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EFFECTS OF ELECTRON DONORS ON Ca^{2+}-DEPENDENT K^+ TRANSPORT IN ONE-STEP INSIDE-OUT VESICLES FROM THE HUMAN ERYTHROCYTE MEMBRANE

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The interactions between reducing agents and Ca^{2+} in the activation of Ca^{2+}-dependent K^+ transport have been studied in one-step inside-out vesicles. The artificial electron donor system ascorbate + phenazine methosulphate increases the apparent sensitivity to Ca^{2+} by about 5-times over control values (half activation constant, about $5 \cdot 10^{-8}$ M) while oxidized cytochrome *c* decreases the sensitivity to about 1/3 of the controls. Using redox buffers at a fixed p*Ca* it is shown that the shift from the low to the high-affinity state can be accounted for by the reduction of a membrane component accepting two electrons and with an apparent standard redox potential (pH 7.5) of 47 mV. The electrons can be transferred directly from reduced PMS or to oxidized cytochrome *c*, but not from ascorbate, NADH or reduced glutathione.

Introduction

In several cell lines, the increase of the cytoplasmic Ca^{2+} concentration elicits a selective increase of the membrane permeability to K^+ by activation of the so-called Ca^{2+}-dependent K^+ channel [1]. The human red cell has been widely used as a model for the study of this transport system [2]. We have previously shown that reducing agents can lead to the activation of the Ca^{2+}-dependent K^+ transport at subliminal cytoplasmic Ca^{2+} concentrations in red cells from man [3] and other species [4] and in the Ehrlich cell [5,6]. Experiments with resealed ghosts showed that the electron donors, acting on the internal side of the membrane, increase the sensitivity to Ca^{2+} of the K^+ channels [3]. The recent availability of an everted vesicle's preparation in which the Ca^{2+}-dependent K^+ transport is preserved [7,8] allows us now to analyze in further detail the interactions between Ca^{2+} and reducing agents in the activation of this transport system. A preliminary communication of some of these results has been presented elsewhere [9].

Materials and Methods

One-step inside-out vesicles from human erythrocyte membranes were prepared essentially as described by Lew and Seymour [7]. Fresh cells washed with isotonic KCl containing 0.1 mM EGTA were lysed (1/40) with an ice-cold solution containing 2.5 mM K-Hepes (pH 7.5) and 0.1 mM EGTA and concentrated to 50–100% haematocrit (in terms of the original cells) by centrifugation (15 min at $30000 \times g$). These ghosts were vesiculated by a 30–45 min incubation at 37°C followed by four passages through a 26G3/8 needle. The vesicles were then washed once with 40 volumes of

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Abbreviations: ascorbate/PMS, 1 mM potassium ascorbate + 0.01 mM phenazine methosulphate; DCIP, dichlorophenolindophenol; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid.

a solution containing (mM): KCl, 18; K-Hepes (pH 7.5), 16.5; Tris-EGTA, 0.1, resuspended in the same solution at 100% equivalent haematocrit and stored on ice until used.

The activity of the Ca^{2+}-dependent K^+ channel was assessed from measurements of either the uptake or the efflux of ^{86}Rb, which behaves similarly to K^+ for this transport system. Uptake experiments were started by mixing 0.2 ml of vesicle suspension with 0.6–1 ml of incubation medium containing tracer amounts $((1-5)\cdot 10^6$ cpm/ml) of ^{86}Rb. After different incubation periods (5 to 60 min), 0.1 ml aliquots of the incubation mixture were deposited on the top of 5 cm long columns containing 1 ml of Na^+-activated Dowex 50-X8-100 resin and eluted immediately with 1.8 ml of an ice-cold solution containing 0.2 M sucrose, 5 mM Na-Hepes (pH 7.5) and 0.5 mg/ml of bovine serum albumin. The eluent containing the vesicles was mixed with 0.1 ml of 1% Triton X-100 and the radioactivity measured by Cerenkov counting. The columns were treated with 0.9 ml of 1% albumin and washed with 1.8 ml of eluting solution prior use. Each column could be used for at least ten samples before measurable amounts of extravesicular ^{86}Rb passed through it.

The efflux of ^{86}Rb was studied in vesicles previously loaded with the tracer by a 15 min incubation in the presence of 0.1 mM Ca^{2+}. The loaded vesicles were sedimented by centrifugation (5 min at $10000 \times g$), the supernatant aspirated and the incubation started by resuspending in medium containing no radioactivity. Samples were taken at different times (0 to 60 min) and processed as described before for uptake experiments.

