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THE EFFECTS OF SEVERAL LIGANDS ON THE POTASSIUM-VANADATE INTERACTION IN THE INHIBITION OF THE ($Na^+ + K^+$)-ATPase AND THE Na^+, K^+ PUMP

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Inhibition by vanadate of the K^+-dependent *p*-nitrophenylphosphatase activity catalyzed by the ($Na^+ + K^+$)-ATPase partially purified from pig kidney showed competitive behavior with the substrate. K^+ and Mg^{2+} acted as cofactors in promoting that inhibition. Ligands which inhibited the K^+-dependent *p*-nitrophenyl phosphate hydrolysis (Na^+, nucleotide polyphosphates, inorganic phosphate) protected against inhibition by vanadate. The magnitude of that protection was proportional to the inhibition produced in the absence of vanadate. In the presence of only *p*-nitrophenyl phosphate and Mg^{2+}, or when the protective ligands were tested alone, the activation of *p*-nitrophenyl phosphate hydrolysis by K^+ followed a sigmoid curve in the presence as well in the absence of vanadate. However, the combination of 100 mM NaCl and 3 mM ATP resulted in a biphasic effect of K^+ on the *p*-nitrophenyl phosphate hydrolysis in the presence of vanadate. After an initial rise at low K^+ concentration, the *p*-nitrophenylphosphatase activity declined at high K^+ concentrations; this decline became more pronounced as the vanadate concentration was increased. This biphasic response was not seen when a nonphosphorylating ATP analog was combined with Na^+ (which favors the nucleotide binding) or with inorganic phosphate (a requirement for K^+-K^+ exchange). Experiments with inside-out resealed vesicles from human red cells showed that in the absence of Na^+ plus ATP, K^+ promoted vanadate inhibition of *p*-nitrophenylphosphatase activity in a nonbiphasic manner, acting at cytoplasmic sites. On the other hand, in the presence of Na^+ plus ATP, the biphasic response of *p*-nitrophenyl phosphate hydrolysis is due to K^+ acting on extracellular sites. In vanadate-poisoned intact red blood cells, the biphasic response of the ouabain-sensitive Rb^+ influx as a function of the external Rb^+ concentration failed to develop when there was no Na^+ in the extracellular media. In addition, in the absence of extracellular Na^+, external Rb^+ did not influence the magnitude of inhibition. The present findings indicate that external K^+ favors vanadate inhibition by displacing Na^+ from unspecified extracellular membrane sites.

Introduction

Vanadate is a powerful inhibitor of the active Na^+,K^+-transport mechanism and the associated ($Na^+ + K^+$)-ATPase activity present in plasma cell membranes (see Ref. 1 for references). To exert its effect, this inhibitor binds to sites located on the intracellular membrane surface [2,3]. Mg^{2+} and K^+ act as co-factors for vanadate inhibition [4,5]. The action of Mg^{2+} takes place intracellularly [6]. The sites at which K^+ must attach and the mechanism involved in its effects are controversial. The basic initial observation [7] was that when the ($Na^+ + K^+$)-ATPase activity in the presence of vanadate-containing ATP was plotted as a function of the K^+ concentration, the curve showed a 'bump' with an initial activation at low K^+ followed by an abrupt inhibition at a K^+

concentration too low for normal Na^+-K^+ competition at intracellular Na^+ sites. In resealed human red cell ghosts with high vanadate-containing ATP and high internal Na^+, inhibition of the ouabain-sensitive ATPase activity was observed only when external K^+ was increased and not when similar changes were made in intracellular K^+ [8]. These effects of external K^+ were confirmed in experiments on Na^+ efflux and K^+ influx in intact red cells [5,6] and on Na^+ efflux in dialyzed squid axons [3]. Typical behavior was a biphasic curve of flux which increased with $[K^+]$ at low K^+ concentration, reached a maximum and decreased at high external K^+ concentration. Because under certain conditions (high external Na^+ and low vanadate) inhibition developed at K^+ concentrations higher than those required to saturate the external K^+-pump sites, it was concluded that the sites at which external K^+ promoted vanadate inhibition could not be the sites, or could not be the only sites, from which external K^+ is transported into the cells [5,6]. In addition, the efflux of Na^+ from cells with normal ATP and high vanadate concentrations showed a reversed K^+-free effect (larger ouabain-sensitive Na^+ efflux in the absence than in the presence of external K^+), as did cells with low ATP content in the absence of the inhibitor. This led to the suggestion [3,5] that vanadate inhibited the release of K^+ from the 'occluded' $E_2(K)$ pump conformation. That vanadate indeed blocks the $E_2(K)$-E_1K conformational change in the $(Na^+ + K^+)$-ATPase was shown by fluorescence studies with the unphosphorylated enzyme [9]. Because, in the absence of enzyme phosphorylation, K^+ is likely to act on intracellular sites [10,11], these experiments also suggested that intracellular K^+ can promote vanadate inhibition. Actually, the experiments just described went even further, for in the absence of K^+, higher concentrations of vanadate induce fluorescence changes similar to those seen for the E_1-E_2 conformational change; i.e., K^+ was not required at all if the vanadate concentration were high enough. Very recently it was shown directly that K^+ acting intracellularly can promote vanadate inhibition; this was seen for the ATP-dependent Na^+ uptake in liposomes with the $(Na^+ + K^+)$-ATPase incorporated [12] and for the strophanthidin-sensitive phosphatase activity in inside-out red cell vesicles [13]. The suggestion was made [12] that the effects of external K^+ on vanadate inhibition were seen only under conditions of ATPase (or pump) turnover due to the formation of the $E_2(K)$ conformation after dephosphorylation and without the need for additional external K^+ sites as originally proposed [5, 6]. The experiments described in this paper were performed to clarify this point. The main conclusion reached is that external K^+ favors vanadate inhibition of the Na^+,K^+-pump only when two conditions are simultaneously satisfied: (i) the system is in state of turnover and (ii) there is sodium in the external medium. This indicates that extracellular K^+ acts by displacing Na^+ from some external site at which Na^+ prevents or antagonizes the vanadate effects.

Materials and Methods

The experiments were performed using purified $(Na^+ + K^+)$-ATPase enzyme, inside-out resealed vesicles from human red blood cells and intact red blood cells. The $(Na^+ + K^+)$-ATPase was partially purified from pig kidney as proposed by Jørgensen [14]; the specific activity obtained varied between 12 and 15 units/mg protein. The inside-out vesicles were prepared as described by Mercer and Dunham [15]: the usual yield was 75–80% and was determined by the acetylcholine esterase activity prior and after treatment with Triton X-100 [16]. The cation loading of the vesicles, as well as the assays for K^+-dependent phosphatase activity and Na^+ uptake followed the method used by Drapeau and Blonstein [17]. Protein was determined by the procedure of Lowry et al. [18] as modified by Markwell et al. [19]. The assays of phosphatase activity used *p*-nitrophenyl phosphate as substrate. The reaction was stopped with 1 vol. 0.2 M NaOH/2.5% SDS/4 mM EDTA [17]. The production of *p*-nitrophenol was estimated by the absorbance at 410 nm. The technique for Rb^+ influx in red cells is published elsewhere [6].

In all experiments precautions were taken to insure that the results expressed real linear velocities of the reactions being measured. This is particularly important in the case of vanadate poisoning, where artifacts due to eventual differences in the onset of inhibition must be ruled out. As a further

safeguard there was always a preincubation with all ligands except the substrate of the reaction being measured. The preincubation time was 10–20 min with the purified enzyme, 10 min with the inside-out vesicles and 2 h with the intact red cells. The longer time in the latter case was to allow vanadate to enter and equilibrate into the cells [6]. More details are given in the figure legends.

All solutions were prepared with bidistilled deionized water. The pH was maintained at 7.4 with Tris-HCl buffer and the temperature was 37°C. The NaCl and KCl salts were Baker Ultrex; all other chemicals were reagent grade. Adenosine, nucleotide polyphosphates and *p*-nitrophenyl phosphate di-tris salt were obtained from Sigma, U.S.A. All nucleotides were transformed into Tris salts after being passed through Dowex 50 columns. Vanadate, as sodium orthovanadate, was from Fisher. Rubidium-86 was purchased from the Comisión Nacional de Energía Atómica of Argentina. A detailed composition of the solutions varied in the different experiments and is given in the corresponding figure and figure legend. Unless otherwise stated, the experimental points are the means of triplicate determinations and each experiment was repeated at least once. The K^+-dependent phosphatase activity was taken as the difference in the activities found in the presence and absence of potassium. In the presence of nonlimiting concentrations of substrate, $MgCl_2$ and KCl, the K^+-dependent fraction was about 99% of the total activity in purified $(Na^+ + K^+)$-ATPase and about 50% in inside-out red cell vesicles.

