Kinetics of Inhibition of NaK-ATPase by Mg^{2+}, K^+, and Vanadate†

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ABSTRACT: This paper describes the kinetics of inhibition of Na^+- and K^+-stimulated adenosine triphosphatase (NaK-ATPase) by Mg^{2+}, K^+, and vanadate in combination and the reversal of this inhibition by Na^+. Inhibition required the simultaneous occupation of inhibitory sites for Mg^{2+}, K^+, and vanadate. Inhibitory sites for Mg^{2+} and K^+ are distinct from their sites for activation of NaK-ATPase. Mg^{2+} increased the apparent affinity for K^+ at its inhibitory site and vice versa. Mg^{2+} also increased the apparent affinity for vanadate. The occupation of inhibitory sites for Mg^{2+} and vanadate obeyed Michaelis-Menten kinetics, but the occupation of the inhibitory site for K^+ was a sigmoid function of K^+ concentration. It appears that at least two K^+ ions may be required to inhibit. Cations which can substitute for K^+ as activators of NaK-ATPase also substituted as inhibitors in the presence of Mg^{2+} and vanadate (e.g., Tl^+, Rb^+, Cs^+, and NH_4^+). The relative affinities of these cations for the activation site and the inhibitory site ranked in about the same order. This result suggests a similarity between sites for activation and inhibition by K^+. In the presence of inhibitory concentrations of Mg^{2+}, K^+, and vanadate, Na^+ freely occupied its site for activation of NaK-ATPase, but the activity generated was limited by the extent of inhibition by Mg^{2+}, K^+, and vanadate. At higher Na^+ concentrations, Na^+ occupied a low-affinity site to antagonize inhibition. Occupation of this low-affinity site was markedly depressed at higher K^+ concentrations, and it appeared that Na^+ antagonized inhibition by displacing K^+ from its inhibitory site. Inhibition of NaK-ATPase by Mg^{2+}, K^+, and vanadate could reflect a mechanism for regulation of NaK-ATPase in vivo.

It was recently shown that ATP obtained from equine muscle (Sigma Chemical Co., St. Louis, MO) contained a potent inhibitor of NaK-ATPase[1] (Charney et al., 1975; Beaugé & Glynn, 1977; Hudgins & Bond, 1977). This inhibitor was isolated from muscle by Josephson & Cantley (1977) and identified as pentavalent vanadium, or vanadate (Cantley et al., 1977).

Inhibition of NaK-ATPase by vanadate is markedly dependent on the concentrations of Mg^{2+} and K^+. Both cations are required for inhibition at concentrations within the range which normally activate NaK-ATPase, and inhibition is therefore a combined effect of Mg^{2+}, K^+, and vanadate (Bond & Hudgins, 1975; Cantley & Josephson, 1976; Cantley et al., 1977; Fagan & Racker, 1977). These papers described a Mg^{2+}- plus K^+-dependent inhibition of NaK-ATPase and did not mention vanadate, which was present as a trace contaminant in the ATP used as a substrate.

Inhibition by Mg^{2+}, K^+, and vanadate is most pronounced at low Na^+ concentrations and is antagonized by Na^+ (Bond & Hudgins, 1975, 1977; Nechay & Saunders, 1978). Thus, all cations required for the activity of NaK-ATPase can modify inhibition by vanadate.

The present paper describes the kinetics of inhibition of NaK-ATPase by Mg^{2+} and K^+ in the presence of vanadate and the antagonism of this inhibition by Na^+. The results indicate that Mg^{2+} and K^+ inhibit at sites which are distinct from their respective sites for activation of NaK-ATPase. Inhibition requires the occupation of both inhibitory sites, in addition to a site for vanadate. Na^+ appears to antagonize inhibition at a site which is also distinct from its site for activation of NaK-ATPase. The occupation of this site by Na^+ may displace K^+ from an inhibitory site.

Vanadate is present in mammalian tissues at concentrations which can inhibit NaK-ATPase (Cantley et al., 1977). Inhibition by Mg^{2+}, K^+, and vanadate and antagonism of inhibition by Na^+ might therefore reflect a mode of regulation of the NaK pump in vivo.

Experimental Section

Preparation of NaK-ATPase. Freshly outdated human blood from the blood bank was used as the source of the enzyme. All operations were carried out at 4 °C. Cells were washed several times in isotonic saline, and white cells were removed by aspiration. Washed cells were lysed in 10 volumes of 1 mM Tris-EDTA (pH 7.8). Membranes were recovered by centrifugation and were washed repeatedly in the same medium to remove visible traces of hemoglobin. After the final wash, membranes were suspended in 10 mM imidazole hydrochloride (pH 7.2) and stored at −20 °C. The protein content of each preparation was measured by the method of Lowry et al. (1951). The results described in this paper were independent of the duration of storage. Freezing and thawing the membranes in dry ice–acetone had no effect on activity, indicating that the membranes were uniformly leaky.

Assay of NaK-ATPase. The ATP used in these experiments was isolated from equine muscle (Sigma Chemical Co., St. Louis, MO), and it contained vanadate as a trace contaminant. In some experiments, the vanadate was contributed by the ATP. In other control experiments, we used equine muscle ATP from which vanadate was removed as described below.

All incubations were carried out in a volume of 2 mL at 38 °C for 60–90 min. Phosphate (P_i) release was linear with time over this interval. Assay conditions common to all experiments were: 2 mM equine muscle ATP (Na^+ or $Tris^+$ salt); 63 mM Tris-HCl (pH 7.4 at 38 °C); 0.25 mM Tris-EGTA; 0.4–0.6 mg of membrane protein. Other conditions are described in figure legends. Mg-ATPase activity was measured as described above, but in the presence of 0.25 mM ouabain and without K^+. We verified in numerous experiments that all the activity reported in this paper is ouabain sensitive. NaK-ATPase activity was calculated by subtracting Mg-ATPase activity from the total activity in the presence of Na^+ and K^+.

