Investigation of Properties of the Ca^{2+} Influx and of the Ca^{2+}-Activated K^+ Efflux (Gárdos Effect) in Vanadate-Treated and ATP-Depleted Human Red Blood Cells

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Abstract. In this study the properties of the $^{45}Ca^{2+}$ influx in human red blood cells (RBC) induced by $NaVO_3$ or ATP-depletion were compared. Both $NaVO_3$-induced and ATP-depletion-induced $^{45}Ca^{2+}$ influxes were in the range 10^{-6}–10^{-5} mol $Ca^{2+} \cdot l_{cells}^{-1} \cdot h^{-1}$. The saturatability of ATP-depletion-induced $^{45}Ca^{2+}$ influx with Ca^{2+} was much less pronounced than that of $NaVO_3$-induced $^{45}Ca^{2+}$ influx. The $NaVO_3$-induced Ca^{2+} influx was sensitive to nifedipine (IC_{50} = 50 μmol/l) and Cu^{2+} (IC_{50} = 9 μmol/l) but these inhibitors had only a marginal effect when ATP-depletion was used as the Ca^{2+} influx inducer. On the other hand, polymyxin B (PXB) (1–5 mg/ml) strongly stimulated the ATP-depletion-induced $^{45}Ca^{2+}$ influx whereas its effect on the $NaVO_3$-induced Ca^{2+} influx was biphasic, with about 10 % stimulation at lower PXB concentrations and an inhibition of 40 % at higher concentrations. SDS-PAGE revealed that both $NaVO_3$ and PXB induced changes in the protein phosphorylation pattern in the presence of Ca^{2+}. $NaVO_3$ stimulated the phosphorylation of several proteins and this effect was counteracted by PXB. The comparison of the kinetics and temperature dependencies of the Gárdos effect induced by $NaVO_3$ and the ATP-depletion showed marked differences. The ability of $NaVO_3$ to induce the Gárdos effect dramatically increased in ATP-depleted cells. These findings indicate that the $^{45}Ca^{2+}$ influxes preceding the activation of the Ca^{2+}-activated K^+ efflux (Gárdos effect) stimulated by $NaVO_3$ and by ATP-depletion, are mediated by different transport pathways. In addition, obtained re-

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sults demonstrate that ATP-depletion and $NaVO_3$-treatment exert additive action in triggering the Gárdos effect.

Introduction

The red blood cell (RBC) Ca^{2+} homeostasis comprises two main components, the low-capacity Ca^{2+} influx and the high-capacity Ca^{2+} efflux, the latter represented by the Ca^{2+} pump (Ca^{2+}-ATPase). In contrast to the knowledge about Ca^{2+} efflux (i.e., Ca^{2+}-ATPase) that has been described in minute details (Carafoli 1994), molecular mechanisms of the calcium influx are still relatively little understood and without any reference to a particular molecular entity(ies).

The study of the Ca^{2+}-dependent K^+ efflux in human RBC (further referred to as the Gárdos effect) contributed significantly to the characterization of the Ca^{2+} influx in RBC. This is known to be induced by a plethora of agents. In addition to NaF treatment, and ATP-depletion (Gárdos 1958; Ferreira and Lew 1977), substances as propranolol (Manninen 1970; Szász and Gárdos 1974; Szász et al. 1977), $NaVO_3$ (Varečka and Carafoli 1982; Fuhrmann et al. 1984), lead salts (Riordan and Passow 1971) or redox-modification (Sanchez et al. 1980; Fuhrmann et al. 1985) and, probably, also prostaglandin E_2 (Li et al. 1996) have been used as inducers of the Ca^{2+} influx in RBC.

In our recent study we have demonstrated that Ca^{2+} influxes induced by $NaVO_3$ and NaF differ in their characteristics to an extent that enables us to conclude that both these Ca^{2+} inward transports are mediated by different pathways (Varečka et al. 1998). The properties of the ATP-depletion-induced (Ferreira and Lew 1977) and of the vanadate-induced Ca^{2+} influx (Varečka and Carafoli 1982; Stimpel et al. 1984; Varečka et al. 1986, 1987, 1995, 1997a,b; Engelmann and Duhm 1989) indicate that the character of Ca^{2+} influx is close to a carrier-mediated transport. Unfortunately, there are no conclusive data about the Ca^{2+} influx induced by other inducers of the Gárdos effect.

