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Divalent Cations and the Phosphatase Activity of the (Na + K)-Dependent ATPase

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Abstract

Phosphatase activity of a kidney (Na + K)-ATPase preparation was optimally active with Mg^{2+} plus K^+. Mn^{2+} was less effective and Ca^{2+} could not substitute for Mg^{2+}. However, adding Ca^{2+} with Mg^{2+} or substituting Mn^{2+} for Mg^{2+} activated it appreciably in the absence of added K^+, and all three divalent cations decreased apparent affinity for K^+. Inhibition by Na^+ decreased with higher Mg^{2+} concentrations, when Ca^{2+} was added, and when Mn^{2+} was substituted for Mg^{2+}. Dimethyl sulfoxide, which favors E_2 conformations of the enzyme, increased apparent affinity for K^+, whereas oligomycin, which favors E_1 conformations, decreased it. These observations are interpretable in terms of activation through two classes of cation sites. (i) At divalent cation sites, Mg^{2+} and Mn^{2+}, favoring (under these conditions) E_2 conformations, are effective, whereas Ca^{2+}, favoring E_1, is not, and monovalent cations complete. (ii) At monovalent cation sites divalent cations compete with K^+, and although Ca^{2+} and Mn^{2+} are fairly effective, Mg^{2+} is a poor substitute for K^+, while Na^+ at these sites favors E_1 conformations. K^+ increases the K_m for substrate, but both Ca^{2+} and Mn^{2+} decrease it, perhaps by competing with K^+. On the other hand, phosphatase activity in the presence of Na^+ plus K^+ is stimulated by dimethyl sulfoxide, by higher concentrations of Mg^{2+} and Mn^{2+}, but not by adding Ca^{2+}; this is consistent with stimulation occurring through facilitation of an E_1 to E_2 transition, perhaps an E_1-P to E_2-P step like that in the (Na + K)-ATPase reaction sequence. However, oligomycin stimulates phosphatase activity with Mg^{2+} plus Na^+ alone or Mg^{2+} plus Na^+ plus low K^+: this effect of oligomycin may reflect acceleration, in the absence of adequate K^+, of an alternative E_2-P to E_1 pathway bypassing the monovalent cation-activated steps in the hydrolytic sequence.

Key Words: (Na + K)-ATPase; phosphatase; calcium; magnesium; manganese; oligomycin; dimethyl sulfoxide.

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Introduction

The reaction sequence of the (Na + K)-ATPase involves a Na^+-activated phosphorylation of the enzyme and a K^+-activated dephosphorylation, separated by conformational transitions linked to the active transport of first Na^+ and then K^+ (Robinson and Flashner, 1979; Cantley, 1981; Schuurmans Stekhoven and Bonting, 1981). In the hydrolytic steps K^+ activates by binding to sites accessible *in vivo* from the extracellular medium, and, probably before dissociation of inorganic phosphate (Robinson, 1985), K^+ then becomes occluded (Post *et al.*, 1972; Beaugé and Glynn, 1979), that is, becomes poorly exchangeable with either cytoplasmic or extracellular media. Entry to the occluded sites may, nevertheless, be gained from the cytoplasmic medium, and this is the route available for K^+ activating the phosphatase reaction catalyzed by this enzyme (Drapeau and Blostein, 1980).

The terminal steps of the ATPase reaction sequence may be studied in terms of phosphoenzyme hydrolysis. This reaction proceeds slowly in the absence of extracellularly available monovalent cation (as in the Na-ATPase reaction and the uncoupled Na^+-efflux mode of the sodium pump (Glynn and Karlish, 1976)); however, phosphoenzyme hydrolysis is greatly accelerated by K^+ and rather less so by extracellular Na^+ (Blostein, 1983). Recently, Post (1985) has shown that Ca^{2+} can also accelerate phosphoenzyme hydrolysis. Divalent cations are required for phosphorylation of the enzyme, and Mg^{2+}, which is the optimal cation, remains bound at least through phosphoenzyme hydrolysis (Fukushima and Post, 1978).

The hydrolytic steps may also be studied in terms of the phosphatase reaction, in which an exogenous substrate is cleaved in lieu of the phosphoenzyme. The experiments described here are directed toward examining the effects of various monovalent and divalent cations on this reaction through their occupying the specific sites available to them. In addition, the effects of these cations on the conformational transitions of the enzyme are considered, as well as how reagents that alter these transitions influence the actions of the cations.

Materials and Methods

The enzyme preparation was obtained from medullae of frozen canine kidneys, following the procedure of Jorgensen (1974). Phosphatase activity was measured in terms of nitrophenol production from incubations at 37°C with *p*-nitrophenyl phosphate as substrate (Robinson, 1969). The standard incubation medium contained enzyme, 30 mM histidine HCl/Tris (pH 7.8), 3 mM nitrophenyl phosphate (Tris salt), 3 mM $MgCl_2$, and 10 mM KCl. The

Table I. Effects of Divalent Cations on Phosphatase Activity[a]

Divalent cations added	Relative activity		
	with 0 KCl	with 10 mM KCl	with 10 mM NaCl
0 $MgCl_2$	< 1	< 1	< 1
3 mM $MgCl_2$	1.9 ± 0.3	100	1.1 ± 0.3
1 mM $CaCl_2$	< 1	< 1	< 1
3 mM $MgCl_2$ plus 1 mM $CaCl_2$	13 ± 1	46 ± 2	5 ± 1
1 mM $MnCl_2$	12 ± 1	26 ± 3	9 ± 1

[a]The incubation media contained 30 mM histidine · HCl/Tris (pH 7.8), 3 mM nitrophenyl phosphate (as the Tris salt), and the concentrations of monovalent and divalent cations indicated. Ouabain-inhibitable phosphatase activity is expressed relative to that with 3 mM $MgCl_2$ and 10 mM KCl.

reaction was initiated by adding the substrate to the otherwise complete medium. Activities reported are those inhibitable by 0.3 mM ouabain and are expressed relative to that in the standard medium, defined as 100. All incubations were performed in duplicate or triplicate at 37°C, and the data presented are averages of four or more such experiments.