All assay constituents except membranes were added to the reaction tubes, and these were placed in an ice bath. Membranes were added last and, after approximately 10 min,

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[1] Abbreviations used: NaK-ATPase, Na^+- and K^+-stimulated adenosine triphosphatase; P_i, inorganic phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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tubes were transferred at timed intervals to a water-bath shaker for incubation. The reaction was stopped by addition of 0.5 mL of cold perchloric acid at timed intervals, and the tubes were returned to an ice bath. Membranes were removed by centrifugation at 4 °C, and an aliquot of the supernatant was taken for P_i assay by a modification of the method of Fiske & SubbaRow (1925).

All data represent the average of three or more experiments performed in duplicate.

Calculation of Free Mg^{2+} Concentration. When the Mg^{2+} concentration was less than the ATP concentration (2 mM), the free Mg^{2+} concentration (Mg^{2+}_f) was calculated on the basis of a dissociation constant of 32 μM for Mg–ATP as reported by Robinson (1974a) under similar assay conditions. When the Mg^{2+} concentration was greater than 2 mM, the free Mg^{2+} concentration was calculated by subtracting the ATP concentration from the total Mg^{2+} concentration.

Preparation of Tris–ATP. In experiments in which the Na^+ concentration was varied, Tris–ATP was used as the substrate. This was prepared from Na_2ATP by ion-exchange chromatography with Dowex 50-X8 cation-exchange resin in the $Tris^+$ form. The column effluent was neutralized to pH 7.8 with Tris base. Vanadate is not removed by this method.

Vanadate was removed from equine muscle ATP by ion-exchange chromatography with Dowex 50-X8 in the H^+ form (Hudgins & Bond, 1977). The success of this procedure depends on the fact that vanadate (VO_4^{3-}) is converted to a cation (VO_2^+) at low pH. The column effluent was neutralized to pH 7.8 with Tris base.

We established that vanadate had been removed on the basis of two tests: (1) there was no evidence of a Mg^{2+}- plus K^+-dependent inhibition of NaK–ATPase when this ATP was used as a substrate, and (2) catecholamines did not stimulate NaK–ATPase in the presence of high concentrations of Mg^{2+} and K^+ (Hudgins & Bond, 1977; Josephson & Cantley, 1977).

Results

Time Course of Inhibition by Mg^{2+}, K^+, and Vanadate. We verified in a number of experiments that P_i release was linear with incubation time under inhibitory conditions. These experiments were performed at both high and low concentrations of Mg^{2+} and K^+, time being measured after addition of enzyme to the complete reaction system at 38 °C. This finding is in apparent disagreement with the results of Cantley & Josephson (1976) and Gibbons et al. (1978), who reported that the onset of inhibition was relatively slow and developed over a period of 5–10 min. The discrepancy may be related to the low specific activity of NaK–ATPase from red cells relative to NaK–ATPase preparations from other tissues. A delay in P_i release over the initial 5 min of incubation would have been barely detectable in our system, and there was certainly no longer delay. The activities reported in this paper, therefore, reflect true rates.

Inhibition as a Function of K^+ Concentration. Figure 1 shows NaK–ATPase activity as a function of K^+ concentration at several fixed Mg^{2+} concentrations. The vanadate concentration was 0.5 μM in this and all subsequent experiments in which equine muscle ATP was used as a substrate. A biphasic response to K^+ was observed at all Mg^{2+} concentrations: activity rose to a peak at about 2 mM K^+ as activation sites were occupied and then declined to a plateau as inhibitory sites were occupied. An increase in Mg^{2+} concentration had the following effects: inhibition began at lower K^+ concentrations, the peak and plateau activities both decreased, and the slope of the inhibitory phase of each curve appeared steeper. This experiment shows that *both* Mg^{2+} and

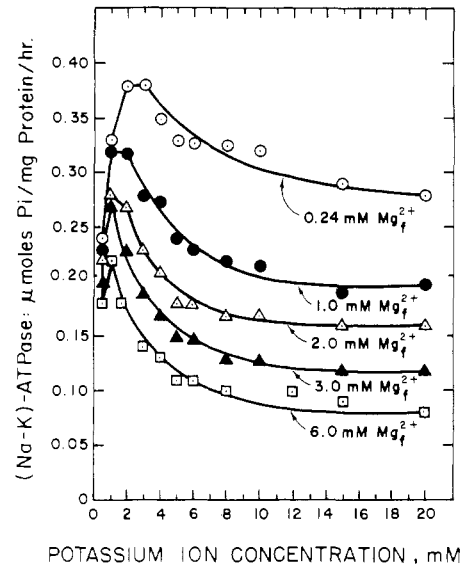

FIGURE 1: NaK–ATPase activity as a function of K^+ concentration at several Mg^{2+} concentrations in the presence of 0.5 μM vanadate (contributed by 2 mM equine muscle ATP). The Na^+ concentration was 34 mM. Mg^{2+}_f refers to free, unchelated Mg^{2+}. This was calculated as described in Experimental Section. The free Mg^{2+} concentration was 0.24 mM in the presence of 2 mM Mg^{2+} and ATP.