The incubations for the transport experiments were performed at room temperature (22–24°C) in a medium of the following composition (mM): KCl, 18; K-Hepes (pH 7.5), 16.5; Tris-EGTA, 0.4; $CaCl_2$, 0 to 0.5 mM. The Ca^{2+} concentrations were calculated using a value of $6.5 \cdot 10^{-8}$ M^{-1} for the dissociation constant of EGTA. In several experiments redox mixtures were added to the incubation medium to obtain fixed redox potentials, as indicated with the results; in these cases the incubations were carried out under anaerobic conditions. In those experiments in which phenazine methosulphate (PMS) was used as a redox carrier, catalase (800 U/ml) was also added to the medium.

$^{86}RbCl$ and $^{22}NaCl$ were purchased from The Radiochemical Centre, Amersham. Chemicals were obtained either from Sigma London Chem. Co. Ltd. or from E. Merck, Darmstadt.

Results

As it has been previously reported [10], one-step inside-out vesicles respond to Ca^{2+} in an all or nothing fashion, with fast and full equilibration of ^{86}Rb both in uptake and efflux experiments. As shown for ^{86}Rb efflux in Fig. 1 (left), the fraction of vesicular space emptied of ^{86}Rb during the rapid equilibratoin phase (initial 10 to 20 min) increased in a graded stepwise fashion with increasing Ca^{2+} concentrations. The addition of 1 mM potassium ascorbate + 0.01 mM phenazine methosulphate (ascorbate/PMS) to the incubation medium (Fig. 1, right) increased the fraction of vesicles activated at submaximal Ca^{2+} concentrations, but did not modify the ^{86}Rb loss in medium without Ca^{2+} or with maximal Ca^{2+} concentrations (p*Ca* 4). Neither ascorbate nor PMS alone had any significant effect. The activation by Ca^{2+} was inhibited by quinine both in the presence as in the absence of ascorbate/PMS (not shown).

In preliminar experiments carried out without catalase in the medium, it was found that ascor-

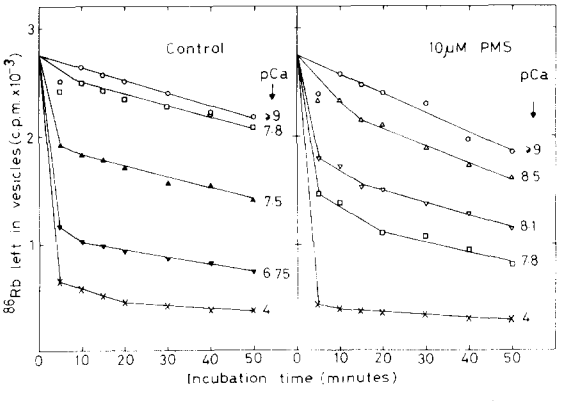

Fig. 1. Effects of ascorbate/PMS on the efflux of ^{86}Rb from inside-out vesicles. Note that the sequence of p*Ca* values ($-\log$ of molar Ca^{2+} concentration) is not the same in both sides of the figure. The incubation medium contained catalase (800 U/ml) and 1 mM ascorbate in all the cases, and in those labelled 'PMS' (right-side panel) 0.01 mM phenazine methosulphate was also added.

bate/PMS produced also a large increase of a Ca^{2+}-independent and quinine-insensitive permeability to ^{86}Rb, this effect being most evident after the first 10 min of incubation; the permeability to ^{22}Na was also increased in these circumstances. The addition to the incubation medium of catalase (800 U/ml) or the performance of incubations under anaerobic conditions prevented largely this non-specific effect of reduced PMS, suggesting that it was due to membrane damage effects of hydrogen peroxide produced by autoxidation of PMS.

Fig. 2 shows that ascorbate/PMS decreased the Ca^{2+} concentration needed to obtain half-maximal activation of ^{86}Rb efflux to about 1/5 of the control values. The effect of ascorbate/PMS had the same extent both in the presence and in the absence of Mg^{2+}, which is known to decrease the sensitivity of the vesicles to Ca^{2+} [10]. In two experiments as those of Fig. 2, ascorbate/PMS shifted the half-activating Ca^{2+} concentration from $4.8 \cdot 10^{-8}$ to $9.5 \cdot 10^{-9}$ M in the absence of Mg^{2+} and from $1.2 \cdot 10^{-7}$ to $3.0 \cdot 10^{-8}$ M in the presence of 0.1 mM Mg^{2+}. The increase of the apparent affinity was then similar in both cases (4–5-times). NADH could replace ascorbate as the bulk electron donor, but the simultaneous presence of PMS was required in order to obtain the effect. Reduced glutathione added to the incubation medium had no effect on the Ca^{2+} sensitivity of the ^{86}Rb uptake.

