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Na^+/K^+-ATPase: modes of inhibition by Mg^{2+}

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Adding 15 mM free Mg^{2+} decreased V_{max} of the Na^+/K^+-ATPase reaction. Mg^{2+} also decreased the $K_{0.5}$ for K^+ activation, as a mixed inhibitor, but the increased inhibition at higher K^+ concentrations diminished as the Na^+ concentration was raised. Inhibition was greater with Rb^+ but less with Li^+ when these cations substituted for K^+ at pH 7.5, while at pH 8.5 inhibition was generally less and essentially the same with all three cations: implying an association between inhibition and ion occlusion. On the other hand, Mg^{2+} increased the $K_{0.5}$ for Na^+-activation of the Na^+/K^+-ATPase and Na^+-ATPase reactions, as a mixed inhibitor. Changing incubation pH or temperature, or adding dimethylsulfoxide affected inhibition by Mg^{2+} and $K_{0.5}$ for Na^+ diversely. Presteady-state kinetic studies on enzyme phosphorylation, however, showed competition between Mg^{2+} and Na^+. In the K^+-phosphatase reaction catalyzed by this enzyme Mg^{2+} was a (near) competitor toward K^+. Adding Na^+ with K^+ inhibited phosphatase activity, but under these conditions 15 mM Mg^{2+} stimulated rather than inhibited; still higher Mg^{2+} concentrations then inhibited with K^+ plus Na^+. Similar stimulation and inhibition occurred when Mn^{2+} was substituted for Mg^{2+}, although the concentrations required were an order of magnitude less. In all these experiments no ionic substitutions were made to maintain ionic strength, since alternative cations, such as choline, produced various specific effects themselves. Kinetic analyses, in terms of product inhibition by Mg^{2+}, require Mg^{2+} release at multiple steps. The data are accommodated by a scheme for the Na^+/K^+-ATPase with three alternative points for release: before MgATP binding, before K^+ release and before Na^+ binding. The latter alternatives necessitate two Mg^{2+} ions bound simultaneously to the enzyme, presumably to divalent cation-sites associated with the phosphate and the nucleotide domains of the active site.

Introduction

The Na^+/K^+-ATPase reaction involves enzyme phosphorylation and dephosphorylation, transitions between two major conformational families, and interactions between enzyme and ATP, ADP, P_i, Na^+, K^+ and Mg^{2+}. Usual formulations of the standard Albers-Post reaction scheme, however, depict interactions with all those ligands except Mg^{2+} [1–5]. Effects of Mg^{2+} on the enzyme have been examined for several decades, yet even recent studies disagree on such basic issues as the number of sites available to Mg^{2+}, which steps in the reaction sequence are affected by Mg^{2+}, and the sensitivity of these responses to other ligands such as K^+ [6–12]. The experiments reported here extend the steady-state kinetic studies on Na^+/K^+-ATPase, Na^+-ATPase and K^+-nitrophenyl phosphatase reactions catalyzed by this enzyme, including examination of interactions among ligands and factors modifying the response to Mg^{2+}. In addition, presteady-state kinetic studies on the ATPase reaction permit evaluation of Mg^{2+} effects during just the initial steps of the reaction sequence. Although an unresolved experimental problem in assessing ionic strength effects precludes a quantitative analysis of the data, these experiments in conjunction with previous results strongly support a reaction sequence for the Na^+/K^+-ATPase in which MgATP and Mg^{2+} (as a product) interact in different order over three alternative pathways, representing alternative points for Mg^{2+} release. This formulation requires that two Mg^{2+} be bound to the enzyme simultaneously in two of the pathways, in accord with proposals [10,12] depicting nucleotide- and phosphate-binding regions for Mg^{2+}, with migration of Mg^{2+} between these sites during the reaction sequence.

Abbreviations: FITC, fluorescein isothiocyanate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IAF, 5-iodoacetamidofluorescein; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethylsulfoxide; Taps, tris(hydroxymethyl)methylaminopropanesulfonic acid.

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Methods and Materials

The enzyme preparation was obtained from the medullae of frozen canine kidneys [13].

Na^+/K^+-ATPase activity was measured in terms of P_i production [14], routinely at 37°C. The standard medium contained 20 mM Hepes/triethylamine (pH 7.5), 0.1 mM EGTA, 3 mM ATP, 3 mM $MgCl_2$, 90 mM NaCl, and 10 mM KCl. Na^+-ATPase activity was measured similarly, in media without KCl and with 10–100 mM NaCl. K^+-nitrophenylphosphatase activity was measured in terms of nitrophenol production [15], routinely at 37°C. The standard medium was the same as that for the Na^+/K^+-ATPase reaction, except that 3 mM nitrophenyl phosphate was substituted for ATP and NaCl was omitted.

Data for the steady-state experiments presented are means of four or more experiments, each performed in duplicate to quadruplicate, and are presented ± S.E. where appropriate. Lines in the figures were fitted by eye.

Presteady-state kinetic measurements were made using enzyme labeled with IAF [16] and a stopped-flow fluorimeter (Kinetic Instruments) interfaced with a Macintosh IIcx computer (Apple Computer) through a MacADIOS interface board (GW Instruments). Excitation was at 492 nm, and emitted light was recorded after passage through a Corning 69 cut-off filter (528 nm); 6–8 traces were recorded and the rate constant for a single-exponential transition calculated. Reaction temperature was 24°C.

Frozen kidneys were obtained from Pel-Freeze; ATP, nitrophenyl phosphate, Hepes, Mes, Taps, and EGTA from Sigma; IAF from Molecular Probes; triethylamine from Aldrich; and Me_2SO and choline chloride from Fisher. The choline chloride was recrystallized from ethanol/water and stored in a desiccator under vacuum; fresh solutions were made daily.

Results

Interactions of Mg^{2+} and K^+ with Na^+/K^+-ATPase activity

Adding 15 mM $MgCl_2$ to the standard incubation medium reduced Na^+/K^+-ATPase activity by a third. This inhibition, however, was not associated with an increased $K_{0.5}$ for K^+-activation (Fig. 1a). Instead, $MgCl_2$ inhibited more at high KCl concentrations that at low, and Dixon plots of Mg^{2+}-inhibition at various KCl concentrations reveal a family of diverging lines (Fig. 1b). This pattern of mixed inhibition conflicts with earlier reports [7,10] of uncompetitive inhibition (in which Dixon plots are parallel); Sachs [10], however, found for Mg^{2+}-inhibition toward K^+ "nearly parallel" Dixon plots. Furthermore, Mg^{2+} inhibition did not have an absolute requirement for K^+, in contrast to a recent report [11], and the sensitivity of inhibition to

