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SUBSTITUTING MANGANESE FOR MAGNESIUM ALTERS CERTAIN REACTION PROPERTIES OF THE ($Na^+ + K^+$)-ATPase

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Summary

$MnCl_2$ was partially effective as a substitute for $MgCl_2$ in activating the K^+-dependent phosphatase reaction catalyzed by a purified ($Na^+ + K^+$)-ATPase enzyme preparation from canine kidney medulla, the maximal velocity attainable being one-fourth that with $MgCl_2$. Estimates of the concentration of free Mn^{2+} available when the reaction was half-maximally stimulated lie in the range of the single high-affinity divalent cation site previously identified (Grisham, C.M. and Mildvan, A.S. (1974) J. Biol. Chem. 249, 3187—3197). $MnCl_2$ competed with $MgCl_2$ as activator of the phosphatase reaction, again consistent with action through a single site. However, with $MnCl_2$ appreciable ouabain-inhibitable phosphatase activity occurred in the absence of added KCl, and the apparent affinities for K^+ as activator of the reaction and for Na^+ as inhibitor were both decreased. For the ($Na^+ + K^+$)-ATPase reaction substituting $MnCl_2$ for $MgCl_2$ was also partially effective, but no stimulation in the absence of added KCl, in either the absence or presence of NaCl, was detectable. Moreover, the apparent affinity for K^+ was increased by the substitution, although that for Na^+ was decreased as in the phosphatase reaction. Substituting $MnCl_2$ also altered the sensitivity to inhibitors. For both reactions the inhibition by ouabain and by vanadate was increased, as was binding of [^{48}V]-vanadate to the enzyme; furthermore, binding in the presence of $MnCl_2$ was, unlike that with $MgCl_2$, insensitive to KCl and NaCl. Inhibition of the phosphatase reaction by ATP was decreased with 1 mM but not 10 mM KCl. Finally, inhibition of the ($Na^+ + K^+$)-ATPase reaction by Triton X-100 was increased, but that by dimethylsulfoxide decreased after such substitution.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

These findings are considered in terms of Mn^{2+} at the divalent cation site being a better selector than Mg^{2+} of the E_2 conformational states of the enzyme, states also selected by K^+ and by dimethylsulfoxide and reactive with ouabain and vanadate; the E_1 conformational states, by contrast, are those selected by Na^+ and ATP, and also by Triton X-100.

Introduction

The enzymatic representation of the plasma membrane sodium pump, the $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3), catalyzes a number of reactions including Na^+-dependent enzyme phosphorylation (phosphokinase), Na^+-dependent ADP/ATP exchange (transphosphorylase), Na^+-dependent ATPase, $(Na^+ + K^+)$-dependent ATPase, and (with various substrates including nitrophenyl phosphate) K^+-dependent phosphatase (phosphohydrolase) activities [1—5]. In these reactions two classes of sites for the substrate ATP are apparent: (i) high-affinity sites, with a K_m value in the range 0.1—1 μM, involved in enzyme phosphorylation, ADP/ATP exchange, and both ATPase reactions, and (ii) low-affinity sites with a K_m value in the range 0.1—0.5 mM, occupancy of which modifies some but not all reactions, for example, markedly stimulating the $(Na^+ + K^+)$-ATPase reaction but inhibiting the ADP/ATP exchange [6—8]. Whether the low-affinity substrate sites serve only as regulatory sites, whether they exist simultaneously with the high-affinity substrate sites, whether in some reaction sequences one is transformed into the other, or indeed whether they are only kinetic manifestations of a single class of sites are issues yet to be resolved. In all these reactions Mg^{2+} is required, optimally equimolar with ATP, but binding of Mg^{2+} in the absence of ATP is with an apparent affinity corresponding to the low-affinity class of substrate sites [8—10]. Moreover, with ATP concentrations sufficient to saturate only the high-affinity sites raising the $MgCl_2$ concentration to saturate its sites variously modifies the reaction processes [7,8,11].

Grisham and associates [9,12,13] have previously examined interactions of Mn^{2+} with the enzyme and found, from binding measurements and kinetic studies on the ATPase reaction, a single high-affinity site with a K_d value of 0.2—1 μM. Mg^{2+} competed with Mn^{2+} with a K_i value near 1 mM, and ATP modified Mn^{2+}-enzyme reactions with a concentration dependence consistent with occupancy of the low-affinity substrate site [9,13].

For the phosphatase reaction, the class of substrate sites available for catalysis is unresolved [1—5]. This report describes effects of substituting $MnCl_2$ for $MgCl_2$, studies begun in the course of examining the role of divalent cations in the phosphatase reaction, and revealing unexpected modification of Na^+, K^+, and inhibitor interactions with the enzyme. The observations thus bear on the sites available both to monovalent and divalent cations in the phosphatase and ATPase reactions, the relationship of these sites to those for ATP, and how binding to such sites affects enzyme properties and conformational states.