The ATP-depletion- or ischemia-induced changes in the Ca^{2+} homeostasis were observed also in other cells. These changes involve an increase in the passive Ca^{2+} permeability, changes in cytoplasmic Ca^{2+} concentration, and an increase in the Ca^{2+} release from the intracellular Ca^{2+} stores. Such changes were observed in endothelial (Arnould et al. 1992), myocycardial (Clague et al. 1993), vascular endothelial (Ziegelstein et al. 1994), neural (Bickler and Hansen 1994; Johnson et al. 1994; Gleitz et al. 1996; Chen et al. 1999) cells, cells of proximal tubules (Weinberg et al. 1997), macrophages (Vemuri and Marchase 1999), and hepatocytes (Gasbarrini et al. 1992; Carini et al. 1994; Crenesse et al. 1999) and smooth muscle cells (Duridanova et al. 1995; Petkov et al. 1998). The increase of the $^{45}Ca^{2+}$ influx upon the ATP-depletion seems to be a general phenomenon, although in some cells opposite effects of ATP-depletion and/or ischemia were observed (Stevens et al. 1994; Rekalov et al. 1997; Peters et al. 1998). Thus, the study of the properties of the ATP-depletion-induced Ca^{2+} influx could not only reveal the nature and regulatory aspects of the Ca^{2+} influx pathway but could also be of interest

from the aspects of comparative biochemistry and physiology. Here we present the evidence that the Ca^{2+} influx and the Ca^{2+}-activated K^+ efflux (Gárdos effect) induced by $NaVO_3$ and by ATP-depletion display different characteristics in RBC.

Materials and Methods

Preparation of Red Blood Cell suspension

Blood from healthy volunteers of both sexes was withdrawn by venipuncture into EDTA-containing medium (5 mmol/l). It was stored at 0–4 °C and used within 3 days. Isolation of RBC: a) centrifugation of the blood (10 min, $600 \times g$, 4 °C); b) aspiration of the supernatant with the buffy coat; c) washing the pellet three times; d) resuspension of isolated RBC in a medium containing (in mmol/l): 20 Tris-HCl (pH 7.4); 130 NaCl; 5 KCl; 10 glucose (further referred to as the suspension medium), to a final haematocrit of 30 %. RBC prepared by this way were immediately used for experiments.

Vanadate-induced Ca^{2+} influx

The Ca^{2+} influx was measured with the radionuclide ^{45}Ca, using the procedure described earlier (Varečka et al. 1997a). In brief: aliquots of 30 % RBC suspension were preincubated with 1 mmol/l $NaVO_3$ for 15 min at 25 °C. $^{45}CaCl_2$ (2.5 mmol/l) was added and incubated for 60 min at the same temperature (if not indicated differently). Incubation was stopped by addition of the same volume of a medium, containing in mmol/l: 20 Tris-HCl (pH 7.3); 75 KCl; 60 NaCl; 10 glucose and 1 EDTA (further referred to as the stopping medium) followed by rapid centrifugation (1 min, $1500 \times g$, 4 °C). Supernatant was sucked off and the pellet was repeatedly washed with the stopping medium for three times to remove the excess of extracellular ^{45}Ca. Finally, the pellet was precipitated with 10 % trichloroacetic acid (TCA) containing 20 mmol/l $LaCl_3$, spun down for 1 min at $1500 \times g$, 4 °C, and the supernatant was taken for the liquid scintillation counting. Control cells without vanadate were run in a parallel line. Inhibitors, if applied, were added in the same volume of solvent (DMSO, methanol, max. 0.5 % v/v). All samples were run in duplicates. Results are given as means of the parallel samples ± S.E.M., from three or more separate experiments.

ATP-depletion-induced Ca^{2+} influx

ATP depletion was achieved by incubation of 30 % RBC suspension (in suspension medium) with 12.5 mmol/l inosine and 5 mmol/l iodoacetamide for 3 h at 37 °C. The influx of $^{45}Ca^{2+}$ was measured similarly as it was described in the previous section.

Measurement of Ca^{2+}-dependent K^+ efflux

The Ca^{2+}-dependent K^+ efflux was monitored by estimating of the net K^+ efflux with the aid of flame photometry in $NaVO_3$-treated and ATP-depleted RBC. Briefly: at time zero, $^{40}CaCl_2$ (2.5 mmol/l) was added and aliquots of suspension were taken after a 50 min incubation at 25 ℃. After spinning down the RBC through a layer of dibutylphtalate, the supernatant was used for flame photometry. Controls were treated in parallel.