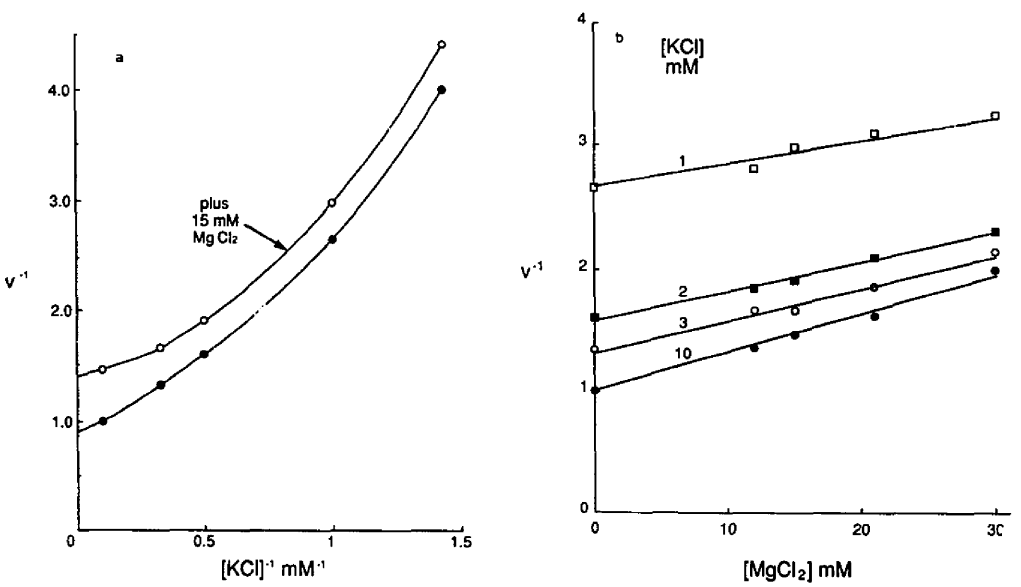

Fig. 1. Effect of $MgCl_2$ on K^+-activation of Na^+/K^+-ATPase. (a) The relative velocities of enzyme incubated at 37°C in the standard medium modified to contain the concentrations of KCl indicated, in the absence (●) or presence (○) of an additional 15 mM $MgCl_2$. Data are presented in double-reciprocal form, with the velocity in the standard medium defined as 1.0. $K_{0.5}$ values are: 1.7 mM (control) and 1.1 mM (15 mM Mg^{2+}). (b) The results of these and comparable experiments, with the concentrations of KCl and added $MgCl_2$ indicated, in the form of a Dixon plot.

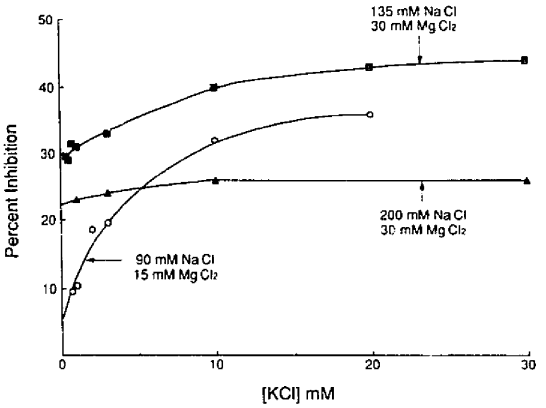

Fig. 2. Effect of K^+ and Na^+ concentrations on Mg^{2+} inhibition. Experiments were performed as in Fig. 1 but with the concentrations of KCl, NaCl and additional $MgCl_2$ indicated. The percent inhibition caused by the added concentration of $MgCl_2$ is plotted against the KCl concentration for three levels of NaCl.

K^+ concentration decreased markedly as the Na^+ concentration was raised (Fig. 2).

Substituting RbCl for KCl reduced ATPase activity but increased inhibition by $MgCl_2$, whereas substituting LiCl reduced both ATPase activity and inhibition (Table I), as previously described [7]. The differential sensitivity that follows substituting Rb^+ or Li^+ for K^+ was

TABLE I

Inhibition by $MgCl_2$ as a function of monovalent cation and pH

Enzyme was incubated at 37°C in the standard medium (10 mM KCl) or media containing in place of KCl 10 mM RbCl or 50 mM LiCl at either pH 7.5 (20 mM Hepes) or pH 8.5 (20 mM Taps), in the absence or presence of an additional 15 mM $MgCl_2$. The relative activity (without additional $MgCl_2$) is expressed relative to incubations with KCl.

Monovalent cation	Incubation pH	Relative activity	Percent inhibition by 15 mM $MgCl_2$
Rb^+	7.5	0.63 ± 0.01	61 ± 3
K^+	7.5	1.00	32 ± 2
Li^+	7.5	0.27 ± 0.01	18 ± 5
Rb^+	8.5	0.93 ± 0.03	32 ± 3
K^+	8.5	1.00	26 ± 2
Li^+	8.5	0.18 ± 0.02	29 ± 5

there attributed to the differential rates of occlusion/deocclusion for those cations in the reaction sequence: the ease of deocclusion follows the sequence $Rb^+ < K^+ \ll Li^+$ [7,9,17]. Raising the incubation pH also speeds deocclusion [9], and at pH 8.5 ATPase activity with RbCl was equivalent to that with KCl, and inhibition by $MgCl_2$ was also nearly the same. Nevertheless, inhibition persisted at pH 8.5, with all three cations, indicating that inhibition involves more than effects on cation occlusion/deocclusion.

