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LOW-AFFINITY Na^+ SITES ON ($Na^+ + K^+$)-ATPase MODULATE INHIBITION OF Na^+-ATPase ACTIVITY BY VANADATE

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Na^+-ATPase activity is extremely sensitive to inhibition by vanadate at low Na^+ concentrations where Na^+ occupies only high-affinity activation sites. Na^+ occupies low-affinity activation sites to reverse inhibition of Na^+-ATPase and (Na^+, K^+)-ATPase activities by vanadate. This effect of Na^+ is competitive with respect to both vanadate and Mg^{2+}. The apparent affinity of the enzyme for vanadate is markedly increased by K^+. The principal effect of K^+ may be to displace Na^+ from the low-affinity sites at which it activates Na^+-ATPase activity.

Introduction

(Na^+, K^+)-ATPase catalyzes a low level of ouabain-sensitive, Mg^{2+}- and Na^+-dependent ATPase activity (Na^+-ATPase) in the absence of K^+ [1–5]. This activity reflects a slow, spontaneous hydrolysis of the phosphoenzyme formed from ATP in the presence of Mg^{2+} and Na^+. This hydrolysis is greatly accelerated by K^+ [3].

Biphasic activation curves are observed when Na^+-ATPase activity is studied as a function of Na^+ concentration. Na^+ first activates the enzyme at high-affinity sites, and activity approaches a plateau. Na^+ then occupies a second class of low-affinity sites to activate once again [3–5]. In the presence of vanadate, (Na^+, K^+)-ATPase activity also exhibits high- and low-affinity sites for Na^+, and the pattern of activation by Na^+ closely resembles that for activation of Na^+-ATPase activity [6]. This similarity led us to the present investigation which examined the role of high- and low-affinity Na^+ sites in the regulation of Na^+-ATPase activity by vanadate.

Materials and Methods

Preparation of (Na^+, K^+)-ATPase. (Na^+, K^+)-ATPase was prepared from the outer medulla of dog kidney by the method of Post and Sen [7]. Protein was determined by the method of Lowry et al. [8] with bovine albumin as the standard.

Na^+-ATPase assay. Na^+-ATPase activity was measured in terms of P_i production by means of the sensitive assay procedure described by Muszbek et al. [9]. Na^+-ATPase activity was calculated by subtracting the activity in the presence of ouabain (0.25 mM) from that in its absence. Conditions common to all experiments were: 150 μM Tris-ATP, 6 mM Mg^{2+}, 0.25 mM EGTA and 63 mM Tris-HCl (pH 7.4 at 38°C). Incubations were carried out in a volume of 2 ml at 38°C. Figures represent the average of two or more assays carried out in duplicate. Other conditions are described in figure legends.

Abbreviation: EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

Results

Fig. 1 shows Na^+-ATPase activity as a function of Na^+ concentration. In the absence of vanadate, biphasic activation curves give clear evidence of the high- and low-affinity Na^+ sites which others have noted [3–5]. In the presence of vanadate, however, only the low-affinity Na^+ sites were evident. Inhibition by vanadate was nearly complete at low Na^+ concentrations, and the occupation of low-affinity Na^+ sites, at high Na^+ concentrations, reversed inhibition. Complete reversal of inhibition was achieved with approximately 120 mM Na^+.

The influence of vanadate concentration on Na^+-ATPase activity is shown in Fig. 2. The concentration of vanadate required for half-maximal inhibition increased from approx. 1 μM with 30 mM Na^+, to 25 μM with 60 mM Na^+, to more than 100 μM with 120 mM Na^+. These results show that Na^+ competes with vanadate to reverse inhibition of Na^+-ATPase activity.

Mg^{2+} is required for vanadate binding [10], and for this reason marked inhibition by Mg^{2+} was evident in the presence of 10 μM vanadate (Fig. 3). Mg^{2+} inhibited more effectively with 30 mM Na^+ than with 60 mM Na^+. This result shows that Na^+ also competes with Mg^{2+} at the site where Mg^{2+} promotes vanadate binding. Mg^{2+} was much less inhibitory in the absence of vanadate, and this inhibition exhibited no Na^+-dependence.