Protein phosphorylation

Red blood cells in the suspension medium (haematocrit 30 %) were preincubated with 10 μCi of ^{32}P-labelled orthophosphate for 30 min at 30 ℃. One ml aliquots of the RBC suspensions containing in addition Ca^{2+} (2.5 mmol/l); $NaVO_3$ (1 mmol/l) and polymyxin B (3 mg/ml) were incubated for 60 min at room temperature (shown in the Fig. 3). After incubation the pellet was lysed with 1 ml of 0.3 mol/l glycerol at 0 ℃ for 10 min. Ghosts were harvested by centrifugation on the microcentrifuge (5 min, $1500 \times g$, 4 ℃). Lysate was discarded and the pellets were dissolved in 200 μl of the medium, containing in mmol/l: 10 Tris-HCl, pH 8; 1 EDTA; further 1 % sodium dodecyl sulphate (SDS); 5 % β-mercaptoethanol; 15 % glycerol and 0.1 % bromophenol blue. The amount of proteins was assessed by the method of Lowry et al. (1953). Suspensions of erythrocyte ghost protein (10 mg/ml) were loaded onto the gel for SDS PAGE. Electrophoresis was carried out according to the method of Fairbanks et al. (1971). Myosin, β-galactosidase, phosphorylase B, bovine serum albumin (BSA) and carboanhydrase were used as the molecular weight standards. Incorporation of ^{32}P was detected by autoradiography (X-ray plate Medix Rapid, Foma, Hradec Králové, Czech Republic).

Materials

$^{45}CaCl_2$ and $^{32}P_{in}$ were obtained from Pharmacia-Amersham (Little Chalfont, U.K.); dibutylphtalate and polymyxin B from Serva (Heidelberg, Germany); $NaVO_3$ was from Reachim (Moscow, Russia). Chemicals for SDS PAGE were purchased from Serva, and all other chemicals (of analytical grade) were purchased from Lachema (Brno, Czech Republic). Nifedipine was synthetized in the Institute of Drug Research (Modra, Slovakia) and was kindly provided by Dr. Zdeno Mahrla.

Results

Dependence of $^{45}Ca^{2+}$ influx on extracellular concentration of calcium

The time course of both $NaVO_3$-treatment and ATP-depletion-induced $^{45}Ca^{2+}$ influx was in accordance with the data published previously (Ferreira and Lew 1977; Varečka and Carafoli 1982). Nevertheless, the dependence of $^{45}Ca^{2+}$ influx on extracellular Ca^{2+} concentration exhibited differences in saturability (Fig. 1). The value of $K_{M(Ca)}$ for $NaVO_3$ was close to 0.5 mmol/l. However, at the same experimental conditions, the ATP-induced $^{45}Ca^{2+}$ influx failed to exhibit saturation in respect to

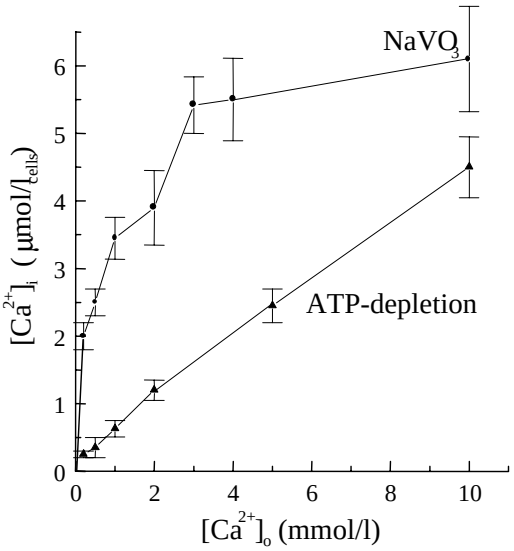

Figure 1. $^{45}Ca^{2+}$ influx in ATP-depleted and $NaVO_3$-treated red blood cells. Dependence on the extracellular Ca^{2+} concentration. $^{45}Ca^{2+}$ influxes were measured as described in Materials and Methods. Data are means ± S.E.M. of three independent experiments. (●), $NaVO_3$-treated and (▲), ATP-depleted RBC. Controls without vanadate or inosine were run in parallel. The radioactivity of control cells was subtracted from the corresponding experimental values. All samples were run in duplicates.

extracellular Ca^{2+} concentration. The average rate of the ATP-depletion-induced $^{45}Ca^{2+}$ influx reached 8.6 ± 2.6 $\mu mol \cdot l_{cells}^{-1} \cdot h^{-1}$, ($n = 3$) and differed significantly from that of the $NaVO_3$-induced $^{45}Ca^{2+}$ influx with values ranging between 5–80 $\mu mol \cdot l_{cells}^{-1} \cdot h^{-1}$, ($n = 62$).

