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Modulation of Ca^{2+}-dependent K^+ transport by modifications of the $NAD^+/NADH$ ratio in intact human red cells

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The effects of variations of the $NAD^+/NADH$ quotient on the uptake of ^{86}Rb by human red cells loaded by non-disruptive means with the chelator Benz2 and different amounts of ^{45}Ca has been examined. The $NAD^+/NADH$ quotient was modified by the addition of pyruvate and/or lactate or xylitol. It was found that the uptake of ^{86}Rb at a given intracellular Ca^{2+} concentration was faster in the reduced state (lactate or xylitol added). Metabolic changes were associated with variations of the redox state. However, glycolitic intermediates did not significantly modify the apparent affinity for Ca^{2+} of the Ca^{2+}-dependent K^+ channel in one-step inside-out vesicles prepared from the erythrocyte membrane. Taken together, these results suggest that modifications of the cytoplasmic redox potential could modulate the sensitivity to Ca^{2+} of the Ca^{2+}-dependent K^+ channel in the human red cells under physiological conditions. This conclusion is consistent with previous findings in inside-out vesicles of human erythrocytes using artificial electron donors.

It has been shown previously that several redox agents can modify the sensitivity to Ca^{2+} of the Ca^{2+}-dependent K^+ channel in the human erythrocyte [1,2]. Using inside-out vesicles we have shown that the reduction of a membrane component with $E'_o = 47$ mV (pH 7.5) conferred high affinity for Ca^{2+} to the K^+ channel [3]. Most of these data were obtained using artificial electron donor systems. In order to test the possible physiological relevance of the above observations we have studied here the effects of variations of the $NAD^+/NADH$ ratio in minimally disturbed intact erythrocytes. These variations were achieved by incubation with lactate or pyruvate or with xylitol, which is able to reduce NAD^+ in the human erythrocyte [4].

The main technical problem of this study was to achieve and measure stable and uniform intracellular Ca^{2+} concentrations at the submicromolar level, where the threshold for activation of the K^+ channel lies. This problem was solved by using cells first loaded by non-disruptive means with the Ca^{2+} chelator Benz2 [5]. The cells were then incubated with ^{45}Ca (the total amount always smaller than that of the chelator) in the presence of the divalent cation ionophore A23187. Under these conditions the intracellular Ca^{2+} concentration can be calculated from the chelator and ^{45}Ca contents [5]. Preliminary experiments showed that the intracellular Ca^{2+} concentration stabilized within the first 2 min of incubation. The activity of the Ca^{2+}-dependent K^+ channel was followed by measuring the uptake of ^{86}Rb added to the incubation medium. The cell contents of ^{45}Ca and ^{86}Rb were measured by differential scintillation counting after several incubation periods terminated by dilution with 10 vols. of ice-cold medium containing 0.1 mM EGTA and 1 mM quinine and inmediate centrifugation through dibutylphthalate oil [6]. Details on the experimental methods are given with the figure legends. The Ca^{2+}-induced uptake of ^{86}Rb could be described by single ex-