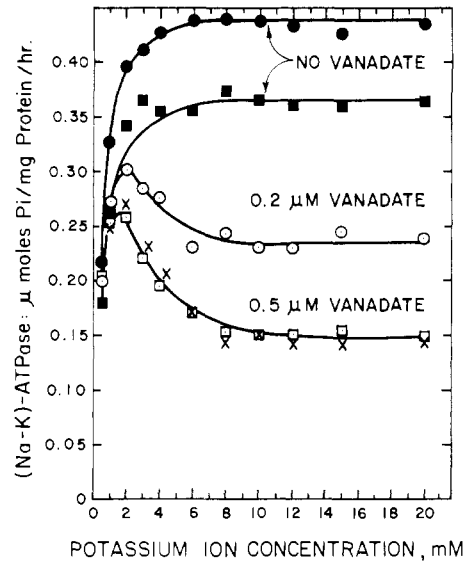

FIGURE 2: NaK–ATPase activity as a function of K^+ concentration with and without vanadate present. The Na^+ concentration was 34 mM. In the upper pair of curves (no vanadate) the concentrations of free Mg^{2+} were 0.24 mM (●) and 6 mM (■), and vanadate-free ATP was the substrate. In the lower curves (vanadate present), the free Mg^{2+} concentration was 2 mM. The curves labeled 0.2 and 0.5 μM vanadate were generated with vanadate-free ATP to which Na_3VO_4 was added. The points (X) were generated with equine muscle ATP which contained vanadate.

K^+ are required for inhibition in the presence of vanadate.

It is significant to note that inhibition by K^+ was evident before its site for activation was fully saturated (compare Figures 1 and 2). When activity reached a peak and began to decline at higher K^+ concentrations, it is likely that activation sites continued to be occupied, but the occupation of inhibitory sites then had the dominant kinetic effect. The inhibitory sites were saturated at a K^+ concentration of 10 mM in all cases, as activity reached a plateau.

The inhibitory sites for Mg^{2+} did not saturate over the range of Mg^{2+} concentrations used in this experiment, and the extent of inhibition at a saturating K^+ concentration was limited by

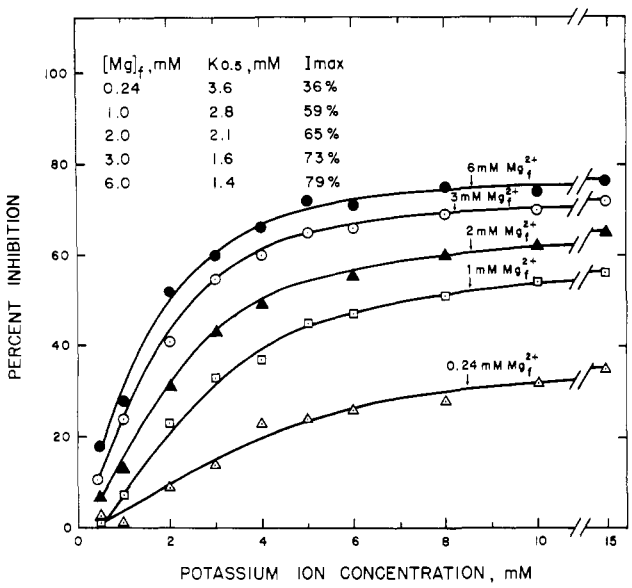

FIGURE 3: Percent inhibition of NaK-ATPase as a function of K^+ concentration in the presence of 0.5 μM vanadate. The data were derived from the curves in Figure 1 and control curves run concurrently, at each Mg^{2+} concentration, in the absence of vanadate. For each point on the curves in Figure 1, percent inhibition was calculated relative to a corresponding control point. These percentages were plotted as experimental points. The maximum inhibition (I_{max}) at a saturating K^+ concentration was estimated by visual inspection of curves that extended to 20 mM K^+. The concentration of K^+ required for half-maximal inhibition ($K_{0.5}$) was calculated from Hill plots. The Hill coefficient was 1.8 for all curves. Values of $K_{0.5}$ and I_{max} are shown on the figure. The solid lines were calculated from the Hill equation with these values.

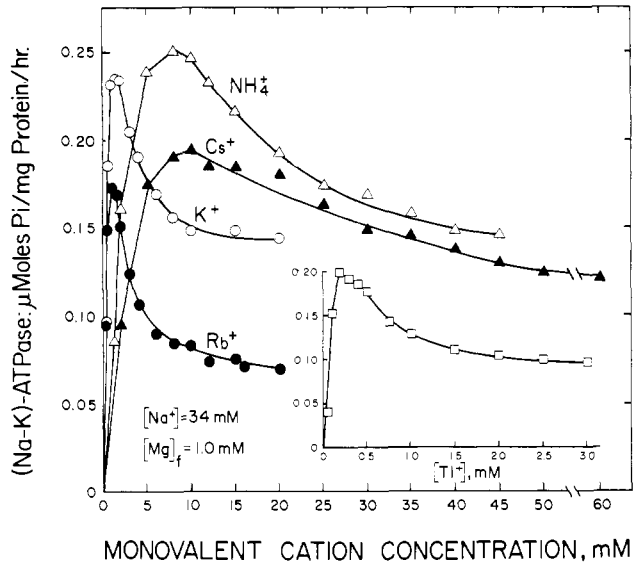

FIGURE 4: NaK-ATPase activity as a function of the concentrations of K^+ and congeners of K^+ in the presence of 0.5 μM vanadate.

the availability of Mg^{2+} and vanadate at their respective inhibitory sites. The kinetics of inhibition by Mg^{2+} and vanadate are described more fully in subsequent sections.

All curves in Figure 1 were run in parallel with controls in which vanadate-free ATP was used as a substrate. Two of these control curves, at the extremes of Mg^{2+} concentration in Figure 1, are shown in Figure 2. In the absence of vanadate, K^+ did not inhibit at concentrations as high as 20 mM. The higher Mg^{2+} concentration was inhibitory, as expected, because NaK-ATPase activity is optimal with equimolar Mg^{2+} and ATP (cf. Dunham & Glynn, 1961).