The addition of oxidized cytochrome *c* (0.032 mM) to the incubation medium increased the Ca^{2+} concentration needed to obtain half-maximal activation of ^{86}Rb uptake, decreasing the apparent affinity for Ca^{2+} to about 1/3 of the control values (Fig. 3). In this case the effect was the same with or without PMS. Oxidized dichlorophenolindophenol (DCIP) (1 mM) decreased also the affinity for Ca^{2+}, but the effect was smaller than with cytochrome *c* and increased by the addition of PMS.

In order to investigate the range of redox potential values (E') at which modifications of the Ca^{2+} affinity took place, experiments were performed at a fixed p*Ca* (7.5, which gives about half-maximal activation under control conditions) and with dif-

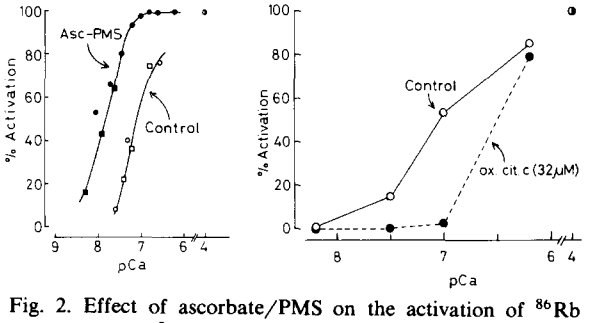

Fig. 2. Effect of ascorbate/PMS on the activation of ^{86}Rb transport by Ca^{2+}. The results of two experiments similar to those of Fig. 1 are shown. The vesicular space sensitive to Ca^{2+}, which equilibrates with the medium during the initial rapid phase (10 to 20 min), was estimated for each p*Ca* by extrapolation of the slow rate phase to zero-time [10]. Per cent activation was then calculated taking the value obtained without Ca^{2+} as 0% and that obtained at p*Ca* 4 as 100%.

Fig. 3. Effects of oxidized cytochrome *c* (0.032 mM) on the activation of Ca^{2+}-dependent ^{86}Rb uptake. Per cent activation was estimated as explained in the legend of Fig. 2.

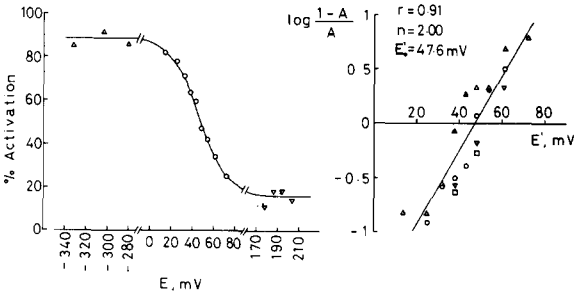

Fig. 4. Activation of ^{86}Rb uptake by a fixed Ca^{2+} concentration (p*Ca* = 7.5) at different redox potentials. The redox potentials tested were accomplished with mixtures (1 mM final concentrations) of NADH/NAD^+, ascorbate/dehydroascorbate or reduced/oxidized DCIP, using for calculations E'_0 values of −303 mV, 43 mV and 187 mV, respectively. The last two mixtures were prepared by oxidation of potassium ascorbate (1 mM) with DCIP or by reduction of DCIP (1 mM) with potassium ascorbate, respectively. In all the cases the medium contained also 0.01 mM phenazine methosulphate and the incubations were performed under anaerobic conditions. On the left-hand side, the results of a typical experiment are shown (per cent activation calculated as explained in the legend of Fig. 2) using different symbols for the three redox mixtures. On the right-hand side, data of four experiments are represented with different symbols for each experiment; for this plot, the activation obtained at each E' value was expressed as a fraction (A) of the whole redox-sensitive activation for the same experiment (taking the value obtained with excess oxidized DCIP as 0% redox activation and that with excess NADH as 100%).