The effect of Cu^{2+} and PXB on $^{45}Ca^{2+}$ influx

The $NaVO_3$-induced Ca^{2+} influx exhibits sensitivity to several inhibitors, including some HS-reagents and divalent cations that exerted biphasic action on the $^{45}Ca^{2+}$ influx in human RBC (Varečka et al. 1986). Present results are in accordance with these findings (Fig. 2A and 2B). Cu^{2+} ions inhibited the $NaVO_3$-induced $^{45}Ca^{2+}$ influx up to 25 μmol/l ($p < 0.05$, Fig. 2A). Higher concentrations of Cu^{2+} caused only a slight stimulation of the $NaVO_3$-induced Ca^{2+} influx. However, when similar concentrations of Cu^{2+} ions were applied to ATP-depleted RBC, the inhibition of the ATP-depletion-induced $^{45}Ca^{2+}$ influx was not observed. This indicates that the ATP-depletion-induced $^{45}Ca^{2+}$ influx is insensitive to Cu^{2+} (Fig. 2A).

The effect of PXB on the $NaVO_3$-induced Ca^{2+} influx also exhibited biphasic character. Nevertheless, in contrast to the effect of Cu^{2+} ions, at lower concentrations PXB stimulated the $^{45}Ca^{2+}$ influx in ATP-depleted cells (Fig. 2B, upper curve). At higher concentrations, however, PXB induced depression of the ATP-dependent Ca^{2+} influx. This result may either indicate that: a) at low PXB concentrations the remaining ATP is counteracting the PXB-induced inhibition of Ca^{2+} influx into ATP-depleted cells; or, b) that higher concentrations of PXB are needed to overcome the activating influence of protein phosphorylation on the influx of calcium (Wen et al. 1984).

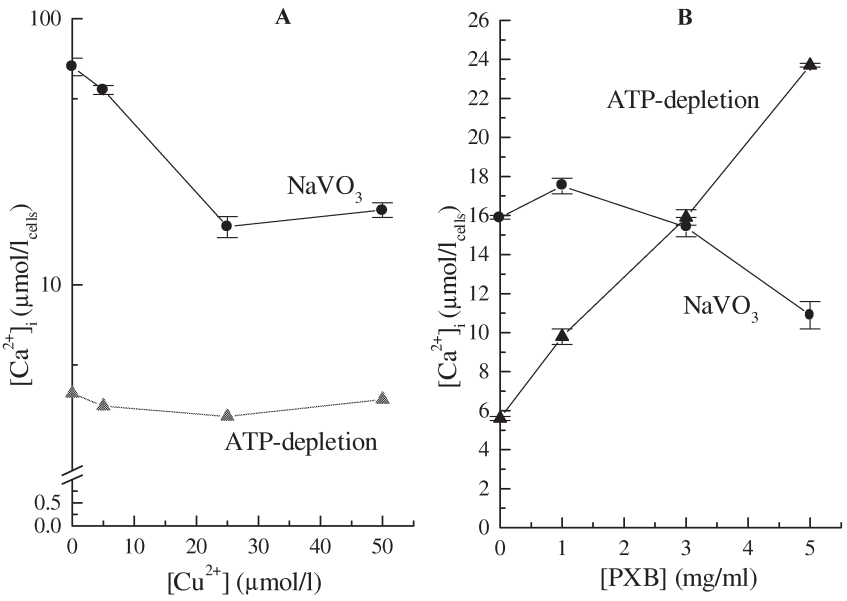

Figure 2. The effect of Cu^{2+} ions (A) and polymyxin B (B) on the $^{45}Ca^{2+}$ influx in ATP-depleted (▲) and $NaVO_3$-treated (●) red blood cells. Control cells were treated in parallel. The radioactivity of control cells was subtracted from the corresponding experimental values. All samples were run in duplicates. **A.** Results are means ± S.E.M. of three experiments. Note the logarithmic scale of the ordinata. **B.** Results are means ± S.E.M. of six ($NaVO_3$) and two (ATP-depletion) experiments.