When necessary, the ionized Mg^{2+} concentrations were calculated using the following Mg^{2+} complexes' dissociation constants, taken from Sillén and Martel [20] and estimated for 37°C (pH 7.4) and ionic strength 100 mM: MgATP, 0.091 mM; MgADP, 0.59 mM; MgEGTA, 5.5 mM; magnesium *p*-nitrophenyl phosphate, 10 mM; magnesium phosphate, 10 mM. For the magnesium adenylyl (β,γ-methylene)diphosphonate and magnesium adenosine tetraphosphate complexes we arbitrarily took the same dissociation constant as for MgATP.

Results

The effects of substrate, magnesium and potassium on the vanadate inhibition of the K^+-dependent p-nitrophenylphosphatase activity

In purified pig kidney $(Na^+ + K^+)$-ATPase, the K^+-dependent *p*-nitrophenylphosphatase activity as a function of the substrate concentration follows Michaelian kinetics. The K_m for *p*-nitrophenyl phosphate ranged from 4 to 6 mM (50 mM KCl/3 mM $MgCl_2$); this is close to the value of 3.4 mM reported for rat brain microsomes [21] and of 2 mM for ox brain enzyme [22] under slightly different conditions. Vanadate inhibition of the K^+-dependent *p*-nitrophenylphosphatase activity shows competitive behavior with the substrate (Fig. 1). With 50 mM KCl/3 mM $MgCl_2$ and in the absence of other ligands the plot of the reciprocal of activities vs. vanadate concentration at two different *p*-nitrophenyl phosphate concentrations (2 mM and 10 mM) gave a K_i for vanadate of about 6 nM. This agrees rather well with a K_i of about 4 nM obtained in ^{48}V binding experiments [23] for a high-affinity site. However, our kinetic

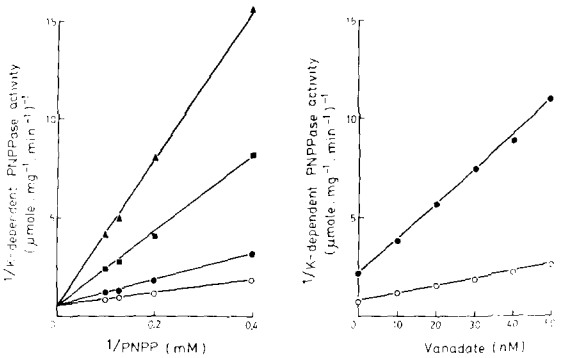

Fig. 1. Left: Reciprocal plots of the K^+-dependent *p*-nitrophenyl phosphatase (PNPPase) activity in purified $(Na^+ + K^+)$-ATPase as a function of the substrate concentration in the absence and presence of different vanadate concentrations: ▲, $1 \cdot 10^{-7}$ M; ■, $5 \cdot 10^{-8}$ M; ●, $1 \cdot 10^{-8}$ M; ○, control. Right: A plot of the reciprocal of the K^+-dependent *p*-nitrophenylphosphatase activity in purified $(Na^+ + K^+)$-ATPase versus vanadate concentration at two different *p*-nitrophenyl phosphate concentrations: ●, 2 mM; ○, 10 mM. The intercept of the straight lines gives an estimated K_i for vanadate of 6 nM. In all cases, the composition of the solutions was as follows: KCl 50 mM; $MgCl_2$ 3 mM; Tris-HCl (pH 7.4; 37°C), 130 mM; EGTA 0.1 mM. See Materials and Methods for details.

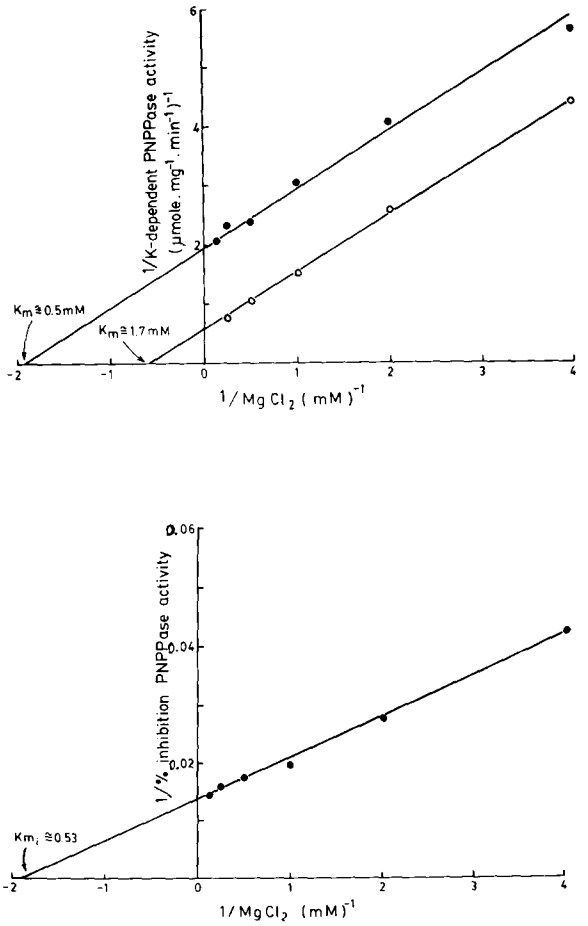

Fig. 2. Top: Reciprocal plots of the K^+-dependent *p*-nitrophenylphosphatase (PNPPase) activity in purified ($Na^+ + K^+$)-ATPase as a function of the $MgCl_2$ concentration in the absence (open circles) and presence (filled circles) of $5 \cdot 10^{-8}$ M vanadate. Note that the addition of vanadate reduced both the maximal velocity and the K_m for $MgCl_2$. Bottom: with data taken from the same experiment the figure shows reciprocal plots of the fractional inhibition by vanadate of the K^+-dependent *p*-nitrophenylphosphatase activity as a function of the $MgCl_2$ concentration. The straight lines through the points were drawn by eye. The meaning of 'K_{mi}' is the apparent affinity for $MgCl_2$ as a co-factor for vanadate inhibition. The composition of the solutions was as follows: KCl, 20 mM; *p*-nitrophenyl phosphate, 5 mM; Tris-HCl (pH 7.4; 37°C), 160 mM; EGTA, 0.1 mM. See text for details.

data do not allow us to detect the proposed second binding site with low affinity.

The activation of the K^+-dependent *p*-nitrophenylphosphatase activity by $MgCl_2$ can be also described by Michaelian kinetics (see Ref.

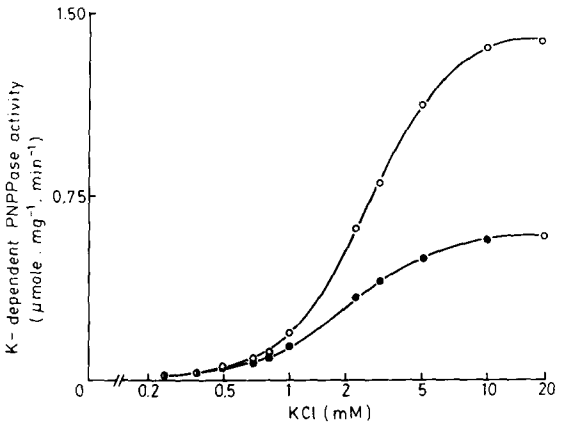

Fig. 3. Potassium activation of *p*-nitrophenyl phosphate hydrolysis in purified ($Na^+ + K^+$)-ATPase enzyme in the absence (open circles) and presence (filled circles) of $5 \cdot 10^{-8}$ M vanadate. The composition of solutions was as follows: $MgCl_2$, 3 mM; *p*-nitrophenyl phosphate, 5 mM; Tris-HCl (pH 7.4; 37°C) (180 − KCl concentration) mM; EGTA, 0.1 mM. Note: (i) the absence of any biphasic effect of K^+ in the presence of vanadate; (ii) the enzymatic activity is plotted against the logarithm of the KCl concentration; (iii) the $K_{0.5}$ for KCl is about 2 mM in the control and around 3 mM in the presence of vanadate.