Figure 2 also shows the effect of vanadate concentration on the kinetics of inhibition by K^+. An increase in vanadate concentration decreased the peak and plateau activities, as did an increase in Mg^{2+} concentration (Figure 1). When the inhibitory sites for K^+ were saturated, the level of inhibition was limited only by the extent to which inhibitory sites for both Mg^{2+} and vanadate were occupied.

The bottom curve in Figure 2 was generated in two separate experiments: one with equine muscle ATP and the other with vanadate-free ATP to which 0.5 μM vanadate was added. The two curves were superimposable, meaning that 2 μM equine muscle ATP contributed 0.5 μM vanadate to the reaction system. This gave a molar ratio of ATP to vanadate of 4000:1, in close agreement with the value reported by Cantley et al. (1977). On the basis of this result, we estimate that the vanadate concentration was 0.5 μM in experiments with 2 mM equine muscle ATP as the substrate.

In order to examine the association of K^+ with its inhibitory sites more directly, we expressed activity at each K^+ concentration in Figure 1 as percent of a control activity at the same Mg^{2+} concentration, but without vanadate. From these data, we generated a family of curves expressing percent inhibition as a function of K^+ concentration at several Mg^{2+} concentrations (Figure 3). Hill plots of these data were linear, and the Hill coefficient was 1.8 for all curves. With increasing Mg^{2+} concentration, the maximal inhibition (I_{max}) at a saturating K^+ concentration increased, and the concentration of K^+ required for half-maximal inhibition ($K_{0.5}$) decreased. The solid lines connecting the experimental points were calculated from the Hill equation with the values shown.

A Comparison of Inhibition by K^+ and Its Congeners. The following cations can substitute for K^+ as activators of NaK-ATPase: Rb^+, Cs^+, NH_4^+, and Tl^+ (Skou, 1960; Britten & Blank, 1968). Figure 4 shows that these cations also substitute for K^+ as inhibitors of NaK-ATPase in combination with Mg^{2+} and vanadate. In the presence of vanadate, biphasic curves describing activity as a function of concentration were obtained for each cation. In the absence of vanadate, none of these cations inhibited at concentrations as high as 20 mM (5 mM for Tl^+). Their relative affinities for activation sites ranked in the following order: $Tl^+ > Rb^+ = K^+$ (approximately) $> NH_4^+ > Cs^+$ (data not shown). It is evident from Figure 4 that their relative affinities for inhibitory sites ranked in the same order.

Inhibition as a Function of Mg^{2+} Concentration. Figure 5A shows the effect of K^+ and vanadate on inhibition of NaK-ATPase by Mg^{2+}. Activity was optimal with equimolar Mg^{2+} and ATP in the absence of vanadate, as others have observed (cf. Dunham & Glynn, 1961). The data in Figure 5A were normalized by expressing all activities at each K^+ concentration as a percent of the optimal activity. Inhibition by Mg^{2+} was independent of K^+ concentration in the absence of vanadate, but there was a marked dependence in the presence of vanadate.

In order to analyze these curves kinetically, vanadate-dependent inhibition was calculated by expressing activity at each point in the presence of vanadate as a percent of the corresponding activity in the absence of vanadate and subtracting this value from 100. The results are shown in Figure 5B. Woolf plots of these data were linear, allowing calculation of the apparent K_i for inhibition by Mg^{2+} and the maximum inhibition (I_{max}) at a saturating Mg^{2+} concentration. The solid lines connecting the experimental points were calculated from the Michaelis-Menten equation with the values shown. As the K^+ concentration was increased, I_{max} increased, and the apparent K_i for inhibition by Mg^{2+} decreased. Mg^{2+} had