Frozen canine kidneys were obtained from Pel Freeze, and nitrophenyl phosphate, ouabain, and oligomycin from Sigma.

Results

For the phosphatase activity of the (Na + K)-ATPase, a divalent cation was required (Table I), and with Mg^{2+} as the divalent cation, activity was greatly enhanced by adding K^+. With 10 mM KCl the optimal $MgCl_2$ concentrations were from 3–15 mM, but with 0.3 mM KCl, a concentration below the $K_{0.5}$ in the presence of 3 mM $MgCl_2$, activity decreased with $MgCl_2$ concentrations above 0.6 mM (Fig. 1). This antagonism is consistent with competition by Mg^{2+} for the K^+ sites.

The slight activity in the absence of added K^+ could be due to contaminating monovalent cations or to K^+ bound tightly to enzyme, but against this interpretation is the lesser decline in activity as the $MgCl_2$ concentrations was raised from 0.6 to 9 mM with no added KCl compared to the greater decline with 0.3 mM KCl (Fig. 1): if Mg^{2+} inhibits competitively then it should inhibit more at lower K^+ concentrations. On this basis, the phosphatase activity in the absence of added KCl could be due either to activity in the actual absence of cations occupying the K^+ sites or to Mg^{2+} occupying the K^+ sites and thereby activating the phosphatase reaction, albeit with lower efficacy than when K^+ occupies those sites.

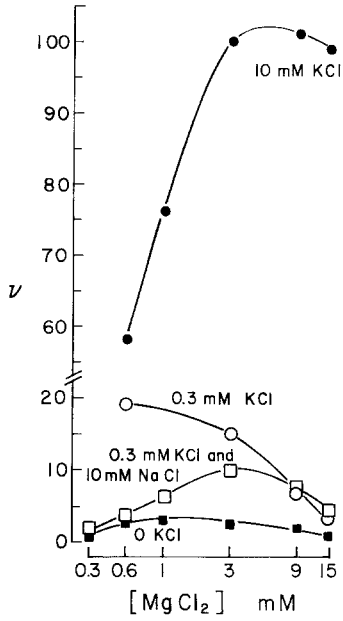

Fig. 1. Effect of $MgCl_2$ concentration on phosphatase activity. Activity was measured in media containing 30 mM histidine HCl/Tris (pH 7.8), 3 mM nitrophenyl phosphate, the concentrations of $MgCl_2$ shown, and either 10 mM KCl (●), 0.3 mM KCl (○), 0.3 mM KCl plus 10 mM NaCl (□), or no added monovalent cations (■); velocities are expressed relative to that with 3 mM $MgCl_2$ and 10 mM KCl, defined as 100.

Analogously, as the KCl concentration was increased at fixed levels of $MgCl_2$, the velocity first increased and then fell (shown in Fig. 2 with 0.6 mM $MgCl_2$, but inhibition also occurred with 3 and 9 mM $MgCl_2$ at KCl concentrations above 50 mM). The $K_{0.5}$ for K^+ also increased with $MgCl_2$ concentration (Fig. 2). These relationships are thus compatible with competition by K^+ for the Mg^{2+} sites, in addition to competition by Mg^{2+} for the K^+ sites (Skou, 1974; Robinson, 1975).

When $CaCl_2$ was substituted for $MgCl_2$, phosphatase activity was not detectable in the absence or presence of KCl (Table I). Adding $CaCl_2$ with 3 mM $MgCl_2$, however, markedly increased activity over that with $MgCl_2$ alone, as previously reported (Tashima *et al.*, 1977; Huang and Askari, 1984). On the other hand, activity with $MgCl_2$ plus KCl was decreased by $CaCl_2$ (Table I). The decline in K^+-stimulated activity with increasing $CaCl_2$ concentration (Fig. 3) was paralleled by an apparent shift in the $K_{0.5}$ for K^+ activation when 1 mM $CaCl_2$ is added to 3 mM $MgCl_2$: this may be seen by first subtracting the K^+-independent activity in the presence of $CaCl_2$ to leave the K^+-activated portion (Fig. 4) and replotting this difference (Fig. 5).

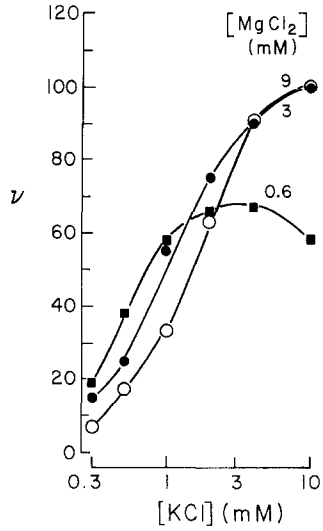

Fig. 2. Effect of $MgCl_2$ concentration on activation by KCl. Experiments were performed and the data are presented as in Fig. 1, except that the KCl concentrations were as shown, with 9 mM (○), 3 mM (●), or 0.6 mM (■) $MgCl_2$.

