Properties of the Ca^{2+} influx reveal the duality of events underlying the activation by vanadate and fluoride of the Gárdos effect in human red blood cells

Ľudovít Varečka[a,*], Elena Peterajová[b], Erika Píšová[b]

[a] *Department of Biochemistry and Microbiology, Slovak University of Technology, Radlinského 9, 81237 Bratislava, Slovakia*
[b] *Pinel Psychiatric Hospital, 90218 Pezinok, Slovakia*

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Abstract The properties of the $^{45}Ca^{2+}$ influx by human red blood cells (RBC) induced by $NaVO_3$ or NaF were compared. The $NaVO_3$-induced $^{45}Ca^{2+}$ influx was slower and less extensive than that induced by NaF. Both processes were saturable with Ca^{2+}. Substitution of Na^+ by K^+ inhibited the $^{45}Ca^{2+}$ influx induced by $NaVO_3$ but stimulated that by NaF. The $NaVO_3$-induced Ca^{2+} influx was sensitive to nifedipine (IC_{50} = 50 mol/l), Cu^{2+} (IC_{50} = 9 mol/l), DTNB (5,5′-dithiobis-(dinitrobenzoic acid)) (IC_{50} = 12 mol/l) (maximal inhibition 16%, 18%, and 28%, respectively, if NaF was used as inducer). On the other hand, tetrodotoxin (TTX) and cyclosporin A inhibited only the NaF-induced $^{45}Ca^{2+}$ influx (IC_{50} = 21 mol/l and 28 mol/l, respectively). Pig RBC, known not to display the $NaVO_3$-induced Ca^{2+} influx, exhibited Ca^{2+} influx induced by NaF. The results show that $NaVO_3$ activates the Ca^{2+} influx via a pathway homologous to the L-type Ca^{2+} channel while the NaF-induced Ca^{2+} influx is mediated via the TTX-sensitive Na^+ channel in the presence of NaF with possible participation of calcineurin or cyclophilin. Thus, the Gárdos effect induced by $NaVO_3$ and NaF represents two phenomena activated by different mechanisms present in the cryptic state in the RBC membrane.

Key words: Red blood cell; $NaVO_3$; NaF; Ca^{2+} influx; Gárdos effect

1. Introduction

Since its discovery in 1958 [1] the Ca^{2+}-dependent K^+ efflux in human red blood cells (RBC) (further referred to as the Gárdos effect) has been extensively studied (see [2,3] for review) and has been helpful in understanding the role of the Ca^{2+}-activated K^+ channels (K(Ca)), especially in excitable cells. The role of K(Ca) in non-excitable cells is much less understood.

A feature of the Gárdos effect is its inducibility by various agents and/or treatments. In addition to NaF and ATP depletion [1,4], propranolol [5–7], $NaVO_3$ [8], lead [10] or redox modification [9,11] and, probably, also PGE2 [12] have been used as inducers. Despite the different molecular character of the individual inducers, their action involves the translocation of extracellular Ca^{2+} across the RBC membrane which, finally, leads to the activation of K(Ca) and massive K^+ loss. Therefore, it is feasible that the common denominator of all inducers (with lead as a possible exception) is their ability to perturb the RBC Ca^{2+} homeostasis which leads to (transient or permanent) accumulation of Ca^{2+} in the RBC cytoplasm.

As the RBC Ca^{2+} homeostasis presumably comprises only two components, the low-capacity Ca^{2+} influx pathway and the high-capacity Ca^{2+} efflux pathway represented by the Ca^{2+} pump, the inducers of the Gárdos effect could serve as tools to characterize the Ca^{2+} influx pathway(s) and, thereby, to unravel the nature of the Gárdos effect. Extensive characteristics of the Ca^{2+} influx induced by ATP depletion [4] and by $NaVO_3$ [8,13–16] are available and suggest that the Ca^{2+} influx is mediated by a carrier. The question of whether NaF and $NaVO_3$ activate the same or different Ca^{2+} influx pathway(s) was addressed in this work.