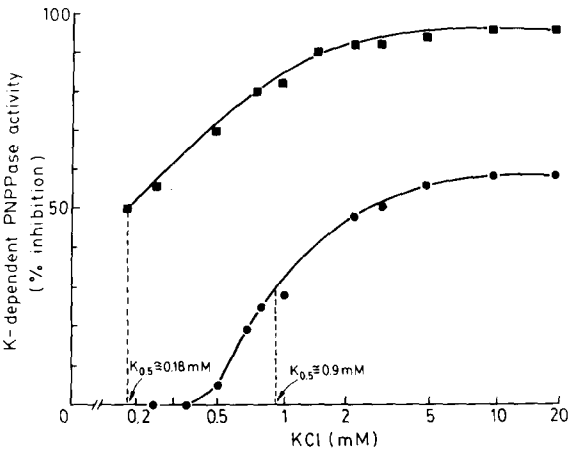

Fig. 4. Inhibition of the K^+-dependent *p*-nitrophenylphosphatase (PNPPase) activity in purified ($Na^+ + K^+$)-ATPase as a function of vanadate and KCl concentrations. The composition of solutions was: $MgCl_2$, 3 mM; *p*-nitrophenyl phosphate, 5 mM; Tris-HCl (pH 7.4; 37°C) (180 − KCl concentration) mM; EGTA, 0.1 mM. ■, $1 \cdot 10^{-6}$ vanadate; ●, $5 \cdot 10^{-8}$ M vanadate. Note: (i) for each vanadate concentration the percentage of inhibition increase with KCl concentration; (ii) the $K_{1/2}$ for KCl as a co-factor for vanadate inhibition decreases when the concentration of the inhibitor is increased, (iii) the KCl concentrations are plotted in logarithmic scale; (iv) the data at $5 \cdot 10^{-8}$ M vanadate correspond to the experiment illustrated in Fig. 3.

20). Fig. 2 (top) indicates that this behavior is maintained for the residual activity in the presence of submaximal concentrations of vanadate, although there is a 3-fold reduction in the K_m value. With fixed concentrations of KCl, *p*-nitrophenyl phosphate and vanadate the fractional inhibition of the K^+-dependent *p*-nitrophenylphosphatase activity increases with $MgCl_2$ concentrations along a hyperbolic curve. Reciprocal plots such as that of fig. 2 (bottom) give a K_{mi} for $MgCl_2$ of about 0.5 mM. This could indicate that magnesium promotes vanadate inhibition of the $(Na^+ + K^+)$-ATPase enzyme by binding to a single site. Similar conclusions were reached in studies on vanadate-manganese binding [24].

In a medium lacking any other ligand besides *p*-nitrophenyl phosphate and $MgCl_2$, K^+ stimulates the hydrolysis of *p*-nitrophenyl phosphate along a sigmoid curve with a $K_{1/2}$ of about 3 mM (see also Ref. 21). In the presence of submaximal concentrations of vanadate, the stimulation of the *p*-nitrophenyl phosphate hydrolysis by KCl shows similar behavoir and the $K_{1/2}$ for KCl remains basically unchanged (Fig. 3). The fraction of the activity inhibited by vanadate increases together with KCl also following a sigmoid relationship (not shown). In addition, Fig. 4 shows that the $K_{1/2}$ for this KCl effect goes from about 0.9 mM at $5 \cdot 10^{-8}$ M vanadate to about 0.18 mM when the concentration of vanadate is 10^{-6} M; i.e., the apparent affinity for KCl as a cofactor for inhibition increases with the concentration of the inhibitor. The counterpart is also true, and the apparent affinity for vanadate increases with KCl concentration. The $K_{1/2}$ for vanadate goes from $2 \cdot 10^{-7}$ M at 1 mM KCl to $3 \cdot 10^{-8}$ M at 20 mM KCl (results not shown). The sigmoidicity of the curve relating fractional inhibition to KCl concentration suggests that potassium favors vanadate inhibition by attaching to more than one enzyme site. It should be noticed that in Figs. 3 and 4 KCl concentrations are plotted on logarithmic scales; the sigmoidicities we refer to apply to KCl concentrations plotted on linear scales (not shown).

The effects of sodium, nucleotides and phosphate on the vanadate inhibition of the K^+-dependent p-nitrophenylphosphatase activity

The results of the previous section show that

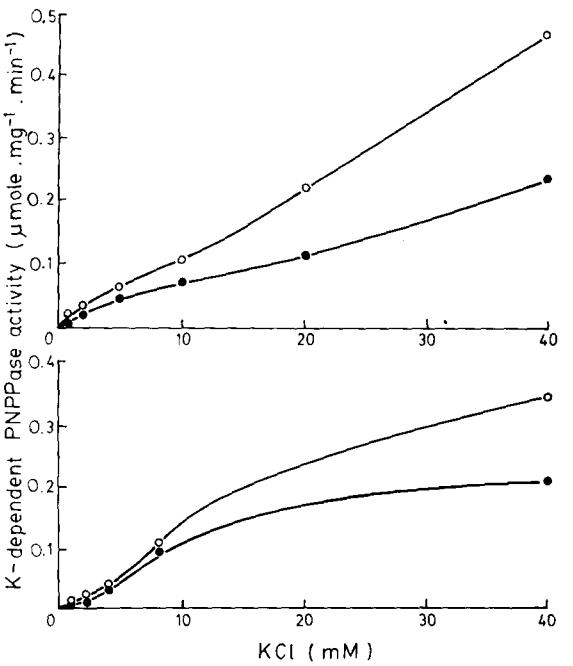

Fig. 5. The effects of 100 mM NaCl (top) and 3 mM ATP (bottom) on the activation of *p*-nitrophenyl phosphate hydrolysis in purified $(Na^+ + K^+)$-ATPase enzyme in the absence (open circles) and presence (filled circles) of $5 \cdot 10^{-8}$ M vanadate. The composition of solutions was as follows: Top: $MgCl_2$, 3 mM; *p*-nitrophenyl phosphate, 5 mM; NaCl, 100 mM; Tris-HCl (pH 7.4; 37°C) (80 − KCl concentration) mM; EGTA, 0.1 mM. Bottom: $MgCl_2$, 3 mM; *p*-nitrophenyl phosphate, 5 mM; ATP, 3 mM; Tris-HCl (pH 7.4; 37°C) (180 − KCl concentration) mM; EGTA, 0.1 mM. Note: (i) the absence of biphasic effect of potassium in the presence of vanadate; (ii) with 100 mM Na^+ the $K_{0.5}$ values for KCl are well above 10 mM in control and vanadate; (iii) with 3 mM ATP the $K_{0.5}$ for KCl is between 10–12 mM in both instances.

K^+ acts as a cofactor for vanadate inhibition of the K^+-dependent *p*-nitrophenylphosphatase activity in purified $(Na^+ + K^+)$-ATPase. This concurs with observations for the $(Na^+ + K^+)$-ATPase activity [5]. However, the curve relating *p*-nitrophenyl phosphate hydrolysis and KCl concentration in the presence of vanadate leads to saturation without biphasic response seen for the hydrolysis of ATP [5] and for Na^+ efflux or K^+ influx in vanadate-poisoned cells [3,5,6]. The experiments described below aimed to study, on the K^+-vanadate interaction, the effects of the ligands, and their analogs or related substances, which in one way or another are related to the

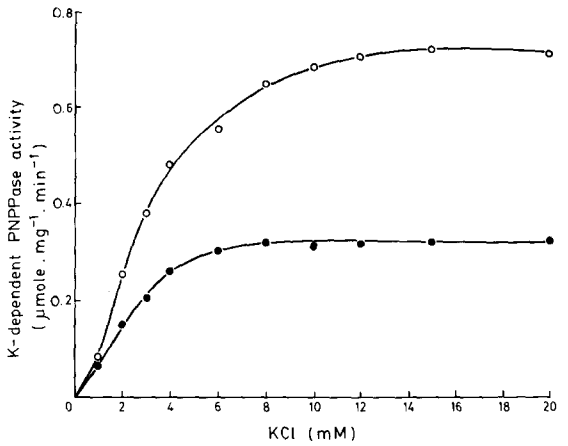

Fig. 6. The effects of 5 mM inorganic phosphate on the K^+ activation of *p*-nitrophenyl phosphate hydrolysis in purified ($Na^+ + K^+$)-ATPase enzyme in the absence (open circles) and presence (filled circles) of $5 \cdot 10^{-8}$ M vanadate. The composition of the solutions was: $MgCl_2$, 3 mM; *p*-nitrophenyl phosphate, 5 mM; Tris-HCl (pH 7.4; 37°C) (180 − KCl concentration) mM; EGTA, 0.1 mM. Note: (i) the absence of 'bump' (biphasic effect of potassium in the presence of phosphate alone or phosphate plus vanadate); (ii) the $K_{0.5}$ for KCl is about 2.4 mM in the control and around 2.8 mM in the presence of vanadate.