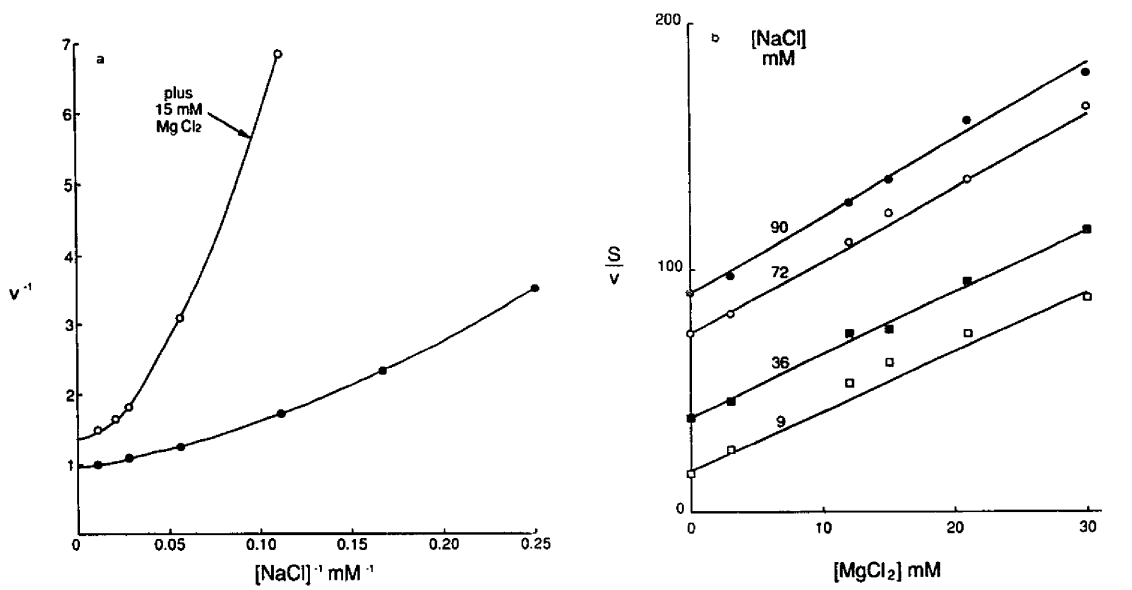

Fig. 3. Effect of $MgCl_2$ on Na^+-activation of Na^+/K^+-ATPase. (a) Corresponding to experiments shown in Fig. 1a, but with 10 mM KCl and the concentrations of NaCl indicated, in the absence (●) or presence (○) of an additional 15 mM $MgCl_2$. $K_{0.5}$ values are: 7.6 mM (control) and 21 mM (15 mM $MgCl_2$). (b) Velocities from similar experiments with the NaCl and additional $MgCl_2$ concentrations indicated, presented as Cornish-Bowden plots of S/v against inhibitor (where S is NaCl concentration); the lines intersect near −90 mM $MgCl_2$.

Interactions of Mg^{2+} and Na^+ with Na^+/K^+-ATPase activity

Adding 15 mM $MgCl_2$ to the incubation media increased the $K_{0.5}$ for Na^+-activation, although Mg^{2+} was not a simple competitor toward Na^+, and inhibition persisted at infinite NaCl concentration (Fig. 3a). Cornish-Bowden plots of inhibition at various NaCl concentrations reveal a family of converging lines (Fig. 3b). This pattern of mixed inhibition contrasts with the pattern of parallel lines that occurs with competitive inhibition. Sachs [10] also found mixed inhibition by Mg^{2+} toward Na^+, in studies on Na^+/K^+-exchange in erythrocytes, when no intracellular K^+ was present and extracellular K^+ was high.

In addition, raising the incubation pH from 6.5 to 8.5 decreased the $K_{0.5}$ for Na^+ (with the standard $MgCl_2$ concentration), but that change in pH increased inhibition by $MgCl_2$ at low NaCl concentrations (Table II). This increased inhibition at pH 8.5 correlates neither with slower K^+ deocclusion nor with a decreased apparent affinity for Na^+. Lowering the incubation temperature to 20°C or adding Me_2SO also markedly reduced inhibition, notably at low NaCl concentrations, although neither of these modifications increased the apparent affinity for Na^+ (Table II).

Solute effects on Na^+/K^+-ATPase activity

In the preceding experiments, the addition of $MgCl_2$ was also accompanied by an increased ionic strength. To control ionic strength, another ion without specific effects on the enzyme should be varied inversely with $MgCl_2$. Unfortunately, searches for such a nonspecific ion have been unsuccessful. Choline has been a frequent choice; triethylamine, which has a pK_a of 10.7 [19], is an obvious alternative. But these ions produce quantitatively and qualitatively different effects that vary with the concentration and ratio of Na^+ and K^+ (Fig. 4). In the standard medium adding 90 mM choline chloride had little effect, whereas adding 90 mM triethylamine chloride inhibited by 30%. But if the NaCl and KCl concentrations were merely reversed, then choline chloride stimulated and triethylamine chloride had little effect. Moreover, stimulation or inhibition by choline chloride was a function of the ratio of Na^+ to K^+ rather than of their absolute amount (i.e., their ionic strength).

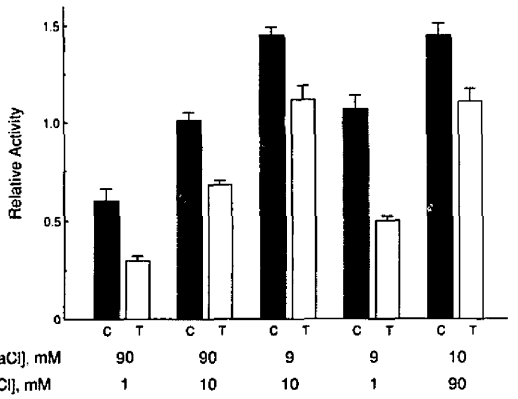

Fig. 4. Effects of choline chloride or triethylamine chloride on Na^+/K^+-ATPase. Activity with the concentrations of NaCl and KCl indicated, and with either 90 mM choline chloride (C) or triethylamine chloride (T), is presented relative to that in the absence of those organic salts.

Consequently, if choline is substituted for Mg^{2+} to maintain a constant ionic strength, then reducing the choline alone would decrease Na^+/K^+-ATPase activity at low Na^+ concentrations more than at high Na^+ concentrations, in addition to any effects of the added Mg^{2+}. Because diverse responses occurred with all the cations tested (including Tris, imidazole, histidine and glucosamine: not shown), no attempt was made in the experiments presented here to maintain a constant ionic strength, as desirable as that goal is in principle.

Mg^{2+} and Na^+-ATPase activity

In the Na^+-ATPase reaction cation occlusion/deocclusion is thought not to be kinetically significant in the E_2 to E_1 transitions [2]. Thus, studies on this reaction should permit examination of inhibition by Mg^{2+} in the absence of its effects on those processes. As in the Na^+/K^+-ATPase reaction, Mg^{2+} inhibited more at low than at high Na^+ concentrations, but significant inhibition occurred with 15 mM $MgCl_2$ even in the presence of 100 mM NaCl (Table III): inhibition by Mg^{2+} appeared to be mixed rather than purely competitive. Sachs [10] reported more inhibition by Mg^{2+} with low than with high Na^+ concentrations, in

TABLE II

Effect of temperature, pH and dimethylsulfoxide on $K_{0.5}$ for Na^+ and inhibition by $MgCl_2$

Enzyme was incubated at 20°C or 37°C in the standard medium (90 mM NaCl) or media containing 9 mM NaCl, in the absence or presence of an additional 15 mM $MgCl_2$, at pH 7.5 (20 mM Hepes), pH 6.5 (20 mM Mes), or pH 8.5 (20 mM Taps), and in the absence or presence of Me_2SO (20%, v/v). The $K_{0.5}$ for Na^+ was measured from incubations in the standard medium containing no additional $MgCl_2$, but with a range of NaCl concentrations, and at the temperatures and pH levels indicated, as well as in the absence and presence of Me_2SO; the $K_{0.5}$ value was calculated using the Kinfit program [18].

Temperature (°C)	pH	Presence of Me_2SO	$K_{0.5}$ for Na^+ (mM)	Percent inhibition by 15 mM $MgCl_2$ with	
				90 mM NaCl	9 mM NaCl
37	6.5	–	13.0±0.8	27±2	36±4
37	7.5	–	7.6±0.1	33±2	81±5
37	7.5	+	9.6±1.8	24±3	43±2
37	8.5	–	6.0±0.1	26±2	98±4
20	7.5	–	9.9±1.8	32±3	47±3

TABLE III

Effect of $MgCl_2$ and choline chloride on Na^+-ATPase activity

Enzyme was incubated at 37°C in the standard medium, without KCl but with 10 or 100 mM NaCl, and with additional $MgCl_2$ or choline chloride as indicated. Dixon plots of Mg^{2+}-inhibition reveal converging lines that intersect near −100 mM $MgCl_2$.