2. Materials and methods

Blood from healthy volunteers of both sexes was withdrawn by venipuncture into EDTA-containing anticoagulant (5 mmol/l), and was used within 3 days, stored at 0–4°C. RBC were isolated after centrifugation of the blood (10 min at 600×*g*) and aspiration of the supernatant with the buffy coat, and three-fold washing with and, finally, suspending into a medium containing (in mmol/l): 20 Tris-HCl (pH 7.3), 130 NaCl, 5 KCl, 10 glucose (further referred to as the suspension medium), to a hematocrit of 30%, and immediately used for experiments. Pig blood was obtained from the local slaughterhouse and was used for experiments on the same day.

The influx of Ca^{2+} was measured with the radionuclide ^{45}Ca, after repetitive washing for removal of extracellular radioactivity, as described previously [8]. Aliquots of 30% suspension were preincubated with 1 mmol/l $NaVO_3$ for 15 min or for 3 min with 10 mmol/l NaF at 25°C, and $^{45}CaCl_2$ (2.5 mmol/l final) was added and incubated for 60 min at the same temperature unless indicated otherwise. The incubation was stopped by addition of the same volume of stopping medium containing (in mmol/l): 20 Tris-HCl (pH 7.3), 75 KCl, 60 NaCl, 10 glucose, and 1 EDTA, and by rapid centrifugation of the sample in a microcentrifuge. The supernatant was sucked off and the pellet was washed with the stopping medium three more times. Finally, the pellet was precipitated with 10% perchloric acid containing 20 mmol/l $LaCl_3$, the precipitate was centrifuged and the pellet was used for liquid scintillation counting. Control cells without $NaVO_3$ were treated in parallel. When inhibitors were tested, the same volume of solvent (DMSO, methanol, max. 0.5% v/v) was added to the control samples. All samples were done in duplicate and the average value of parallel samples (±standard error) is given in the figures. The standard error is indicated by bars when it exceeded the dimension of the symbol. The results presented in the figures represent typical experiments obtained independently from each other. The number of experiments performed varied from three to nine. The values were corrected for the radioactivity obtained in control cells without inducer.

$^{45}CaCl_2$ was from the Radiochemical Centre (Amersham, UK), $NaVO_3$ was from Reachim (Moscow, Russia). Tetrodotoxin was purchased from Calbiochem, DTNB from Merck, Darmstadt, Germany. Nifedipine was synthesized in the Institute of Drug Research, Modra, Slovakia and was kindly provided by Dr. Zdeno Mahrla. Cyclosporin A was kindly provided by Prof. V. Betina (Slovak University of Technology, Bratislava). Menthol and camphor were purchased from Me-

*Corresponding author. Fax: (42) (1) 7393198.
E-mail: varecka@checdek.chtf.stuba.sk

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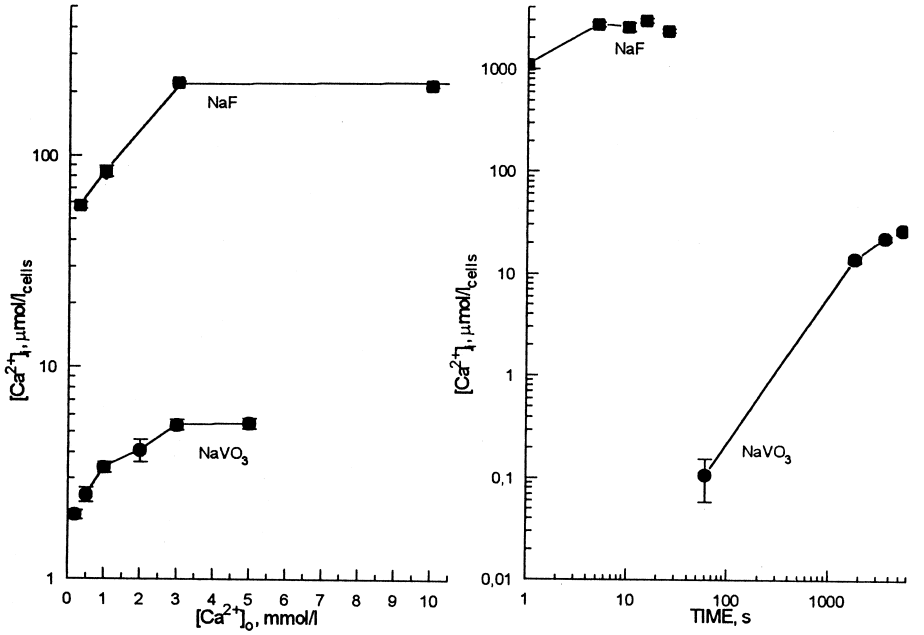

Fig. 1. The Ca^{2+} influx by human RBC induced by 1 mmol/l $NaVO_3$ (circles) or 10 mmol/l NaF (squares). Left panel: $[Ca^{2+}]_o$ dependence. Right panel: Time course.

dika (Bratislava, Slovakia). Other chemicals (all of analytical grade) were purchased from Lachema (Brno, Czech Republic).