Methods and Materials

The enzyme preparation was obtained from the medullae of frozen canine kidneys by a modification [14] of the procedure of Jorgensen [15]. The specific activity of the $(Na^+ + K^+)$-ATPase reaction ranged from 13 to 19 μmol P_i liberated/mg protein per min; the specific activity of K^+-nitrophenyl phosphatase reaction was one-fifth that of the ATPase reaction. $(Na^+ + K^+)$-ATPase activity was measured in terms of the production of inorganic phosphate, as previously described [16], and calculated as the difference between incubations in the presence of 0.3 mM ouabain but no added NaCl and KCl and those without ouabain but containing added NaCl and KCl; ATPase activity in the presence of ouabain was less than 2% of the optimal $(Na^+ + K^+)$-ATPase activity. The standard medium contained 30 mM histidine-HCl/Tris (pH 7.8), 3 mM $MgCl_2$, 3 mM ATP (as the Tris salt), 90 mM NaCl, and 10 mM KCl. K^+-nitrophenyl phosphatase activity was measured in terms of the production of nitrophenol, as previously described [17], and calculated as the difference between incubations in the presence of 0.3 mM ouabain and no added KCl and those without ouabain (and generally with added KCl); nitrophenyl phosphatase activity in the presence of ouabain was less than 2% of the optimal K^+-nitrophenyl phosphatase activity. The standard medium contained 30 mM histidine-HCl/Tris (pH 7.8), 3 mM $MgCl_2$, 3 mM *p*-nitrophenyl phosphate (as the Tris salt), and 10 mM KCl. Incubations were performed at 37°C. Data presented are averages of four or more determinations, each performed in duplicate.

[^{48}V]Vanadate binding was calculated as the difference between binding during incubations in the presence and absence of divalent cations. The standard medium contained 30 mM histidine-HCl/Tris (pH 7.8), 3 mM $MgCl_2$, and 10 nM [^{48}V]orthovanadate. The enzyme concentration was 0.6—0.8 μg protein/ml of medium. After incubation for 15 min at 37°C the 0.5 ml incubation mixtures were filtered through the Gelman Metricel membrane filters (0.45 μm pore size) and each filter then washed twice with 1.0 ml of ice-cold 0.3 mM histidine-HCl/Tris (pH 7.8); filtration and washing were complete within 15 s. The filters were air-dried and the radioactivity remaining on them measured by liquid scintillation counting.

Frozen canine kidneys were obtained from Pel Freeze; ATP, nitrophenyl phosphate, ouabain, and Triton X-100 from Sigma, and $MgCl_2$, $MnCl_2$, and ammonium orthovanadate from Fisher. [^{48}V]Vanadyl chloride was obtained from Amersham, and converted to orthovanadate as described by Cantley et al. [18].

Results

$MnCl_2$ as well as $MgCl_2$ activated the K^+-dependent phosphatase reaction catalyzed by the $(Na^+ + K^+)$-dependent ATPase enzyme, but the maximal velocity attainable with $MnCl_2$ was lower than that with $MgCl_2$ (Fig. 1). With 3 mM nitrophenyl phosphate as substrate and 10 mM KCl, the maximal velocities, in relative units, were 0.3 with $MnCl_2$ and 1.27 with $MgCl_2$, and the concentrations for half-maximal velocity, $K_{0.5}$, were 0.14 mM $MnCl_2$ and

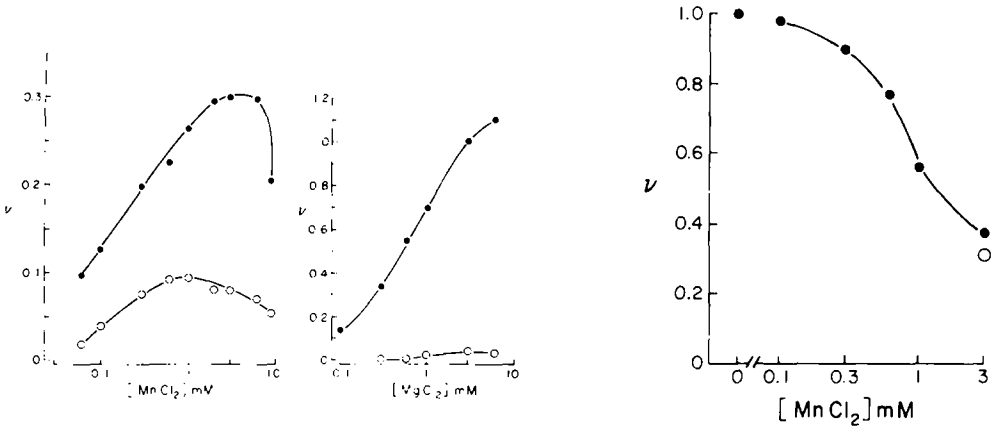

Fig. 1. (Left-hand two figures.) Effect of divalent cations on ouabain-inhibitable phosphatase activity. Phosphatase activity was measured, as described in Methods and Materials, in the standard medium but with the concentrations of divalent cations shown, in the presence of (a) 10 mM KCl, (b) no added KCl, and (c) 0.3 mM ouabain. Plotted for both $MnCl_2$ and $MgCl_2$ are (a—c), the increment in activity on adding 10 mM KCl over activity with ouabain (•), and (b — c), the increment in activity on removing ouabain but without adding KCl (○). Velocity in all cases is presented relative to that with 10 mM KCl and 3 mM $MgCl_2$ less that with no KCl and 0.3 mM ouabain, defined as 1.0.