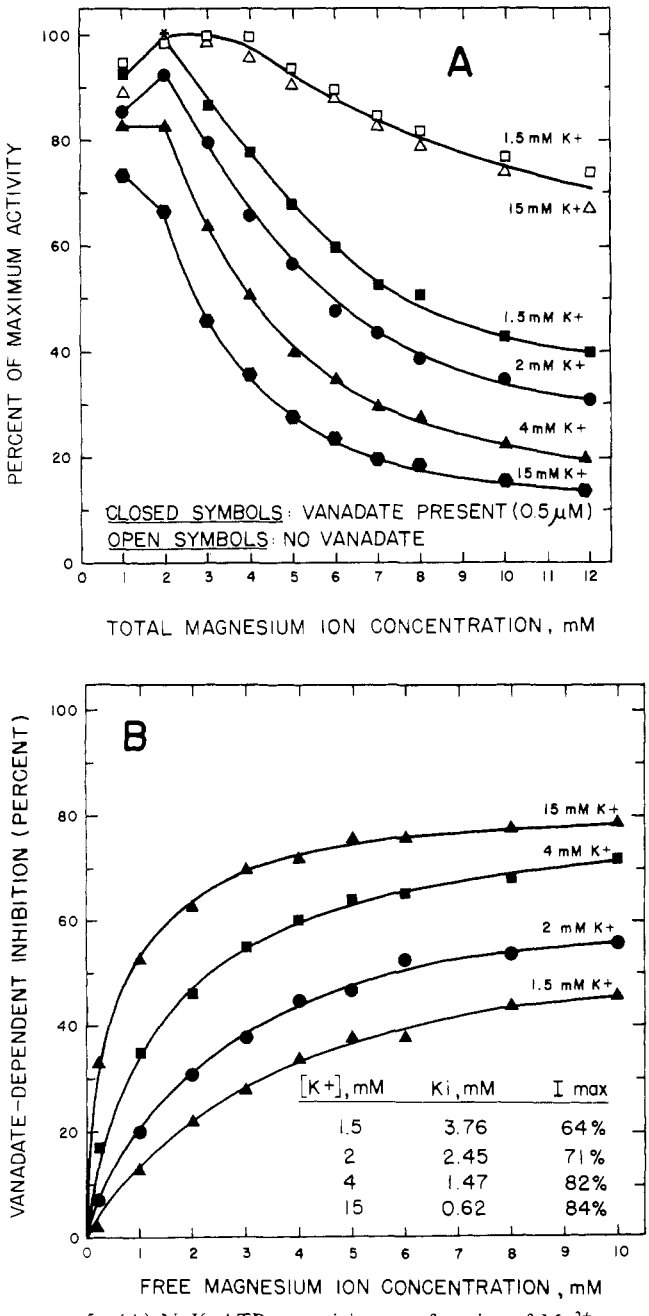

FIGURE 5: (A) NaK-ATPase activity as a function of Mg^{2+} concentration at several K^+ concentrations with and without vanadate present. At all K^+ concentrations, activity is expressed as a percent of the activity with equimolar Mg^{2+} and ATP (2 mM) in the absence of vanadate (*). The Mg^{2+} concentrations shown on the abscissa refer to *total* Mg^{2+}. (This was done in order to decompress the plot at the lowest Mg^{2+} concentrations.) In the absence of vanadate (open symbols) only the data for 1.5 and 15 mM K^+ are shown. The values at other K^+ concentrations fell between these points. The Na^+ concentration was 34 mM. (B) Vandate-dependent inhibition as a function of Mg^{2+} concentration at several K^+ concentrations. The points were derived from the data in A as described in the text. In this experiment, free Mg^{2+} concentration is plotted on the abscissa. Woolf plots were constructed from these data (i.e., plots of [Mg]/% I vs. [Mg], where % I is vanadate-dependent inhibition). These plots were linear. The abscissa intercept gave the apparent K_i for Mg^{2+} and the reciprocal of the slope gave I_{max} at saturating Mg^{2+}. These calculated values are indicated on the figure. The solid lines were calculated by substituting these values into the Michaelis-Menten equation.

similar effects on the kinetics of inhibition by K^+ (Figure 3). Therefore, each cation increases the apparent affinity for the other at its inhibitory site, and I_{max} as either cation is varied

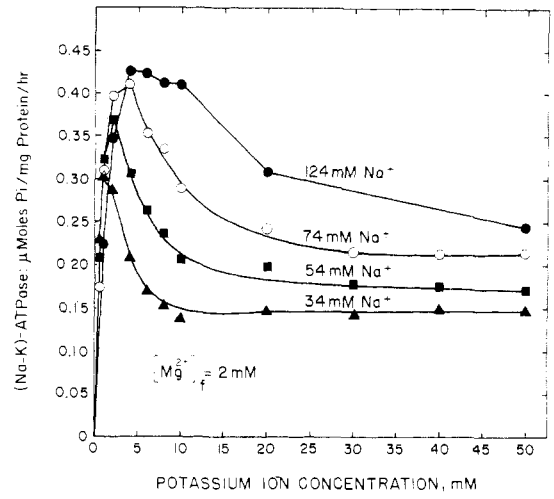

FIGURE 6: NaK-ATPase activity as a function of K^+ concentration at several Na^+ concentrations in the presence of 0.5 μM vanadate.

can be limited by the fixed concentration of the other, or of vanadate (Figure 2).

Inhibition as a Function of Vanadate Concentration. From the data of Figure 5B it was possible to estimate the apparent K_i for vanadate, at saturating concentrations of Mg^{2+} and K^+, according to the following reasoning. The inhibitory sites for K^+ were saturated at 15 mM K^+ (Figures 1 and 3), and inhibition approached 84% as inhibitory sites for Mg^{2+} approached saturation at this K^+ concentration (Figure 5B). The extent of inhibition should then be limited only by the vanadate concentration (0.5 μM). If inhibition is complete when vanadate is also saturating, then 84% of the vanadate sites must be occupied in the presence of 0.5 μM vanadate and saturating Mg^{2+} and K^+. If vanadate binds according to Michaelis-Menten kinetics, the percent occupancy of vanadate sites is $100/(1 + K_i/[V])$, and the calculated K_i is 0.095 μM.

In order to test the reliability of the data and the validity of the assumptions on which this estimate was based, we measured the apparent K_i for vanadate in the presence of 15 mM K^+ and 10 mM free Mg^{2+} (not shown). The value obtained was 0.11 μM, in close agreement with the estimated value. The apparent K_i for vanadate increased as the Mg^{2+} concentration decreased: it was 1.2 μM in the presence of 0.24 mM free Mg^{2+}. The apparent K_i for vanadate was also higher at K^+ concentrations below saturation (not shown). There appears to be a symmetrical interaction between the three inhibitory sites such that each ion increases the apparent affinities for the others. Similar conclusions were reached by Cantley et al. (1977).

These experiments also revealed one notable difference in the kinetics of inhibition by vanadate as compared with Mg^{2+}. Inhibition by vanadate approached completion at Mg^{2+} concentrations of 0.24 and 10 mM. It does not appear, therefore, that inhibition by vanadate can be limited by the Mg^{2+} concentration, whereas inhibition by Mg^{2+} can be limited by the vanadate concentration (Figure 5B).

