Vanadate and Fluoride Activate Red Cell Na^+ Permeability by Different Mechanism

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Abstract. Fluoride and vanadate are known to induce the Ca^{2+}-dependent K^+ efflux. We found that both agents concomitantly induced the Ca^{2+}-dependent $^{22}Na^+$ influx. The extent of the Ca^{2+}-dependent $^{22}Na^+$ influx induced by vanadate was very small in human red blood cells but clearly visible in guinea-pig red blood cells. The effect of fluoride has been studied in human red blood cells only. The $^{22}Na^+$ influx induced by vanadate was inhibited by amiloride but was resistant to tetrodotoxin, whereas that induced by fluoride was resistant to amiloride but sensitive to tetrodotoxin. The effects of inhibitors indicate that vanadate activates the Na/H antiporter and that fluoride opens a tetrodotoxin-sensitive Na^+ channel in red blood cells in the Ca^{2+}-dependent manner. The results also indicate that both agents activate the Ca^{2+}-dependent Na^+ permeabilities by unknown auxiliary mechanisms.

Key words: Red blood cells — $^{22}Na^+$ uptake induction — Fluoride — Vanadate

Introduction

The activation of the Ca^{2+}-dependent K^+ efflux (the so-called Gárdos effect) from human red blood cells (RBC) has been demonstrated by many experimental treatments (for review see Lew and Ferreira 1978; Schwarz and Passow 1983; Varečka and Carafoli 1982). It is generally accepted that the common denominator of all the treatments is their debilitating action on the energetic metabolism which leads

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finally to the loss of the cell ability to keep the ionic, especially Ca^{2+} homeostasis. Although the opening of the Ca^{2+}-dependent K^+ channel is a prevalent event following the increase of the cytoplasmic Ca^{2+} concentration, possible changes in the Na^+ permeability were considered in the early stages of the study of the Gárdos effect (for review see Passow 1964; Lew and Ferreira 1978). The increase of the Na^+ permeability in ATP-depleted cells seems to be extremely small, if any, when compared to the K^+ one (Romero and Whittam 1971; Lew and Ferreira 1976, 1978; Simons 1976) but it could occur during the late phase of the Gárdos effect as a consequence of the RBC shrinkage due to the KCl efflux (Passow 1964). Here, the Na^+ permeability may play a role in the recovery of the cell volume (Passow 1964). Escobales and Canessa (1985) studied the Na^+ permeability changes induced by increased $[Ca^{2+}]_i$ in intact RBC treated with Ca^{2+} ionophore A23187. They found that it could be ascribed to the activation by Ca_i^{2+} of the Na/H antiporter which does not operate in resting conditions.

On the other hand, two reports are known which demonstrated the appearance of the tetrodotoxin (TTX)-sensitive Na^+ permeability in human RBC. Huestis (1977) rendered the human red cell membrane permeable for Na^+ upon pretreatment of intact red cells with phosphatidylcholine liposomes. Szász and Gárdos (1974) reported that carbachol induced the TTX-sensitive $^{22}Na^+$ uptake by human RBC. The mechanisms of activation of these changes remained unexplained.

In this paper we present evidence that amiloride (AMD)-and TTX-sensitive $^{22}Na^+$ uptake could be elicited by different treatments used for the activation of the Ca^{2+}-dependent K^+ efflux (the Gárdos effect) *via* the increase of the cytoplasmic Ca^{2+} concentration.

Materials and Methods

Red blood cells (RBC)

Human blood was obtained from healthy and drug-free volunteers of both sexes and was used within 3 days, being stored at 0–4 °C. As anticoagulant, EDTA, K salt was used at a final concentration of 5 $mmol.l^{-1}$. Red cells were prepared immediately before experiments by differential centrifugation (500 $\times g$ for 10 min at ambient temperature). RBC were centrifuged, the buffy coat aspirated, and the pellet was washed three times in a medium containing (in $mmol.l^{-1}$): Tris-HCl (pH 7.3) – 20; NaCl – 130; KCl – 5; glucose – 10; (further referred to as the medium) and, finally, suspended in the same medium. Guinea-pig blood was obtained by the cardial punction of adult, non-anaesthetized animals as described above, and was used immediately for experiments.