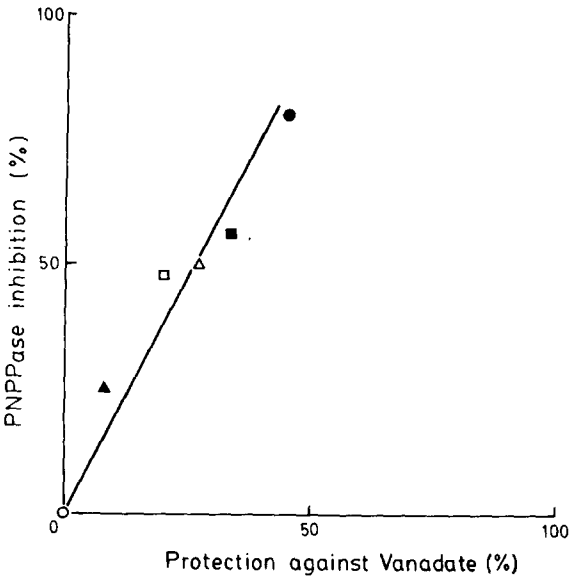

Fig. 7. Comparative effect of several nucleotide polyphosphates (ATP (●), adenylyl(β,γ-methylene)diphosphonate (■), adenosine tetraphosphate (△), ADP (□)) adenosine (○) and inorganic phosphate (▲) as inhibitors of the *p*-nitrophenylphosphatase (PNPPase) activity and as protective agents against inhibition by vanadate of that activity in purified ($Na^+ + K^+$)-ATPase enzyme. The composition of solutions was: KCl, 40 mM; *p*-nitrophenyl phosphate, 5 mM; Tris-HCl (pH 7.4; 37°C) 140 mM; EGTA, 0.1 mM. To obtain an ionized Mg^{2+} concentration of 2 mM, enough $MgCl_2$ was added in accordance with the dissociation constant for the different Mg complexes given in the text. The concentration of all tested ligands was 2 mM, and the solutions were nominally free of sodium. When used, vanadate was at $5 \cdot 10^{-8}$ M. The ordinate indicates the percentage inhibition of the *p*-nitrophenyl phosphate hydrolysis produced by the tested ligands in the absence of vanadate. The abscissa indicates the protection against vanadate inhibition, expressed as a percentage, calculated as follows:

$$100\left(1 - \frac{\text{fractional vanadate inhibition in the presence of the ligand}}{\text{fractional vanadate inhibition in the absence of the ligand}}\right)$$

cycle of ATP hydrolysis by the ($Na^+ + K^+$)-ATPase. In each case, the tested ligand was added alone to the reaction mixture containing fixed amount of $MgCl_2$ and *p*-nitrophenyl phosphate and variable concentration of KCl in the absence and presence of submaximal doses of vanadate.

The effects of sodium are illustrated in the upper part of Fig. 5. At 100 mM concentration, NaCl inhibits the K^+-dependent *p*-nitrophenyl phosphate hydrolysis in the unpoisoned enzyme with a large reduction in the apparent affinity for potassium (see also Ref. 21). At the same time, the enzyme becomes less sensitive to the K^+-promoted inhibition by vanadate. From this and other experiments the $K_{1/2}$ for KCl as a cofactor for inhibition went from about 0.9 mM in Na^+-free medium to 3 mM with 10 mM NaCl and about 12 mM with 100 nM NaCl. The lower part of Fig. 5 shows that similar results can be seen with ATP. On the other hand (Fig. 6), 5 mM inorganic phosphate inhibits the K^+-dependent *p*-nitrophenylphosphatase activity without affecting the $K_{1/2}$ for KCl. At the same time, phosphate has a smaller protective action against vanadate than that observed with NaCl and ATP; 5 mM inorganic phosphate increased the $K_{1/2}$ for the K^+-promoted inhibition from 0.9 mM to about 1.5 mM (not shown). The other important observation illustrated by Fig. 5 and 6 is that in the presence of vanadate and either of the three ligands tested alone (NaCl, ATP, phosphate) the rate of *p*-nitrophenyl phosphate hydrolysis as a function of

KCl concentration failed to show the biphasic relationship seen for the $(Na^+ + K^+)$-ATPase activity and the Na^+ and K^+ active transport.

Fig. 7 summarizes several experiments where ATP, other adenosine polyphosphates (hydrolyzable and nonhydrolyzable), adenosine and inorganic phosphate, were tested in their effect as inhibitors of the *p*-nitrophenylphosphatase activity and as a protective agents against vanadate inhibition. *p*-Nitrophenyl phosphate, Mg^{2+} and KCl (at saturating concentrations) were kept constant. The figure indicates that the more effective a nucleotide is as a *p*-nitrophenylphosphatase inhibitor, the more effective it becomes as a protector against vanadate inhibition. It is interesting that adenosine is completely ineffective in both aspects, suggesting that a phosphoryl group must be attached to the nucleotide. On the other hand, phosphate alone is effective, but more as a *p*-nitrophenylphosphatase inhibitor than against vanadate. The differences between ATP (the most powerful of the nucleotides) and the others could be due to steric reasons; however, action due to a some phosphorylation from ATP can not be ruled out. The implications of these findings for the mechanism of *p*-nitrophenyl phosphate hydrolysis will be treated in the Discussion.

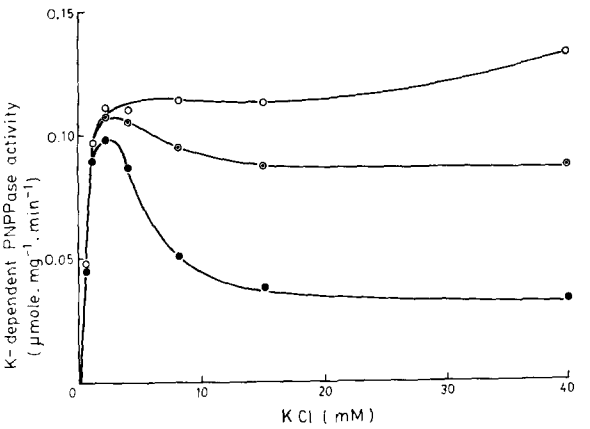

Fig. 8. Potassium activation of *p*-nitrophenyl phosphate hydrolysis in purified $(Na^+ + K^+)$-ATPase enzyme in the absence and presence of vanadate (●, $5 \cdot 10^{-7}$ M, ⊙, $5 \cdot 10^{-8}$ M) when ATP and NaCl are simultaneously present in the incubation media. The composition of the solutions was: $MgCl_2$, 3 mM; *p*-nitrophenyl phosphate, 5 mM; ATP, 3 mM; NaCl, 100 mM; Tris-HCl (pH 7.4; 37°C) (80 − KCl concentration) mM; EGTA, 0.1 mM. Note: (i) the high apparent affinity for KCl; in the absence of vanadate (○) the curve relating *p*-nitrophenylphosphatase activity to KCl concentration reaches a plateau at about 2 mM KCl; (ii) in the presence of vanadate, the *p*-nitrophenylphosphatase activity as a function of KCl follows a biphasic curve with an activation at low KCl followed by inhibition at high KCl; (iii) as the concentration of vanadate is increased, inhibition develops at lower KCl concentration, whereas the slope and magnitude of the decline in activity are increased.