Fig. 2. Effect of $MnCl_2$ on phosphatase activity with 3 mM $MgCl_2$. As in Fig. 1, ouabain-inhibitable phosphatase activity (the difference between incubations with 10 mM KCl and those with 0.3 mM ouabain (a—c) was measured in the standard medium containing, in addition, the concentrations of $MnCl_2$ shown (•). For comparison, ouabain-inhibitable phosphatase activity in the absence of $MgCl_2$ but the presence of 3 mM $MnCl_2$ is also shown (○).

0.8 mM $MgCl_2$.

This $K_{0.5}$ value for $MnCl_2$, however, does not reflect the actual affinity of the enzyme for Mn^{2+} because chelation reduced the concentration of free Mn^{2+} far below this level. The major chelator was the 30 mM histidine present as a buffer; since the K_d value for the Mn^{2+}-histidine complex is 0.263 mM [19], the actual concentration of free Mn^{2+} in the presence of 30 mM histidine and 0.14 mM $MnCl_2$ was 1.2 μM. Moreover, the concentration of free Mn^{2+} was further reduced by the presence of 3 mM nitrophenyl phosphate. The K_d value for the Mn^{2+}-nitrophenyl phosphate complex has not been reported, but may be roughly estimated from that for the Mg^{2+}-nitrophenyl phosphate complex, 5.88 mM [17], by comparison with the ratios of the K_d values between Mg^{2+}- and Mn^{2+}-complexes of ATP, ADP, and AMP, which are all in the range 5—6 [20—23]. On this basis the dissociation constant for the Mn^{2+}-nitrophenyl phosphate complex would be on the order of 1 mM, and thus the concentration of free Mn^{2+} in the presence of both 30 mM histidine and 3 mM nitrophenyl phosphate would be roughly 0.3 μM. This value is in close agreement with the K_d value for Mn^{2+} at the single high-affinity site of the enzyme, 0.2 μM reported by O'Connor and Grisham [12] from studies of Mn^{2+} binding using electron paramagnetic resonance. (Because the dissociation constant for the Mg^{2+}-histidine complex is not available, similar estimates of the corrected $K_{0.5}$ value for Mg^{2+} are not possible.)

The influence of the histidine buffer on the concentration of free Mn^{2+} was confirmed by substituting 30 mM Hepes/Tris (pH 7.8). In this case the $K_{0.5}$

value for $MnCl_2$ as activator of the phosphatase reaction in the presence of 10 mM KCl and 3 mM nitrophenyl phosphate was 33 μM. Applying the above estimate for the further reduction in free Mn^{2+} due to complexing by nitrophenyl phosphate gave a $K_{0.5}$ of 8 μM.

Adding $MnCl_2$ in the presence of near-optimal concentrations of $MgCl_2$ inhibited the phosphatase reaction (Fig. 2), and this inhibition was consistent with mutual competition between the divalent cations as activators of the reaction, according to the formula:

$$v_t = \frac{V}{1 + \frac{K_{0.5}}{[MgCl_2]}\left(1 + \frac{[MnCl_2]}{K'_{0.5}}\right)} + \frac{V'}{1 + \frac{K'_{0.5}}{[MnCl_2]}\left(1 + \frac{[MgCl_2]}{K_{0.5}}\right)}$$

where V and $K_{0.5}$ are the parameters for $MgCl_2$ and V' and $K'_{0.5}$ those for $MnCl_2$. For example, with the values above for $MgCl_2$ and $MnCl_2$, the total velocity, v_t, with 3 mM $MgCl_2$ and 1 mM $MnCl_2$ is calculated to be 0.58 whereas the measured value (Fig. 2) was 0.56. (Converting the $K_{0.5}$ value for $MnCl_2$ to the above estimate for the $K_{0.5}$ value for free Mn^{2+}, together with the corresponding estimate for free Mn^{2+} with 1 mM $MnCl_2$, gives with this formula essentially the same result, a calculated value of 0.59; however, a quantitative pursuit of this interaction is probably not justified since additional factors may also be involved, such as effects of elevated divalent cation concentrations on activation by K^+ [17,24,25].)

Ouabain is a specific inhibitor of the ($Na^+ + K^+$)-ATPase, so that inhibition by ouabain identifies reactions catalyzed by the enzyme [1—5]. In the absence of added KCl the phosphatase activity that was inhibited by 0.3 mM ouabain, was, with $MgCl_2$, at most 2—3% of the phosphatase activity in the presence of 10 mM KCl (Fig. 3). When $MnCl_2$ was substituted for $MgCl_2$, however, the ouabain-inhibitable phosphatase activity in the absence of added KCl was far greater (Fig. 1), both in absolute terms (3—4-fold greater than with $MgCl_2$) and in relation to the activation with 10 mM KCl (approx. one-third that with optimal $MgCl_2$ concentrations). The $K_{0.5}$ value for $MnCl_2$ was nearly the same in the absence of KCl, 0.13 mM, as in the presence of 10 mM KCl.