3. Results

The dependence of the $^{45}Ca^{2+}$ influx induced by $NaVO_3$ and NaF on the extracellular Ca^{2+} showed saturability (Fig. 1) with $K_{M(Ca)} = 0.5$ mmol/l for both $NaVO_3$ and NaF. The time courses of the $^{45}Ca^{2+}$ influx induced by either agent (Fig. 1) showed marked differences. That induced by $NaVO_3$ reached the half-maximal value in several tens of minutes and gradually increased until the end of measurement, in accordance with previously published data [8,16]. On the other hand, NaF-induced Ca^{2+} influx reached the maximal values within about 30 s and then (in most experiments) slightly decreased. The NaF-induced $^{45}Ca^{2+}$ influx was different from that induced by $NaVO_3$ also in the extent (expressed as μmol Ca^{2+}/l_{cells}) at the end of experiments (1 and 60 min, respectively). The former exceeded the latter by an order of magnitude and in some experiments values were obtained (50–3000 μmol/l_{cells}) that suggested equilibration of Ca^{2+} across the RBC membrane (and possible binding and/or precipitation in the cytoplasm) upon the action of NaF in some experiments. In $NaVO_3$-treated cells values between 5 and 80 μmol/

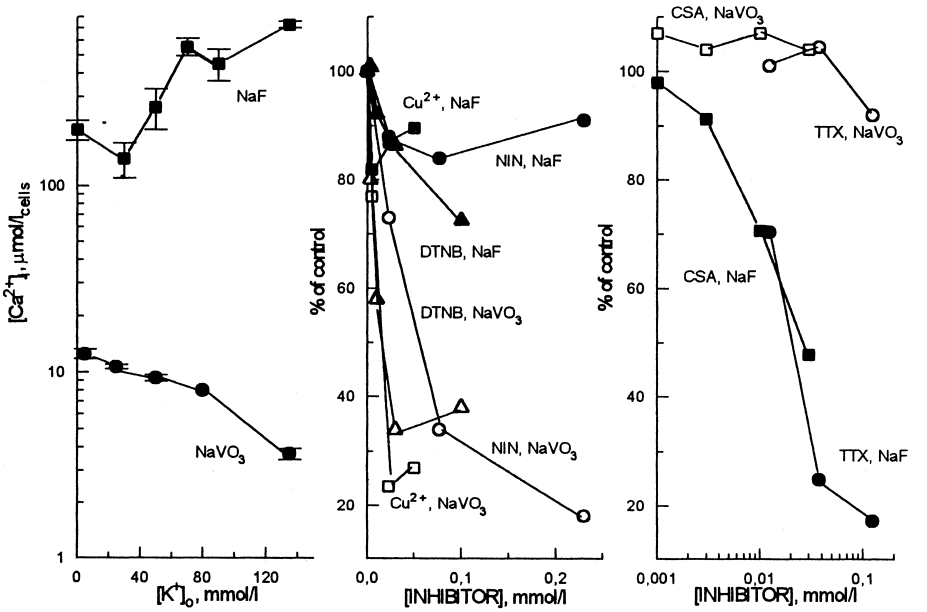

Fig. 2. The effects of inhibitors on the $NaVO_3$- and NaF- (closed symbols) induced $^{45}Ca^{2+}$ influx by human RBC. Left panel: The effect of isotonic Na^+ substitution by K^+. Middle panel: The effect of Cu^{2+} (squares), DTNB (triangles) and nifedipine (circles) ($NaVO_3$, open symbols; NaF, closed symbols). Right panel: The effect of tetrodotoxin (TTX) (circles) and cyclosporin A (CSA) (squares).