Effect of Na^+ on Inhibition by Mg^{2+}, K^+, and Vanadate. The experiments described to this point were all carried out with a relatively low Na^+ concentration (34 mM) in order to maximize inhibition. Na^+ antagonizes inhibition by Mg^{2+}, K^+, and vanadate as shown in Figure 6. At higher Na^+ concentrations inhibition by K^+ was less pronounced; inhibition required higher K^+ concentrations, the peak and plateau activities increased, and the slope of the inhibitory phase of each curve was less steep.

The dependence of activity on Na^+ concentration under

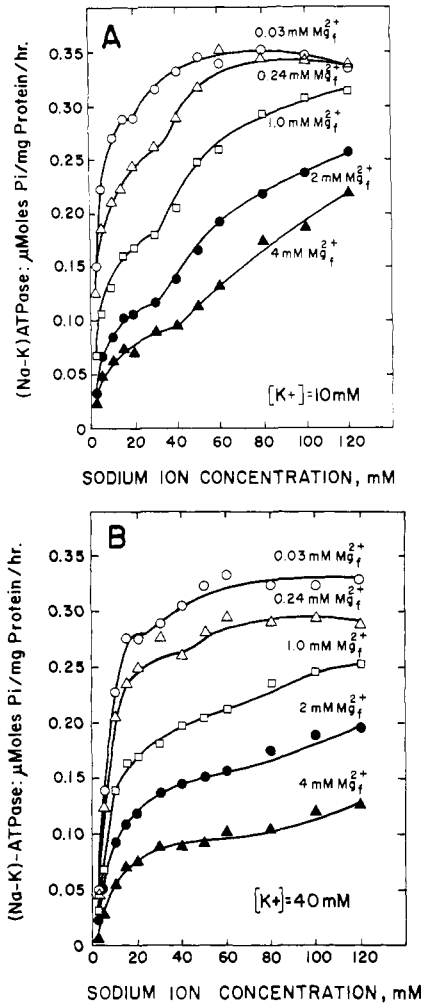

FIGURE 7: (A) NaK-ATPase activity as a function of Na^+ concentration in the presence of 10 mM K^+, 0.5 μM vanadate, and several free Mg^{2+} concentrations. (B) Assay conditions were the same as in A, except for a higher K^+ concentration (40 mM).

inhibitory conditions is shown more fully in Figures 7A and 7B. The inhibitory sites for K^+ were saturated in these experiments, and the extent of inhibition was controlled by varying the Mg^{2+} concentration. In Figure 7A the K^+ concentration was 10 mM, and curves describing NaK-ATPase activity as a function of Na^+ concentration showed distinct intermediary plateaus. As the Mg^{2+} concentration increased, the curves were uniformly depressed, the intermediary plateaus were more pronounced, and two classes of Na^+ sites were evident: high-affinity sites and low-affinity sites. High-affinity sites were present under all conditions, but low-affinity sites were most evident with increasing inhibition. These observations support the identification of high-affinity sites as activation sites for Na^+ and low-affinity sites as sites which Na^+ can occupy to antagonize inhibition.

An experiment bearing on the mechanism of this antagonism is shown in Figure 7B. The conditions were identical with those in Figure 7A except that the K^+ concentration was higher (40 mM). The result was a marked depression in the occupation of low-affinity Na^+ sites. These results, together with those in Figure 6, show that there is a competitive interaction between Na^+ at low-affinity sites and K^+ at inhibitory sites.

Table I summarizes the results of a kinetic analysis of the initial portions of the curves in Figures 7A and 7B, describing the approach to intermediary plateaus as Na^+ occupied high-affinity activation sites. Woolf plots were constructed from the data and K_m and V_{max} were determined from these

Table I: Kinetics of Activation of NaK-ATPase at High-Affinity Sites as Activity Approaches an Intermediary Plateau[a]

$[Mg^{2+}]_f$ (mM)	K_m for Na^+ (mmol/L) 10 mM K^+	K_m for Na^+ (mmol/L) 40 mM K^+	V_{max}[b] (μmol of P_i (mg of protein)$^{-1}$ h^{-1}) 10 mM K^+	V_{max}[b] (μmol of P_i (mg of protein)$^{-1}$ h^{-1}) 40 mM K^+
0.03	3.9	6.3	0.37	0.36
0.24	3.7	5.6	0.29	0.31
1.0	4.7	6.7	0.21	0.23
2.0	5.8	9.9	0.14	0.18
4.0	7.8	8.7	0.11	0.11

[a] Woolf plots were constructed from the data of Figures 7A and 7B. These were linear up to 30 mM Na^+ (Figure 7A) and 50 mM Na^+ (Figure 7B). The indicated kinetic constants were obtained. [b] V_{max} is an upper limit to the activity at the intermediary plateau.

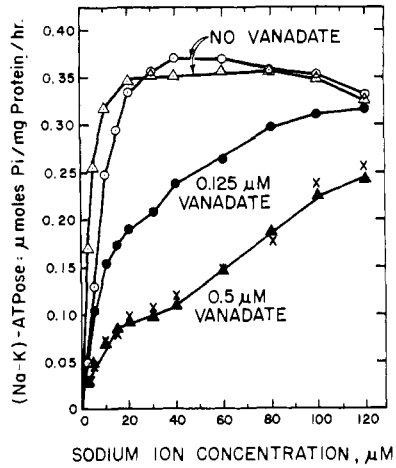

FIGURE 8: NaK-ATPase activity as a function of Na^+ concentration with and without vanadate present. The K^+ concentration was 10 mM throughout. In the upper pair of curves (no vanadate), the concentrations of free Mg^{2+} were 0.03 mM (⊙) and 4 mM (△). A *total* Mg^{2+} concentration of 1 mM gives a free Mg^{2+} concentration of 0.03 mM. In the lower curves (vanadate present), the free Mg^{2+} concentration was 4 mM. The curves labeled 0.125 and 0.5 μM vanadate were generated with vanadate-free ATP to which Na_3VO_4 was added. The points (X) were generated with equine muscle ATP which contained vanadate.

plots. V_{max} is an upper limit to the activity reached at the intermediary plateau at each Mg^{2+} concentration as high-affinity sites approached saturation and before there was significant occupation of low-affinity sites.