To determine if K^+ contaminating the reagents could account for the ouabain-inhibitable phosphatase activity in the absence of added KCl, the incubation media containing $MgCl_2$ or $MnCl_2$ were examined by atomic absorption spectroscopy: the K^+ content was less than 1 μM. Although K^+ tightly bound to the enzyme at the proper site could be present below this concentration and still activate the enzyme, the disparity between such activation in the presence of $MgCl_2$ vs. $MnCl_2$ remains. ($MnCl_2$, when compared to $MgCl_2$, had no effect on the color yield of the phosphatase reaction as determined by adding exogenous *p*-nitrophenol.)

The $K_{0.5}$ value for KCl as activator of the phosphatase reaction was 1.1 mM in the presence of 3 mM $MgCl_2$ (Fig. 3). In the presence of 3 mM $MnCl_2$ estimation of the $K_{0.5}$ value is complicated by the relatively high activity in the absence of added KCl, but when the increment in velocity due to KCl, Δv, was plotted, then a $K_{0.5}$ of 1.7 mM was obtained (Fig. 3). This difference in apparent sensitivity to K^+ on substituting $MnCl_2$ for $MgCl_2$ was

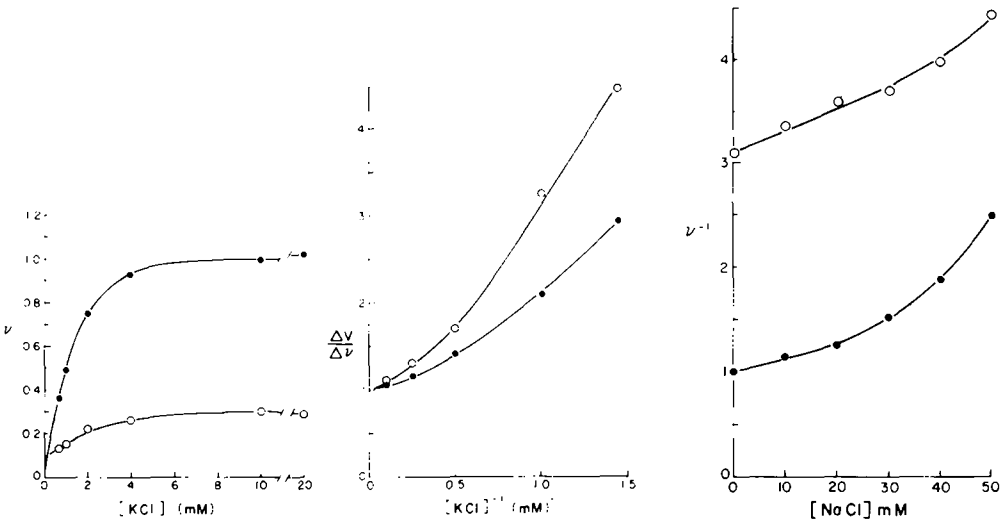

Fig. 3. (Left-hand two figures.) Effect of KCl on the phosphatase reaction. Ouabain-inhibitable phosphatase activity, calculated as in Fig. 1, was measured in the standard media containing the concentrations of KCl indicated and either 3 mM $MgCl_2$ (•) or 3 mM $MnCl_2$ (○). In the left-hand panel is plotted the total difference in velocity between incubations with 0.3 mM ouabain and no KCl and those without ouabain but with the KCl concentrations shown (equivalent to a—c in Fig. 1). In the right-hand panel is plotted the increment in velocity, Δv, between incubations with KCl and those without added KCl; in this case the Δv value is divided by the maximal increment in velocity, ΔV, and the data are presented in double-reciprocal form.

Fig. 4. Effect of NaCl on the phosphatase reaction. Ouabain-inhibitable phosphatase activity (a—c) calculated as in Fig. 1, was measured in the standard media containing the concentrations of NaCl shown and either 3 mM $MgCl_2$ (•) or 3 mM $MnCl_2$ (○). Data are presented in the form of a Dixon plot.

paralleled by a difference in apparent sensitivity to Na^+ as well. In the presence of 10 mM KCl and 3 mM $MgCl_2$, 40 mM NaCl inhibited the phosphatase reaction by half, whereas, after substituting the 3 mM $MnCl_2$ for $MgCl_2$, 40 mM NaCl inhibited by only one-fifth (Fig. 4).

The K_m value for the substrate nitrophenyl phosphate was the same, 3 mM, with either 3 mM $MnCl_2$ (data not presented) or 3 mM $MgCl_2$ (as previously shown [6]). With both $MnCl_2$ and $MgCl_2$ the double-reciprocal plots were linear.