The biphasic effect of K^+ on p-nitrophenyl phosphate hydrolysis in vanadate-poisoned $(Na^+ + K^+)$-ATPase enzyme

In the experiments where the rate of ATP hydrolysis, Na^+ efflux or K^+ influx in the presence of vanadate were measured [3,5,6], the ligands Mg^{2+}, K^+, Na^+ and ATP were all present together. In broken membrane preparations and purified $(Na^+ + K^+)$-ATPase both sides of the membrane were simultaneously expossed to all ligands [5,6]. In resealed red cell ghosts, intact red cells and dialyzed squid axons [3,5,6] the intracellular side was in contact with Na^+, ATP, Mg^{2+} and K^+ and the extracellular surface was bathed with K^+- and Na^+-containing solutions. It seemed possible, then, that, in the experiments described so far, we had failed to observe the biphasic effect of K^+ activation on *p*-nitrophenyl phosphate hydrolysis because the simultaneous presence of Na^+ and ATP is needed for that effect to occur. The experiment of Fig. 8 indicates that this is just the case. In the absence of vanadate, the addition of 100 mM NaCl and 3 mM ATP decreases the *p*-nitrophenylphosphatase activity but increases the apparent affinity for potassium (see also Refs. 21, 22); the curve relating *p*-nitrophenyl phosphate hydrolysis to KCl concentration reaches a plateau at about 2 mM KCl and shows a tendency to a further and slight increase above 15 mM KCl. On the other hand, in the presence of vanadate an unequivocal biphasic behavior is observed. As the concentration of vanadate is increased, the KCl concentration at which inhibition begins to develop is lower, whereas the slope and magnitude of the decline are increased. It should be also stressed here that, as was explained in Material and Methods, all points in the figure correspond to steady-state velocities, that is, the shapes of the curves are

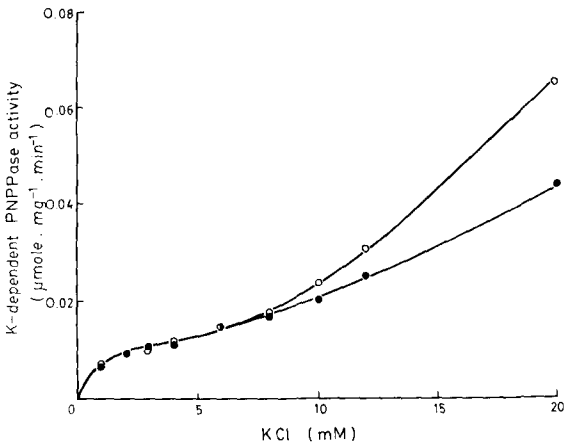

Fig. 9. The effects of NaCl plus adenylyl (β,γ-methylene)diphosphonate on the potassium activation of *p*-nitrophenyl phosphate hydrolysis by purified ($Na^+ + K^+$)-ATPase in the absence (open circles) and presence (filled circles) of $5 \cdot 10^{-8}$ M vanadate. The composition of solutions was as follows: $MgCl_2$, 3 mM; *p*-nitrophenyl phosphate, 5 mM; NaCl, 100 mM; adenylyl (β,γ-methylene)diphosphonate, 1 mM; Tris-HCl (pH 7.4; 37°C) (80 − KCl concentration) mM; EGTA, 0.1 mM. See text for details.

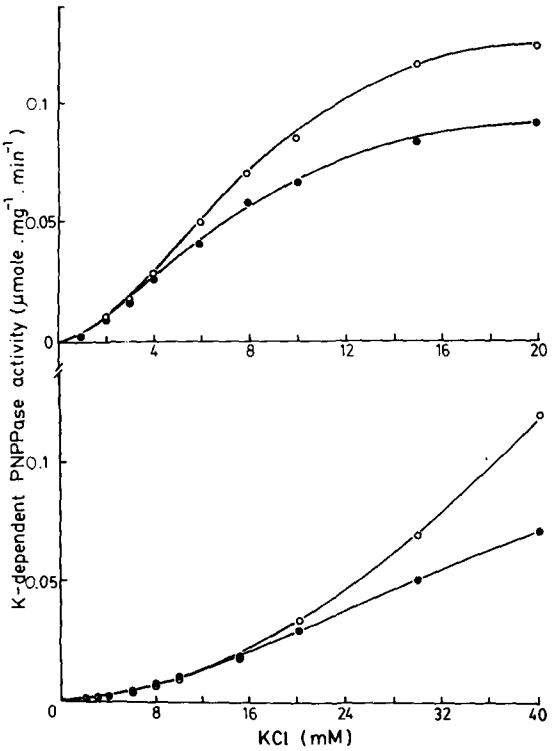

Fig. 10. The effects of inorganic phosphate plus adenylyl (β,γ-methylene)diphosphonate (top) and the combination of inorganic phosphate, adenylyl (β,γ-methylene)diphosphonate and NaCl (bottom) on the potassium activation of *p*-nitrophenyl phosphate hydrolysis by purified ($Na^+ + K^+$)-ATPase enzyme in the absence (open circles) and presence (filled circles) of vanadate. The composition of solutions was: (Top) $MgCl_2$, 3 mM; *p*-nitrophenyl phosphate, 5 mM; inorganic phosphate, 5 mM; adenylyl (β,γ-methylene)diphosphonate, 2 mM; Tris-HCl (pH 7.4; 37°C) (180 − KCl concentration) mM; EGTA, 0.1 mM. When present, vanadate was at $5 \cdot 10^{-8}$ M. (Bottom): $MgCl_2$, 3 mM; *p*-nitrophenyl phosphate, 5 mM; inorganic phosphate, 5 mM; adenylyl (β,γ-methylene)diphosphonate, 1 mM; NaCl, 50 mM; Tris-HCl (pH 7.4; 37°C) (130 − KCl concentration) mM; EGTA, 0.1 mM. When present, vanadate was at $1 \cdot 10^{-7}$ M.

not due to time differences in the onset of vanadate inhibition.

The sodium and ATP requirements for the biphasic K^+-activation of *p*-nitrophenyl phosphate hydrolysis in the presence of vanadate could have several explanations. One possibility is that what is needed is the binding of the nucleotide in the presence of sodium, regardless of the enzyme phosphorylation. This hypothesis was tested in the experiment of Fig. 9, where ATP was replaced with the non-hydrolyzable ATP analog, adenylyl (β,γ-methylene)diphosphonate. This analog has been shown to support K^+-K^+ exchange in red cell ghosts [26] and to induce the same conformational changes as ATP does in purified ATPase, both acting with low apparent affinity [27]. The concentration of the analog used in this case was high enough to replace ATP in that respect [27]. It is clear from the figure that, despite the noticeable inhibition produced in the unpoisoned enzyme and the protection against the effects of vanadate (inhibition does no develop at KCl concentrations lower than 10 mM), no biphasic response of the *p*-nitrophenylphosphatase activity as a function of KCl concentration is observed in the presence of vanadate. The shape of the control curve, with its early plateau at low potassium followed by an increase at higher KCl, is intriguing, but we can offer no explanation for it.

Another possibility for the need of sodium and ATP in the biphasic response is that the enzyme must be phosphorylated. As the same enzyme residue seems to be involved in phosphorylation from ATP or from inorganic phosphate [28], it might be that phosphorylation from phosphate

can work. From the available evidence [28] it seems that 5 mM inorganic phosphate will induce some enzyme phosphorylation even in the presence of other ligands such as K^+, Na^+ and nucleotides. This is also supported by the fact that intact red cells have a large component of K^+-K^+ exchange through the Na^+ pump which, among other ligands, seems to require phosphorylation by intracellular phosphate. If the above assumptions are correct the experiment of Fig. 6 clearly indicates that phosphorylation by itself (at least from inorganic phosphate) is not enough to induce the biphasic response of the vanadate-poisoned enzyme to potassium. In addition, the experiments illustrated in Fig. 10 indicate that in purified enzyme the biphasic response just mentioned cannot be produced by the simultaneous presence of inorganic phosphate and ATP analogs (top) or even by the simultaneous presence of inorganic phosphate, ATP analog and sodium (bottom). The importance of these results will be treated in the Discussion.

