Biochimica et Biophysica Acta, 648 (1981) 145–150
Elsevier/North-Holland Biomedical Press

BBA 79422

VANADATE INHIBITION OF ACTIVE Ca^{2+} TRANSPORT ACROSS HUMAN RED CELL MEMBRANES

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(Received April 8th, 1981)

Key words: Ca^{2+} transport; Vanadate inhibition; (Human red cell)

(1) Vanadate (pentavalent vanadium) inhibits with high affinity ($K_{0.5}$ = 3 μM) the ATP-dependent Ca^{2+} efflux in reconstituted ghosts from human red cells. (2) To inhibit Ca^{2+} efflux vanadate has to have access to the inner surface of the cell membrane. (3) The inhibitory effect of vanadate is potentiated by intracellular Mg^{2+} and by intracellular K^+. (4) Ca^{2+} in the external medium antagonizes the inhibitory effect of vanadate.

Introduction

The Ca^{2+}-ATPase from human red cell membranes is inhibited with high affinity by vanadate (pentavalent vanadium) [1,2]. Inhibition depends on Mg^{2+} and is stimulated by K^+ [1,2]. Mg^{2+} and K^+ excert their effects by increasing the apparent affinity of the Ca^{2+}-ATPase for vanadate [1,2]. Inhibition by vanadate is independent of Ca^{2+} at concentrations up to 50 μM. Higher concentrations of Ca^{2+} progressively release the inhibitory effect of vanadate [2].

This paper reports experiments on reconstituted ghosts from human red cells designed to test the effects of vanadate on active Ca^{2+} efflux and to see on which of the surface of the cell membrane vanadate and the ligands that interact with vanadate promote or modify inhibition of the Ca^{2+} pump. Results show that vanadate acting intracellularly inhibits with high affinity active Ca^{2+} efflux. The effect of vanadate is promoted by intracellular Mg^{2+} and K^+ and is antagonized by extracellular Ca^{2+}.

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Materials and Methods

Preparation of resealed ghosts

The procedure used was essentially similar to that of Mualem and Karlish [3]. Fresh human red cells were washed three times with 10 vol. of a solution containing: 10 mM KCl, 130 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EDTA and 10 mM Tris-HCl (pH 7.7 at 25°C).

1 vol. washed cells was lysed at 2–3°C in 10 vol. of a solution containing: 2 mM ATP (disodium salt), 10 mM phosphocreatine (diTris salt), 5 unit/ml creatine phosphokinase, 5 mM $^{45}CaCl_2$ (spec. act. 1 Ci/mol), 0.1 mM ouabain, 1 mM EDTA, pH 7.7, at 25°C, plus $MgCl_2$ and NH_4VO_3 in the concentrations indicated in figures and tables. After 2 min enough of a 2 M solution of KCl, NaCl or LiCl was added to give a final concentration of 130 mM. The ghost suspension was incubated at 37°C during 10 min and then the resealed ghosts were washed three times with 10 vol. of a solution containing: 140 mM NaCl, 1 mM EGTA, 10 mM Tris-HCl (pH 7.7 at 25°C). When indicated, KCl or LiCl were added instead of NaCl. When KCl was present, the solution also contained 70 mM sucrose to prevent lysis of the ghosts due to the increase in the permeability of the cell membrane to K^+ induced by intracellular Ca^{2+} (see Ref. 4).

0005-2736/81/0000–0000/$02.50

Comparison of the concentration of ^{45}Ca and ATP in the ghosts with that in the lysing solution allowed us to estimate that more than 90% of the ghosts were resealed to ATP and Ca^{2+}.

Measurements of Ca^{2+} efflux

The washed and resealed ghosts were suspended in enough of the final wash solution at 0°C to give a hematocrit of approx. 50%. Efflux was initiated by adding 0.5 ml ghost suspension to 9.5 ml incubation media at 37°C. The suspension was incubated for 5 min and then every minute for 10 min 0.25-ml samples of the suspension were removed into 0.5 ml ice-cold incubation medium containing 0.2 mM $LaCl_3$. The samples were spun down at $10000 \times g$ for 1 min in an Eppendorf microcentrifuge and the radioactivity in the supernatant was measured. The plots of the amount of radioactivity in the supernatant against the incubation time were linear during the first 12 min. The value of the Ca^{2+} efflux was calculated from this initial slope of the plot, the specific activity of ^{45}Ca in the ghosts and the hematocrit values.