The activity in the absence of added KCl increased with higher $CaCl_2$ concentrations, even with those that antagonized the K^+-stimulated activity (Fig. 3), but this (Ca + Mg)-phosphatase activity then declined at still higher $CaCl_2$ concentrations. The optimal $CaCl_2$ concentration for phosphatase activity in the absence of added KCl was decreased at a lower $MgCl_2$ concentration (0.6 mM) and increased at a higher $MgCl_2$ concentration (9 mM), consistent with competition by Ca^{2+} for the Mg^{2+} sites as well (data not shown). Such competition by Ca^{2+} toward Mg sites may also occur in the presence of added KCl (Robinson, 1974; Beaugé and Campos, 1983), but here be masked by the more apparent competition between Ca^{2+} and K^+.

When $MnCl_2$ was substituted for $MgCl_2$, greater phosphatase activity was detectable in the absence of KCl, but less with near-optimal (10 mM) KCl (Table I), as previously reported (Robinson, 1981). With $MnCl_2$ as the sole divalent cation, activity with 10 mM KCl increased with $MnCl_2$ concentrations to 3 mM (Fig. 6), but declined by one-fourth with 9 mM $MnCl_2$ (data not shown). The decline in K^+-stimulated activity with higher $MnCl_2$ concentrations was more pronounced at the lower KCl concentration, 0.3 mM (Fig. 6), again consistent with the divalent cation competing for the K^+ sites. As with $CaCl_2$, calculating the increment in activity due to K^+ (Fig. 4) revealed that substituting Mn^{2+} for Mg^{2+} increased the $K_{0.5}$ for K^+ (Fig. 5). By contrast, in the absence of added KCl the decline in phosphatase

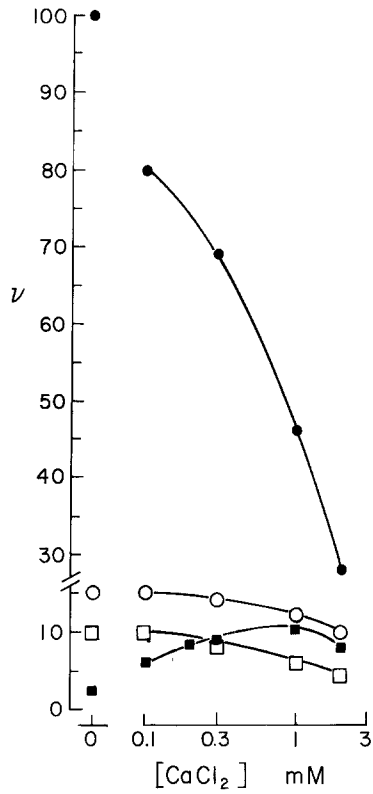

Fig. 3. Effect of $CaCl_2$ concentration on phosphatase activity. Experiments were performed and data are presented as in Fig. 1, except that 3 mM $MgCl_2$ was present throughout, together with the $CaCl_2$ concentrations shown, in the presence of either 10 mM KCl (●), 0.3 mM KCl (○), 0.3 mM KCl plus 10 mM NaCl (□), or no added monovalent cations (■).

activity at high $MnCl_2$ concentrations (Fig. 6) cannot be explained in terms of competition between cations since Mn^{2+} was the only inorganic cation added.

Adding NaCl inhibited phosphatase activity in the presence of 10 mM KCl (Fig. 7); as the concentration of $MgCl_2$ was increased, however, inhibition by NaCl diminished. Analogously, adding 0.2–2 mM $CaCl_2$ to 3 mM $MgCl_2$ decreased inhibition due to NaCl (Fig. 8), and substituting 1 mM $MnCl_2$ for 3 mM $MgCl_2$ decreased inhibition even more (Fig. 8).

Adding NaCl in the absence of KCl, either with $MgCl_2$, $MgCl_2$ plus $CaCl_2$, or $MnCl_2$, decreased phosphatase activity (Table I). On the other hand, adding NaCl with 0.3 mM KCl, a concentration well below the $K_{0.5}$ for K^+, did not inhibit as much as expected from inhibition by NaCl with 10 mM

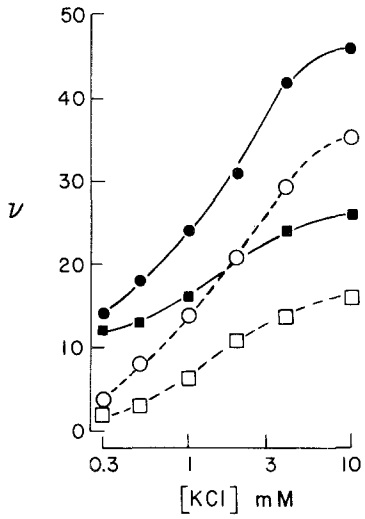

Fig. 4. Effect of divalent cations on activation by KCl. Experiments were performed as in Fig. 2 with the concentrations shown of KCl in the presence of either 3 mM $MgCl_2$ plus 1 mM $CaCl_2$ (●) or 1 mM $MnCl_2$ (■). In addition, the increment in velocity due to added KCl (subtracting for each KCl concentration the activity without KCl) is also plotted for the experiments with $MgCl_2$ plus $CaCl_2$ (○) and $MnCl_2$ (□).

