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DIVALENT CATIONS AS ALLOSTERIC MODIFIERS OF THE $(Na^+ + K^+)$-DEPENDENT ATPase

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SUMMARY

With a $(Na^+ + K^+)$-dependent ATPase preparation from rat brain, equimolar $MgCl_2$ or $MnCl_2$ increased similarly the cooperative response to activation by Na^+, measured in terms of the slope of the Hill plot, n, although their effects on other kinetic parameters differed markedly. In like manner, both $MgCl_2$ and $MnCl_2$ increased similarly the sensitivity of the enzyme to ouabain, in contrast to their dissimilar effects on activity in the absence of ouabain. These data suggest that one role of these cations is to act at specific sites on the enzyme to favor certain conformational state(s), perhaps by influencing subunit interactions, *i.e.* as heterotropic allosteric modifiers.

INTRODUCTION

Kinetic studies[1–3] of the $(Na^+ + K^+)$-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) suggest that cooperative interactions occur between the binding sites for each species of activating monovalent cations, Na^+ and K^+, with these interactions in turn sensitive to the binding to the enzyme of certain other substances[2,4,5] ("heterotropic modifiers"), in accord with allosteric processes[6]. This formulation is supported by the recent report[7] of a subunit structure of the ATPase, for interactions between subunits are the basis of the cooperative and heterotropic allosteric responses[5]. It seemed of interest to reconsider a role of divalent cations (in addition to their being a component of the cation–ATP substrate complex) in this vein: as heterotropic allosteric modifiers influencing (a) the cooperative responses to activating monovalent cations, and (b) the inhibition by ouabain, which appears to bind preferentially to one major conformational form of the enzyme[8–10].

METHODS AND MATERIALS

The $(Na^+ + K^+)$-dependent ATPase was obtained from a rat brain microsomal preparation by treatment with deoxycholate and then NaI, as previously described[2].

$(Na^+ + K^+)$-dependent ATPase activity was measured in terms of the production of P_i, as previously described[2]. The standard medium contained 50 mM histidine–

HCl (pH 7.8 with Tris), 3 mM $MgCl_2$, 3 mM ATP (as the Tris salt), 90 mM NaCl, 10 mM KCl, and the enzyme preparation (0.1 mg protein/ml). Incubation was for 4–8 min at 37°; activity was linear with time during these periods. Activity in the absence of Na^+ and K^+ ("Mg^{2+}-ATPase") was measured concurrently; such activity averaged only a few percent of the ($Na^+ + K^+$)-dependent ATPase activity[2], and was subtracted from the total activity in the presence of Na^+ and K^+ to give the ($Na^+ + K^+$)-dependent activity. Because of variations in the absolute activity of different enzyme preparations, enzyme velocities are expressed relative to the ($Na^+ + K^+$)-dependent ATPase activity of a concurrent control incubation in the standard medium, defined as 1.0.

ATP was purchased from Sigma Chemical Co. as the sodium salt, and converted to the Tris salt. All solutions were made in water that had been redistilled from an all-glass still. Protein was measured by the biuret method, using bovine serum albumin as a standard.

Experimental points are the average of five or more experiments performed in duplicate. Values from the Hill plots of n and $K_{0.5}$ were calculated from the equations for the straight lines obtained by the method of least squares, with standard deviations calculated as previously described[2].

RESULTS AND DISCUSSION

As the concentration of either Na^+ or K^+ is increased in the incubation medium, the activity of the ($Na^+ + K^+$)-dependent ATPase increases in a sigmoidal fashion, to give Lineweaver–Burk plots that are concave upward and Hill plots with slopes n greater than 1.0; this pattern of response has been interpreted in terms of cooperative interactions between the binding sites for each cation[1–3]. To examine the influence of divalent cations on such cooperative responses of the ATPase the effects on Na^+ kinetics were selected because of the lesser possibility that with Na^+ apparent changes in cooperativity might instead reflect competition between the ions for the monovalent cation site[3]. Thus, in accord with previous experiments[3], as the concentration of one activating cation, K^+, was raised, $K_{0.5}$ for Na^+, the concentration of Na^+ for half-maximal activation, increased (Fig. 1), as would be expected if K^+ were competing for the Na^+ site. However, the index of cooperativity between the Na^+ sites, n, the slope of the Hill plot, fell as the K^+ concentration was raised (Fig. 1), which would not be expected[3] if competition between these cations were the only process influencing n.

In this framework the effects of $MgCl_2$ concentration were studied in terms of the kinetic parameters for Na^+ activation: $K_{0.5}$, n, and V. As the concentration of $MgCl_2$ was raised, n increased (Figs. 1 and 2), in contrast to the effect of raising the concentration of K^+ (Fig. 1). However, $K_{0.5}$ for Na^+ also increased, indicating that competition between Mg^{2+} and Na^+ for the Na^+ site apparently occurred as well. Thus, although both Mg^{2+} and K^+ seem to act as competitors with Na^+ for the Na^+ site, as demonstrated by their both increasing the $K_{0.5}$ for Na^+, the divalent cation Mg^{2+}, presumably by acting at its own site, increased the cooperative response to Na^+, whereas K^+ did not.