For the $(Na^+ + K^+)$-dependent ATPase reaction substituting $MnCl_2$ for $MgCl_2$ also reduced enzyme activity (Fig. 5), although the maximal velocity attainable with $MnCl_2$ was somewhat closer to that with $MgCl_2$ than for the phosphatase reaction. On the other hand, inhibition by higher concentrations of $MnCl_2$ was more apparent (Fig. 5). With 30 mM histidine, 3 mM ATP, and 2 mM $MnCl_2$ the concentration of free Mn^{2+} would be roughly 0.3 μM, based on dissociation constants for Mn^{2+}-ATP and Mn^{2+}-histidine of 0.0166 mM and 0.263 mM, respectively [19,23]. The disparity in ATPase activity between $MnCl_2$ and $MgCl_2$ at concentrations well below 2 mM is consistent with occupancy of sites for free Mn^{2+} having $K_{0.5}$ values in the submicromolar range, similar to those demonstrable in the phosphatase assay (Figs. 1 and 2), and different efficacies between the Mn^{2+}-ATP and Mg^{2+}-ATP complexes.

In the absence of added NaCl and KCl, ouabain-inhibitable ATPase activity was not detectable with either $MgCl_2$ or $MnCl_2$. In the presence of 90 mM

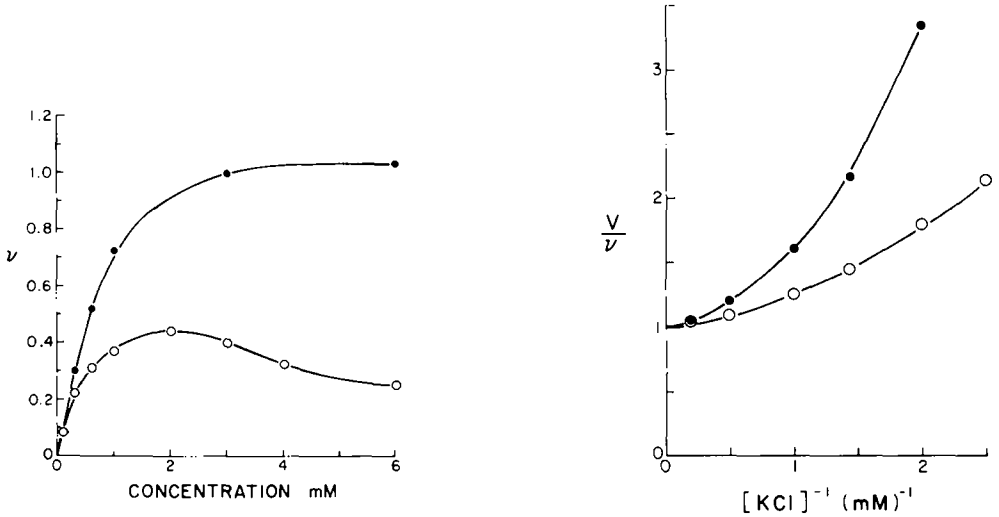

Fig. 5. Activation of the $(Na^+ + K^+)$-ATPase reaction by divalent cations. $(Na^+ + K^+)$-ATPase activity was measured as described in Methods and Materials in the standard media containing the concentrations of $MgCl_2$ (•) or $MnCl_2$ (○) shown. Velocity is presented relative to that with 3 mM $MgCl_2$ defined as 1.0.

Fig. 6. Effect of KCl on the $(Na^+ + K^+)$-ATPase reaction. Experiments were performed as in Fig. 5 except that the incubation media contained the concentrations of KCl shown and either 3 mM $MgCl_2$ (•) or 3 mM $MnCl_2$ (○). The observed velocity, divided by the maximal velocity with divalent cation plus saturating KCl, v/V, is plotted in double-reciprocal form.

NaCl and the absence of added KCl, ouabain-inhibitable ATPase activity was present, corresponding (at least in part) to the Na^+-ATPase reaction catalyzed by this enzyme [1—5]. This Na^+-ATPase activity was the same fraction of the $(Na^+ + K^+)$-ATPase activity with either $MgCl_2$ or $MnCl_2$. Consequently, if there was K^+ (or a K^+-like cation) tightly bound to the enzyme then in the ATPase reaction process substituting Mn^{2+} for Mg^{2+} did not increase either its availability or efficacy.

Activation of the ATPase reaction by KCl in the presence of 90 mM NaCl was altered by $MnCl_2$, but oppositely to the effect of $MnCl_2$ with the phosphatase reaction. Substituting $MnCl_2$ for $MgCl_2$ decreased the $K_{0.5}$ value for KCl by half, from 0.8 mM to 0.4 mM (Fig. 6). On the other hand, substituting $MnCl_2$ increased the $K_{0.5}$ value for NaCl (measured in the presence of 10 mM KCl) drastically, from 7 mM to 29 mM (Fig. 7). This decrease in apparent affinity for Na^+ in the ATPase reaction thus paralleled the decrease in apparent affinity for Na^+ as an inhibitor in the phosphatase reaction (Fig. 4).