ferent redox mixtures giving E' values ranging from -340 to $+210$ mV. PMS (0.01 mM) was always present in these experiments as an electron carrier, and the incubations were performed under anaerobic conditions in order to prevent changes in the E' values, which were checked spectrophotometrically from the reduced/oxidized PMS ratio [11]. Fig. 4 (left) shows that there were large changes in the fraction of activated vesicles with variations of E' in the 0 to 100 mV range. Using mixtures of NADH/NAD^+ (tested down to 1/10) the activation was always maximal, while with mixtures of reduced/oxidized DCIP at a ratio smaller than 1 the activation was always minimal. Another series of experiments was performed using variable concentrations of either ascorbate and DCIP (1 to 5 mM) or PMS (5 to 20 μM) but at a fixed redox potential (25 mV); the activation values obtained were always the same in all these cases, indicating that the observed effects were not dependent on the absolute concentrations of the redox mixtures but on their E' values, which presumably affect the redox state of some membrane component involved in K^+ channel activation.

If it is assumed that the fraction of this membrane component which is present in its reduced form at a given E' value correlates with the fraction (A) of the redox sensitive activation obtained, then the ratio oxidized/reduced membrane component could be estimated from the ratio $(1-A)/A$ and, from the Nernst equation, it could be written:

$$\log\{(1-A)/A\} = n/59(E' - E'_0) \quad (1)$$

where E' and E'_0 (mV) represent the actual and the standard redox potentials of the membrane component and n the number of electrons exchanged. Fig. 4 (right) shows that our data fits adequately ($r = 0.91$) to Eqn. 1, the values derived for E'_0 and n being 47 mV and 2.00 electrons, respectively.

Discussion

Our results show that the affinity for Ca^{2+} of the Ca^{2+}-dependent K^+ channels of one-step inside-out vesicles depends on the redox state of a membrane component able to exchange two electrons and with an apparent standard redox potential (E'_0, pH 7.5) of about 47 mV. The reduced state displays 'high-affinity' characteristics while the oxidized one shows 'low-affinity', the difference between these two situations amounting to a 15-fold variation in the Ca^{2+} concentration needed to obtain half-maximal effect.

These results in inside-out vesicles confirm and extend those previously reported in intact cells and resealed ghosts [3], although several differences deserve to be noted. In the vesicles the reducing agents do not increase the maximal effect of Ca^{2+}, while both the affinity and the maximal effect were increased in resealed ghosts [3]. On the other hand, a broad range of reducing agents, including ascorbate, NADH, NADPH and HS-glutathione, were effective without further additions when they were loaded into resealed ghosts [3]; in contrast, these agents needed the presence of PMS to act in the inside-out vesicles, where only PMS and cytochrome *c* were apparently able to connect as effective electron carriers with the membrane redox system. These differences could reflect an altered behavior of the channel in the everted preparation caused by the loss of loosely bound components during the vesicle's preparation steps, as suggested also by the fact that oligomycin, an effective channel inhibitor in intact cells or ghosts [3,12], does not act in the vesicles (unpublished observations). Additionally, some 'soluble' factor, washed out from the vesicles, might be normally involved in the redox modulation of the channels in intact cells. Preliminary results obtained in this laboratory encourage this last possibility.

The nature of the membrane redox components responsible for the effects of reducing agents remains unknown. The inability of glutathione to act directly suggests that redox changes of HS-groups are not the basis for the changes of the affinity for Ca^{2+}. The estimated E'_0 of about 47 mV falls within the range of E'_0 values assigned in other systems to either *b* type cytochromes of flavins, and both of them have been detected in erythrocyte membranes [4,13]. It has also been shown previously that several oxido-reductase inhibitors, notably flavin antagonists, can prevent the activation of the Ca^{2+}-dependent K^+ channel [3,14]; however, in another study carried out in erythrocytes of several species [4] no correlation was found between membrane-linked NADH dehydro-

genase, which is very sensitive to some of those drugs, and the Ca^{2+}-dependent K^+ channel activity.

A pertinent question is whether or not redox modulation of the K^+ channel could be relevant under physiological conditions. It has often been postulated that the cell metabolic state could modify the sensitivity of the K^+ channels to Ca^{2+} although the mechanism remains obscure [15,16]. On the basis of the present results, it is tempting to speculate that a change of the cell redox state could be the factor involved. The observation that pyruvate and other oxidizing agents prevent the Ca^{2+}-induced K^+ transport in fluoride-poissoned cells [17] is consistent with this view, since fluoride increases the $NADH/NAD^+$ ratio [18]. However, direct evidence of redox modulation of Ca^{2+}-dependent K^+ transport under physiological conditions is still lacking.

Acknowledgement

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