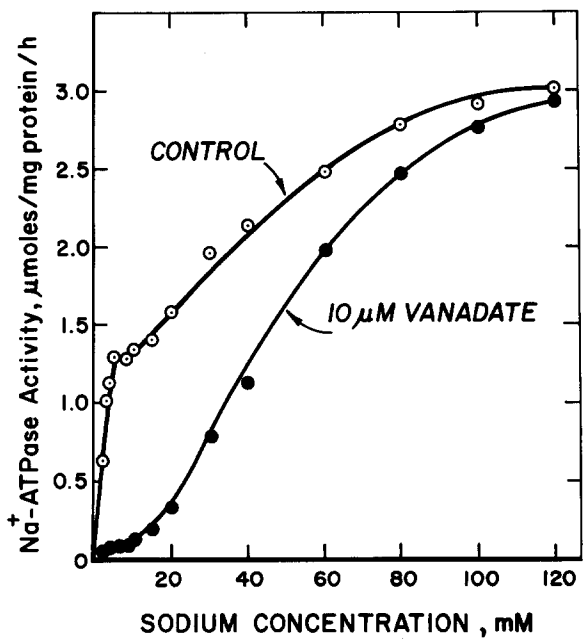

Fig. 1. Na^+-ATPase activity as a function of Na^+ concentration in the presence and absence of vanadate. Other conditions are described in Materials and Methods.

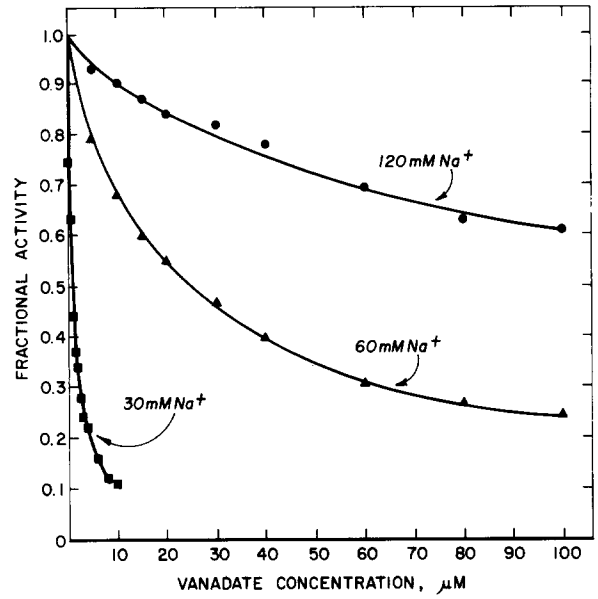

Fig. 2. A comparison of the influence of vanadate on fractional Na^+-ATPase activity at Na^+ concentrations of 30, 60 and 120 mM. Na^+-ATPase activity is expressed as a fraction of control activity (in the absence of vanadate) at each Na^+ concentration.

Mg^{2+} increases the apparent affinity for vanadate [6] and Fig. 3 shows that vanadate also increases the apparent affinity for Mg^{2+}: the curve describing inhibition by Mg^{2+} with 60 mM Na^+ and 10 μM vanadate was shifted to the left in the presence of 40 μM vanadate. Because of the nature of this interaction between Mg^{2+} and vanadate, it follows that Na^+ cannot compete with either ion alone, but must compete functionally with both vanadate and Mg^{2+}. The outcome of this competition is reversal of inhibition.

The effect of K^+ on enzyme activity in the presence of vanadate was examined in Fig. 4. With 10 μM vanadate, the tendency for K^+ to inhibit, by promoting vanadate binding, outweighed its tendency to activate (Na^+, K^+)-ATPase. With