Substituting $MnCl_2$ for $MgCl_2$ also altered the sensitivity of the enzymatic reactions to inhibitors. The concentration for 50% inhibition, the I_{50}, for ouabain was decreased in the presence of $MnCl_2$ (Table I). In these experiments the assay was initiated by adding the enzyme to the reaction mixture; inhibition by ouabain did not vary appreciably during the usual 8 min incubation since the percentage inhibition was the same after 4 and 8 min of incubation at 37°C. To measure inhibition by vanadate, however, the enzyme was first preincubated at 37°C with vanadate for 10 min and the assay then initiated by adding the substrate. As in the experiments with ouabain the I_{50} for

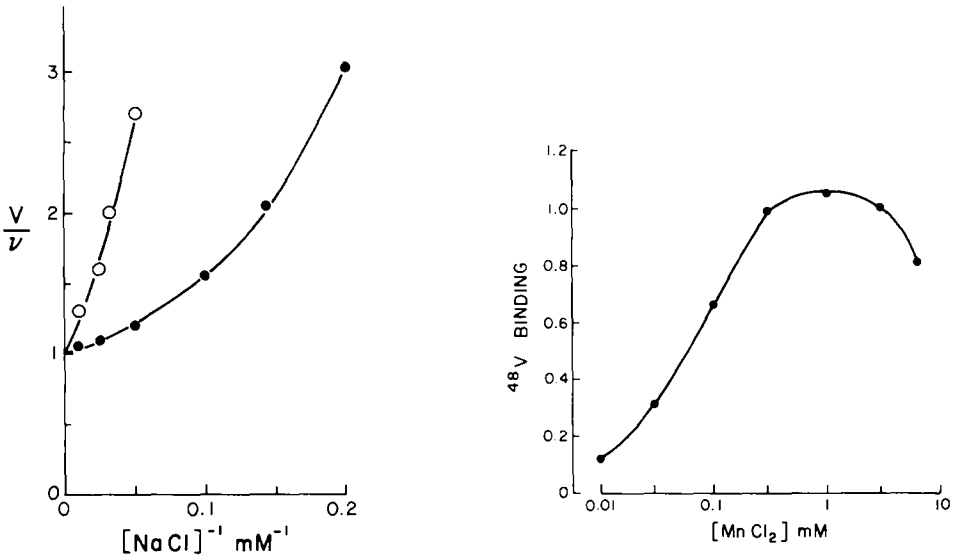

Fig. 7. Effect of NaCl on the $(Na^+ + K^+)$-ATPase reaction. The experiments were performed and the data are presented as in Fig. 6, except that the NaCl concentration was varied as shown in the presence of 3 mM $MgCl_2$ (●) or 3 mM $MnCl_2$ (○). The KCl concentration was 10 mM.

Fig. 8. Effect of $MnCl_2$ on [^{48}V]vanadate binding. Binding of [^{48}V]vanadate was measured as described in Methods and Materials but with the concentrations of $MnCl_2$ shown. Specific binding is presented relative to that with 3 mM $MnCl_2$ defined as 1.0; this averaged 1.2 nmol/mg protein.

vanadate was also decreased by substituting $MnCl_2$ for $MgCl_2$, although the difference was greater when the enzyme was preincubated with vanadate in the absence of KCl (reaction started by adding KCl and substrate) than in the

TABLE I

EFFECT OF SUBSTITUTING $MnCl_2$ FOR $MgCl_2$ ON SENSITIVITY TO INHIBITORS

The concentration of inhibitor for 50% inhibition, the I_{50}, was measured for the K^+-phosphatase and $(Na^+ + K^+)$-ATPase reactions in their standard media containing either 3 mM $MgCl_2$ or 3 mM $MnCl_2$ as indicated. Inhibition of the K^+-phosphatase reaction by ATP was also measured in incubations containing 1 mM KCl. To measure sensitivity to vanadate the enzyme was first preincubated for 10 min at 37°C in the standard media containing all reactants but KCl and substrate ('preincubated without KCl') or all reactants but substrate ('preincubated with 10 mM KCl'); the assay incubation was then initiated by adding the missing reactants.

Inhibitor	I_{50} for inhibitor (μM)			
	K^+-phosphatase reaction		$(Na^+ + K^+)$-ATPase reaction	
	With $MgCl_2$	With $MnCl_2$	With $MgCl_2$	With $MnCl_2$
Ouabain	3	0.3	0.9	0.3
Vanadate				
Preincubated without KCl	0.052	0.021	0.21	0.022
Preincubated with 10 mM KCl	0.031	0.022	0.11	0.022
ATP				
Incubated with 10 mM KCl	210	170	—	—
Incubated with 1 mM KCl	41	62	—	—

presence of 10 mM KCl (reaction started by addition of substrate).

Inhibition of the phosphatase reaction by ATP varies also with the KCl concentration of the medium, in this case KCl antagonizing the inhibition [8,26]. At a high KCl concentration, 10 mM, the I_{50} for ATP was higher in the presence of $MnCl_2$ (Table I), consistent with $MnCl_2$-diminishing activation by KCl (Fig. 3). On the other hand, at a low KCl concentration, 1 mM, the reverse was true (Table I); this latter observation is thus consistent with the ability of $MnCl_2$ to induce a K^+-like activation of the phosphatase reaction in the absence of KCl (Fig. 1), $MnCl_2$ at the K^+ sites having effects akin to a pharmacological partial agonist.