The apparent K_m for Na^+ increased as the concentrations of either Mg^{2+} or K^+ increased, because these cations compete with Na^+ at its site for activation of NaK-ATPase (cf. Bader et al., 1970; Post et al., 1960). This competition made a negligible contribution to inhibition, however, because activity nearly reached V_{max} at the intermediary plateau at all Mg^{2+} concentrations (Figures 7A and 7B; Table I).

The lack of dependence of V_{max} on K^+ concentration (Table I) can be explained on the basis that inhibitory K^+ sites were saturated at 10 mM K^+ (Figure 1), and inhibition was then limited only by the concentrations of Mg^{2+} and vanadate. In other experiments (not shown), we found that V_{max} increased at K^+ concentrations below 10 mM. Thus the position of the intermediary plateau depends only on the extent of inhibition, regardless of which ion is varied.

Figure 8 shows that there is little effect of Mg^{2+} concentration on the kinetics of activation of NaK-ATPase by Na^+ in the absence of vanadate. The apparent K_m for Na^+ increased slightly at the higher Mg^{2+} concentration, but there was no evidence of the low-affinity Na^+ sites seen in the

presence of vanadate (Figure 7A). The lower curve in Figure 8 shows that the effect of equine muscle ATP can be duplicated by 0.5 μM vanadate. As the vanadate concentration increased, the position of the intermediary plateau shifted downward, as when the Mg^{2+} concentration increased (Figures 7A and 7B).

Discussion

Inhibition of NaK–ATPase by Mg^{2+}, K^+, and vanadate appears to involve sites for Mg^{2+} and K^+ which are distinct from their respective sites for activation. These cations have higher affinities for activation sites than for inhibitory sites, so that activation predominates at low concentrations of Mg^{2+} and K^+ and inhibition at higher concentrations. Vanadate probably occupies sites from which inorganic phosphate is normally released (Cantley et al., 1977).

The occupation of inhibitory K^+ sites was a sigmoid function of K^+ concentration and the Hill coefficient was 1.8 at all Mg^{2+} concentrations tested (Figure 3). It appears, therefore, that these sites are actually a complex of at least two interacting sites, and two K^+ ions are required for inhibition. The occupation of inhibitory sites for Mg^{2+} and vanadate obeyed Michaelis–Menten kinetics, suggesting that one ion binds at each site.

It is of interest to note a number of similarities between sites for activation and inhibition by K^+: (1) two K^+ ions are required at the activation site (Sen & Post, 1964); two K^+ ions also appear to be required at the inhibitory site (Figure 3). (2) The activation sites will accept Tl^+, Rb^+, NH_4^+, and Cs^+ as substitutes for K^+ (Skou, 1960; Britten & Blank, 1968); the inhibitory sites will also accept these cations, and with the same relative ranking of affinities (Figure 4). (3) K^+ activates NaK–ATPase at a site accessible on the external surface of the membrane (Whittam, 1962); K^+ also inhibits at an external site (Beaugé & Glynn, 1978). Finally, (4) Na^+ can displace K^+ from its activation site to inhibit NaK–ATPase (Post et al., 1960); Na^+ appears to antagonize inhibition in the presence of Mg^{2+}, K^+, and vanadate by displacing K^+ from an inhibitory site (Figures 7A and 7B).

These similarities strongly suggest a relationship between sites for activation and inhibition by K^+. For example, these sites might reflect alternate states of a single site which is a high-affinity activation site on the phosphoenzyme and becomes a site of lower affinity on the dephosphoenzyme (cf. Glynn & Karlish, 1975). Robinson (1974b) has demonstrated sites with different affinities for K^+ using a method based on the fact that NaK–ATPase can be irreversibly inactivated by either Be^{2+} or F^-, provided both Mg^{2+} and K^+ are present. By studying the rate of inactivation as a function of K^+ concentration, he was able to calculate the dissociation constant for K^+ at the site controlling inactivation. Sites of high- and low-affinity were demonstrated on the phospho and dephospho forms of the enzyme, respectively. The dissociation constant at low-affinity sites varied with conditions but was of the same magnitude as the K^+ concentrations which we found to be required for half-maximal inhibition (Figure 3).

Cantley & Josephson (1976) proposed a model for inhibition of NaK–ATPase by Mg^{2+}, K^+, and vanadate (present as a contaminant in the ATP). They observed that inhibition developed relatively slowly upon mixing enzyme with inhibitory ions and suggested that the binding of Mg^{2+}, K^+, and vanadate per se does not inhibit. Rather, the three ions bind to form a ternary complex, and a slow reversible conformational change converts the complex to an inactive form. The inhibitory ions were assumed to bind independently, in random order. This model predicts that an increased concentration of one ion will lead to an increase in apparent affinities for the others at inhibitory sites, as we observed (Figures 3 and 5B). This mutual increase in apparent affinities occurs because an increased concentration of one ion can complete the ternary complex, and the subsequent conformational change shifts all binding equilibria in favor of the complex. The model also predicts that inhibitory sites for one or two of the ions can be fully saturated with inhibition limited by the extent to which remaining sites are occupied. This result was observed when K^+ (Figures 1 and 2) or Mg^{2+} (Figure 5B) was varied. In each case the extent of inhibition was limited by the concentrations of vanadate and the other cation. When vanadate was varied in the presence of 15 mM K^+, on the other hand, inhibition approached 100% at a Mg^{2+} concentration as low as 0.24 mM, which is well below saturation at the inhibitory site for Mg^{2+} (Figure 5B). These observations suggest an ordered addition of Mg^{2+} and vanadate with vanadate binding after Mg^{2+}. However, more experiments in which vanadate is treated as a variable are needed to resolve this point.