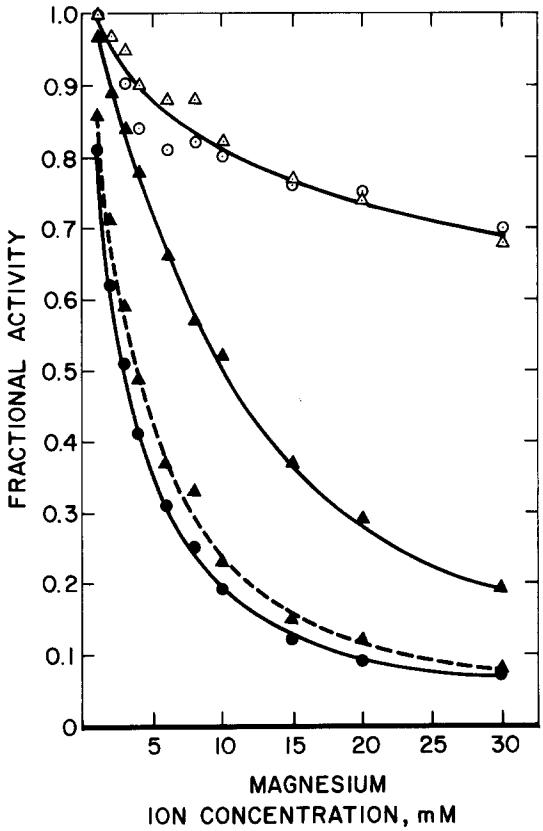

Fig. 3. A comparison of the influence of Mg^{2+} on fractional Na^+-ATPase activity in the presence and absence of vanadate. At each Na^+ concentration, activity is expressed as a fraction of the activity with 1 mM Mg^{2+} in the absence of vanadate. Open symbols: no vanadate. Closed symbols: vanadate present. (○, ●)=30 mM Na^+. (△, ▲)=60 mM Na^+. Curves with solid lines and closed symbols were obtained with 10 μM vanadate; the curve with the dashed line was obtained with 40 μM vanadate.

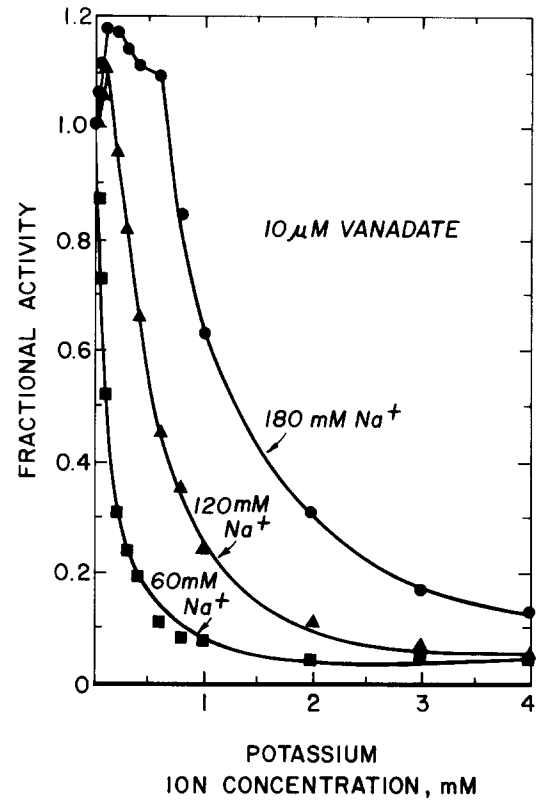

Fig. 4. A comparison of the influence of K^+ on fractional ATPase activity in the presence of 10 μM vanadate. At each Na^+ concentration, activity with K^+ is expressed as a fraction of the Na^+-ATPase activity in the absence of K^+.

120 and 180 mM Na^+ there was slight activation as K^+ occupied high-affinity activation sites. Activation was followed by inhibition as K^+ occupied sites of lower affinity to promote vanadate binding. With 60 mM Na^+, K^+ was only inhibitory. These curves illustrate Na^+-K^+ competition at sites where K^+ promotes vanadate binding; higher K concentrations were required for inhibition at higher Na^+ concentrations.

Fig. 4 also shows the magnitude of the influence of K^+ on inhibition by vanadate. With 60 and 120 mM Na^+, enzyme activity was 95% inhibited at 4 mM K^+. There was little inhibition in the absence of K^+ under these conditions (see (Fig. 1). In other experiments (not shown), we measured the apparent dissociation constant for vanadate as an inhibitor of (Na^+, K^+)-ATPase with 120 mM Na^+, 6 mM Mg^{2+}, 150 μM ATP and 20 mM K^+. A value of 0.05 μM was obtained. Except for the K^+, conditions were identical to those for the assay shown in the upper curve of Fig. 2, where 100 μM vanadate inhibited by less than 50%. Therefore, 20 mM K^+ increased the apparent affinity for vanadate more than 2000-fold. This comparison should be valid even though different enzyme activaties were being measured if it is assumed that vanadate inhibits the two activities at a common site, and that half-maximal inhibition is achieved when 50% of the sites are occupied by vanadate.

Discussion

Na^+ activates Na^+-ATPase at two classes of sites with high- and low-affinity. Our results demonstrate that Na^+ acts at the low-affinity activation sites to decrease the apparent affinity for vanadate as an inhibitor of Na^+-ATPase activity. These sites appear to be identical to the low-affinity sites where Na^+ competes with K^+ to reverse inhibition of (Na^+, K^+)-ATPase activity by vanadate [6], and to the low-affinity sites where Na^+ displaces [^{48}V]vanadate from purified (Na^+, K^+)-ATPase [10].