Measurements of Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity was measured on fragmented membranes of human red blood cells [5] following the procedure described previously [6].

Materials

ATP (substantially vanadium-free), phosphocreatine, creatinephosphokinase and norepinephrine were from Sigma Chem. Co. Other reagents were of analytical grade. Vanadate solutions were prepared by dissolving NH_4VO_3 in a solution of 50 mM Tris-HCl (pH 7.7). $^{45}CaCl_2$ was provided by Comisión Nacional de Energía Atómica, Argentina.

Results and Discussion

Preliminary experiments

The experiments in this paper were performed with ghosts containing non-limiting concentrations of ATP and Ca^{2+}. Control experiments (not shown) demonstrated that under these conditions the active transport of Ca^{2+} is so fast that the phosphocreatine and creatine phosphokinase have to be included in the ghosts to regenerate the ATP hydrolyzed by the pump. To see if vanadate affects the ATP regenerating system, resealed ghosts containing 0, 0.1 and 1.0 mM vanadate were incubated at 37°C during 30 min under conditions identical to those used for the Ca^{2+}-efflux experiments. After incubation the ATP content of the ghosts measured by ion-exchange chromatography [7] was 2.15, 2.14 and 2.19 mM for the ghosts containing 0, 0.1 and 1.0 mM vanadate, respectively. The concentrations found are close to those expected from the concentration of ATP in the lysing solution. This suggests that the regeneration of ATP is not impaired by vanadate. Therefore the effects of vanadate on Ca^{2+} transport to be discussed in this paper cannot be attributed to changes in the supply of ATP to the pump.

Norepinephrine prevents vanadate inhibition of the (Na^+, K^+)-ATPase [8,9], presumably by chelating vanadate [10]. To see if norepinephrine also protects Ca^{2+}-ATPase, the activity of the enzyme was measured at different norepinephrine concentrations in media with and without 10 μM vanadate. Results in Table I show that norepinephrine in concentrations higher than 0.8 mM prevents inhibition by vanadate without having any effect on Ca^{2+}-ATPase activity.

TABLE I

THE EFFECT OF NOREPINEPHRINE ON VANADATE INHIBITION OF THE Ca^{2+}-ATPase ACTIVITY

The incubation medium contained ATP (disodium salt) 1 mM, KCl 100 mM, $CaCl_2$ 0.6 mM, EGTA 0.5 mM, $MgCl_2$ 5 mM, Tris-HCl (pH 7.7 at 37°C) 50 mM and red cell membranes (0.6 mg protein · ml^{-1}). The Ca^{2+}-ATPase activity is measured as the difference between the activity in this medium and in a medium with identical composition but without $CaCl_2$. Norepinephrine was added at the beginning of the incubation at 37°C.

Norepinephrine concentration (mM)	Ca^{2+}-ATPase activity (μmol P_i/mg protein per h)	
	0 μM vanadate	10 μM vanadate
0.0	1.28	0.24
0.8	1.31	1.12
1.5	1.29	1.22
3.0	1.28	1.20
6.0	1.15	1.12

Sidedness of inhibition by vanadate

In the experiments in Fig. 1, Ca^{2+} efflux from reconstituted ghosts with and without ATP was measured as a function of increasing concentrations of either intra- or extracellular vanadate. Results show that vanadate is without effect on the small efflux of Ca^{2+} from ATP-free ghosts. As the concentration of vanadate in ATP-containing ghosts increases, the Ca^{2+} efflux decreases, approaching the value observed in the absence of ATP. This result suggests that vanadate at the inner surface of the cell membrane is effective in inhibiting active transport of Ca^{2+} since the low hematocrit used precludes any significant accumulation in the extracellular media of vanadate leaking from the cells. The concentration of intracellular vanadate for half-maximal inhibition is about 3 μM, a value which is very close to that for half-maximal inhibition of the Ca^{2+}-ATPase by vanadate in the presence of Mg^{2+} and K^+ [1,2].