[NaCl] (mM)	Relative activity	Percent change in Na^+-ATPase activity with			
		$MgCl_2$		choline chloride	
		15 mM	30 mM	45 mM	90 mM
10	0.55±0.08	−21±4	−35±5	+2±6	−11±7
100	1.00	−14±4	−24±3	−6±4	−5±3

studies on uncoupled Na^+-efflux in erythrocytes, and concluded that the inhibition was at least mixed.

Presteady-state kinetics of Mg^{2+}/Na^+ interactions

To avoid the multiple sites of Mg^{2+} interactions occurring over the entire reaction sequence, the presteady-state kinetics of the Mg^{2+}/Na^+ interactions with the E_1 enzyme conformation were examined. In these experiments the rates of the transition from the E_1 to E_2 conformation of the IAF-labeled enzyme were followed fluorimetrically [16]. Enzyme in buffer containing 5 to 40 mM NaCl was mixed rapidly with buffer containing ATP, the same concentrations of NaCl, and a range of $MgCl_2$ concentrations. The subsequent fluorescence changes reflect the transition from E_1 to E_2 conformations, require ATP, Mg^{2+} and Na^+, and thus correspond to E_2P formation from E_1 [20]. The rate constant for this transition is decreased by 30 mM $MgCl_2$ in the presence of 5 mM NaCl but not 40 mM NaCl (Table IV), consistent with competitive inhibition.

TABLE IV

Effect of Na^+ and Mg^{2+} on the rate of fluorescence change of IAF-labeled enzyme

IAF-labeled enzyme, in the presence of the NaCl concentrations indicated and 0.1 mM $MgCl_2$, was rapidly mixed with equal concentrations of NaCl, sufficient $MgCl_2$ to give the final concentrations listed, and 0.1 mM ATP; each syringe also contained 20 mM Hepes/triethylamine (pH 7.5). The subsequent fluorescence change was monitored, as described under Methods. The rate constant for that change, attributable to the transition from E_1 to E_2P, is listed as a function of final concentrations of NaCl and $MgCl_2$.

[$MgCl_2$] (mM)	Rate constant for fluorescence changes (s^{-1}) with		
	5 mM NaCl	10 mM NaCl	40 mM NaCl
0.1	7.8±0.8	7.0±1.0	7.6±0.5
1.0	6.3±1.0	6.8±0.5	7.7±1.2
10.0	3.7±0.2	7.7±0.6	8.8±1.0
30.0	1.4±0.2	3.1±0.2	7.8±0.5

Mg^{2+} and K^+-phosphatase activity

Studies on the K^+-nitrophenylphosphatase reaction provide several advantages for studying Mg^{2+} interactions with the enzyme. There is no phosphorylated intermediate [21] and divalent cation occlusion presumably does not occur; in addition, K^+ occlusion/

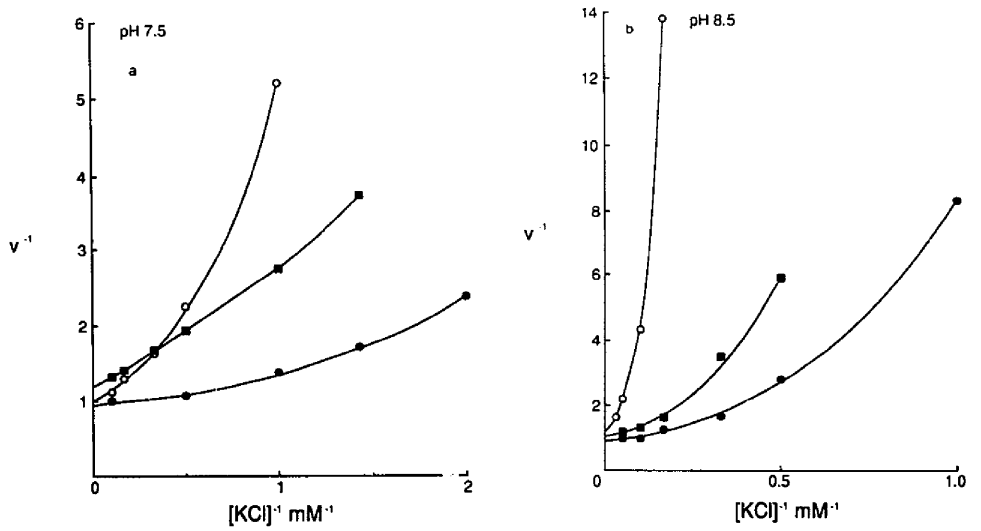

Fig. 5. Effects of $MgCl_2$ and choline chloride on K^+-activation of phosphatase reaction. (a) Relative velocities of enzyme incubated in the standard medium for the phosphatase reaction modified to contain the concentrations of KCl indicated (●), or containing additionally 15 mM $MgCl_2$ (○) or 45 mM choline chloride (■). Data are presented in double-reciprocal form, with the velocity in the standard medium defined as 1.0. $K_{0.5}$ values are: 0.7 mM (control), 2.3 mM (15 mM $MgCl_2$) and 1.3 mM (45 mM choline chloride). (b) Velocities from corresponding experiments at pH 8.5 (Taps buffer in place of Hepes, same symbols as in (a)). $K_{0.5}$ values are: 1.4 mM (control), 9 mM (15 mM $MgCl_2$), and 3.6 mM (45 mM choline chloride).

TABLE V

Effect of $MgCl_2$ and choline chloride on phosphatase activity

Phosphatase activity was measured in the standard medium modified to contain the concentrations of monovalent cations indicated, in the absence and presence of an additional 15 mM $MgCl_2$ or 45 mM choline chloride

Cations	Relative activity	Percent change in activity with	
		15 mM $MgCl_2$	45 mM choline chloride
10 mM KCl	1.00	−8±2	−21±3
10 mM RbCl	0.98±0.01	−7±1	−24±2
10 mM CsCl	0.52±0.02	−83±2	−28±2
100 mM KCl	0.53±0.01	+5±3	−13±3
100 mM RbCl	0.52±0.01	+6±8	−11±2
100 mM CsCl	0.54±0.03	−18±5	−20±3
100 mM LiCl	0.06±0.01	−61±6	−43±5
10 mM KCl plus 90 mM NaCl	0.10±0.03	+62±6	−39±6

deocclusion does not seem to participate in the reaction cycle [22]. Na^+ is not present in the standard medium, but K^+ activates the reaction at cytoplasmically-accessible sites that correspond to the sites at which Na^+ activates the Na^+/K^+-ATPase reaction [23]. Accordingly, 15 mM Mg^{2+} increased the $K_{0.5}$ for K^+-activation (Fig. 5a) and decreased the V_{max} little or not at all. Mg^{2+} thus appears to act as a mixed (or possibly a competitive) inhibitor toward K^+.