KCl (Robinson *et al.*, 1983). Moreover, this (Na + K)-phosphatase activity rose relative to that with K^+ alone as the $MgCl_2$ concentration was increased, and surpassed it at 9 mM $MgCl_2$ (Fig. 1). The (Na + K)-phosphatase activity also rose as the $MnCl_2$ concentration was increased (Fig. 6). By contrast, the (Na + K)-phosphate activity fell as the $CaCl_2$ concentration was increased in the presence of 3 mM $MgCl_2$ (Fig. 3).

With 10 mM KCl and Mg^{2+} as the sole divalent cation, the K_m for the substrate, nitrophenyl phosphate, changed little from 0.6 to 15 mM $MgCl_2$ (Table II). Adding 1 mM $CaCl_2$ with 3 mM $MgCl_2$, however, halved the K_m, and substituting $MnCl_2$ for $MgCl_2$ reduced the K_m even further (Table II).

As previously shown with $MgCl_2$ (Robinson, 1972; Albers and Koval, 1972), adding dimethyl sulfoxide increased phosphatase activity with 10 mM KCl and increased it even more with 0.3 mM KCl (Table III), decreasing the $K_{0.5}$ for K^+. On the other hand, dimethyl sulfoxide increased activity less with no added KCl than with 0.3 mM KCl (Table III), again arguing against the activity in the absence of added KCl being due solely to low levels of contaminating or bound K^+. Dimethyl sulfoxide, however, stimulated activity with 10 mM NaCl but no added K^+ (Table III).

With 1 mM $CaCl_2$ plus 3 mM $MgCl_2$ or with 1 mM $MnCl_2$ substituted for $MgCl_2$, dimethyl sulfoxide also stimulated, and more so with 0.3 mM KCl

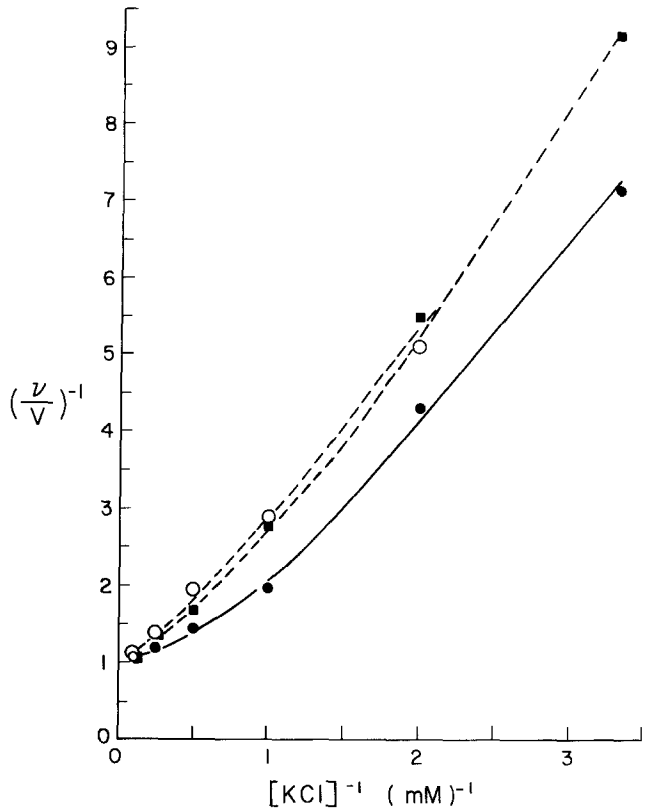

Fig. 5. Activation by KCl. The increments in velocity due to added KCl in Fig. 4 are replotted in double-reciprocal form from the experiments with $MgCl_2$ plus $CaCl_2$ (○) and $MnCl_2$ (■), for comparison with comparable experiments in which the sole divalent cation added was 3 mM $MgCl_2$ (●).

Table II. Effects of Divalent Cations on K_m for Substrate[a]

Divalent cations added	K_m for nitrophenyl phosphate (mM)
0.6 mM $MgCl_2$	2.6
3 mM $MgCl_2$	2.9
15 mM $MgCl_2$	2.7
3 mM $MgCl_2$ plus 1 mM $CaCl_2$	1.3
1 mM $MnCl_2$	0.7

[a]The K_m values were estimated from double-reciprocal plots of enzyme velocities measured in incubation media containing 30 mM histidine · HCl/Tris (pH 7.8), 10 mM KCl, the concentrations of divalent cations indicated, and nitrophenyl phosphate concentrations from 0.5 to 6 mM.

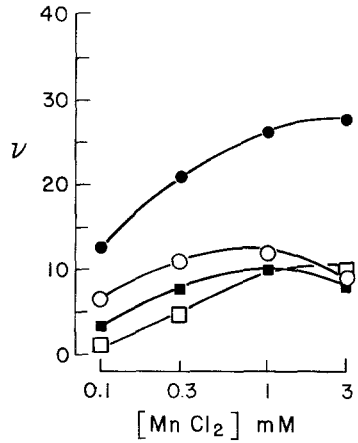

Fig. 6. Effect of $MnCl_2$ concentration on phosphatase activity. Experiments were performed and data are presented as in Fig. 1, except that the sole divalent cation was $MnCl_2$, at the concentrations shown, in the presence of either 10 mM KCl (●), 0.3 mM KCl (○), 0.3 mM KCl plus 10 mM NaCl (□), or no added monovalent cations (■).