Changes in membrane protein phosphorylation

SDS PAGE of RBC membrane proteins treated with $^{32}P_{in}$ revealed several differences in their phosphorylation (Fig. 3). Non-treated samples as well as samples treated with Ca^{2+}, PXB and $NaVO_3$ alone exhibited no apparent changes in protein phosphorylation patterns. However, when added together with PXB or $NaVO_3$, Ca^{2+} ions exerted considerable influence on phosphorylation pattern of RBC membrane proteins: a) In presence of PXB (3 mg/ml) plus Ca^{2+} much less $^{32}P_{in}$ was incorporated into all bands; except for two bands with molecular weights over 200 kDa which became, in contrast, more phosphorylated. b) In presence of $NaVO_3$, Ca^{2+} ions enhanced the phosphorylation of proteins considerably. Nevertheless, the bands also become more diffuse, an effect that might be ascribed to proteolysis. c) $NaVO_3$, Ca^{2+} and PXB (3 mg/ml) when acting simultaneously caused an overall decrease in the incorporated radioactivity. On the basis of these findings it was concluded that the effect of PXB on membrane protein phosphorylation might exceed that of inhibition of the protein kinase C (PKC)-mediated processes.

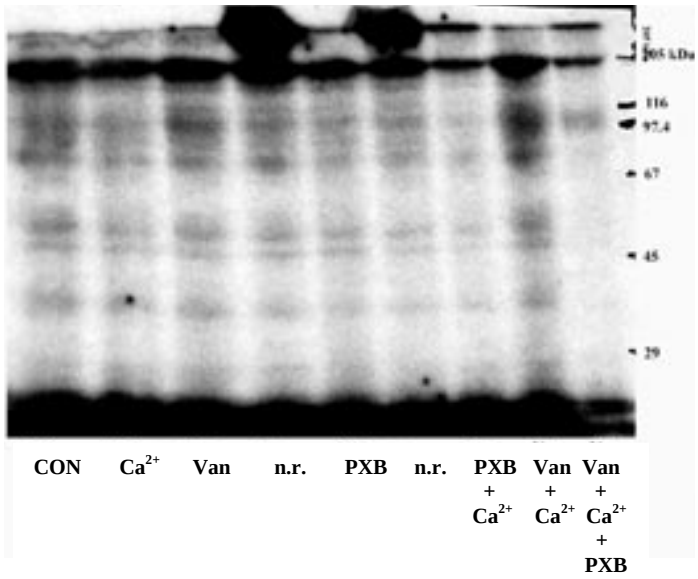

Figure 3. Effect of $CaCl_2$ (Ca^{2+} ions), vanadate (Van) and polymyxin B (PXB) on phosphorylation of the RBC membrane proteins. RBC suspensions were pre-incubated with 10 μCi of ^{32}P-labelled orthophosphate for 30 min at 30°C. Aliquots of the RBC suspensions containing in addition $CaCl_2$ (Ca^{2+}) (2.5 mmol/l); $NaVO_3$ (Van) (1 mmol/l); PXB (3 mg/ml) were incubated for 60 min at room temperature. Instead of Ca^{2+} ions vanadate and PXB, the controls were treated with the same volumes of solvents (distilled water or methanol, max. 0.5% v/v) and were run in parallel. n.r., non-related lane.

The effect of nifedipine on $^{45}Ca^{2+}$ influx in RBC

The sensitivity of the $NaVO_3$-induced or ATP-depletion-induced $^{45}Ca^{2+}$ influx to dihydropyridines was examined by means of nifedipine (IC_{50} = 50 μmol/l). In $NaVO_3$-treated cells nifedipine inhibited the Ca^{2+} influx by about 79%, while the Ca^{2+} influx induced by ATP-depletion was found not to be sensitive to similar concentrations of nifedipine (Fig. 4). On the other hand, an addition of $NaVO_3$ to ATP-depleted cells led to a slight inhibition of the $^{45}Ca^{2+}$ influx (about 28%).