Recent studies [27] showed that certain reagents act to favor one or the other major conformational states of the enzyme, E_1 or E_2, as indicated by altered sensitivity to state-dependent inhibitors; non-ionic detergents such as Triton X-100 favoring the E_1 states, and polar aprotic solvents such as Me_2SO the E_2 states. Substituting $MnCl_2$ for $MgCl_2$ affected the sensitivity to these two classes of reagents as inhibitors of the ($Na^+ + K^+$)-ATPase reaction as well: decreasing the inhibition by Triton X-100 (0.08 μl/ml of medium) markedly, from 31 to 4%, while increasing the inhibition by 100 μl/ml Me_2SO slightly, from 32 to 41%.

To examine further the possibility of $MnCl_2$ producing a K^+-like effect, the binding of [^{48}V]vanadate was measured since it affords the opportunity to study ligand effects in the absence of reactants required for enzymatic activity. Preliminary experiments established that binding of ^{48}V was rapid in the presence of either $MgCl_2$ or $MnCl_2$, reaching a plateau in 5—10 min, and that the complex could be filtered and washed to a constant level of retention in 15 s, well within the half-life for the dissociation of the enzyme-vanadate complex even at 37°C, 210 s [18]. Cantley et al. [18] previously demonstrated that vanadate binding required Mg^{2+}, and was increased by KCl but decreased by NaCl. In accord with that report, binding of 10 nM [^{48}V]vanadate in the absence of added $MgCl_2$ was equivalent to that in the presence of 1 mM unlabeled vanadate (here equated to nonspecific binding), whereas adding 3 mM $MgCl_2$ increased binding 7-fold. The K_d value for this Mg^{2+}-dependent vanadate binding was 96 nM. In the absence of added $MgCl_2$ neither 10 mM NaCl nor KCl affected vanadate binding appreciably, but 10 mM KCl increased

TABLE II

EFFECT OF SUBSTITUTING $MnCl_2$ FOR $MgCl_2$ ON [^{48}V]VANADATE BINDING

Binding of [^{48}V]vanadate was measured as described in Methods and Materials during incubations at 37°C in media containing 30 mM histidine — HCl/Tris (pH 7.8), 3 mM $MgCl_2$ or 3 mM $MnCl_2$, 10 nM [^{48}V]vanadate, and the other additions as indicated. Specific binding of vanadate is expressed relative to that with 3 mM $MgCl_2$ defined as 1.0 (equivalent to 0.3 nmol vanadate/mg protein).

Additions	Binding of vahadate	
	With $MgCl_2$	With $MnCl_2$
None	1.00	4.14
10 mM KCl	3.32	4.10
10 mM NaCl	0.40	3.89

binding 3-fold in the presence of 3 mM $MgCl_2$ and 10 nM vanadate, whereas 10 mM NaCl decreased it by more than half (Table II).

By contrast, substituting 3 mM $MnCl_2$ increased the affinity for vanadate, the K_d value decreasing to 12 nM. Moreover, with 3 mM $MnCl_2$ and 10 nM vanadate, binding was not affected appreciably by either 10 mM KCl or NaCl (Table II). The $K_{0.5}$ value for $MnCl_2$ as an activator of vanadate binding was 0.06 mM (Fig. 8). When this $K_{0.5}$ value for $MnCl_2$ is corrected for chelation by histidine, the concentration of free Mn^{2+} at half-maximal binding was 0.5 μM, near the estimate for free Mn^{2+} as activator of the phosphatase reaction. When 30 mM Hepes/Tris (pH 7.8) was substituted for the histidine buffer, the $K_{0.5}$ value for $MnCl_2$ was 2 μM.

Maximal binding, calculated for a saturating vanadate concentration, was the same with either 3 mM $MgCl_2$ or 3 mM $MnCl_2$, averaging 3 nmol vanadate/mg protein. This value is consistent with an enzyme purity of 75—90% and in accord with sodium dodecyl sulfate gel electrophoretic patterns of the enzyme preparation (Robinson, J.D., unpublished results). On the other hand, the specific activity of the $(Na^+ + K^+)$-ATPase reaction, while within the range reported [5], is about half that of the most active enzyme preparation. The reason for this discrepancy is uncertain; perhaps vanadate binding survives better than the overall ATPase reaction, as ouabain binding also may [5].

Discussion

$MnCl_2$, when substituted for $MgCl_2$, was partially effective as an activator of both the K^+-phosphatase and $(Na^+ + K^+)$-ATPase reactions. Although a definitive evaluation of the affinity for Mn^{2+} was not possible in the absence of dissociation constants for all the possible Mn^{2+} complexes in the incubation media, the estimated $K_{0.5}$ values for free Mn^{2+}, 0.3—0.5 μM, lie within the range previously determined by kinetic studies on the $(Na^+ + K^+)$-ATPase reaction and by binding studies, 0.2—0.9 μM [9,12]. Activation by Mn^{2+} of the reactions described here thus are considered to be mediated through the single high-affinity divalent cation site per functional enzyme oligomer, previously identified [9], although inhibition occurring at higher concentrations suggests occupancy of additional sites as well. From antagonism to Mn^{2+} this activating site is equated with a Mg^{2+} site having a $K_{0.5}$ value of 0.1—1 mM [8—10,24,25], a site through which Mg^{2+} activates the phosphatase reaction [8,10,24,25], inhibits the ADP/ATP exchange reaction [7], increases the $K_{0.5}$ value for Na^+ as activator of enzyme phosphorylation and the Na^+-ATPase reaction [11], and potentiates enzyme inactivation by reagents such as Be^{2+} and acetic anhydride [8].