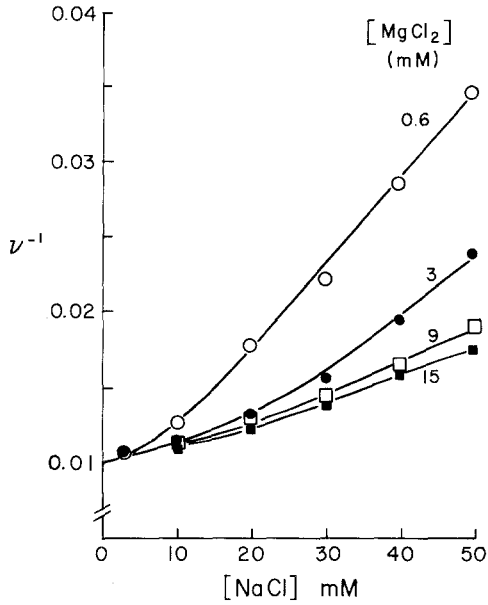

Fig. 7. Effect of $MgCl_2$ concentration on inhibition by NaCl. The incubation media contained 30 mM histidine HCl/Tris, 3 mM nitrophenyl phosphate, 10 mM KCl, the concentrations of NaCl shown, and either 0.6 mM (○), 3 mM (●), 9 mM (□), or 15 mM (■) $MgCl_2$. Data are presented in the form of a Dixon plot, with activity in the absence of added NaCl normalized to 100.

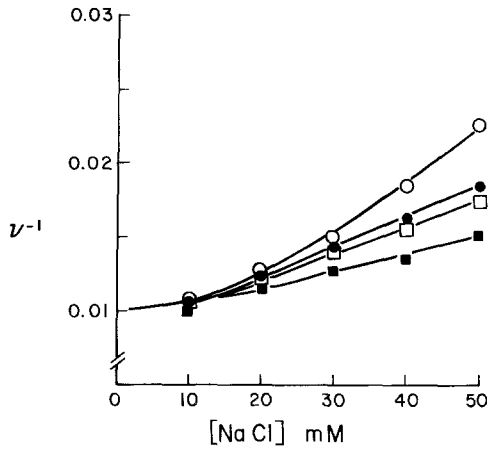

Fig. 8. Effect of divalent cations on inhibition by NaCl. Experiments were performed and the data are presented as in Fig. 7, except that the divalent cations added were either 3 mM $MgCl_2$ plus 0.2 mM $CaCl_2$ (○), 3 mM $MgCl_2$ plus 1 mM $CaCl_2$ (●), 3 mM $MgCl_2$ plus 2 mM $CaCl_2$ (□), or 1 mM $MnCl_2$ (■). Data are presented in the form of a Dixon plot, with activity in the absence of added NaCl normalized to 100.

than with either 10 mM KCl or no added KCl (Table III). On the other hand, the stimulation throughout was less than with 3 mM $MgCl_2$ alone, and with these divalent cations no stimulation of activity occurred with NaCl in the absence of KCl (Table III, columns 2 and 3).

The (Na + K)-phosphatase activity was stimulated by dimethyl sulfoxide with $MgCl_2$ (Robinson *et al.*, 1984), $MgCl_2$ plus $CaCl_2$, and $MnCl_2$ (Table III).

To determine the effect of dimethyl sulfoxide on the apparent affinity for Mg^{2+}, incubations were performed with 10 mM nitrophenyl phosphate and

Table III. Effect of Dimethyl Sulfoxide on Phosphatase Activity[a]

Monovalent cations added	Percent change due to dimethyl sulfoxide		
	with 3 mM $MgCl_2$	with 3 mM $MgCl_2$ + 1 mM $CaCl_2$	with 1 mM $MnCl_2$
10 mM KCl	+ 45	+ 13	+ 18
0.3 mM KCl	+126	+ 43	+ 47
0.3 mM KCl plus 10 mM NaCl	+161	+145	+133
10 mM NaCl	+135	− 2	− 3
None	+ 66	+ 18	+ 13

[a]The percent increases or decreases in phosphate activity due to adding dimethyl sulfoxide (10% v/v) to incubation media containing the indicated monovalent and divalent cations are presented.

Table IV. Effect of Oligomycin on Phosphatase Activity[a]

Monovalent cations added	Percent change due to oligomycin		
	with 3 mM $MgCl_2$	with 3 mM $MgCl_2$ plus 1 mM $CaCl_2$	with 1 mM $MnCl_2$
10 mM KCl	+ 2	− 3	+ 1
0.3 mM KCl	− 34	− 28	− 31
0.3 mM KCl plus 10 mM NaCl	+123	+109	+ 89
10 mM NaCl	+176	− 12	+ 91
None	− 78	− 47	− 39

[a]The percent increases or decreases in phosphatase activity due to adding oligomycin (10 μg/ml) to incubation media containing the indicated monovalent and divalent cations are presented.

30 mM KCl to minimze effects of the reagent through increased affinity for these ligands. With these near-saturating concentrations of nitrophenyl phosphate and KCl, adding dimethyl sulfoxide reduced the K_m for $MgCl_2$ from 1.7 to 0.6 mM.