The $K_{0.5}$ for K^+-activation of the phosphatase reaction, however, responded to pH oppositely from the $K_{0.5}$ for Na^+-activation of the Na^+/K^+-ATPase reaction (Fig. 5;Table II): raising the pH increased the $K_{0.5}$ for K^+, although it also increased the inhibition by Mg^{2+} as it did toward Na^+.

When Rb^+ was substituted for K^+ at pH 7.5, inhibition by Mg^{2+} remained the same (Table V), in contrast to substituting Rb^+ for K^+ in the Na^+/K^+-ATPase reaction (Table I), and consistent with K^+ or Rb^+ occlusion/deocclusion not participating in the phosphatase reaction. The $K_{0.5}$ for Cs^+ is higher than that for K^+ or Rb^+ [24], and inhibition by Mg^{2+} was greater with 10 mM CsCl. With 100 mM concentrations of these salts, where velocities are equivalent, 15 mM $MgCl_2$ inhibited only with CsCl (Table V).

The $K_{0.5}$ for Li^+ is also higher than that for K^+ [24], but even with 100 mM LiCl the velocity is only a tenth that with the other cations examined, and inhibition by $MgCl_2$ was far greater (Table V). This greater sensitivity to Mg^{2+} with Li^+, compared to the other monovalent cations, also distinguishes between the phosphatase and ATPase reactions.

Adding 90 mM NaCl to 10 mM KCl reduced K^+-phosphatase activity about the same as substituting 100 mM LiCl for 10 mM KCl (Table V). But with K^+ plus Na^+, adding 15 mM $MgCl_2$ markedly stimulated phosphatase activity (Table V). This stimulation decreased with 30 mM $MgCl_2$ (Fig. 6), corresponding qualitatively to the increased inhibition by 30 mM Mg^{2+} occurring with K^+ (Fig. 6) and with Rb^+ and Cs^+ (not shown).

A quantitative interpretation of these data is hampered by uncertainty about effects of ionic strength. Raising the K^+ concentration from 10 to 100 mM halved the phosphatase activity (Table V) and this inhibition may be attributable, in part, to effects of ionic strength. Adding 90 mM choline chloride to 10 mM KCl reduced activity also by half (Fig. 6). Similarly, adding 30 mM $MgCl_2$ (equivalent in ionic strength to 90 mM KCl or choline chloride) to 10 mM KCl reduced activity by nearly half (Fig. 6). But inhibition by $MgCl_2$ is not solely due to effects of ionic strength: at low KCl concentrations 15 mM $MgCl_2$ inhibited far more than 45 mM choline chloride (Figs. 5, 7). Moreover, with 1 mM KCl plus 29 mM NaCl the stimulation by MgCl, was apparent at far lower concentrations (Fig.

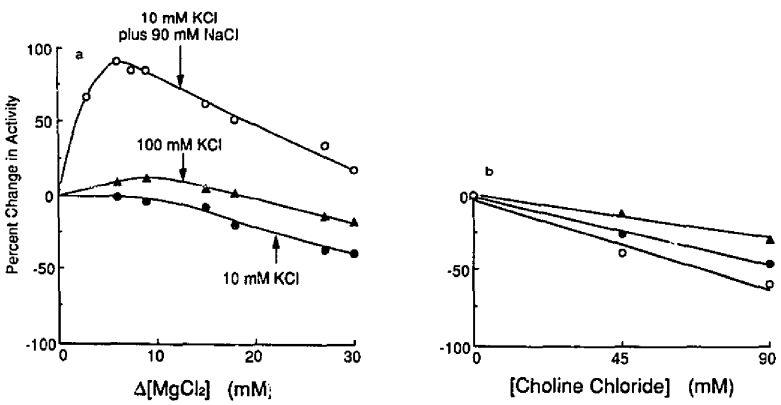

Fig. 6. Effect of $MgCl_2$ and choline chloride on phosphatase reaction with high KCl and NaCl concentrations. The percent change in activity, stimulation or inhibition, is presented as a function of additional $MgCl_2$ ($\Delta MgCl_2$, (a)) or choline chloride (b), in the standard medium containing 10 mM KCl (●) or in media modified to contain 100 mM KCl (▲) or 10 mM KCl plus 90 mM NaCl (○).

TABLE VI

Effect of $MgCl_2$ and choline chloride on phosphatase activity in the presence of dimethylsulfoxide

Phosphatase activity was measured in the standard medium modified to contain the concentrations of monovalent cations indicated, in the absence and presence of an additional 15 mM $MgCl_2$ or 45 mM choline chloride, and in all cases with 20% (v/v) Me_2SO added. The effect of Me_2SO (in the absence of additional $MgCl_2$ and choline chloride) is listed relative to the activity with the particular monovalent cations in the absence of Me_2SO.

Cations	Relative effect of Me_2SO	Percent change in activity with	
		15 mM $MgCl_2$	45 mM choline chloride
10 mM KCl	1.58±0.07	+4±2	−9±3
100 mM KCl	2.82±0.12	−4±2	−1±4
100 mM LiCl	1.73±0.07	−54±5	−58±4
10 mM KCl plus 90 mM NaCl	4.10±0.27	+8±1	−17±3

7): 5 mM $MgCl_2$ then doubled the activity, whereas with 1 mM KCl in the absence of NaCl 5 mM $MgCl_2$ halved the activity. The inhibition by $MgCl_2$ in the presence of 1 mM KCl with and without NaCl was far greater than that with comparable choline chloride (Fig. 7). On the other hand, with 100 mM KCl, $MgCl_2$ either stimulated, or inhibited less, than did comparable amounts of choline chloride (Fig. 6).

Since Mn^{2+} both activates and inhibits at far lower concentrations than Mg^{2+} [25], experiments with Mn^{2+} permit examination of divalent cation effects with little change in ionic strength. Raising the $MnCl_2$ concentration from 0.1 to 3 mM reduced activity with 10 mM KCl by half, but tripled activity with 10 mM KCl plus 90 mM NaCl (data not shown). Nevertheless, inhibition in the presence of K^+ plus Na^+ did occur with $MnCl_2$ also: activity with 5 mM $MnCl_2$ was less than half that with 1 mM $MnCl_2$.

Adding Me_2SO was previously shown to decrease inhibition of the K^+-phosphatase reaction by Mg^{2+} [26], in accord with its ability to reduce inhibition of the Na^+/K^+-ATPase activity at low Na^+ concentrations (Table II). Me_2SO stimulated K^+-phosphatase activity with 100 mM KCl more than that with 10 mM KCl, in the presence of the standard concentration of $MgCl_2$ in the incubation medium, 3 mM (Table VI). In the presence of an additional 15 mM $MgCl_2$, however, Me_2SO did not inhibit with either 10 or 100 mM KCl.