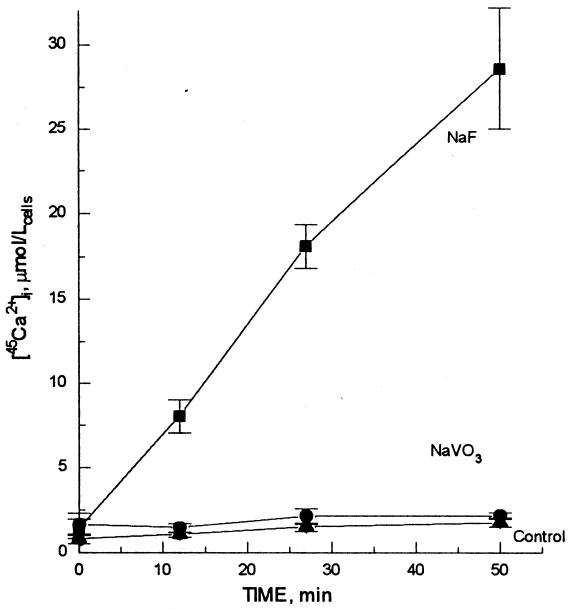

Fig. 3. The effect of $NaVO_3$ (circles) and NaF (squares) on the $^{45}Ca^{2+}$ influx in pig RBC. Control RBC without addition were treated in parallel (triangles).

l_{cells} were obtained (values from several tens of experiments with either inducer).

The prominent feature of the Ca^{2+} influx induced in $NaVO_3$ [8] (and ATP-depleted RBC [4]) is its inhibition by isotonic substitution of Na^+ for K^+ but not for choline. In NaF-treated cells, the Ca^{2+} influx was influenced by Na^+ substitution in the opposite direction (Fig. 2). Moreover, the effect was not specific for K^+ because the substitution of Na^+ by choline$^+$ had a similar, even more pronounced, effect (not shown).

It was found previously [13] that the $NaVO_3$-induced Ca^{2+} influx displays sensitivity to several inhibitors, e.g. to HS reagents and divalent cations creating mercaptide bonds which exert a biphasic action on the Ca^{2+} influx [13]. NaF-induced Ca^{2+} influx was less sensitive to these compounds, represented by Cu^{2+} and DTNB (Fig. 2). At submillimolar concentrations, the maximal inhibition by Cu^{2+} was less than 20% and by DTNB less than 30% (75 and 65%, for the $NaVO_3$-induced Ca^{2+} influx, with $IC_{50} = 9$ μmol/l and 12 μmol/l, respectively).

In accordance with the data of Stimpel et al. [17], the $NaVO_3$-induced Ca^{2+} influx was sensitive to dihydropyridine Ca^{2+} antagonists. It was found to be fully suppressed by nifedipine as an inhibitor (up to 140 μmol/l) (Fig. 2) ($IC_{50} = 50$ μmol/l). On the other hand, the Ca^{2+} influx induced by NaF was less sensitive to nifedipine (Fig. 2) and, in the same concentration range, the degree of inhibition did not reach 20%.

NaF was found to increase the membrane permeability for Na^+ ions which was sensitive to the nerve poison tetrodotoxin (TTX) [18]. The Ca^{2+} influx elicited by NaF was also sensitive to TTX in the same concentration range ($IC_{50} = 21$ μmol/l) (Fig. 2). However, the $NaVO_3$-induced Ca^{2+} influx was not sensitive to TTX (Fig. 2). Also other agents which are known to affect the function of CNS, menthol and camphor, in millimolar concentrations, in part inhibited the NaF-induced Ca^{2+} influx but not that induced by $NaVO_3$ (not shown). The calcineurin inhibitor [19] and RBC cyclophilin ligand [27,28] cyclosporin A (CSA) was another compound which inhibited the effect of NaF ($IC_{50} = 28$ μmol/l) but it exerted a rather stimulatory effect on the $NaVO_3$-induced Ca^{2+} influx (Fig. 2).

It is known that $NaVO_3$ is unable to elicit the Ca^{2+} influx and the Gárdos effect in pig RBC [13] (Fig. 3). However, NaF under conditions similar to those used for human RBC was still able to induce the $^{45}Ca^{2+}$ influx, although to a lesser extent, in pig RBC (Fig. 3).

