Investigation of Properties of the Ca\textsuperscript{2+} Influx and of the Ca\textsuperscript{2+}-Activated K\textsuperscript{+} 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 45Ca\textsuperscript{2+} influx in human red blood cells (RBC) induced by NaVO\textsubscript{3} or ATP-depletion were compared. Both NaVO\textsubscript{3}-induced and ATP-depletion-induced 45Ca\textsuperscript{2+} influxes were in the range 10\textsuperscript{−6}–10\textsuperscript{−5} mol Ca\textsuperscript{2+}/l\textsuperscript{−1}cells\textsuperscript{−1}h\textsuperscript{−1}. The saturatability of ATP-depletion-induced 45Ca\textsuperscript{2+} influx with Ca\textsuperscript{2+} was much less pronounced than that of NaVO\textsubscript{3}-induced 45Ca\textsuperscript{2+} influx. The NaVO\textsubscript{3}-induced Ca\textsuperscript{2+} influx was sensitive to nifedipine (IC\textsubscript{50} = 50 \textmu mol/l) and Cu\textsuperscript{2+} (IC\textsubscript{50} = 9 \textmu mol/l) but these inhibitors had only a marginal effect when ATP-depletion was used as the Ca\textsuperscript{2+} influx inducer. On the other hand, polymyxin B (PXB) (1–5 mg/ml) strongly stimulated the ATP-depletion-induced 45Ca\textsuperscript{2+} influx whereas its effect on the NaVO\textsubscript{3}-induced Ca\textsuperscript{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\textsubscript{3} and PXB induced changes in the protein phosphorylation pattern in the presence of Ca\textsuperscript{2+}. NaVO\textsubscript{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\textsubscript{3} and the ATP-depletion showed marked differences. The ability of NaVO\textsubscript{3} to induce the Gárdos effect dramatically increased in ATP-depleted cells. These findings indicate that the 45Ca\textsuperscript{2+} influxes preceding the activation of the Ca\textsuperscript{2+}-activated K\textsuperscript{+} efflux (Gárdos effect) stimulated by NaVO\textsubscript{3} and by ATP-depletion, are mediated by different transport pathways. In addition, obtained re-

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results 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), myoccardial (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 × 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 × 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 × 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°C. After spinning down the RBC through a layer of dibutylphthalate, 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 \(\mu\)Ci of \(^{32}\)P-labelled orthophosphate for 30 min at 30°C. 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°C for 10 min. Ghosts were harvested by centrifugation on the microcentrifuge (5 min, 1500 \(\times\) g, 4°C). Lysate was discarded and the pellets were dissolved in 200 \(\mu\)l of the medium, containing in mmol/l: 10 Tris-HCl, pH 8; 1 EDTA; further 1% sodium dodecyl sulphate (SDS); 5% \(\beta\)-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, \(\beta\)-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.); dibutylphthalate 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
extracellular Ca\(^{2+}\) concentration. The average rate of the ATP-depletion-induced \(^{45}\text{Ca}^{2+}\) influx reached 8.6 ± 2.6 µmol-l\(^{-1}\)\text{cells}\cdot h^{-1}, (n = 3) and differed significantly from that of the NaVO\(_3\)-induced \(^{45}\text{Ca}^{2+}\) influx with values ranging between 5–80 µmol-l\(^{-1}\)\text{cells}\cdot h^{-1}, (n = 62).

The effect of Cu\(^{2+}\) and PXB on \(^{45}\text{Ca}^{2+}\) 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 \(^{45}\text{Ca}^{2+}\) 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 \(^{45}\text{Ca}^{2+}\) influx up to 25 µmol/l \((p < 0.05, \text{Fig. } 2A)\). Higher concentrations of Cu\(^{2+}\) ions 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 \(^{45}\text{Ca}^{2+}\) influx was not observed. This indicates that the ATP-depletion-induced \(^{45}\text{Ca}^{2+}\) 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 \(^{45}\text{Ca}^{2+}\) 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).
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 CaCl₂ (Ca²⁺ ions), vanadate (Van) and polymyxin B (PXB) on phosphorylation of the RBC membrane proteins. RBC suspensions were pre-incubated with 10 μCi of ³²P-labelled orthophosphate for 30 min at 30°C. Aliquots of the RBC suspensions containing in addition CaCl₂ (Ca²⁺) (2.5 mmol/l); NaVO₃ (Van) (1 mmol/l); PXB (3 mg/ml) were incubated for 60 min at room temperature. Instead of Ca²⁺ 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 ⁴⁵Ca²⁺ influx in RBC

The sensitivity of the NaVO₃-induced or ATP-depletion-induced ⁴⁵Ca²⁺ influx to dihydropyridines was examined by means of nifedipine (IC₅₀ = 50 μmol/l). In NaVO₃-treated cells nifedipine inhibited the Ca²⁺ influx by about 79%, while the Ca²⁺ 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 NaVO₃ to ATP-depleted cells led to a slight inhibition of the ⁴⁵Ca²⁺ influx (about 28%).

Properties of the Ca²⁺-dependent K⁺ efflux

The Ca²⁺-dependent K⁺ efflux (i.e., of the Gárdos effects) induced by NaVO₃ 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 Ca²⁺) in both the NaVO₃-treated as well as in the ATP-depleted cells. The lag phase duration was shorter in the NaVO₃-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 NaVO₃ reached maximal values at about 25°C, while in ATP-depleted cells the K⁺ effluxes
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 NaVO₃ 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 NaVO₃ for induction of the Gárdos effect (Fig. 6). In comparison to 100 μmol/l NaVO₃ required to induce the Gárdos effect in the absence of ATP-depletion, after 5 h of ATP-depletion, the Gárdos effect could be induced by 3 μmol/l NaVO₃ only.

Discussion

Previous results (Ferreira and Lew 1977; Varečka and Carafoli 1982) indicated that the Ca²⁺ influxes into RBC induced either by NaVO₃ or by ATP-depletion may have some common characteristics (e.g., they may be inhibited to similar extent by external [K⁺]₀). In contrast, the present study revealed that the Ca²⁺ influxes elicited by these inducers exhibit several different properties, such as affinity to Ca²⁺ (Fig. 1), or sensitivity to inhibitors. Most conspicuous features seem to be the differences in sensitivity of the NaVO₃-induced or ATP-depletion-evoked Ca²⁺ influxes to the Ca²⁺-channel blocking agent nifedipine (Fig. 4) to PXB (Fig. 2B) and to Cu²⁺ (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 NaVO₃ may exert some specific action on the Ca²⁺ 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.

d 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 dibuthylphthalate 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 (Rodríguez-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 concomitant 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 millimolar 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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