With 10 mM KCl plus 90 mM NaCl adding Me_2SO increased activity markedly, but then increasing the $MgCl_2$ concentration by 15 mM stimulated little further (Table VI), in contrast to adding 15 mM $MgCl_2$ in the absence of Me_2SO (Fig. 6). With 100 mM LiCl adding Me_2SO had relatively little effect on inhibition by $MgCl_2$.

Discussion

These data are here considered in terms of steady-state kinetic analyses that require distinct patterns of product inhibition as a function of the specific reaction step at which that product is released. Two limitations to these analyses are apparent. First, Mg^{2+} could inhibit by binding to the enzyme at other sites, and this

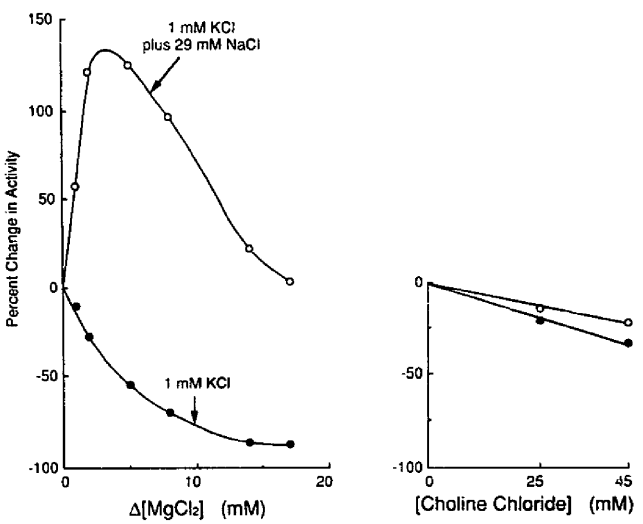

Fig. 7. Effect of $MgCl_2$ and choline chloride on phosphatase reaction with low KCl and NaCl concentrations. Experiments were performed and data are presented as in Fig. 6, except that the media contained either 1 mM KCl (●) or 1 mM KCl plus 29 mM NaCl (○) and the basic $MgCl_2$ concentration, to which the various $\Delta[MgCl_2]$ shown were added, was 1.0 mM.

issue is addressed in the final section of this discussion. Second, in these experiments Mg^{2+}-inhibition is measured without compensation for changes in the ionic strength due to the added $MgCl_2$. It seems likely that at least some steps in the sequence are sensitive to ionic strength. Unfortunately, there is no obvious non-specific cation that can be added and subtracted reciprocally with Mg^{2+} to keep the ionic strength constant. Choline is frequently used, but the experiments here show that choline chloride concentrations comparable in ionic strength to 30 mM $MgCl_2$ stimulate Na^+/K^+-ATPase activity in low Na^+/high K^+ media and inhibit in high Na^+/low K^+ media. And since various cations affect the reaction differently, there is no obvious alternative to choline.

Steps at which Mg^{2+} could interact

Fig. 8 depicts four steps at which Mg^{2+}, as a product of the ATPase reaction, might interact as an inhibitor. This scheme thus provides a framework for considering the data presented here as well as results reported earlier; in the text the symbols (K), (Mg), and (K,Mg) represent those cations 'occluded' by the enzyme, i.e., bound so that exchange with the medium is quite slow.

(i) Mg^{2+} bound with the phosphorylated enzyme [27] could be released immediately after dephosphorylation, as the next step in the reaction sequence. Recent experiments with the Mg^{2+} analogs ^{60}Co and ^{54}Mn showed tight binding that followed enzyme phosphorylation and release that followed dephosphorylation [28,29], but the time course was not examined in sufficient detail to demonstrate at which of the succeeding steps that release occurred.

Mg^{2+} is known to react with the enzyme, in the absence and presence of K^+, to promote phosphorylation by P_i [30,31] and binding of vanadate [32,33]. Mg^{2+} binding directly to $(K)E_2$ corresponds to the proposed release of Mg^{2+} from $(K,Mg)E_2$.

For the reaction sequence:

$$(Mg)E_2P \overset{+K}{\rightleftharpoons} (K,Mg)E_2P \overset{-P_i}{\rightleftharpoons} (K,Mg)E_2 \overset{-Mg}{\rightleftharpoons} (K)E_2 \overset{+MgATP}{\rightleftharpoons} (K)E_2MgATP$$

Mg^{2+} should be an uncompetitive inhibitor toward K^+ (assuming P_i release is irreversible: no P_i is present under initial velocity conditions). Uncompetitive inhibition is characterized by parallel lines in Dixon plots, although it can be experimentally difficult to distinguish between parallel and nearly-parallel but intersecting lines. The diverging lines found here (Fig. 1b) conflict with reports by Pedemonte and Beaugé [7] and Sachs [10], who did find uncompetitive inhibition. On the other hand, Rossi and Garrahan [11] reported instead an absolute dependence on K^+ for inhibition by Mg^{2+}, and argued that inhibition resulted from Mg^{2+} interacting with a branch pathway involving K^+ at sites not associated with dephosphorylation. In that study, Mg^{2+} was added to the medium in exchange for choline; here, choline (Fig. 4) inhibited at low but not high K^+-concentrations, so that removing choline could cause an apparent stimulation at low but not high K^+-concentrations: effects that could appear as an increased inhibition by Mg^{2+} as K^+-concentrations were raised, thereby producing the artefactual appearance of an absolute dependence of inhibition on K^+-concentrations.

In any case, an early kinetic finding is inconsistent with the reaction scheme above. With Mg^{2+} release immediately preceding MgATP binding, Mg^{2+} must be a competitive inhibitor toward MgATP. Instead, it is a mixed inhibitor [34]. Consequently, Mg^{2+} must bind at another step in the sequence: instead or in addition.

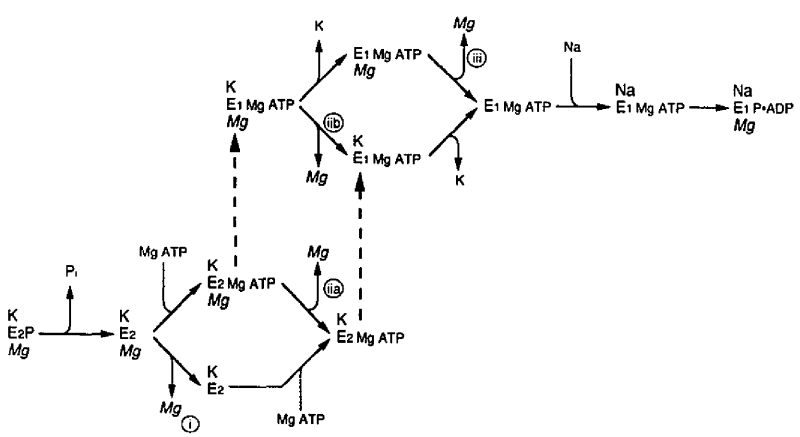

Fig. 8. Pathways for the Na^+/K^+-ATPase reaction, between $(K,Mg)E_2P$ and $(Na,Mg)E_1P \cdot ADP$. Four alternative sites for Mg^{2+} release after enzyme dephosphorylation are indicated.