The sidedness of the K^+-vanadate interactions in the inhibition of the K^+-dependent p-nitrophenylphosphatase activity

So far, experiments reported here have been performed on purified ($Na^+ + K^+$)-ATPase enzyme. The main advantage of that preparation (very high fractional K^+-dependent *p*-nitrophenylphosphatase activity) is spoiled by the fact that all ligands in the incubation solutions are simultaneously in contact with both membrane surfaces; this means that although some inference about sidedness of interactions could be made, no definite proof about it could be obtained. To attack the problem of sidedness we decided to use inside-out resealed vesicles from human red cells, which in our hands showed a percentage of inversion (70–80%) and an ATP-dependent Na^+ uptake (about 50%) similar to those reported by other laboratories [15,29]. In the interpretation of the results we followed the criteria [17] that the fraction of the *p*-nitrophenyl phosphate hydrolysis stimulated by potassium is the expression of the *p*-nitrophenylphosphatase activity related to the ouabain-sensitive (or Na^+ plus K^+) ATPase activity of the Na^+,K^+ pump. It must be stressed that there was enough EGTA in the incubation media and during the preparation of vesicles to exclude any interference due to a Ca^{2+}-dependent phosphatase activity. Preliminary experiments indicated that in the absence of ATP and Na^+, potassium stimulates *p*-nitrophenyl phosphate hydrolysis by acting on cytoplasmic (extravesicular) sites

TABLE I

ACTIVATION BY CYTOPLASMIC K^+ OF THE *p*-NITROPHENYL PHOSPHATE HYDROLYSIS IN INSIDE-OUT RED CELL VESICLES AND THE EFFECTS OF CYTOPLASMIC Na^+ AND ATP IN THE ABSENCE AND PRESENCE OF VANADATE

The composition of solutions was as follows. (a) Intravesicular (extracellular) side: Tris-glycylglycine (pH 7.4; 37°C), 36 mM; choline chloride, 10 mM; $MgCl_2$, 4 mM. (b) Extravesicular (cytoplasmic) side: Tris-glycylglycine (pH 7.4; 37°C), 15 mM or 5 mM (in the presence of NaCl); $MgCl_2$, 4 mM; *p*-nitrophenyl phosphate, 5 mM; EGTA, 0.1 mM; KCl + choline chloride, 10 mM. The K^+-dependent *p*-nitrophenylphosphatase activity was taken as the difference between the activity found in the presence and in the absence of potassium on the extravesicular (cytoplasmic) side (there was no potassium in the intravesicular (extracellular) side). The table represents one of three experiments carried out in quadruplicate. Similar results were obtained when the EGTA concentration was 0.5 mM. Note: (i) under all experimental conditions, including the presence of vanadate, the *p*-nitrophenylphosphatase activity was higher with 10 mM KCl than with 1 mM KCl in the cytoplasmic side; (ii) in the absence of cytoplasmic Na^+ and cytoplasmic ATP, $1 \cdot 10^{-8}$ M vanadate produced inhibition of the *p*-nitrophenyl phosphate hydrolysis with 10 cytoplasmic mM K^+ but not with 1 mM cytoplasmic K^+; (iii) the simultaneous presence of 10 mM cytoplasmic Na^+ and 3 mM ATP inhibited the *p*-nitrophenylphosphatase activity in the absence of vanadate and protected against inhibition by $1 \cdot 10^{-8}$ M vanadate; (iv) the solute concentration in the intravesicular (extracellular) side refers to the concentrations present in the loading solutions of the vesicles.

Cytoplasmic composition				K^+-dependent *p*-nitrophenylphosphatase activity (nmol·mg^{-1}·min^{-1})
Vanadate ($1 \cdot 10^{-8}$ M)	Na^+ (mM)	ATP (mM)	K^+ (mM)	
–	–	–	1	0.67 ± 0.04
–	–	–	10	2.62 ± 0.04
+	–	–	1	0.59 ± 0.06
+	–	–	10	1.47 ± 0.07
–	10	3	1	0.17 ± 0.07
–	10	3	10	0.64 ± 0.05
+	10	3	1	0.20 ± 0.03
+	10	3	10	0.75 ± 0.07

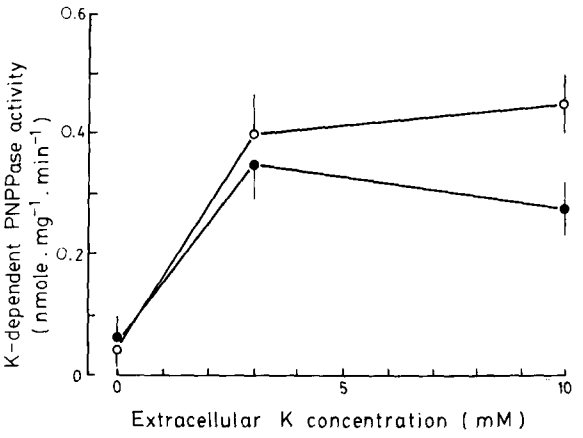

Fig. 11. Effects of extracellular potassium on the *p*-nitrophenyl phosphate hydrolysis in inside-out red cell vesicles in the absence (open circles) and presence (filled circles) of $1 \cdot 10^{-8}$ M vanadate with NaCl plus ATP in the cytoplasmic side and NaCl in the extracellular side. The composition of solutions was as follow: (a) Intravesicular (extracellular) side: Tris-glycylglycine (pH 7.4; 37°C), 36 mM; $MgCl_2$, 4 mM; NaCl, 40 mM; KCl + choline chloride, 10 mM. (b) Extravesicular (cytoplasmic) side: Tris-glycylglycine (pH 7.4; 37°C), 30 mM; $MgCl_2$, 4 mM; *p*-nitrophenyl phosphate, 5 mM; ATP, 3 mM NaCl, 40 mM; KCl, 2 mM; EGTA, 0.1 mM. The K^+-dependent *p*-nitrophenylphosphatase activity was taken as the difference between the activity found in the presence and absence (on both vesicular sides) of potassium. The figure represents one of three experiments carried out in quadruplicate. Similar results were obtained when 0.5 mM EGTA was used. Note: (i) in the absence of vanadate *p*-nitrophenyl phosphate hydrolysis seems to be monotonically activated by extracellular potassium; (ii) $1 \cdot 10^{-8}$ M vanadate has little or no effect on the K^+-dependent *p*-nitrophenylphosphatase activity with 3 mM extracellular K^+ but produced a 40% inhibition of that activity at 10 mM extracellular K^+ ($P < 0.02$); (iii) although not statistically significant, in the presence of vanadate the K^+-dependent *p*-nitrophenylphosphatase activity is lower at 10 mM than at 3 mM extracellular K^+. This tendency was observed in all experiments performed; (iv) the solute concentrations in the intravesicular (extracellular) side refer to those used in the loading solutions of the vesicles.

and that there was no K^+ effect on extracellular (intravesicular) sites. With ATP and Na^+ on the cytoplasmic side, intracellular K^+ still stimulates *p*-nitrophenyl phosphate hydrolysis; in addition, a noticeable stimulation by extracellular K^+ is observed, but this effect of external K^+ does not takes place if there is no cytoplasmic K^+ as well. These findings fully reproduce recent reports by Drapeau and Blonstein [17].

Regarding the K^+-vanadate interactions, Table I shows that in the absence of cytoplasmic Na^+ and ATP, vanadate inhibition is promoted by potassium acting on intracellular sites. In the table, no inhibition was detected at 1 mM K^+_{cyt}, whereas about 40% inhibition was seen when K^+_{cyt} was 10 mM. An exceedingly important additional observation is that in the presence of vanadate, the *p*-nitrophenylphosphatase activity was still higher at 10 mM K^+_{cyt} than at 1 mM K^+_{cyt}. Although only two K^+_{cyt} concentrations were tested, other experiments with different vanadate concentrations gave similar results, suggesting that in vanadate-poisoned vesicles no biphasic effect of cytoplasmic potassium is observed (see also Ref. 13). It is also important to notice that we found no effect of extracellular K^+ on vanadate inhibition in the absence of cytoplasmic Na^+ and ATP (not shown). This concurs with recent finding in an other laboratory [13]. Table I also shows that the presence of 10 mM Na^+ and 3 mM ATP on the cytoplasmic side inhibits the K^+_{cyt}-dependent *p*-nitrophenylphosphatase activity and at the same time overcomes the K^+_{cyt}-promoted vanadate inhibition, at any rate over the range of concentrations studied.

On the other hand (Fig. 11), in the presence of 3 mM ATP in the cytoplasmic side and 40 mM NaCl on both membrane surfaces, vanadate inhibition of *p*-nitrophenyl phosphate hydrolysis was promoted by potassium acting on extracellular sites. With 3 mM extracellular K^+ there was no significant difference between the control values and the values in the presence of vanadate, whereas with 10 mM extracellular K^+, inhibition by vanadate amounted to 40% ($P < 0.02$). Similar results were obtained in three other experiments. It is important that in the presence of vanadate the rate of K^+-dependent *p*-nitrophenylphosphatase activity with 10 mM K^+_{ext} was smaller (although not statistically significant) than with 3 mM external K^+. This was consistently seen in all experiments, suggesting that it is external K^+ that is responsible for the biphasic K^+ effects on *p*-nitrophenyl phosphate hydrolysis by purified $(Na^+ + K^+)$-ATPase in the presence of vanadate, Na^+ and ATP (Fig. 8).