Oligomycin inhibits the (Na + K)-ATPase reaction, and this inhibition decreases with increasing concentrations of $MgCl_2$ (Robinson, 1971). By contrast, inhibition of the phosphatase activity of this enzyme by oligomycin was detectable only at low KCl concentrations and in the absence of added KCl (Table IV). Moreover, with 0.3 mM KCl, raising the $MgCl_2$ concentration from 0.6 to 15 mM increased inhibition from 33 to 67% (data not shown), opposite to the effect of Mg^{2+} on inhibition of the (Na + K)-ATPase activity.

On the other hand, oligomycin, like dimethyl sulfoxide, stimulated the (Na + K)-phosphatase activity (Table IV), as previously reported (Robinson *et al.*, 1984). Although this activity declined at high $MgCl_2$ concentrations in the presence and absence of oligomycin, the stimulation produced by oligomycin, expressed as that ratio, increased with $MgCl_2$ concentration (Fig. 9). Oligomycin also stimulated (Na + K)-phosphatase activity in the presence of $MgCl_2$ plus $CaCl_2$ and of $MnCl_2$ (Table IV), although less than with 3 mM $MgCl_2$ alone. Finally, oligomycin stimulated phosphatase activity in the presence of Na^+ but absence of K^+ when Mg^{2+} or Mn^{2+} was the sole divalent cation (Table IV).

Discussion

The simplest model for the phosphatase activity of the (Na + K)-ATPase requires two classes of cation sites, both accessible *in vivo* from the cytoplasm (Drapeau and Blostein, 1980), one for divalent cations and one for monovalent cations. Greatest activity is achieved when these two classes of

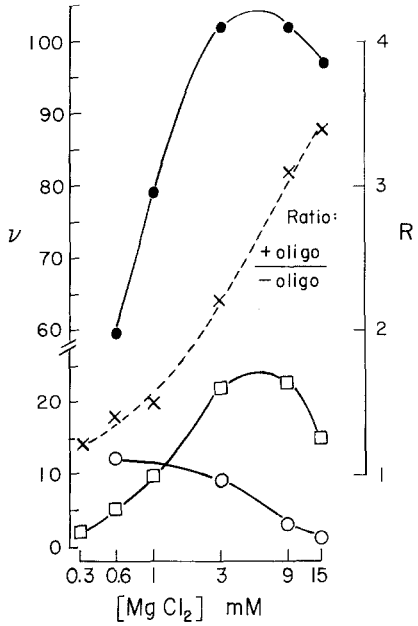

Fig. 9. Effect of oligomycin on phosphatase activity. Experiments were performed and are presented against the left-hand ordinate as in Fig. 1, except that oligomycin (10 μg/ml) was present throughout, in the presence of either 10 mM KCl (●), 0.3 mM KCl (○), or 0.3 mM KCl plus 10 mM NaCl (□). In addition, against the right-hand ordinate is plotted the ratio of the activity with 0.3 mM KCl plus 10 mM NaCl in the presence of oligomycin to that in the absence of oligomycin (x).

sites are occupied by, respectively, Mg^{2+} and K^+. At the divalent cation sites Mn^{2+} may substitute for Mg^{2+}, but Ca^{2+} is ineffective as an activator of catalysis and acts as a competitor toward Mg^{2+}; moreover, monovalent cations also can compete. At the monovalent cation sites K^+ and its usual congeners are effective. Although Na^+ stimulates phosphatase activity of a rat brain enzyme (Robinson, 1969), with this kidney enzyme it was less effective than no added monovalent cation (Table I). Activity without added monovalent cations strongly suggests that Ca^{2+}, Mn^{2+}, and (weakly) Mg^{2+} can activate through occupying the monovalent cation sites (when appropriate divalent cations occupy the divalent cation sites); however, as less effective activators than K^+, these divalent cations can also inhibit through competition with K^+ for the monovalent cation sites. From these experiments it is not necessary to postulate additional cation sites, but further classes have been proposed (Albers and Koval, 1973: Skou, 1974). Although the monovalent cation sites of the phosphatase reaction do differ in some significant properties from those activating phosphoenzyme hydrolysis,

including sidedness of accessibility *in vivo* (Drapeau and Blostein, 1980), apparent affinity for K^+ (Robinson, 1975), and relative efficacy of Na^+ (Blostein, 1983 vs. Table I here), such differences may reflect the divergent properties of the opposite faces of the same cation-translocating channel.