In preliminary experiments (not shown) we observed that addition of vanadate to the incubation media of vanadate-free ghosts led to a time-dependent inhibition of Ca^{2+} efflux. It was difficult to decide whether this was an effect of external vanadate or of vanadate entering the ghosts [11]. To prevent the effects of a possible vanadate leak into the cells during the assay time, extracellular vanadate was tested on ghosts containing 1.5 mM norepinephrine. It can be seen (Fig. 1) that the initial rate of Ca^{2+} efflux in norepinephrine containing ghosts remains unaffected by vanadate in the incubation media. Results in Fig. 1 strongly suggest that the Ca^{2+} pump is inhibited by combination of vanadate at a site on the internal surface of the cell membrane. A similar sidedness for the effect of vanadate has been reported for the Na^+ pump [11,12].

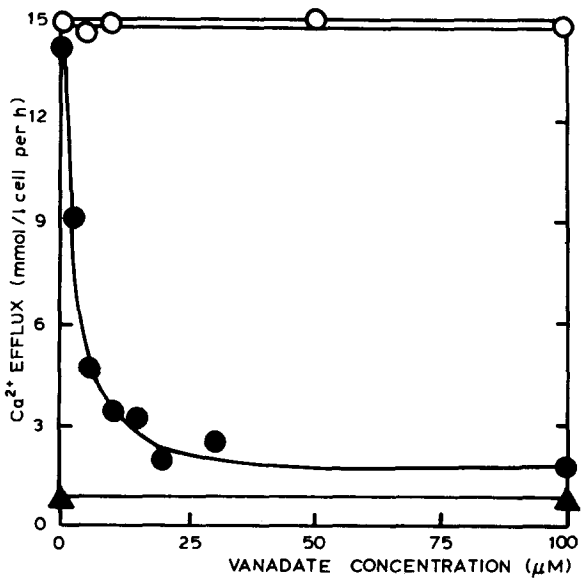

Fig. 1. The efflux of Ca^{2+} from reconstituted ghosts as a function of the concentration of intra- (●, ▲) or extracellular (○) vanadate. Ghosts contained either 2 (○, ●) or 0 mM (▲) ATP. The effect of extracellular vanadate was tested in ghosts containing 1.5 mM norepinephrine. All ghosts contained 130 mM KCl and 11.5 mM $MgCl_2$. The incubation media contained 140 mM KCl.

Sidedness of the effects of cations

As mentioned before, in disrupted membranes as the concentration of Ca^{2+} raises above 50 μM it progressively prevents inhibition of the Ca^{2+}-ATPase by vanadate [2]. Results in Fig. 1 show that vanadate fully inhibits the Ca^{2+} efflux in ghosts containing 5 mM $CaCl_2$. If tested on the Ca^{2+}-ATPase activity of disrupted membranes this concentrations of $CaCl_2$ would have abolished almost completely the effect of vanadate. Table II shows the results of one experiment in which inhibition of Ca^{2+} efflux by vanadate was measured in ghosts suspended in media with and without 1 mM $CaCl_2$. It can be seen that extracellular Ca^{2+} reduces from 63 to 31% the inhibitory effect

TABLE II

THE EFFECT OF EXTERNAL Ca^{2+} ON THE INHIBITION OF Ca^{2+} EFFLUX BY INTRACELLULAR VANADATE

The gosts contained 11.5 mM $MgCl_2$ and 130 mM NaCl. The incubation media contained 140 mM NaCl.

	Ca^{2+} efflux (mmol/l cell per h)	
	in medium without $CaCl_2$	in medium with 1 mM $CaCl_2$
Ghosts containing 0 μM vanadate	11.0	10.8
Ghosts containing 50 μM vanadate	4.1	7.6

of 50 μM vanadate. Similar effects of external Ca^{2+} were observed in two other independent experiments. This effect of external Ca^{2+}, together with the lack of effect of intracellular Ca^{2+} (Fig. 1). suggests that inhibition by vanadate is released by Ca^{2+} acting at sites oriented toward the external surface of the cell membrane. A similar sidedness for the protective effect of Ca^{2+} on vanadate inhibition has been reported for the Ca^{2+}-ATPase of sarcoplasmic reticulum [13,14].