The need for external Na^+ in the biphasic effect of external K^+ on the Na^+ pump in the presence of vanadate

In the experiments referred to before [3,5,6], the effects of external K^+ on vanadate inhibition in intact cells and resealed red cell ghosts were investigated in the presence of external sodium. In addition to the biphasic effect of external K^+ on the Na^+ pump and $(Na^+ + K^+)$-ATPase activity

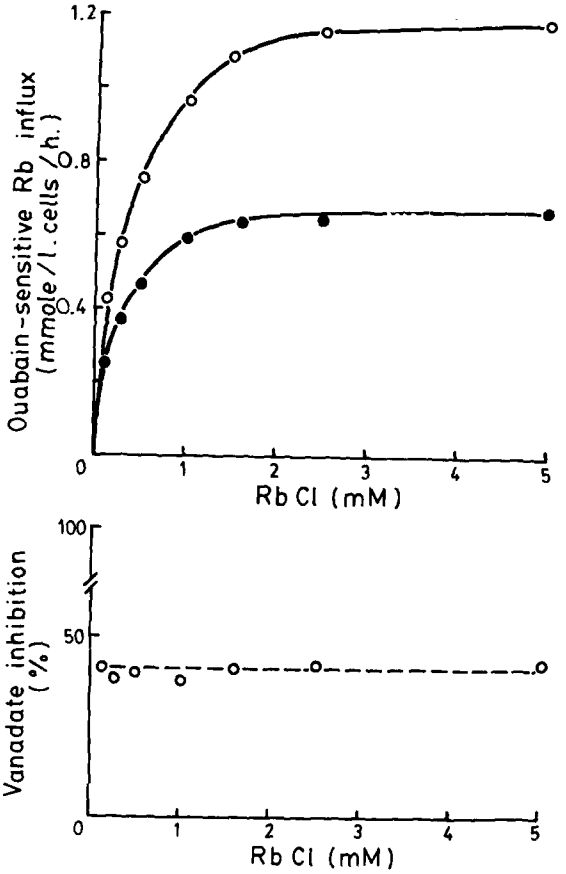

Fig. 12. Ouabain-sensitive Rb^+ influx as a function of extracellular Rb^+ concentration in human red blood cells incubated in the absence (top figure, open circles) and presence (top figure, filled circles) of $5 \cdot 10^{-7}$ M vanadate and in solutions nominally free of external sodium. The composition of the incubation media was the following: Tris-HCl (pH 7.4; 37°C), 10 mM; $MgCl_2$, 1 mM; choline chloride + RbCl, 150 mM; EGTA, 0.1 mM. Note: (i) the influx of Rb^+ is monotonically activated by external Rb^+ both in the presence and absence of vanadate; i.e., there is no biphasic effect of external Rb^+ on Rb^+ influx in vanadate-poisoned cells (top figure); (ii) the percentage of vanadate-inhibited Rb^+ influx is the same at all Rb^+ concentrations tested (bottom figure). For experimental details see text.

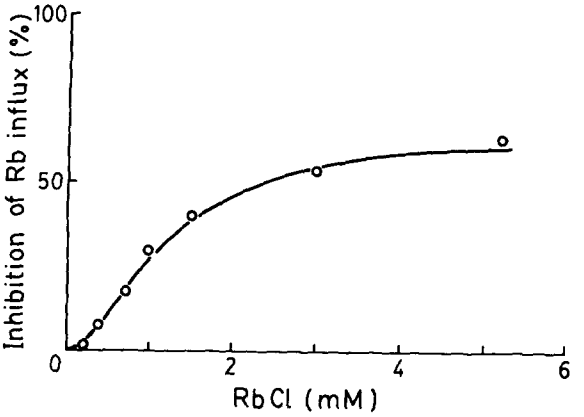

Fig. 13. Vanadate inhibition of the ouabain-sensitive Rb^+ influx as a function of the external Rb^+ concentration in intact human red blood cells incubated in the presence of 15 mM external Na^+. The composition of the incubation solutions was as follows: $MgCl_2$, 1 mM; Tris-HCl (pH 7.4; 37°C), 10 mM; NaCl, 15 mM; RbCl + choline chloride, 135 mM. See text for experimental details. Note that the curve relating percentage of vanadate inhibition to external Rb^+ concentration is markedly sigmoid.

in the presence of vanadate, those experiments suggest a competitive antagonism between external Na^+ and K^+, for (i) as the external Na concentration was reduced, the peak of the maximal K^+-activation was shifted to lower external K^+ concentrations, and (ii) the same magnitude of inhibition was attained at high external K^+ regardless of the external Na^+ concentration. It was suggested [6] that this external K^+ effect could be due to a displacement of Na^+ from some external site at which Na^+ in some way prevented vanadate from acting.

If the hypothesis just studied is correct, then in the absence of external Na^+, extracellular K^+ should lose its power as a co-factor in the inhibition by vanadate. This possibility was tested in the experiment illustrated in Fig. 12. It can be clearly seen (top) that in the absence of external Na^+ (choline was used as a replacement) the activation of the ouabain-sensitive Rb^+ influx by external Rb^+ followed a hyperbolic curve both in the absence and presence of vanadate. In other words, the biphasic response of the Na^+,K^+ pump to external K^+ (Rb) activation in vanadate-poisoned cells disappears if external Na^+ is removed. In addition, the bottom of Fig. 12 shows that with no external Na^+ the percentage of vanadate inhibi-

tion was the same at all external Rb^+ concentrations tested.

Fig. 13 is a plot of vanadate inhibition of the ouabain-sensitive rubidium influx (expressed as percentage) as a function of the external Rb^+ concentration in the presence of 15 mM extracellular Na^+. It is obvious that (i) the fractional inhibition increases together with the Rb^+ concentration, and (ii) the relationship between vanadate inhibition and Rb^+ concentration is strongly sigmoid.

Discussion

Magnesium-vanadate interactions

Mg^{2+} is required for vanadate inhibition of the phosphatase activity in purified $(Na^+ + K^+)$-ATPase (Fig. 2). This concurs with the Mg^{2+} requirement for vanadate inhibition of the $(Na^+ + K^+)$-ATPase activity [4], the active Na^+,K^+ transport [6] and the binding of vanadate to the purified $(Na^+ + K^+)$-ATPase enzyme [13,24]. The intracellular location of the Mg^{2+}-binding sites has already been established [6]. Our kinetic data indicate that Mg^{2+} exerts its effects by binding to a single site as previously suggested [13,24]; the K_{mi} for $MgCl_2$ in our hands (0.5 mM) seems reasonably close to the $K_{0.5}$ of 0.25 mM reported in ^{48}V-binding experiments [13]. On the other hand, the K_m for Mg^{2+} activation of *p*-nitrophenyl phosphate hydrolysis is more than 3-times the K_{mi} in promoting vanadate inhibition of that hydrolysis (Fig. 2). This might suggest that these two Mg^{2+} effects take place at different sites. The K_m for the Mg^{2+} activation of *p*-nitrophenyl phosphate hydrolysis at submaximal doses of vanadate is smaller than in the unpoisoned enzyme (Fig. 2). This does not necessarily means a change in the true affinity of the enzyme for the ligand, because this type of result is to be expected in a kinetic scheme as simple as that proposed by Briggs and Haldane (a reduction in the catalytic rate constant produces a reduction in both V_{max} and K_m).

p-Nitrophenyl phosphate-vanadate competition

The competitive *p*-nitrophenyl phosphate-vanadate interaction (Fig. 1) could be interpreted in the sense that vanadate and *p*-nitrophenyl phosphate bind to the same enzyme site or that, by binding to different sites, they displace the enzyme to forms with lower affinity for the other ligand. If the former hypothesis was correct, the fact that phosphate is both a vanadate antagonist and an inhibitor of the K^+-dependent *p*-nitrophenyl phosphate hydrolysis (Figs. 6 and 7) by competing with *p*-nitrophenyl phosphate would indicate that the three aforementioned ligands share the same binding site. In this scheme *p*-nitrophenyl phosphate binding occurs, at least in part, via its phosphoryl group. The fact that both vanadate and phosphate compete with *p*-nitrophenyl phosphate is in accordance with the view first proposed by Lopez et al. [31] that vanadate inhibits the alkaline phosphatase in *Escherichia coli* by specifically attaching to the phosphate-binding site of the enzyme (see Ref. 23 for $(Na^+ + K^+)$-ATPase).