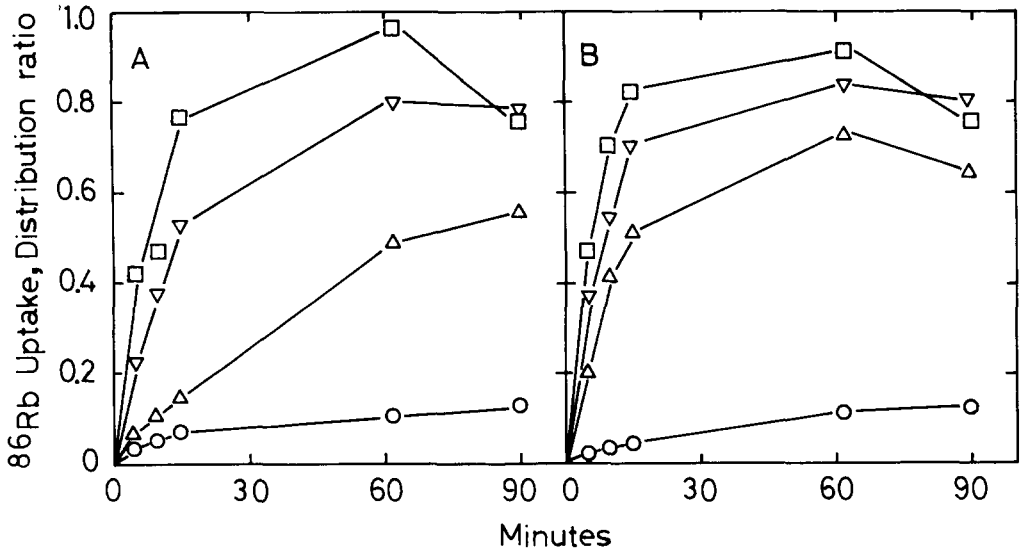

Fig. 1. Effects of variations of $NAD^+/NADH$ ratio on Ca^{2+}-dependent $^{86}Rb^+$ uptake by human red cells. Cells were first loaded with Benz2 [5] by incubation at 10% haematocrit and 37°C in standard medium containing 0.1 mM EGTA, 10 mM glucose, 10 mM pyruvate and 0.2 mM Benz2-acetoxymethyltetraester (a generous gift of Dr. V.L. Lew, Physiological Laboratory, Cambridge, U.K.) during 60 min. Standard medium had the following composition (mM): 75 NaCl, 75 KCl, 0.2 $MgCl_2$, 10 K-Hepes (pH 7.5). Pyruvate was added in order to prevent ATP depletion on loading with the Ca^{2+} chelator [7,8]. After washing, the cells were resuspended at 10% haematocrit in standard medium containing either 10 mM pyruvate (oxidized state, panel A) or 10 mM lactate, 0.7 mM NADH and 0.5 units/ml lactate dehydrogenase (reduced state, panel B). In the last condition NADH and lactate dehydrogenase were added to the medium to secure that any produced pyruvate is quickly reduced to lactate. Extracellular lactate or pyruvate enter the cells and modifies the $NAD^+/NADH$ ratio by mediation of the intracellular lactate dehydrogenase [4]. After 60 min at 37°C 1-ml aliquots of the cell suspension were distributed in Eppendorf tubes containing ^{86}Rb (about $5 \cdot 10^5$ cpm/ml) and different ^{45}Ca concentrations (specific activity about 10^6 cpm/μmol). The uptake experiment was started by the addition of the ionophore A23187 (final concentration 10 μM). After different incubation periods, 0.1-ml samples were mixed with 1 ml of ice-cold standard medium containing 0.2 mM EGTA and 1 mM quinine and centrifuged over dibutylphthalate oil [6]. The cell pellets were extracted with 0.4 N perchloric acid and counted for ^{86}Rb and ^{45}Ca radioactivity by differential liquid scintillation counting. The content of Benz2 in the cells was estimated by titration of cell lysates with $CaCl_2$ under a Ca^{2+}-selective electrode. It ranged between 0.4 and 1 mmol/l cells in different experiments. The Ca^{2+} concentration inside the cells was calculated from the ^{45}Ca and Benz2 contents assuming for the chelator a dissociation constant of 60 nM [5]. The ^{45}Ca content of the cells remained constant for each condition in all the samples taken a different times and it was the same for pyruvate or lactate-treated cells. Symbols represent the following Ca^{2+} concentrations (pCa): < 9 (○); 7.40 (△); 6.80 (▽), 3.20 (□).

ponentials from which the first-order rate constant (k) can be calculated. The amount of ^{86}Rb taken up at equilibrium was the same in all the cases, but the rate of uptake increased with increasing Ca^{2+} concentrations.