^{22}Na uptake measurement

RBC (40% haematocrit) suspended in the medium at 25 °C were pipetted into test tubes containing unmixed aliquots of stock solutions of all radionuclide, NaF, and $CaCl_2$ on their bottom of the test tubes so that the final activity/concentration was as follows: $^{22}NaCl$ (about 100 $MBq.ml^{-1}$), $NaVO_3$ (1 $mmol.l^{-1}$), NaF (10 $mmol.l^{-1}$), $CaCl_2$ (2.5

$mmol.l^{-1}$), then the suspension was vigorously vortexed. At time intervals indicated in the Figures (otherwise 60 min), 0.5 ml aliquots were withdrawn from the suspension, and centrifuged through a layer of silicone oil. The individual phases were separated. The radioactivity of supernatants and pellets was measured by liquid scintillation counting (corrected for quenching) after precipitating proteins with trichloracetic acid (5% (w/v) final concentration). Control cells without NaF and without Ca were treated in parallel. The experimental points represent the mean value of duplicate measurements ± standard error. The latter is depicted by bars whenever exceeded the dimension of symbol. Each experiment is a representative of at least two, but, as a rule, three experiments.

Materials

$^{22}NaCl$ was purchased from the Institute of Radionuclides of the Academy of Sciences of the (former) USSR, Moscow, tetrodotoxin from Koch-Light (Sankyo), ionomycin from Calbiochem, Tris base and dibutyl phtalate from Serva, and the methyl-phenyl silicone oil from Lučební závody Kolín, Czech Republic. Amiloride was a generous gift of Merck, Sharp & Dohme, U.S.A. Sodium metavanadate was the product of Reachim, Moscow, Russia. Other used chemicals (all of analytical grade) were purchased from Lachema, Brno, Czech Republic.

Results

When the suspension of human RBC was incubated in the conditions usually used for the measurement of the vanadate-induced Ca^{2+} uptake (Varečka et al. 1986) (25 °C, medium containing (in $mmol.l^{-1}$): Tris-HCl (pH 7.3) – 20; NaCl – 130; KCl – 5; glucose – 10; $CaCl_2$ – 2.5 but with 40% haematocrit instead of 30%) with $^{22}Na^+$ it became labelled in a time dependent manner. (Fig. 1*A*). The label equilibrated within 15 minutes or less at 25 °C and the equilibration was not significantly influenced by the addition of EGTA instead of Ca^{2+} (both 2.5 $mmol.l^{-1}$). When the RBC suspension was pre-incubated with sodium vanadate (1 $mmol.l^{-1}$) for 15 min in the presence of EGTA (2.5 $mmol.l^{-1}$), the $^{22}Na^+$ uptake did not differ from controls without vanadate. When 2.5 $mmol.l^{-1}$ Ca^{2+} was added, the rate and the steady state value of the labelling increased by 50–100% (Fig. 1*A*). The same experiment performed in parallel in guinea-pig RBC yielded similar results with interesting quantitative differences. The comparable samples (0.2 ml of packed cells) of guinea-pig RBC were labelled in the absence of vanadate similarly as were human RBC. In the presence of vanadate the rate of labelling was twice as high as in the EGTA-treated cells and one order of magnitude higher in the presence of both vanadate and Ca^{2+} (Fig. 1*B*). A similar increase of $^{22}Na^+$ labelling was observed when RBC were pretreated with ionomycin (1 $\mu g.ml^{-1}$) instead of vanadate in the presence of Ca^{2+} (2.5 $mmol.l^{-1}$). In an experiment not detailed here we measured the volume of extracellular space retained in the RBC pellet after its separation from the suspension (46 $\mu l.ml^{-1}_{\text{packed cells}}$). We calculated that the radioactivity of RBC pellets withdrawn at 0 min or in controls without $^{22}Na^+$