Potassium-vanadate interactions

K^+ promotes vanadate inhibition of the K^+-dependent *p*-nitrophenylphosphatase activity in the absence of any other ligands besides Mg^{2+} and *p*-nitrophenyl phosphate (Fig. 4). The sigmodicity of the curve relating inhibition to KCl lineal concentration scales (not shown) indicates that more than one K^+-binding site are involved. In addition, we have seen that potassium decreases the apparent K_i for vanadate; this agrees with reports that potassium increases the affinity of the enzyme to vanadate but without affecting the maximal amount of vanadium being bound [24]. This in turn concurs with previous work where, even in the absence of potassium, high vanadate concentrations were found to be able to produce fluorescence changes, indicating a shift of the E_1 into E_2 enzyme conformation [9]. Our results also show that the apparent affinity for potassium as a cofactor in vanadate inhibition of the K^+-dependent *p*-nitrophenylphosphatase activity increases at high vanadate concentrations (Fig. 4). In the case of purified $(Na^+ + K^+)$-ATPase it was reported that vanadate increased the equilibrium binding affinity for Rb^+ [23]. Although the data could not accurately distinguish between two or three Rb^+ sites per ouabain site, the authors (see also Ref. 32) favor the idea that those were external Rb^+ sites. However, if we consider the experiments in inside-out red cell vesicles (Table I, see also Ref. 13 for similar results) where there were

K^+ and Mg^{2+} on the intracellular membrane surface and only buffer on the extracellular side, we must conclude that the results of Figs. 3 and 4, as well as those on Rb^+ binding, represent the attachment of potassium to intracellular sites. A possible contamination with potassium inside the vesicles would not alter the conclusion, because it has been shown [13] that extracellular K^+ has no effect on vanadate inhibition under these conditions (see also Fig. 12). Table I (and other results not shown where Na^+ or ATP were tested alone) indicate that Na^+ and ATP can antagonize the internal K^+-promoted vanadate inhibition of the K^+-dependent *p*-nitrophenyl phosphate hydrolysis acting on the intracellular membrane surface. It is likely that the protective effects exerted by other nucleotides and by inorganic phosphate also take place intracellularly. Taking together the fluorescence [9] and the ^{48}V-binding [24] studies, it is logical to assume that intracellular K^+ promotes vanadate inhibition as a consequence of the shift in the equilibrium from the E_1 to the E_2 enzyme form. On this basis, the protective effects of intracellular Na^+ and nucleotides can be a consequence of their tendency to push the equilibrium into the E_1 form of the enzyme [24]. Inorganic phosphate and *p*-nitrophenyl phosphate can protect just by displacing vanadate from its binding site, but more complex effects cannot be disregarded. The experiments summarized in Figs. 5 and 7 indicate that all ligands having an inhibitory effect on the K^+-dependent hydrolysis also protect against vanadate inhibition of that hydrolysis. Based on the conclusion that vanadate inhibition requires the enzyme in the $E_2(K)$ conformation, these results support the hypothesis that the $E_2(K)$ form is involved in the phosphatase activity of the $(Na^+ + K^+)$-ATPase [17].

If, in addition to Mg^{2+} and *p*-nitrophenyl phosphate, Na^+ plus ATP are included in the incubation media, the response to K^+ of *p*-nitrophenyl phosphate hydrolysis in vanadate-poisoned purified $(Na^+ + K^+)$-ATPase shows biphasic behavior. The experiments performed in inside-out red cell vesicles (Fig. 11) allow us to assert that in this case the K^+ effects take place on extracellular membrane sites. We see then that, under certain experimental conditions, external K^+ can act as a co-factor in vanadate inhibition of $(Na^+ + K^+)$-ATPase activity [8], Na^+ and K^+ transport [3,5,6] and also *p*-nitrophenylphosphatase activity (this paper) by the Na^+ pump. Relevant questions to answer are (i) why are ATP and Na^+ required and (ii) on what side of the membrane does Na^+ act? First of all, Na^+ plus ATP must be included together, for Fig. 5 shows that the biphasic response to K^+ is not obtained when either of them is present alone. In addition, Na^+ plus adenylyl (β,γ-methylene)diphosphonate (Fig. 10) do not give the biphasic K^+ effect, suggesting that this type of interation is not just due to the binding of the nucleotide to the enzyme in the presence of Na^+. Furthermore, the impossibility of obtaining that biphasic response to K^+ with vanadate in the presence of inorganic phosphate, either alone or in combination with Na^+ or with Na^+ plus adenylyl (β,γ-methylene)diphosphonate, could suggest that phosphorylation of the enzyme per se is not the required, or at least the only required, event. However, to be certain about this an evaluation of the amount of phosphoenzyme formed from ATP compared with that formed from inorganic phosphate would be necessary, since the results may represent quantitative differences between both conditions. One possibility is that what is needed is to have the enzyme (or Na^+ pump) under turnover conditions. This has been suggested before, but in a quite different context. It was proposed [12] that external K^+ promotes vanadate inhibition on the Na^+ pump as a consequence of the $E_2(K)$ conformation that followed dephosphorylation by external K^+. However, if this were the case, each pump unit after being dephosphorylated by external K^+ should bind vanadate very tightly and consequently should become inhibited. The results would be: (a) for Na^+ efflux, a monotonically decreasing curve of Na^+ efflux vs. K^+ concentration and (b) for K^+ influx, a nonbiphasic relation between influx and external K^+ with the addition that the fractional inhibition of K^+ influx should be independent of the external K^+ concentration. These predictions are not borne out, because in the presence of vanadate both Na^+ efflux and K^+ influx displayed a biphasic response to external K^+, while the fractional vanadate inhibition increased together with the external K^+ concentrations [3,5,6]. This is a very important point, because it

indicates that in vanadate poisoning there is no dissociation between the active transport of K^+ and the active transport of Na^+. In addition, if turnover of the pump is required for the external K^+ effects just described, Fig. 12 shows that Na^+ is not only necessary to phosphorylate the enzyme but that it must be present at extracellular sites as well. It could be argued that, even in the absence of external Na^+, with low enough external Rb^+ concentrations a vanadate inhibition changing with external Rb^+ might have been obtained. This possibility cannot be completely ruled out. However, in cells poisoned with lower vanadate concentrations (which would reduce the apparent affinity for external Rb^+ as cofactor in inhibition (see Ref. 5)) we found no external Rb^+-dependent inhibition in the absence of external Na^+ (not shown). At first sight, the results of Fig. 12 seem to disagree with those of Robinson and Mercer [13] who found that in inside-out red cell vesicles extracellular (intravesicular) K^+ promoted vanadate inhibition of the $(Na^+ + K^+)$-ATPase activity in the apparent absence of extracellular Na^+. However, it must be remembered that in the inside-out vesicle system the extracellular side is the intravesicular side. The existence of Na^+ in the incubation media was needed in their experiments to stimulate the ATP hydrolysis; this in turn stimulates Na^+ transport. Due to the very small vesicular volume, and considering also that they preincubated their system prior to measuring the ATPase activity, it is not unlikely that some Na^+ entered and built up an Na^+ concentration high enough to produce the observed effects. In this regard it should be stressed that as Fig. 13 shows, a small Na^+ concentration can induce the biphasic external K^+ effects we are dealing with. Findings similar to those of Fig. 13 have been recently reported by Sachs [33].

The need for pump turnover in order to observe the biphasic effects of external K^+ on the *p*-nitrophenylphosphatase activity could be explained on the basis of the following considerations: (i) the biphasic effect requires that external K^+ should stimulate phosphatase activity at low concentrations and inhibit it at moderate concentrations; (ii) the inhibition by external K^+ at moderate concentrations can be explained by the hypothetical displacement of sodium ions from external sites at which they prevent vanadate from acting [6]; (iii) the biphasic effect will therefore be seen if external K^+ stimulated the *p*-nitrophenylphosphatase activity at low concentration. Such stimulation is seen only in the presence of ATP and intracellular Na^+, presumably because only in these conditions does external K^+ lead to the formation of $E_2(K)$, the enzyme form which catalyzes the hydrolysis of *p*-nitrophenyl phosphate.

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