Another aspect of the reaction mechanism must also be included: the transitions of the enzyme between two major families of conformational states, E_1 and E_2, (Jorgensen, 1975; Karlish *et al.*, 1978; Robinson, 1981; Robinson *et al.*, 1983). That Na^+ favors E_1 and K^+ favors E_2 conformations seems unequivocal, but the effects of divalent cations on the equilibria between these conformations is less clear-cut. Adding a ligand that binds to both conformations will favor a distribution between E_1 and E_2 reflecting their differential affinity for the ligand; furthermore, the *change* in distribution between E_1 and E_2 induced by that ligand will depend on the prior conformation (i.e., if the enzyme population is entirely in either E_1 or E_2 conformations, then adding such a ligand will inevitably shift the distribution toward the alternative conformational family). Moreover, the initial distribution depends not only on Na^+ and K^+, but also on pH, buffer composition, ionic strength, and temperature (Skou and Esmann, 1980; Swann, 1983). These considerations may explain some apparent discrepancies about reported effects of divalent cations on enzyme conformations. Thus, at lower temperatures (16–25°C) and in the presence of K^+, both of which favor E_2 conformations, adding $MgCl_2$ shifted the fluorescence of fluorescein isothiocyanate-labeled enzyme toward that identified with E_1 conformations (Karlish, 1980); similarly at lower pH, in the absence of Tris buffer, and at 22°C, all of which favor E_2 conformations, adding $MgCl_2$ shifted the fluoresence of eosin-labeled enzyme toward that identified with E_1 conformations (Skou and Esmann, 1980). On the other hand, at 37°C, adding $MgCl_2$ changed the tryptic digestion pattern from that identified with the E_1 conformation to the E_2 (Castro and Farley, 1979), and at 37°C in the buffer system used here $MnCl_2$ was more effective than $MgCl_2$ in changing the tryptic digestion pattern from E_1 to E_2 (Robinson, 1982). Adding $CaCl_2$ stimulated the ADP/ATP exchange reaction of this enzyme, dependent on E_1-P, whereas adding $MgCl_2$ and $MnCl_2$ inhibited the exchange reaction (Tobin *et al.*, 1973; Robinson, 1983); correspondingly, the preferential sequence for promoting binding of cardiac glycosides and vanadate, which is predominantly to E_2 conformations, was $Mn^{2+} > Mg^{2+} \gg Ca^{2+}$ (Schwartz *et al.*, 1968; Robinson and Mercer, 1981): such effects of cations would seem particularly relevant. Finally, dimethyl sulfoxide here increased the apparent affinity for both Mg^{2+} and K^+. From such considerations it is proposed that under the experimental conditions here, and in the absence of Na^+, K^+, oligomycin, and dimethyl sulfoxide, the enzyme will be predominantly in the E_1 conformations. Adding Mg^{2+} would then shift the distribution toward E_2

conformations; Mn^{2+}, by virtue of greater differential affinity, will shift the distribution further toward E_2 [although neither as far as K^+ (Robinson, 1982)], whereas Ca^{2+} will maintain the enzyme predominantly in the E_1 conformations.

In the scheme proposed here

$$\begin{array}{ccccc} & & E_1(Mg) & \overset{K^+}{\rightleftharpoons} & E_1(Mg)K & \rightleftharpoons & E_2(Mg)K \\ & & \downarrow\uparrow Mg^{2+} & & \downarrow\uparrow Mg^{2+} & & \downarrow\uparrow Mg^{2+} \\ E_1Na & \underset{Na^+}{\rightleftharpoons} & E_1 & \overset{K^+}{\rightleftharpoons} & E_1K & \rightleftharpoons & E_2K \end{array}$$

the catalytically active form is $E_2(Mg)K$ (the binding of substrate is not shown). Na^+ acts as a competitor toward K^+. On the other hand, both Mg^{2+} and Mn^{2+}, which favor E_2 conformations under these conditions, activate when occupying the divalent cation sites, whereas Ca^{2+}, which favors E_1 conformations, is thus ineffective as the sole divalent cation.

This scheme also readily accounts for the antagonism to inhibition by Na^+ that occurs when the Mg^{2+} concentration is raised or when Mn^{2+} is substituted for Mg^{2+}: according to the proposal developed above, these alterations favor E_2 conformations while Na^+ bind to E_1 conformations. Conversely, adding Ca^{2+} with Mg^{2+} might then be expected to increase inhibition by Na^+, since, according to the proposal above, Ca^{2+} favors E_1 conformations; instead, the opposite occurs. A quantitative examination of the model, however, shows that this effect on inhibition by Na^+ can result from the following changes: if Ca^{2+} increases the apparent K_d for both Na^+ and K^+ (by competing at the monovalent cation sites) and if Ca^{2+} also binds to the divalent cation site on E_1 to form an unreactive complex (competing with Mg^{2+}) but has negligible effect on the transition from $E_1(Mg)K$ to $E_2(Mg)K$, then inhibition by Na^+ will be less when Ca^{2+} is added.

Inhibition by high concentrations of Mg^{2+} or Mn^{2+} could occur not only through competition with K^+ for the monovalent cation sites but also through product inhibition if obligatory cation release is required in the reaction sequence (Robinson, 1981); however, there is no independent evidence that this is the case. A further mode of inhibition could occur through Mg^{2+} and Mn^{2+} preferentially stabilizing an enzyme conformation other than the catalytic one, either through the cation sites discussed here or some additional sites (Robinson, 1983; Pedemonte and Beaugé, 1983). Apparent competition between divalent cations and K^+ could also result from these cations favoring conformations different from those binding K^+ optimally.

The effects of dimethyl sulfoxide and oligomycin may also be accounted for by these reagents favoring, respectively, E_2 and E_1 conformations

(Robinson *et al.*, 1984). Thus, dimethyl sulfoxide decreases the $K_{0.5}$ for K^+ whereas oligomycin increases it. The greater inhibition by oligomycin as the Mg^{2+} concentration is raised, however, might seem paradoxical if Mg^{2+} favors E_2 conformations, but this also may be accommodated by the scheme if the effect of Mg^{2+} as a competitor toward K^+ for the monovalent cation sites is greater than the effect of Mg^{2+} on shifting the enzyme toward E_2 conformations.

Stimulation by dimethyl sulfoxide of phosphatase activity with Mg^{2+} plus Na^+ (Table III) is, in this scheme, explainable as Na^+ at the monovalent cation site promoting catalysis, assisted by dimethyl sulfoxide holding the enzyme in the E_2 conformation (against the tendency for Na^+ to favor the E_1 conformation). In this explanation E_2(Mg)Na would be catalytically effective, as is E_2(Mg)K.