Properties of the Ca^{2+}-dependent K^+ efflux

The Ca^{2+}-dependent K^+ efflux (i.e., of the Gárdos effects) induced by $NaVO_3$ or by ATP-depletion exhibited different kinetic characteristics (Fig. 5). At temperatures between 22 and 27°C, the onset of the K^+ efflux was delayed (after the addition of Ca^{2+}) in both the $NaVO_3$-treated as well as in the ATP-depleted cells. The lag phase duration was shorter in the $NaVO_3$-treated cells (approx. 3 min *vs.* 10 min). Moreover, the temperature dependencies of the Gárdos effects showed also remarkable differences. Another difference was that the K^+ effluxes induced by $NaVO_3$ reached maximal values at about 25°C, while in ATP-depleted cells the K^+ effluxes

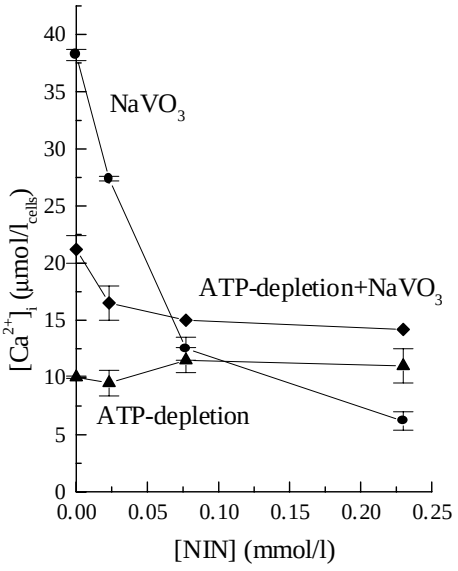

Figure 4. Effect of nifedipine (NIN) on $NaVO_3$-induced and ATP-depletion-induced Ca^{2+} influx in RBC. The measurements were performed as described in Materials and Methods in the presence of indicated concentrations of nifedipine and, where indicated, 1 mmol/l $NaVO_3$. Control cells (without nifedipine) were treated with equal volumes of solvent (0.5% (v/v) dimethylsulfoxide). (●), $NaVO_3$; (▲), ATP-depletion; (♦), $NaVO_3$ and ATP-depletion. Experimental data were corrected for the presence of radioactivity in control RBC. All samples were run in duplicates. Results are means ± S.E.M. of three experiments.

increased with temperature. In order to obtain more information about a possible interaction between both ways of triggering the Gárdos effect, the dependence of the latter on $NaVO_3$ concentration was studied in RBC that were also depleted from ATP to various extent. Results revealed that ATP depletion decreased progressively the required concentration of $NaVO_3$ for induction of the Gárdos effect (Fig. 6). In comparison to 100 μmol/l $NaVO_3$ required to induce the Gardos effect in the absence of ATP-depletion, after 5 h of ATP-depletion, the Gárdos effect could be induced by 3 μmol/l $NaVO_3$ only.

Discussion

Previous results (Ferreira and Lew 1977; Varečka and Carafoli 1982) indicated that the Ca^{2+} influxes into RBC induced either by $NaVO_3$ or by ATP-depletion may have some common characteristics (e.g., they may be inhibited to similar extent by external $[K^+]_o$). In contrast, the present study revealed that the Ca^{2+} influxes elicited by these inducers exhibit several different properties, such as affinity to Ca^{2+} (Fig. 1), or sensitivity to inhibitors. Most conspicuous features seem to be the differences in sensitivity of the $NaVO_3$-induced or ATP-depletion-evoked Ca^{2+} influxes to the Ca^{2+}-channel blocking agent nifedipine (Fig. 4) to PXB (Fig. 2B) and to Cu^{2+} (HS-reagent) (Fig. 2A). These results may indicate that a physiological cytoplasmic concentration of ATP may be indispensable for the inhibitory action of nifedipine (and also for other inhibitors). Another possible interpretation is that $NaVO_3$ may exert some specific action on the Ca^{2+} influx pathway and