The latter alternative seems more likely in light of the strong indication from studies on Mg^{2+}-promoted phosphate and vanadate interactions with $(K)E_2$, cited above, that Mg^{2+} binds to $(K)E_2$.

(iia) An alternative, additional step for Mg^{2+} release, and for Mg^{2+} binding as an inhibitor, would be that following MgATP binding to $(K,Mg)E_2$. This possibility was not considered in other recent proposals [6,7,10,11], but was explicitly examined by Forbush [9] in studies on ^{86}Rb deocclusion: there, Mg^{2+} inhibited deocclusion promoted by MgATP. Those data could be accounted for quantitatively by a scheme in which MgATP could bind to either (K,Mg)E or (K)E and Mg^{2+} to either (K)E or (K)EMgATP (presumably the enzyme conformation is E_2, the form usually depicted as bearing occluded K^+: but see below). This formulation obviously requires two Mg^{2+} bound simultaneously, one with ATP and one not, and is in accord with proposals by Sachs [10] but not those of Pedemonte and Beauge [7] and Rossi and Garrahan [11].

Forbush calculated a K_d for Mg^{2+}-binding to (K)EMgATP of 8 mM, using a rapid-equilibrium model that seems appropriate for those experiments. However, concentrations of Mg^{2+} several-fold higher than 8 mM inhibit ATPase activity relatively little in the presence of optimal concentrations of Na^+ and K^+ (Fig. 1–3). Analysis of a steady-state kinetic model, with very fast binding and release of product, reveals that the observed K_i for product inhibition (calculated from standard kinetic plots) will be smaller than the K_i if the slow step in the reaction sequence follows binding of the inhibitor, but will be larger than the K_i if the slow step precedes. Thus, a K_i larger than 8 mM implies that a slow step in the reaction sequence precedes Mg^{2+} binding to that site. If Mg^{2+} binds to $(K)E_2MgATP$ then the slow step in the reaction sequence, by this analysis, must *precede* that step. However, the conventional viewpoint is that the slow step in the reaction sequence *follows* $(K)E_2MgATP$ formation, as shown by fluorescence studies on enzyme conformational changes [35].

(iib) The third possibility indicates that Mg^{2+} dissociates from $(K,Mg)E_1MgATP$, and binds back as a product inhibitor. With the slow step preceding that transition the observed K_i would then be larger than the K_d, as noted above. Data are not available on conformational transitions from $(K)E_2MgATP$ to E_1MgATP as a function of Mg^{2+} concentration, and making such measurements would be technically quite difficult. However, Karlish et al. [36] found no effect of 1 mM Mg^{2+} on the rate of the E_2 to E_1 conversion measured with 4 μM formycin diphosphate, but Hegyvary and Jorgensen [37] reported that 1–4 mM Mg^{2+} slows the transition from $(K)E_2$ to E_1Na of the FITC-labeled enzyme. On the basis of fluorescence criteria, Mg^{2+} was found to stabilize a conformation distinct from E_2 or E_1 [37,38], whereas, using the criteria of tryptic digestion patterns, both E_2 [39,40] and E_1 [41] conformations have been described with Mg^{2+}.

The rate of the E_2 to E_1 conformational change, measured fluorimetrically with IAF-labeled enzyme, corresponds closely to the rate of ^{86}Rb deocclusion [35], consistent with alternative (iib): a slow conversion of $(K,Mg)E_2MgATP$ to $(K,Mg)E_1MgATP$ followed by rapid release of Mg^{2+} and then K^+. (In those experiments, however, no Mg^{2+} was present; still, the rate of fluorescence change was 10% faster than the rate of deocclusion. Adding Mg^{2+} should stabilize $(K,Mg)E_1$ MgATP, according to this formulation, affecting the rate of ^{86}Rb deocclusion more than the rate of fluorescence change.)

With either (iia) or (iib), i.e., with Mg^{2+} release occurring solely between MgATP binding and K^+ release, two kinetic consequences follow (assuming the absence of P_i and saturation with MgATP). First, Mg^{2+} will be an uncompetitive inhibitor toward K^+ when K^+ as a product is absent. However, when K^+ is present, as in the experiments reported here, then Dixon plots of Mg^{2+}-inhibition as a function of K^+ concentration will diverge slightly: as found here (Fig. 1b). Second, Mg^{2+} will be an uncompetitive inhibitor toward Na^+: this was not the case (Fig. 3). Consequently, another site of Mg^{2+} interaction must be involved.

(iii) The fourth possibility depicts Mg^{2+} release following K^+ release from $(K,Mg)E_1MgATP$. If this were the sole site for Mg^{2+} release, then (again assuming the absence of P_i and saturating MgATP) Mg^{2+} once more should be an uncompetitive inhibitor toward K^+ in the absence of K^+ as product. And in the presence of such K^+, Mg^{2+}-inhibition at this point will give rise to Dixon plots that diverge, as found here (Fig. 1b). On the other hand, Mg^{2+} acting at this site should be a competitive inhibitor toward Na^+. That Mg^{2+}, in the presence of saturating MgATP, is not a simple competitor (Fig. 3) makes a strong argument for Mg^{2+}-inhibition of pathway (ii) as well as (iii).

Furthermore, Mg^{2+} acted as a competitor toward Na^+ in presteady-state experiments where the potential sites in pathways (i) and (ii) are not available (Table IV). And Mg^{2+}-inhibition with a decreasing dependence on K^+ as the Na^+-concentration is raised (Fig. 2) is consistent with inhibition in pathway (iii) but not (ii).

Thus, kinetic evidence argues strongly for all three sites for Mg^{2+} release (and Mg^{2+} binding as a product inhibitor). These are shown in Fig. 9 as a Cleland diagram; enzyme isomerizations are not indicated and thus scheme (iia) is not distinguished from (iib).