Fig. 1 shows the uptake of ^{86}Rb by Benz2-loaded cells first incubated with either pyruvate (A) or lactate (B) at different Ca^{2+} concentrations. The preincubation with pyruvate or lactate did not significantly modify the intracellular Ca^{2+} concentration obtained. At maximal Ca^{2+} or without Ca^{2+} (upper and lower curves) the rate of uptake was the same in both conditions, but at intermediate Ca^{2+} concentrations the uptake was faster in the cells incubated with lactate. In another set of experiments the decrease of the $NAD^+/NADH$ ratio was accomplished using xylitol instead of lactate. Xylitol is able to reduce NAD^+ through xylitol dehydrogenase and NADH remains reduced if no other substrates are added to the incubation medium [4]. The results of one of such experiments are shown in Fig. 2. The uptake was faster in xylitol-treated cells at all the Ca^{2+} concentrations tested.

In order to obtain a continuous range of redox potentials, aliquots of a cell suspension were first

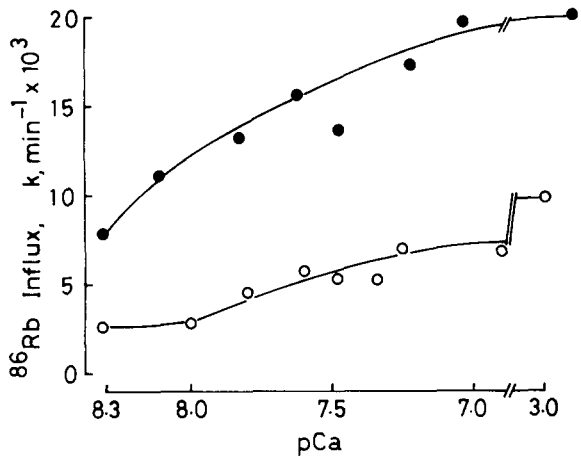

Fig. 2. Effects of treatment with xylitol on the rate of $^{86}Rb^+$ uptake at different cell Ca^{2+} concentrations. Cells were loaded with Benz2 as described in the legend to Fig. 1 and incubated either with 10 mM pyruvate (open symbols) or 10 mM xylitol (closed symbols) during 60 min at 37°C before the uptake experiments were started. Other details as in Fig. 1.

incubated with lactate and pyruvate at different ratios. Then a fixed amount of Ca^{2+} giving submaximal activation was added to all the cell suspensions and the uptake of ^{86}Rb was measured. Fig. 3A shows the changes of the rate of uptake as a function of the ratio lactate/pyruvate. The rate of uptake tended to increase with the increase of this ratio, the measured calcium content of the cells remaining unmodified (not shown). The cell ATP contents of these cells by the end of the experiment were also measured, and were found to decrease as the ratio lactate/pyruvate increased (Fig. 3B).

All the above observations support the idea that changes of the $NAD^+/NADH$ ratio that could take place under physiological circumstances can modigy the sensitivity to Ca^{2+} of the Ca^{2+}-dependent K^+ channel. These modifications should not be expected, in principle, from the E_o' of 47 mV (pH 7.5) previously determined for the membrane-associated component in inside-out vesicles [4], since E_o' of the lactate/pyruvate pair is much more negative (-210 mV at pH 7.5). These results, however, are not necessarily conflicting if additional electron transfer steps were involved in the intact cell or the membrane carrier were somehow modified during the preparation of inside-out vesicles. It should be noted, however, that the effects of changes of the $NAD^+/NADH$ ratio on Ca^{2+}-dependent ^{86}Rb transport reported