Inhibition of the Ca^{2+}-ATPase by vanadate requires Mg^{2+}, which produces a large increase in the affinity of the Ca^{2+}-ATPase for vanadate [1,2]. The effect of Mg^{2+} on the affinity of the Ca^{2+}-ATPase for vanadate is half-maximal at about 1 mM [2]. Results in Table III show that in ghosts containing 2.5 mM $MgCl_2$ and suspended in Mg^{2+}-free media 200 μM vanadate lowers to half the Ca^{2+} efflux. Under these conditions, addition of 6 mM $MgCl_2$ to the incubation media has no further effect on the inhibition by vanadate. When intracellular $MgCl_2$ is raised to 11.5 mM, in the absence of external Mg^{2+}, 50 μM vanadate lowers to one-third the Ca^{2+} efflux. These results indicate that, in contrast to the lack of effect of extracellular $MgCl_2$, increasing the concentration of intracellular $MgCl_2$ from 2.5 to 11.5 mM lowers more than 4-times the concentration of vanadate for half-maximal inhibition of Ca^{2+} efflux. It seems, therefore, that for inhibition of the Ca^{2+} pump by vanadate, Mg^{2+} has to have access to the intracellular surface of the cell membrane. Since the reconstituted ghosts used for these studies contain about 12 mM of Mg^{2+}-complexing compounds, the concentration of Mg^{2+} in the ghosts has to be a small fraction of the total intracellular magnesium. This probably explains why inhibition by vanadate and stimulation of Ca^{2+} efflux in reconstituted ghosts require high concentrations of $MgCl_2$.

In disrupted membranes K^+, but not Li^+, increases about 20-times the apparent affinity of the Ca^{2+}-ATPase for vanadate [1,2]. Studies of this effect of K^+ in reconstituted ghosts are hampered by the large increase that intracellular Ca^{2+} induces in the membrane permeability to K^+ (see Ref. 14). It has been reported that quinine sulfate prevents this increase [15]. Control experiments (not shown) demonstrated that, in our preparation, inclusion of 1 mM quinine sulfate in the incubation media lowers from 5.52 to 0.276 h^{-1} the rate constant of net K^+ loss from the resealed ghosts. After this, experiments on the sidedness of the effect of K^+ were performed using incubation media containing 1 mM quinine sulfate. To study the effects of K^+ on vanadate inhibition of Ca^{2+} efflux, two kinds of resealed ghosts were used: 'high-K^+' ghosts containing 115 mM KCl and 'low-K^+' ghosts containing 20 mM KCl and 95 mM LiCl. Results in Fig. 2 show that the efflux of Ca^{2+} from 'low-K^+' ghosts into a medium containing 140 mM LiCl and initially no K^+ decreases with intracellular vanadate along a curve which is half-maximal at 30 μM vanadate. When KCl replaces LiCl in the external medium a small drop in the concentration of

TABLE III

THE EFFECT OF INTRA- AND EXTRACELLULAR $MgCl_2$ ON INHIBITION OF THE Ca^{2+} EFFLUX BY VANADATE

The ghosts contained 130 NaCl and the incubation media contained 140 mM NaCl. The ghosts contained about 12 mM of Mg^{2+}-complexing compounds so that the actual concentration of intracellular Mg^{2+} is considerably lower than that of $MgCl_2$.

Intracellular vanadate (μM)	Initial intracellular $MgCl_2$ (mM)	Initial extracellular $MgCl_2$ (mM)	Ca^{2+} efflux (mmol/l cell per h)	Inhibition (%)
0	2.5	0	7.85	0
200	2.5	0	3.80	52
0	2.5	6	7.85	0
200	2.5	6	3.30	56
0	11.5	0	14.00	0
50	11.5	0	5.07	64
200	11.5	0	2.90	79