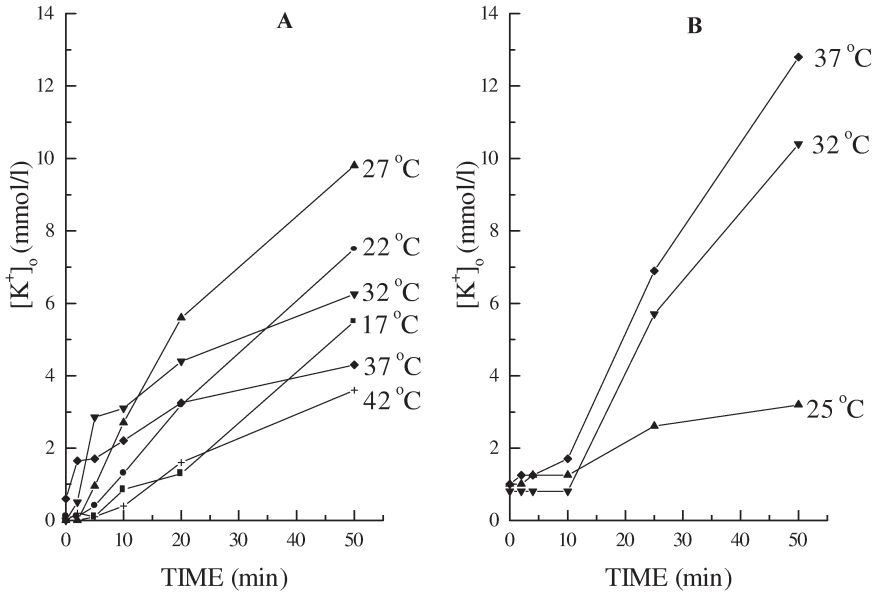

Figure 5. The time- and temperature-dependence of the Gárdos effects induced by $NaVO_3$ (A) or by ATP-depletion (B). The Gárdos effect induced by $NaVO_3$ (A), or ATP-depletion (B) were monitored by the measurement of the K^+ concentration in the external medium as described in Materials and Methods at temperatures indicated in the figure. Values were corrected for the concentration of K^+ ions present in time 0. Single points represent means of 3 (in panel A) or 2 (in panel B) separate measurements made in triplicates.

this may evoke changes in its sensitivity to nifedipine, PXB and Cu^{2+} (Varečka et al. 1997b). The second possibility is favoured also by the experiment shown in the Fig. 4 which indicates that the presence of $NaVO_3$ not only increased the $^{45}Ca^{2+}$ influx but also increased its sensitivity to nifedipine. No specific phosphorylation patterns associated with these changes which could be regarded as homologous to those found in sarcolemma (Hosey et al. 1986; Mundina-Weilenmann et al. 1991) were found so far. At this point it should be mentioned that the non-stimulated (basal) Ca^{2+} influx in intact RBC displays very low, if any, sensitivity to nifedipine (Varečka et al. 1997b). Thus, the differences between properties of the $^{45}Ca^{2+}$ influx in $NaVO_3$-treated and ATP-depleted RBC seem to be due to the presence of $NaVO_3$. Mechanism by which vanadate influences the Ca^{2+} homeostasis include the direct modification of properties of the Ca^{2+} transport (Varečka et al. 1997a). Results presented here confirm the suggestion by Fuhrmann et al. (1984) of multiple target sites of vanadate in the exhaustive explanation of the vanadate-induced $^{45}Ca^{2+}$ influx.