Also shown is the Na^+-ATPase reaction in which deocclusion of Na^+ from an E_2 conformation and MgATP binding to low-affinity substrate sites on E_2 are not kinetically apparent; the formulation depicts alternative orders for Mg^{2+} release and MgATP bind-

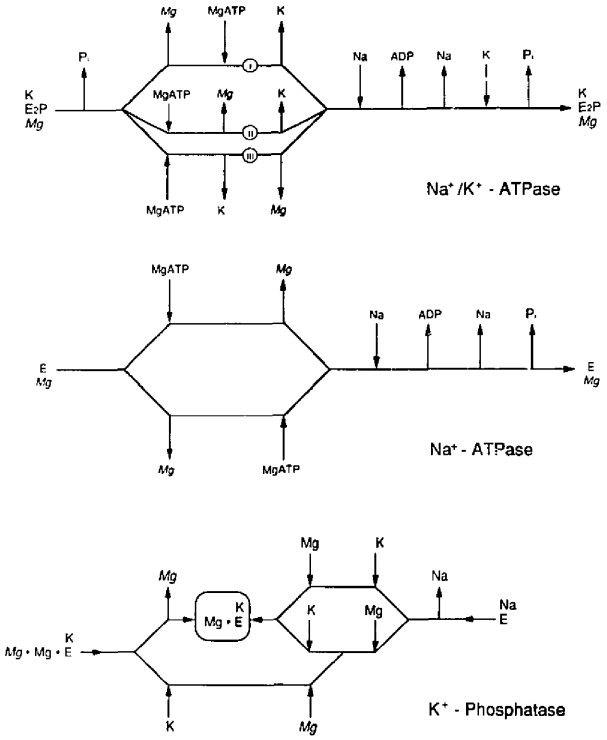

Fig. 9. Reaction schemes for Na^+/K^+-ATPase, Na^+-ATPase, and K^+-phosphatase reactions. For description see text.

ing, in accord with mixed inhibition between Mg^{2+} and Na^+ (Table III), and again implying that Mg^{2+} and MgATP can bind simultaneously. Mixed inhibition by Mg^{2+} toward Na^+ could also occur with a sequence in which Mg^{2+} release precedes a random order binding of Na^+ and MgATP.

In the K^+-nitrophenyl phosphatase reaction enzyme phosphorylation does not occur [21] and thus presumably divalent cation occlusion does not occur either. Mg^{2+} is depicted binding alternatively with K^+ to form the complex that is catalytically active when substrate is bound [42], $E \cdot K \cdot Mg$ (Fig. 9). Mg^{2+} binding to a second site, with lower affinity, will act as a mixed inhibitor toward K^+, as observed. (If K^+ could not bind to $E \cdot Mg \cdot Mg$, or Mg^{2+} to $E \cdot K \cdot Mg$, then Mg^{2+} would be a competitive inhibitor toward K^+, as has been reported [43,44].) Lower concentrations of Mg^{2+} would antagonize inhibition by Na^+ (Figs. 6, 7) through favoring the $E \cdot K \cdot Mg$ complex, whereas higher concentrations would inhibit by forming the catalytically impaired complex $E \cdot K \cdot Mg \cdot Mg$, as also observed (Figs. 6, 7). Moreover, inhibition of vanadate binding by high Mg^{2+}-concentrations [33] is attributable to vanadate reacting with $E \cdot Mg$ but not (or less well) with $E \cdot Mg \cdot Mg$. Mg^{2+} apparently can bind to $(Mg)E_2P$, however, to produce an intermediate insensitive to K^+-activated hydrolysis [30].

Sites at which Mg^{2+} could bind

Mg^{2+} might inhibit by competing directly with Na^+ or K^+ for their specific sites on the cytoplasmic surface of the enzyme (extracellular Mg^{2+} does not inhibit [45]). Thus, Mg^{2+} could act by occupying the Na^+-activating sites of the ATPase reactions, the K^+-activating sites of phosphatase reaction, the K^+ sites that promote vanadate binding, and the monovalent cation sites that accelerate [9] deocclusion.

In the K^+-phosphatase reaction, the inhibition by Na^+ (Table V) is attributable to direct competition between Na^+ and K^+ for the activating sites (Fig. 9), since the K^+ sites are believed to be identical to the cytoplasmically accessible sites at which Na^+ activates the ATPase reactions [23]. Consequently, Mg^{2+} cannot be stimulating the phosphatase reaction in the presence of Na^+ and K^+ (Figs. 6,7) by binding to their monovalent cation sites. However, inhibition by Na^+ might also involve occupancy of the activating sites for Mg^{2+}. Stimulation by Mg^{2+} could then represent displacement of Na^+ from those Mg^{2+}-sites. Me_2SO increases the affinity for Mg^{2+} at those Mg^{2+} sites [26], and in

the presence of Me_2SO both inhibition by Na^+ and stimulation by higher Mg^{2+} concentrations are less (Table VI). Me_2SO also stimulates more with 100 mM KCl than 10 mM KCl (Table VI), as would be expected if K^+ were competing for Mg^{2+} sites. Nevertheless, Me_2SO decreases inhibition by Mg^{2+} with little effect on the $K_{0.5}$ for K^+-activation of the phosphatase reaction [26]. Similarly, in the Na^+/K^+-ATPase reaction Me_2SO has little effect on the $K_{0.5}$ for Na^+ activation, yet it decreases inhibition by Mg^{2+} at low Na^+ concentrations (Table II). Moreover, when the medium pH is lowered the $K_{0.5}$ for K^+ is decreased (Fig. 4), the $K_{0.5}$ for Na^+ is increased (Table II), but in both cases inhibition by Mg^{2+} is decreased (Refs. 26 and Table II).

The strongest argument for Mg^{2+} acting competitively toward Na^+ in pathway (iii), rather than competing directly at the Na^+ sites, comes from the experiments of Fig. 2. There Mg^{2+}-inhibition was increased by higher K^+ concentrations when Na^+ concentrations were low but not when Na^+ concentrations were high: K^+ promotes Mg^{2+} antagonism toward Na^+, which would not be expected if Mg^{2+} inhibited by binding to the Na^+ sites (since the K^+ sites and the Na^+ sites are identical). (This analysis is comparable to the proposal [11] that K^+ acting at sites other than those activating E_2P hydrolysis promotes inhibition by Mg^{2+}, but it requires that the K^+-sites be not in a dead-end branch pathway but in the reaction sequence as the K^+-release sites.)

Although direct competition by Mg^{2+} for Na^+ and K^+-sites may also occur, these arguments support schemes involving separate, distinct sites through which Mg^{2+} acts, as depicted in Figs. 8 and 9. Evidence that two Mg^{2+} can bind simultaneously (here and Refs. 9,10) supports studies on interactions between enzyme, divalent cation, and Cr- or Co-complexes of ATP [8,12], also in accord with this formulation. Thus, the occluded-Mg^{2+} site of $(Mg)E_2P$ may be distorted after dephosphorylation, allowing release of Mg^{2+}, yet coexist with the Mg^{2+}-binding region of the low-affinity nucleotide site on $(K)E_2P$ to which MgATP binds. (It is of interest that Mg^{2+} promotes deocclusion with ATP but not ADP [9], suggesting that the Mg^{2+}-binding region is associated with the gamma phosphate.) These lower-affinity, distorted Mg^{2+}-sites would then continue as sites for Mg^{2+}-inhibition, at least through K^+-release from $(K)E_1MgATP$ (Figs. 8, 9). With the transfer of phosphate from ATP to enzyme, Mg^{2+} from MgATP would follow, to occupy those sites as the occluded Mg^{2+} of $(Mg)E_1P$. On the other hand, divalent cation inhibition of vanadate binding [33] and E_2P hydrolysis [30] would be mediated through the Mg^{2+} binding region of the nucleotide site.

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