4. Discussion

According to the generally accepted notion which originated from the study of ATP-depleted cells the changes in Ca^{2+} homeostasis induced by ATP depletion and leading to the activation of the Gárdos effect are due to the inhibition of Ca^{2+} pumping, and the subsequent entry of Ca^{2+} through the membrane 'resting' permeability mechanism(s). Also, the effect of NaF was explained by its inhibition of glycolysis, the major catabolic pathway in human RBC [2]. This way of thinking was adopted when the study of the effect of $NaVO_3$ on the RBC Ca^{2+} homeostasis has been initiated [8,13] where similar numerical values of the $^{45}Ca^{2+}$ influx were observed. Apparently, this is not the case when NaF is used as an inducer (Fig. 1). The Ca^{2+} influx induced by NaF is not only much more extensive but also much faster than that induced by $NaVO_3$. It should be mentioned that its time course resembles the NaF-induced and Ca^{2+}-dependent Na^+ influx which we described previously [18] and both processes clearly precede the onset of the Gárdos effect which is delayed by about 5 min after addition of Ca^{2+} to both NaF- and $NaVO_3$-treated RBC ([1,8], unpublished observation).

In addition, NaF- and $NaVO_3$-induced Ca^{2+} influxes exhibit striking differences (Fig. 2) in the sensitivity to inhibitors. The inhibitory action of Cu^{2+} and DTNB is largely reduced in NaF-treated RBC as compared with $NaVO_3$ treatment, suggesting only a minor role of HS groups in facilitating the NaF-induced Ca^{2+} influx. On the other hand, the effect of CSA suggests a selective involvement of calcineurin or cyclophilin in mediating the NaF-induced Ca^{2+} influx. It should be mentioned at this point that Co^{2+} inhibits both processes with approximately equal efficiency (not shown).

The experiment with the most explanatory power is the effect of TTX which is an inhibitor of the NaF-induced Ca^{2+} influx only. This (together with the time course) strongly suggests that Ca^{2+} shares the influx pathway with Na^+ [18] in the same manner as suggested by Baker for the nerve and/or chromaffin cell Na^+ channel almost three decades ago [20,21]. The delayed appearance of the Ca^{2+}-dependent K^+ efflux which follows Na^+ and Ca^{2+} influxes is similar to the time course of these ions during the action potential in nerve cells. Phenomenologically, the effect of NaF and Ca^{2+} could be regarded as an extremely slow chemically induced action potential with negligible inactivation (if any). The stimulatory effect of Na^+ substitution on the NaF-induced $^{45}Ca^{2+}$ influx (Fig. 1) could hence be explained by the elimination of the competition of Na^+ for the common transport pathway.

It is conspicuous that the inhibitory efficiencies of several inhibitors tested (nifedipine, TTX, CSA) were less than those observed in other (e.g. excitable) cells. We could not provide any experiments which could explain these results. The recent theoretical model by Bray et al. [29] based on the study of

bacterial chemotaxis suggests that the sensitivity of the signalling pathway is a variable of receptor clustering. This concept, which is amenable to experimental analysis, may be useful for explaining the differences in inhibitor sensitivities also in animal cells.

The results shown in Figs. 1 and 2 indicate that the properties of the Ca^{2+} influx induced by $NaVO_3$ and NaF are different. The interspecies difference experiment shown in Fig. 3 compellingly suggests that both effects are mediated by independent mechanisms. In experiments published in our recent papers [15,16] we have shown that $NaVO_3$-induced Ca^{2+} influx may be preceded by changes in inositol phospholipid metabolism rather than by changes in G-protein activation whereas the effect of NaF implies the involvement of G-protein.

The extensive shifts in ion concentrations and the effects of inhibitors presented above enable us to answer the question whether the Gárdos effect induced by NaF is a inique event or has some homology in other cells and/or organs. Such extensive shifts in concentrations of Ca^{2+}, K^+, and Na^+ occur in brain during ischemia and spreading depression [22], or severe hypoglycemia [23] and were recently found to be antagonized (among other inhibitors) by TTX [24–26]. Although the details of the mechanisms in RBC and brain have still to be found in the future, perturbations of Ca^{2+} homeostasis seem to have central roles in triggering these events.

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