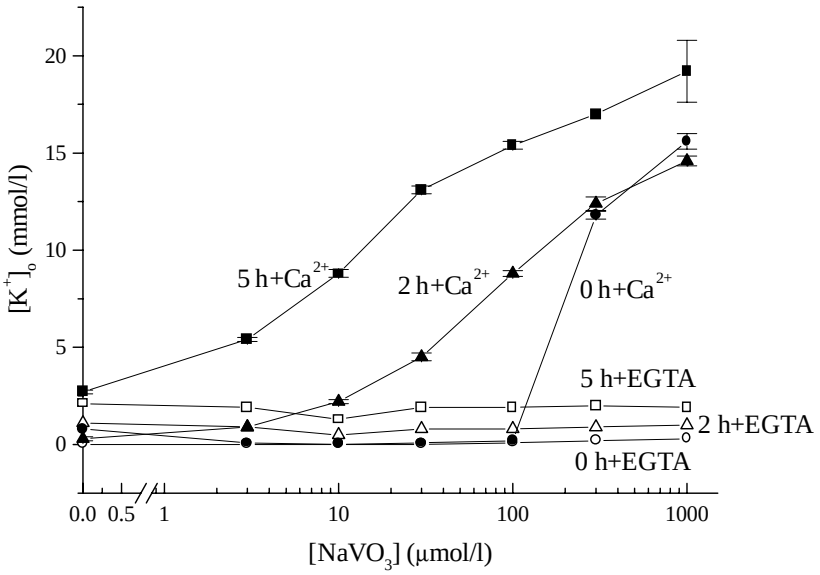

Figure 6. Effect of ATP depletion on the $NaVO_3$-induced Ca^{2+}-dependent K^+ efflux in human RBC. RBC suspensions depleted from ATP for 0 (●), 2 (▲) and 5 (■) hours (for details see Materials and Methods), further treated with different concentrations of $NaVO_3$ (as indicated in the figure) and preincubated for 15 min at room temperature. Subsequently, $CaCl_2$ (2.5 mmol/l) was added to initiate the Ca^{2+}-dependent K^+ efflux and suspensions were incubated for 50 min at 25 °C. EGTA (2.5 mmol/l) was added to the controls (open symbols). Samples were stopped by centrifugation through the dibuthylphtalate layer and the supernatants were taken for estimation of the K^+ content. All samples were run in duplicates. Results are means of three independent experiments ± S.E.M. Note the logarithmic scale on the abscissa.

The modifying effect of $NaVO_3$ on Ca^{2+} homeostasis in lymphocytes and mast cells (without any reference to nifedipine) has been recently described by Ehring et al. (2000). According to their data $NaVO_3$ triggers changes in Ca^{2+} homeostasis by oxidation of the HS-groups. This is at variance with the effect of $NaVO_3$ on the RBC Ca^{2+} homeostasis where the stimulation of the Ca^{2+} influx has been inhibited by HS-reagents and stimulated by dithiothreitol (Varečka et al. 1986). Therefore, the Cu^{2+}-sensitivity of Ca^{2+} influx in the $NaVO_3$-treated RBC observed in the present study might be explained by the presence of HS-groups in the target molecule. However, the character of this target molecule as well as the reason for the loss of Cu^{2+} sensitivity of $^{45}Ca^{2+}$ influx in ATP-depleted cells still remain to be explained.

PXB has been reported in many experimental models including purified systems (Rodriguez-Paris et al. 1989) to act as a PKC inhibitor with some additional anti-calmodulin activity (Hegemann et al. 1991). It was also described as an in-

hibitor of the Gárdos effect with a mechanism of action that differs from those of many other inhibitors. For example, inhibition of the Ca^{2+}-induced K^+ efflux occurs without a concommitant inhibition of the Ca^{2+} influx (Varečka et al. 1987), see also Fig. 2. The loss of biphasic action of PXB on $^{45}Ca^{2+}$ influx in ATP-depleted cells (Fig. 2B) suggests that ATP may be required for the descending (inhibitory) phase of PXB action. This is in accordance with the fact that ATP-depletion itself induces an influx of Ca^{2+} (Ferreira and Lew 1977). On the other hand, it seems to be feasible that the inhibitory effect of PXB on Ca^{2+}-induced K^+ efflux might also be mediated by inhibition of protein phosphorylation (Fig. 3) as has been demonstrated by Wen et al. (1984) in sarcolemmal preparations.

The Gárdos effects induced either by $NaVO_3$ or ATP-depletion exhibit several common features, such as sensitivity to some inhibitors; both processes are sensitive to quinine, oligomycin (Lew and Ferreira 1977; Varečka et al. 1997a), PXB (Varečka et al. 1987). In human RBC, $NaVO_3$ activates the $^{45}Ca^{2+}$ influx and the Gárdos effect in milimolar concentrations (Varečka and Carafoli 1982; Fuhrmann et al. 1984, 1985) exceeding by orders of magnitude the $NaVO_3$ concentrations required for inhibition of the Ca^{2+}-ATPase activity (Barrabin et al. 1989) and the active transport of calcium (Rossi et al. 1981).

The ATP-depletion is expected to affect the Ca^{2+} efflux mediated by the Ca^{2+}-ATPase which is an important target for the action of $NaVO_3$. Therefore, the ATP-depletion is expected to act in an additive manner with the effect of $NaVO_3$. The progressive decrease of the effective $NaVO_3$ concentration necessary for activating the Gárdos effect resulting from the ATP-depletion (Fig. 6) confirms this expectation.

In summary, the effects of $NaVO_3$ and ATP-depletion on RBC revealed differences in the properties of both $^{45}Ca^{2+}$ influx and Gárdos effect elicited by these inducers and indicate that both, the presence of $NaVO_3$ and cellular ATP, contribute to these differences. Taking into account that fluoride induces the Ca^{2+} influx *via* the tetrodotoxin-sensitive Na^+ channel (Varečka et al. 1998), there may exist at least three pathways mediating the Ca^{2+} influx in human RBC which provide Ca^{2+} for activation of the Gárdos effect.

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