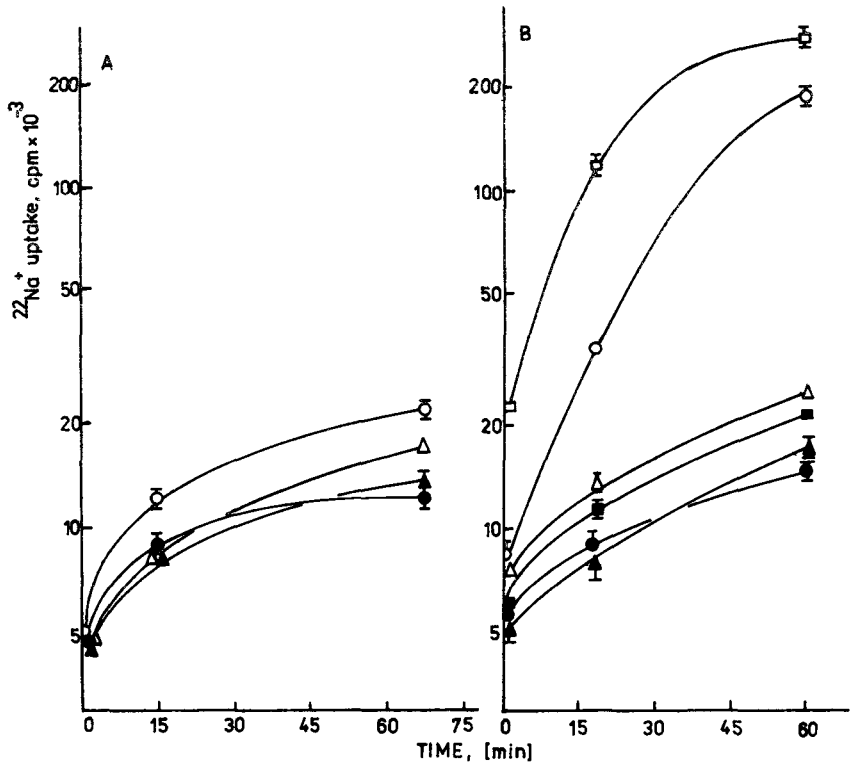

Figure 1. The vanadate- and ionomycine-induced Na^+ uptake in human (*A*), and guinea-pig (*B*) RBC. RBC were preincubated for 15 min at 25 °C with 1 mmol.l^{-1} vanadate (open circles and triangles) in the presence of 0.2 mmol.l^{-1} EGTA (triangles) and ^{22}NaCl (1.5 MBq.ml^{-1}). The addition of 2.5 mmol.l^{-1} Ca^{2+} (circles) followed, and aliquots were withdrawn at indicated time intervals, and treated as described in Materials and Methods. Closed symbols – controls without vanadate. Samples labelled with squares (guinea-pig RBC only) were treated with ionomycin (1 μg.ml^{-1}) instead of vanadate. EGTA – closed squares; Ca^{2+} – open squares. Note the logarithmic scale of the ordinate.

uptake inducer corresponded within experimental error to the radioactivity of the medium entrapped in the cell pellet (data not shown).

We tested also the effect of drugs known as inhibitors of Na^+ transport (amiloride and tetrodotoxin) and found that the $^{22}Na^+$ uptake induced by vanadate in guinea-pig RBC is sensitive to AMD at concentrations over 10^{-6} mol.l^{-1} (Fig. 2). TTX did not influence the $^{22}Na^+$ uptake below 10^{-6} mol.l^{-1}. Above this concentration, hovewever, a slight (about 15% at 10^{-4} mol.l^{-1}) stimulation of the uptake was observed (Fig. 2).

The addition of sodium fluoride (10 mmol.l^{-1}) instead of vanadate also stim-

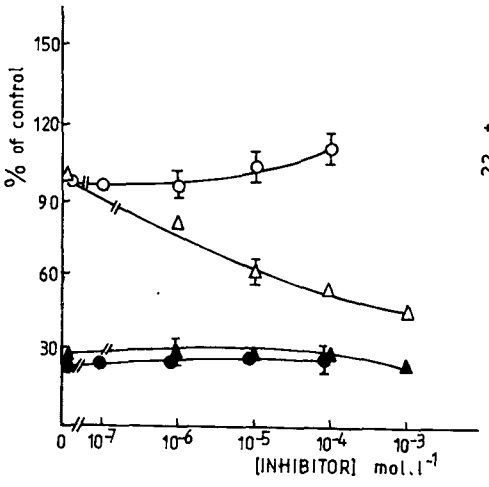

Figure 2. The uptake of $^{22}Na^+$ by guinea-pig RBC induced by vanadate. The $^{22}Na^+$ uptake was measured for 30 min in the presence of vanadate and Ca^{2+} and of indicated concentrations of either TTX (circles), or amiloride (triangles). Open symbols – 1 mmol.l^{-1} $NaVO_3$, closed symbols – controls without vanadate). Results are expressed as % of the controls with vanadate and without inhibitors.