Although the K_m for nitrophenyl phosphate is decreased by dimethyl sulfoxide (Robinson *et al.*, 1984), implying that the substrate binds to the E_2 conformation, the K_m is increased both by K^+, which also favors E_2 conformations, and by Na^+, which favors E_1 conformations. The substrate-binding conformation is thus not easily categorized by such global terminology. Nevertheless, the decrease in K_m when Ca^{2+} is added to Mg^{2+} or when Mn^{2+} is substituted for Mg^{2+} can be attributed to Ca^{2+} and Mn^{2+} competing with K^+ and thereby diminishing the antagonism between K^+ and substrate. Indeed, the decline in velocity on adding Ca^{2+} or substituting Mn^{2+} (Table I) parallels both the decline in K_m due to these divalent cations (Table II) and the declines in both velocity and K_m when K^+, in the presence of 3 mM $MgCl_2$, is decreased (Robinson *et al.*, 1984).

The proposed scheme can thus account for a variety of interactions between monovalent and divalent cations, substrate, and modifiers. But it clearly cannot account for the stimulation by Na^+ when added in the presence of low concentrations of K^+ (Figs. 1, 6, 9; Table IV), termed (Na + K)-phosphatase activity (Robinson, 1970; Robinson *et al.*, 1983). For this activity additional factors must be invoked, including stimulation through K^+ sites accessible *in vivo* from the extracellular medium (Robinson *et al.*, 1983). The most obvious explanation would thus be a reaction pathway like that for the (Na + K)-ATPase, with a Na^+-stimulated phosphorylation of the enzyme to form E_1-P, a conformational transition to E_2-P with its extracellularly accessible sites, a K^+-stimulated dephosphorylation to E_2, and a conformational transition back to E_1. With this pathway, in the presence of a relatively high Na^+ concentration and a low K^+ concentration, the enzyme will be poised far toward the E_1 conformations, and a role for Mg^{2+}, Mn^{2+}, and dimethyl sulfoxide in promoting (Na + K)-phosphatase activity can be understood. It is notable that increasing the Ca^{2+} concentration (favoring E_1 conformations) does not stimulate (Na + K)-phosphatase

activity (Fig. 3) whereas increasing Mg^{2+} or Mn^{2+} (favoring E_2 conformations) does (Figs. 1 and 6). The likely site of these actions in the pathway would thus be on the transition from E_1-P to E_2-P. This assignment is, therefore, in accord with observations, cited above, that the ADP/ATP exchange reaction, dependent on the E_1-P form, is favored by Ca^{2+} but inhibited by Mg^{2+} and Mn^{2+}. Nevertheless, a possible alternative explanation would be that if Ca^{2+} inhibits Na^+ and K^+ binding more effectively than do Mg^{2+} and Mn^{2+}, then higher Ca^{2+} concentrations would inhibit the (Na + K)-phosphatase activity more than Mg^{2+} and Mn^{2+} inhibit.

Stimulation of the (Na + K)-phosphatase activity by oligomycin, on the other hand, is inexplicable by these formulations, particularly since this stimulation is promoted by increasing concentrations of Mg^{2+} (Fig. 9). If dimethyl sulfoxide and higher concentrations of Mg^{2+} and Mn^{2+} stimulate through accelerating a slow step, as argued above, oligomycin might act by stimulating another step which had become rate limiting after divalent cation stimulation of the previously slow step. Possibilities for such a site of action include substrate binding [although no major effect of oligomycin in reducing the K_m for nitrophenyl phosphate could be determined (J. D. Robinson, unpublished observations)] and K^+ binding to the extracellularly accessible K^+ sites of E_2-P [akin to the oligomycin-induced decrease in $K_{0.5}$ for K^+ in the (Na + K)-ATPase reaction (Robinson, 1971)].

The ability of oligomycin to stimulate phosphatase activity in the presence of Na^+ but absence of K^+ (Table IV) suggests a further mode by which oligomycin can stimulate and that may account for these phenomena. An alternative route for the decomposition of E_2-P is a K^+-independent dephosphorylation to E_2, followed by the transition to E_1, steps involved in Na-ATPase activity (Glynn and Karlish, 1976) and uncoupled K^+ efflux (Sachs, 1985). Consequently, oligomycin, by favoring in the absence of bound monovalent cation the conversion of E_2-P to E_1, could open a new pathway for the phosphatase reaction, a pathway effective with Na^+ and either no K^+ or only very low concentrations of K^+.

Finally, these studies may also bear on another aspect of regulating the (Na + K)-ATPase and sodium pump *in vivo*, that is, through competition between Mg^{2+} and Mn^{2+}. Although millimolar $MnCl_2$ was added in these experiments, the concentration of free Mn^{2+} was, due largely to complexing by the buffer, on the order of 3 μM (Robinson, 1981), in accord with studies showing that micromolar Mn^{2+} competes with millimolar Mg^{2+} for binding to the enzyme (Grisham and Mildvan, 1974). In light of the recent demonstration that the cytoplasmic concentration of free Mn^{2+} is roughly 0.8 μM (Ash and Schramm, 1982), then Mn^{2+} would be an effective competitor *in vivo*, with a significant fraction of the enzyme binding Mn^{2+} instead of Mg^{2+}. Thus, as recently suggested by Hansen (1985), the altered enzymatic

properties seen when Mn^{2+} is substituted for Mg^{2+} *in vitro* (Robinson, 1981) may represent alternative modes of enzymatic function *in vivo*.

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