg/l		(—2) ± 1	(—8) ± 2	12 ± 6
Dinitrophenol [c]	54 ± 2 [b]	58 ± 1	24 ± 4	68 ± 1
Dicoumarol, 10 μM	18 ± 7 [b]			
Oligomycin, 20 mg/l	95 ± 2 [a]	61 ± 2	70 ± 1	62 ± 18
Ethacrynic acid, 0.5 mM	75 ± 5 [b]			
Furosemide, 1 mM	(—9) ± 12 [b]			
Ouabain, 1 mM	(—2) ± 13	(—3) ± 2		0 ± 9
Quinine sulphate, 1 mM	98 ± 2			

ethacrynic acid, dinitrophenol, antimycin and dicoumarol. Ouabain, furosemide and rotenone had no significant effect. The inhibition by atebrin of the exodus of $^{86}Rb^+$ elicited by asc-PMS has been shown without comment in Fig. 4.

The effects of the inhibitors on the stimulation of $^{86}Rb^+$ uptake by asc-PMS in normal non-depleted erythrocytes were similar to those found in depleted cells and ghosts (data not shown).

Discussion

Our results show that several electron donors accelerate the movements of Rb^+ (and presumably K^+) through the red cell membrane. An unexpected finding was the striking similarity between the Rb^+ fluxes elicited by the electron donors and the fluxes of $K^+(Rb^+)$ induced by an increase of the intracellular Ca^{2+} concentration (Gardos effect, for reviews see Refs. 20 and 21). In addition to being dependent on the presence of internal Ca^{2+}, the $Rb^+(K^+)$ transport induced by electron donors reported here has the following characteristics that have been also reported for the Ca^{2+}-induced transport: (i) the effect is most prominent after ATP depletion [22—25], unless the internal Ca^{2+} is increased by other procedures [26]; (ii) the increase of the fluxes of K^+ is exceptionally large, exceeding the controls by 1—2 orders of magnitude [22],

whereas the fluxes of Na^+ are much less affected [14,23,25,27,28]; (iii) both the inward and the outwardly directed fluxes are increased, showing a counter-transport-like behavior [27]; (iv) the rate constants for these fluxes are modified by K in a complex fashion, particularly in the low K^+ concentration ranges (as in Fig. 3), suggesting that the transport mechanism is not a simple one [21, 27,29—31]; (v) under conditions of physiological ion distribution, the net effect is a loss of cellular K^+ [21,23,28—30,32] and the generation of a membrane hyperpolarization [33], giving place (as in Fig. 1) to distribution ratios higher than 1 for an externally added tracer [29,34]; (vi) the induced fluxes are sensitive to oligomycin [35] and quinine [36]. Furthermore, it is shown in this paper that the $^{86}Rb^+$ uptake induced by Ca^{2+} and that elicited by electron donors present the same sensitivity pattern to several other inhibitors tested (Table IV). All these similarities point to the conclusion that the effect of electron donors takes place through the Ca^{2+}-activated K^+ channel.

The foregoing conclusion raises the question of the mechanism by which the electron donors activate the Ca^{2+}-dependent K^+ channel. The first possibility to be considered should be whether these agents act by increasing the intracellular Ca^{2+}, either by promoting its uptake from the medium or by liberating bound Ca. There are, however, several reasons for suspecting that this is not the mechanism by which the electron donors bring about their effect: (i) electron donors were effective in Ca^{2+}-containing resealed ghosts in the absence of extracellular Ca^{2+}, when no uptake of Ca^{2+} from the medium could take place, (ii) the amount of Ca bound to the cells is so small [37] that its liberation would not significantly change the Ca^{2+} concentration in ghosts loaded with Ca^{2+} buffers, and (iii) the electron donors increased the $Rb^+(K^+)$ fluxes even in ghosts that were already maximally stimulated by Ca^{2+}, so that a further stimulation by additional Ca^{2+} should not be expected under these conditions (Fig. 5, left).

An alternative possibility is that the activation of the K^+ channel requires some membrane component(s) to be in a given redox state and that, by maintaining this redox state, electron donors increase the effectiveness of the Ca^{2+} already present in the cytoplasm. In fact, if the activation of the K^+ channel implies the interaction of Ca^{2+} with a membrane receptor, the data of Fig. 5 (left) suggest that both the affinity of the receptors for Ca^{2+} and the number of responsive receptors are increased under the redox state brought about by the electron donors. The observation that oxidizing agents prevent the Ca^{2+}-induced K^+ transport [32] and the fact that the effects of both electron donors and Ca^{2+} are prevented by several oxidoreductase and electron transport inhibitors (Table IV), are consistent with the above interpretation.

The nature and location of the primary electron acceptor systems that interact with the electron donor remains to be clarified. Our results suggest that they are accessible only from the intracellular side, since all the non-penetrating electron donors tested were effective only when included in the ghosts. It is tempting to speculate that some of the oxidoreductase activities found in the plasma membrane [1] could be involved in the process. The results with inhibitors lend some support to this suggestion. The observation that atebrin, a potent inhibitor of the NADH-dehydrogenase activities of mammalian plasma membrane [38], prevented the effect of the electron donors and also that of

Ca^{2+} alone could be more than causal, as chlorpromazine, the other flavoenzyme inhibitor tested, did also. On the other hand, the fact that antimycin was also an effective inhibitor could suggest the occurrence of electron transfer steps other than those involving flavoproteins. The action mechanism of the other inhibitors tested is not clear at the moment.

The finding that HS-glutathione, NADH and NADPH were effective in activating the Ca^{2+}-dependent K^+ channel could be significant in relation to the possible physiological role of the Gardos effect. On the other hand, the changes of sensitivity to Ca^{2+} observed upon various modifications of cell metabolism [21] could be related to changes in the cell levels of physiological electron donors. The possible relation of the $Rb^+(K^+)$ fluxes induced by electron donors in red cells to the atebrin-sensitive stimulation of cation and amino acid transport by asc-PMS reported in the Ehrlich ascites-tumor cells [4] remains to be established.

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