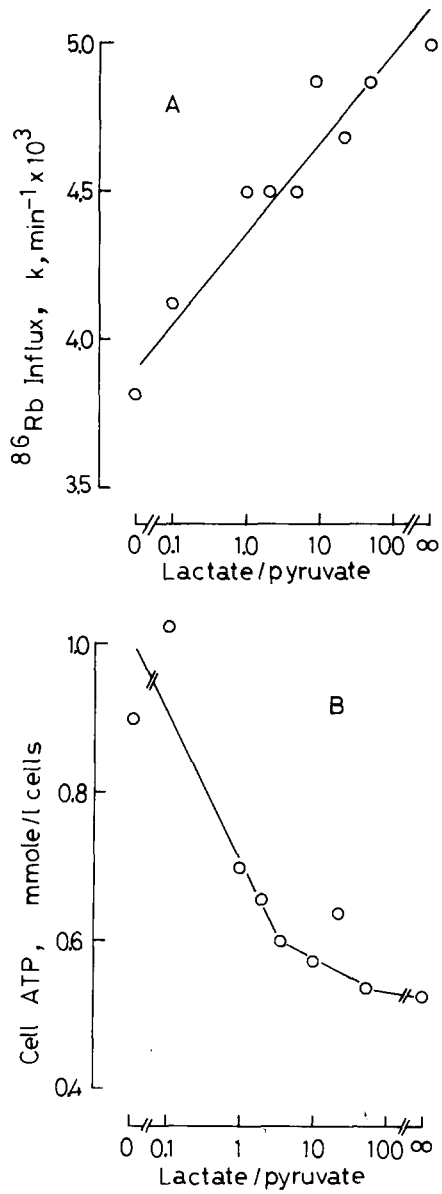

Fig. 3. Effects of variations of the lactate/pyruvate ratio on the rate of uptake of $^{86}Rb^+$ (A) or the ATP content (B) of human red cells at a fixed intracellular Ca^{2+} concentration. Cells loaded with Benz2 were incubated during 60 min at 37°C at different lactate/pyruvate ratios as indicated in the figure. The same amount of Ca was added for the uptake experiment in all the cases, and the measured Ca^{2+} concentration in the cells did not show any trend of variation in the different conditions. Mean ± S.D. of all the nine p*Ca* values was 7.65 ± 0.04.

here are much weaker than those reported previously using artifitial electron donors [1–3]. The results reported here are consistent with the older observation that pyruvate prevents the activation of K^+ transport in fluoride-poisoned cells [9].

Unfortunately, there was no way to avoid other metabolic changes associated to the variation of the $NAD^+/NADH$ quotient. For example, a decrease of the cell ATP level associated to the decrease of the pyruvate/lactate ratio was shown in Fig. 3B. Such changes could be responsible of the observed modifications of the Ca^{2+}- sensitivity. In order to test this point, the effect of several glycolitic intermediates on the Ca^{2+}- sensitivity of ^{86}Rb uptake was tested in one-step inside-out vesicles [10]. The methodology previously described [3,11] was used, except that Mg^{2+} was routinely included in the incubation medium at 5 mM. The following metabolic intermediates, tested at concentrations of 200 to 400 μM, were without effect on Ca^{2+}-dependent ^{86}Rb uptake by inside-out vesicles: glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, 3-phosphoglyceric acid, 2-phosphoglyceric acid, phospho*enol*pyruvic acid, ATP, ADP, AMP, cyclic AMP, GTP and UTP. 2,3-Diphosphoglycerate at 4 mM decreased the rate of uptake of ^{86}Rb at submaximal Ca^{2+} concentrations to about 1/3 of the control value without modifying the fraction of activated vesicles reached at the steady state. This inhibitory effect of diphosphoglycerate was not observed when the Mg^{2+} concentration of the medium was lowered to 0.5 mM.

The changes of Ca^{2+}-dependent K^+ transport observed on treatment of the intact cells with pyruvate, lactate or xylitol could not be, however, attributed to modifications of the cell levels of 2,3-diphosphoglycerate since: (i) changes of 2,3-diphosphoglycerate levels should be very small under our experimental conditions [4], and (ii) at the cellular Mg^{2+} level of about 0.5 mM, which should apply to our experiments [12], little or no effect of 2,3-diphosphoglycerate was observed in inside-out vesicles. These results reinforce our conclusion that the Ca^{2+}-dependent K^+ channel of the human erythrocyte can be subjected to redox regulation under physiological conditions.

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