Inhibition of NaK–ATPase by Mg^{2+}, K^+, and vanadate is antagonized by Na^+ through an action at low-affinity sites (Figures 6, 7A, and 7B). Under inhibitory conditions, low-affinity sites coexist with high-affinity sites. Under noninhibitory conditions, only high-affinity sites can be demonstrated (Figure 8), and they must therefore be activation sites for Na^+. It appears that all activation sites approached saturation at the intermediary plateaus of Figures 7A and 7B, but activity was generated only by that fraction of enzyme with inhibitory sites for Mg^{2+} and vanadate unoccupied. All K^+ sites were occupied in these experiments.

The occupation of low-affinity sites for Na^+ made a larger contribution to activity at higher levels of inhibition, supporting the interpretation that the only effect of Na^+ at these sites is to antagonize inhibition. Na^+ appeared to interfere with the occupation of inhibitory K^+ sites (Figure 6), and, conversely, the occupation of low-affinity Na^+ sites was markedly depressed in the presence of 40 mM K^+ (Figure 7B) as compared with 10 mM K^+ (Figure 7A). The two cations may compete for a common site or act at separate, interacting sites. In either case, the occupation of low-affinity Na^+ sites by Na^+ and inhibitory K^+ sites by K^+ is probably mutually exclusive. Note that an increased concentration of either Mg^{2+} (Figure 7A) or vanadate (Figure 8) did not interfere with the occupation of low-affinity Na^+ sites.

Vanadate is present in tissues at concentrations which might inhibit NaK–ATPase in vivo (Cantley et al., 1977), and this inhibition could reflect a significant regulatory mechanism. The answer to this question requires a knowledge of the sidedness of action of Mg^{2+}, K^+, vanadate, and Na^+. It was recently shown that K^+ inhibits at a site accessible only on the outer surface of the red cell (Beaugé & Glynn, 1978) and that vanadate inhibits from inside (Cantley et al., 1978). The location of sites for Mg^{2+} and Na^+ has not been established.

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Acyl-Coenzyme A Dehydrogenase from Pig Kidney. Purification and Properties†

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ABSTRACT: Comparatively large amounts of an acyl-CoA dehydrogenase have been obtained from pig kidney by a procedure which does not involve prior isolation of mitochondria. The pattern of substrate specificity and the extent of substrate-induced bleaching of the flavin chromophore, using butyryl-, octanoyl-, and palmitoyl-CoA, suggest that the enzyme be classified as a general acyl-CoA dehydrogenase. The purified flavoprotein exhibits absorbance ratios at 272, 373, and 446 nm of 5.7:0.65:1.0, respectively, with an extinction coefficient for bound flavin adenine dinucleotide (FAD) of 15.4 mM^{-1} cm^{-1} at 446 nm. Gel filtration, $NaDodSO_4$ gel electrophoresis, and amino acid analysis indicate that the enzyme is a tetramer, comprised of subunits of about 42 000 molecular weight, containing 3–4 molecules of FAD as isolated. The blue, neutral flavosemiquinone is formed during anaerobic titration of the enzyme with dithionite (observed ϵ_{560} = 2.8 mM^{-1} cm^{-1}). A similar level of semiquinone is formed during deazaflavin/light reduction, but no long-wavelength intermediates are observed on reduction with borohydride. Full reduction of the flavin requires 1.2 mol of dithionite. Back titration of the fully reduced enzyme with ferricyanide yields considerable levels of semiquinone (observed ϵ_{560} = 4.1 mM^{-1} cm^{-1}) and suggests that the extinction coefficient of this species is approximately 5.9 mM^{-1} cm^{-1}. The disproportionation equilibrium between semiquinone, oxidized, and fully reduced flavin forms is attained very slowly in the absence of mediators.

Mammalian fatty acyl-CoA dehydrogenases are flavoproteins participating in the first dehydrogenation step of fatty acid β-oxidation which leads to the formation of α,β-unsaturated acyl-CoA derivatives (Beinert, 1963). The reducing equivalents are then passed to a second component, electron transfer flavoprotein (ETF;[1] Crane et al., 1956), which interacts with the respiratory chain, possibly at the level of a recently identified iron–sulfur flavoprotein (Ruzicka & Beinert, 1977). Three categories of acyl-CoA dehydrogenases have been described with overlapping substrate specificity patterns. Short-chain or butyryl-CoA dehydrogenase acts on C_4–C_6 acyl-CoA (Green et al., 1954), general or acyl-CoA dehydrogenase acts on C_4–C_{16} acyl-CoA with peak activity toward C_{10} acyl-CoA (Crane et al., 1956), and long-chain or palmitoyl-CoA dehydrogenase acts on C_6–C_{16} acyl-CoA with maximal activity toward C_{12} acyl-CoA (Hauge et al., 1956). These three dehydrogenases are thought to be closely associated in vivo (Crane et al., 1956; Stanley & Tubbs, 1975). Despite the widespread belief that β-oxidation in mammals occurs exclusively intramitochondrially, recent evidence suggests that long-chain fatty acids are also oxidized by a

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[1] Abbreviations used: ETF, electron transferring flavoprotein; DCI, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; PMSF, phenylmethanesulfonyl fluoride; FAD, flavin adenine dinucleotide; M_r, relative molecular mass (molecular weight).

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