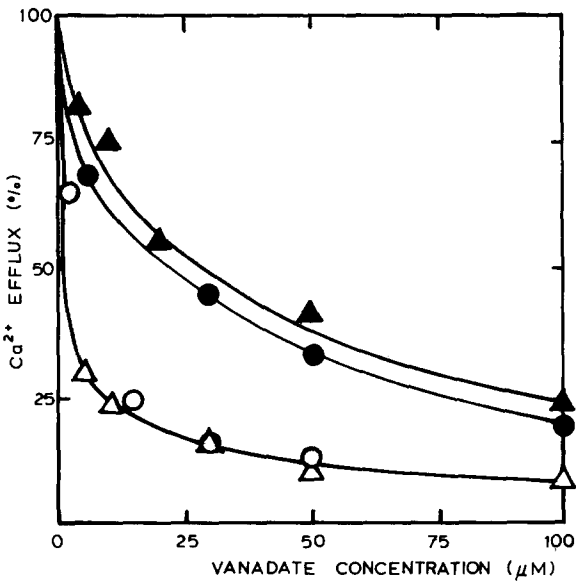

Fig. 2. The effect of K^+ on vanadate inhibition of Ca^{2+} efflux from reconstituted ghosts. The effect of intracellular vanadate was tested in 'high-K^+' ghosts (containing 115 mM KCl) (○, △) and in 'low-K^+' ghosts (containing 20 mM KCl and 95 mM LiCl) (●, ▲) suspended in media containing 140 mM KCl (○, ●) or 140 mM LiCl (△, ▲). All ghosts contained 11.5 mM $MgCl_2$. Fluxes are expressed as the percentage of the control in the absence of vanadate.

vanadate for 50% inhibition of Ca^{2+} efflux is observed. In 'high-K^+' ghosts suspended in initially K^+-free media, the concentration of vanadate for half-maximal effect is 3 μM, a value which is characteristic of vanadate inhibition of the Ca^{2+}-ATPase in the presence of K^+ [1,2]. In 'high-K^+' ghosts the concentration of vanadate for half-maximal inhibition is not changed when KCl replaces LiCl in the external medium, suggesting that intracellular K^+ suffices for vanadate to inhibit with high affinity the Ca^{2+} efflux. Although the experiments in Fig. 2 do not preclude a small effect of extracellular K^+, they strongly suggest that the site at which K^+ combines with the Ca^{2+}-ATPase to increase its affinity for vanadata faces the intracellular surface of the cell membrane. In the Na^+ pump it has been reported that extracellular K^+ is needed to promote inhibition by vanadate [10].

Results in this paper demonstrate that vanadate inhibits active transport of Ca^{2+} across red cell membranes. Comparison of inhibition of Ca^{2+} efflux with that of Ca^{2+}-ATPase indicates that both processes share a number of common features, as follows: (i) the concentration of vanadate for half-maximal inhibition is 3 μM for the Ca^{2+} efflux and 1.2 μM for the Ca^{2+}-ATPase; (ii) for both phenomena the apparent affinity for vanadate is increased by Mg^{2+} and by K^+ in the presence of Mg^{2+}; (iii) inhibition of both Ca^{2+}-ATPase and Ca^{2+} transport is released by Ca^{2+} at concentrations much higher than those which saturate the Ca^{2+} pump. Since Ca^{2+}-ATPase is measured at a micromolar concentration of Ca^{2+} it would seem that the high intracellular concentration of Ca^{2+} used during the efflux experiments does not subtantially modify the response of the Ca^{2+} pump for vanadate. To inhibit Ca^{2+} efflux vanadate has to have access to the inner surface of the cell membrane. Mg^{2+} and K^+ potentiate vanadate intracellularly and Ca^{2+} prevents inhibition of Ca^{2+} efflux when present in the suspending media. Asymmetric requirements for Mg^{2+}, Ca^{2+} and K^+ during vanadate inhibition of Ca^{2+} efflux indicate that the Ca^{2+} pump posseses sites for Mg^{2+} and K^+ on the internal, and a site for Ca^{2+} on the external, surface of the cell membrane. Further studies are needed to ascertain whether these are sites of the Ca^{2+} pump at which Mg^{2+}, Ca^{2+} and K^+ combine during the transport reaction.

Note added in proof: (Received September 7th, 1981)

White this paper was in press results similar to ours on the sidedness of vanadate-cation interactions during vanadate inhibition of calcium transport in squid axons were reported [16].

Acknowledgments

This work was supported by grants from the CONICET and the SECYT (Argentina), the PNUD-UNESCO RLA 78/24, the Program Regional de Desarrollo Científico y Tecnológico of the OEA and the Fundación Alberto J. Roemmers. A.F.R. and P.J.G. are established investigators of the CONICET.

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