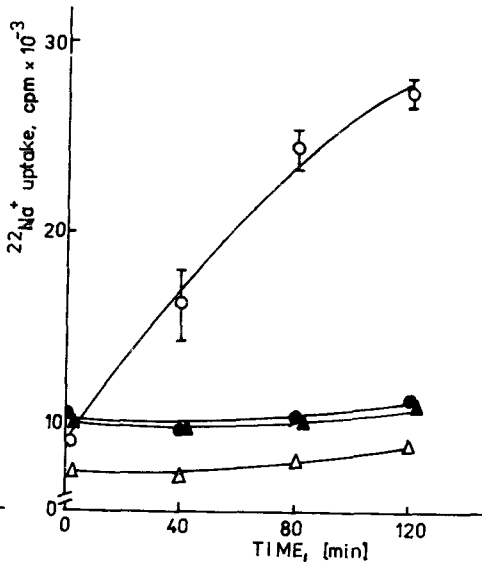

Figure 3. The effect of NaF and Ca^{2+} on the Na^+ uptake by human RBC. The experiment was performed as described in Materials and Methods. NaF-containing test tubes – open symbols, Ca^{2+}-containing test tubes – circles. Controls without NaF (closed symbols), and/or without Ca^{2+} (triangles) were treated in parallel.

ulated the $^{22}Na^+$ uptake in human RBC as compared to control without fluoride. The effect of fluoride on the $^{22}Na^+$ uptake was dependent on the presence of millimolar concentrations of Ca^{2+} in the medium, whereas in the presence of the same concentration of EGTA no stimulation of the $^{22}Na^+$ uptake by fluoride was observed (Fig. 3). It should be mentioned at this point that the time course varied from experiment to experiment under apparently identical conditions. Often the uptake was very rapid starting within seconds after triggering the reaction by the addition of Ca^{2+} or NaF to the suspension. In these experiments the uptake approached the steady-state within few minutes (Fig. 4). So far, we do not understand in detail the causes of these variations.

When we tested the effect of TTX and AMD on the fluoride-induced $^{22}Na^+$ uptake in human red blood cells, we surprisingly, found that it was not sensitive to AMD even at concentration of 0.3 mmol.l^{-1} (Fig. 4*A*) but was sensitive to TTX

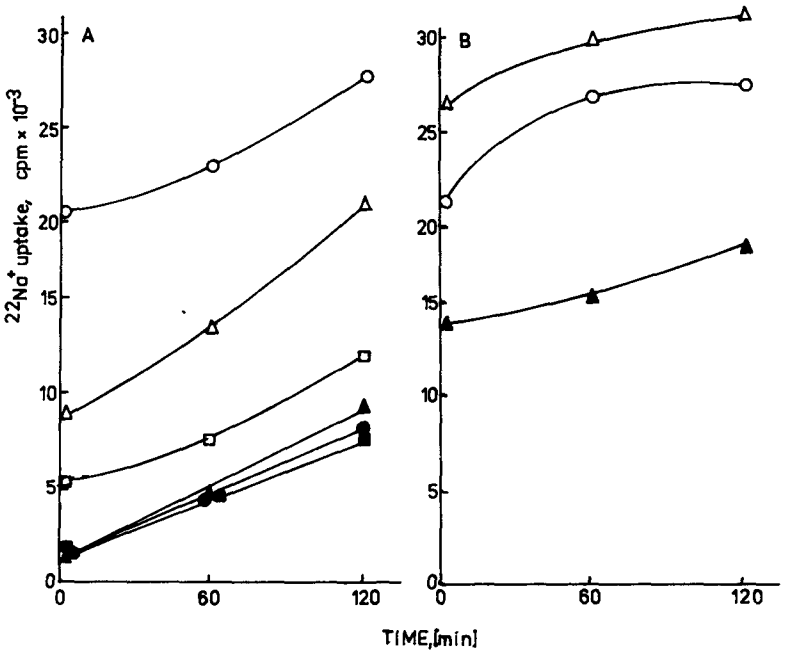

Figure 4. The effect of tetrodotoxin (A, B) and amiloride (B) on the NaF plus Ca^{2+}-induced Na^+ uptake by human RBC. *A*. The kinetics of the NaF-induced Na uptake in the presence of 0.31 μmol.l^{-1} (open triangles), or 3.1 μmol.l^{-1} (open squares) TTX. Controls without TTX – open circles. Closed symbols – NaF omitted. *B*. The kinetics of the NaF – induced Na^+ uptake was measured in the presence of 0.3 mmol.l^{-1} amiloride (open triangles), or in the presence of 3.1 μmol.l^{-1} TTX (closed triangles). Controls without inhibitors – open circles. Controls without NaF were not done in this experiment.