In experiments with intact and reconstituted red cells, it has been shown that the high- and low-affinity sites are internal and external, respectively [11,12]. The high-affinity sites are those at which Na^+ catalyzes enzyme phosphorylation from ATP in the presence of Mg^{2+}. This reaction is an obligatory part of the reaction sequence of both Na^+-ATPase and (Na^+, K^+)-ATPase activities [13]. In contrast, the function of the low-affinity sites and the mechanism of activation by Na^+ at these sites is not well established. Na^+ may have a dual effect at these sites to increase Na^+-ATPase activity by stimulating (1) hydrolysis of the E_2 conformation of the phosphoenzyme (E_2P) (Refs. 3, 14), and (2) the $E_2 \rightleftharpoons E_1$ conformational transition of the dephosphoenzyme [14,15]. This conformational change would also decrease the apparent affinities for Mg^{2+}, vanadate and K^+, all of which bind preferentially to E_2 and shift the $E_2 \rightleftharpoons E_1$ equilibrium toward E_2 [16,17]. Thus, the competition between Na^+ on the one hand, and Mg^{2+}, vanadate and K^+ on the other, may not involve direct competition for common sites, but an indirect competition due to ligand-induced shifts toward alternate enzyme conformations. This interpretation is consistent with observations that sites for Mg^{2+} and vanadate are internal [18], whereas low-affinity Na^+ sites are external [19]. Since K^+ acts at low-affinity external sites to promote inhibition by vanadate [19], some direct competition between Na^+ and K^+ at these external sites may occur.

Post et al. [20] showed that Na^+ inhibits phosphorylation of (Na^+, K^+)-ATPase by P_i. This Na^+-P_i competition is probably related to the Na^+-vanadate competition described here, since vanadate is a transition-state analog of P_i and occupies a P_i site on E_2 [21]. However, a point of apparent conflict with the present results requires comment. Post et al. [20] found Na^+ to be an effective inhibitor at low concentrations (~ 1 mM) when the enzyme was preincubated with Na^+ prior to initiating phosphorylation by P_i. This result suggests that, under these conditions, Na^+ occupies high-affinity internal activation sites to shift the $E_2 \rightleftharpoons E_1$ equilibrium toward E_1, which binds both P_i and vanadate with low affinity. In the presence of vanadate, however, or when the enzyme has been phosphorylated, the E_2 form predominates, and E_2 exhibits low-affinity external sites for Na^+. Taniguchi and Post [15] showed that Na^+ can act at low-affinity sites to reverse the normal reaction sequence of (Na^+, K^+)-ATPase (E_2-$P \rightleftharpoons E_1P$) with a resultant synthesis of ATP from P_i and ADP. Apparently Na^+ can act at either high- or low-affinity sites to generate E_1 depending on experimental conditions.

The influence of vanadate on Na^+-ATPase activity was studied by Cantley et al. [21] and Beaugé et al. [19] with different results. Cantley et al. [21] reported vanadate to be relatively ineffective as an inhibitor of Na^+-ATPase. It is now clear that the Na^+ concentration in their experiments was high enough to allow for substantial occupation of the low-affinity Na^+ sites. Beaugé et al. [19] showed that Na^+-ATPase activity in reconstituted red cells is markedly sensitive to inhibition by vanadate when cells are incubated in Na^+-free media. These workers suggested that Na^+ acted at external low-affinity sites to reverse inhibition by vanadate.

Our results concerning the magnitude of the influence of K^+ on inhibition of the enzyme are in contrast to those of Smith et al. [10]. Under the conditions of our experiments, 20 mM K^+ increased the apparent affinity of the enzyme for vanadate more than 2000-fold. Smith et al. [10] found that K^+ increased vanadate binding by approximately 5-fold. They measured vanadate binding directly, while our measurements were made under steady-state conditions. This difference might account, in part, for the discrepancy. However, Na^+ was not present in their experiments, and it may be that Na^+-K^+ competition at low-affinity sites is the principal mechanism by which K^+ promotes vanadate binding.

In conclusion, the present results demonstrate that the apparent affinity for vanadate decreases markedly as Na^+ occupies low-affinity sites where it activates Na^+-ATPase. These sites may be the low-affinity sites where Na^+ competes with K^+ to reverse inhibition of (Na^+, K^+)-ATPase by vanadate. They may also be the same sites at which external K^+ promotes inhibition by vanadate. The function of these sites in the absence of vanadate remains to be established.

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