The placement of this divalent cation site in the reaction mechanism, however, is less straightforward. (i) The site could be part of the high-affinity substrate site, its lower affinity for Mg^{2+} merely reflecting looser binding when Mg^{2+} is alone than in the presence of the tighter binding and strongly complexing ATP. Against this formulation are effects such as a decreased affinity for nucleotide binding [28] and a decreased $K_{0.5}$ value for Na^+ as activator of enzyme phosphorylation [11] when micromolar ATP and millimolar $MgCl_2$ are compared to micromolar concentrations of both. In such cases Mg^{2+} must

be acting beyond, in space or in the reaction sequence, the high-affinity substrate site. (ii) The site could be that from which Mg^{2+} is discharged in the reaction sequence. Recent studies [3,5,29] indicate that Mg^{2+} is still bound to the enzyme after dephosphorylation of the acyl phosphate reaction intermediate. If release of Mg^{2+} is required for the reaction to proceed, then binding of Mg^{2+} at this point would inhibit, as millimolar Mg^{2+} can inhibit the ADP/ATP exchange and Na^+-ATPase reactions [7,11]. Lack of inhibition of the ($Na^+ + K^+$)-ATPase reaction by millimolar Mg^{2+} could be attributed to ATP saturating the low-affinity substrate site and driving the reaction forward. (iii) The site could be associated with the low-affinity substrate site. The apparent affinity for Mg^{2+} is of the same magnitude as the K_m value for MgATP at that site, and this formulation is consistent with inhibition of the ADP/ATP exchange reaction both by millimolar Mg^{2+} and MgATP [7].

The most striking observation described here, which also bears on these considerations, is the ability of Mn^{2+} to produce effects on the reaction properties similar to those of K^+. Thus, phosphatase activity sensitive to the specific inhibitor ouabain occurred in the absence of added KCl when $MnCl_2$ was substituted for $MgCl_2$; with $MgCl_2$ the ouabain-inhibitable phosphatase reaction requires K^+ at moderate-affinity α sites [24] accessible, in vivo where the enzyme spans the plasma membrane, from the cytoplasmic milieu [30]. K^+-like activity was also manifested by $MnCl_2$ in terms of increased binding of and inhibition by vanadate in the absence of added KCl, when compared to control incubations containing $MgCl_2$, and decreased inhibition by ATP of the phosphatase reaction in the presence of low (1 mM) KCl, again when compared to corresponding incubations with $MgCl_2$. A K^+-like action of Mn^{2+} is also seen when examining the fluoresence of the enzyme reacted with fluorescein isothiocyanate: Mn^{2+} produced a K^+-like decrease in fluorescence and hindered the Na^+-associated fluorescence increase (Flashner, M.S. and Sen, P.C., personal communication).

Although the possibility remains that Mn^{2+} produces such effects through release of K^+ tightly bound to the enzyme, or through occupancy of the K^+ sites themselves, these observations may represent differential selection of alternative conformational states of the enzyme. Current schemes for the reaction sequence feature two major conformational states, E_1 and E_2, each of which may exist in phosphorylated form:

$$E_1 \rightleftharpoons E_1 - P \rightleftharpoons E_2 - P \rightleftharpoons E_2 \rightleftharpoons E_1$$

In the absence of ligands, or with Na^+ and/or ATP, the enzyme appears to be predominantly in the E_1 states, which bear high-affinity sites for ATP and Na^+ plus low-affinity discharge sites for K^+; conversely, in the presence of K^+ the enzyme appears to be predominantly in the E_2 states, which bear moderate-affinity α sites for K^+ plus the low-affinity substrate sites [6,24,26,31—35]. Thus, adding ATP or Na^+ would favor E_1 whereas adding K^+ would favor E_2 states. Formulation (i) above implies that divalent cations would favor E_1, whereas formulation (iii) implies that divalent cations would favor E_2.

These experiments suggest that Mn^{2+} is a more potent selector of the E_2 states than is Mg^{2+}, as manifested by: the greater K^+-like effects; the greater sensitivity to inhibition by vanadate, ouabain, and Me_2SO, and the lesser

sensitivity to inhibition by Na^+, ATP, and Triton X-100. Such selection could result from the two divalent cations having finite affinities for both enzyme states, but with Mn^{2+}, having a differential affinity for E_2 over E_1 greater than does Mg^{2+}. This proposal, in turn, suggests that formulations (i) and (iii) above may both be partially correct in that both high- and low-affinity substrate sites can bind divalent cations, although with quite different affinities, and even that the substrate sites are interconverted in the reaction sequence [3,5,31,32]. Alternatively, the high-affinity and low-affinity substrate sites could coexist, as could E_1 and E_2 states on different protomers of the functional enzyme oligomer [3,36—38].

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