(Fig. 4*B*). We found that TTX concentrations 3.10^{-7} mol.l^{-1} were already effective in the inhibition of the $^{22}Na^+$ uptake (Fig. 4*B*).

The activation of the fluoride-induced $^{22}Na^+$ uptake was observed also in human RBC isolated freshly from blood stored up to one week at 0–4°C but after isolating RBC into the salt medium the effect of fluoride diminished rapidly within 2 hours (data not shown). In contrast, the effect of vanadate persisted also after overnight storage of the suspension of human RBC. We did not measure the effect of fluoride on the $^{22}Na^+$ uptake in guinea-pig RBC so far.

Discussion

Our results showed, in accordance with the data of Taub and Saier (1979) and Escobales and Canessa (1985), that the increase of the cytoplasmic Ca^{2+} activates

the RBC membrane Na^+ permeability. In addition, we found that the characteristics of the Na^+ transport pathway are dependent on the way by which the Ca^{2+} loading was achieved. The effect of vanadate on the Na^+ uptake seems to be similar to that of A23187 (Escobales and Canessa 1985) because it could be mimicked by ionomycin (Fig. 1) and is sensitive to amiloride. The effect of vanadate on the Na^+ uptake was more pronounced in guinea-pig than in human RBC and, therefore, we used guinea-pig RBC as a model for the analysis of this phenomenon. It is interesting that the difference in the Na^+ uptake between human and guinea-pig RBC correlated with the differences in the extent of the vanadate-induced Ca^{2+} uptake (data not shown). Another interesting difference which we found but do not present here in detail is that the guinea-pig RBC, unlike human RBC, possess the Na/Ca antiporter. The causative relationship among these parameters could be experimentally tested in the future.

The results presented above confirmed by an independent approach the data published by other authors (Huestis 1977; Szász and Gárdos 1974) that RBC, cells which demonstrated the presence of the transport system that catalyzes the TTX-sensitive Na^+ transport. Thus, human RBC extend the list of non-excitable cells possessing this transport system (Frelin et al. 1984; Reiser and Hamprecht 1983; Lombet et al. 1982; Villegas et al. 1988), although we know no function which could be ascribed to the TTX-sensitive Na^+ channel in the RBC membrane. The mechanisms by which the latter is activated remain obscure. Unlike the observations of Huestis (1977) and Szász and Gárdos (1974) our method for the activation of the TTX-sensitive $^{22}Na^+$ uptake seems to be more amenable to the experimental analysis because of its simplicity and of many data available in the literature about the various aspects of the fluoride action. The comparison of the increase of the Na^+ permeability by fluoride and vanadate brings some hints concerning the mechanism of action of these agents. First, the generalized increase of the Ca^{2+} concentration in the RBC cytoplasm does not seem to be important for the activation of the Na^+ permeability. Rather, the fact that fluoride and vanadate activate different Na^+ transporting systems in the Ca^{2+}-dependent manner suggests that the way by which Ca^{2+} ions are involved in the activation process is decisive for the final effect. Second, the effects of vanadate and fluoride ("fluoroaluminate") could not be reduced to mimicking the phosphate group as suggested Chabre (1990). Third, the activation of both pathways might not be absolutely independent as AMD and TTX exert an opposite actions in both vanadate- and fluoride-induced $^{22}Na^+$ uptake (Figs. 2, 4). This may be in accordance with the fact that amiloride and its derivatives non-competitively inhibited the binding of batrachotoxin on the neuronal membranes (Velly et al. 1988). We have studied the effects of both fluoride and vanadate on the phosphorylation of both membrane proteins and inositol phospholipids in RBC under conditions corresponding with those used in the experiments described above. So far, we did not observe any changes which

could be relevant to the mechanism of the Na^+ permeability increase (data not shown) so that its explanation remains to be elaborated in the future. Likewise, the relationship of our results to the changes of permeability for sodium induced by ferricyanide and iodoacetate remains to be established (Fuhrmann et al. 1989).

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