The increase in n with rising $MgCl_2$ concentrations was not secondary to the effect of the divalent cation on the turnover rate of the enzyme, since at higher

concentrations of $MgCl_2$ V declined although n was increased further (Fig. 2). For a given NaCl concentration the decline in velocity at higher $MgCl_2$ concentrations could result from increasing competition with Na^+ in the presence of a saturating concentration of Mg^{2+} at its own site; however, mechanisms in addition to competition

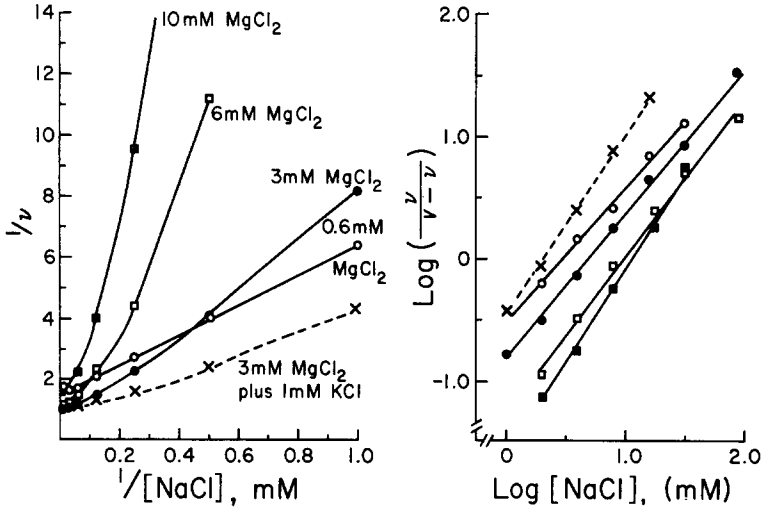


Fig. 1. Effects of $MgCl_2$ on activation by Na^+. The ATPase preparation was incubated in the standard medium, but with the concentration of NaCl shown and in the presence of 0.6 (○), 3.0 (●), 6.0 (□), and 10.0 (■) mM $MgCl_2$. Data are presented in the left-hand panel in the form of a Lineweaver–Burk plot, and in the right-hand panel in the form of a Hill plot. For comparison with the control values, in the presence of 10 mM KCl and 3 mM $MgCl_2$, data from experiments with 1 mM KCl and 3 mM $MgCl_2$ are included (×), in this case with maximal velocities equated to emphasize differences and simplify the plot; kinetic parameters for Na^+ activation with 1 mM KCl and 10 mM KCl were, respectively: V, 0.83 and 1.03; $K_{0.5}$, 2.2 and 5.0 mM; and n, 1.49 and 1.19.

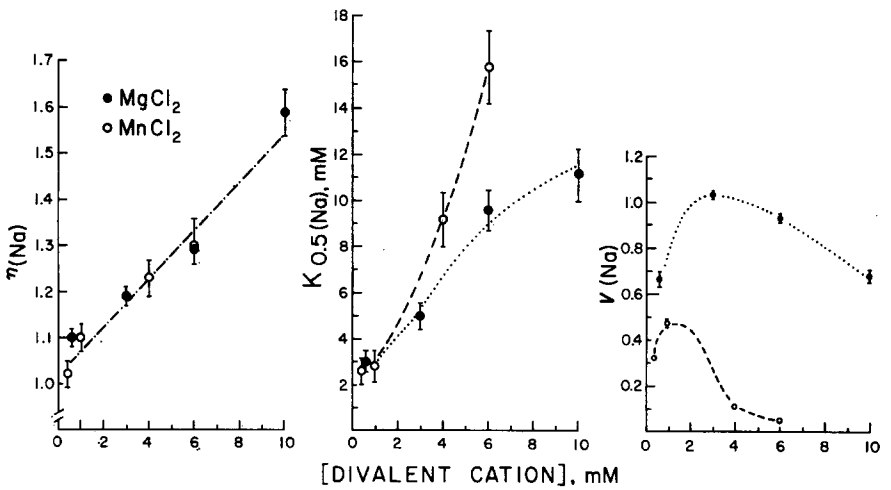


Fig. 2. Effect of $MgCl_2$ and $MnCl_2$ on the kinetic parameters for Na^+ activation. Data from Fig. 1 on $MgCl_2$ concentration (●), and from analogous experiments at varying $MnCl_2$ concentrations (○), are plotted; standard deviations are shown except where smaller than the symbol for the point. Lines were fitted by eye.

with Na^+ are required to account for the decreased V for Na^+, the most probable being antagonism between free Mg^{2+} and the Mg^{2+}–ATP complex at the substrate site[11].

To distinguish further between simple competition and more complex interactions as the basis for the change in n, the effects of $MnCl_2$ on Na^+ kinetics were explored. It has been shown[12] with the ATPase that $MnCl_2$ may be substituted for $MgCl_2$, although lower velocities are achieved. This lesser efficacy of $MnCl_2$ was attributed[12] to its greater potency in competing with Na^+; however, to account for its reduction in V for Na^+ additional mechanisms obviously must be involved (*e.g.* a less effective divalent cation–ATP substrate complex with Mn^{2+}, and Mn^{2+} antagonizing Mn–ATP binding). In any case, greater apparent competition between Mn^{2+} and Na^+ for the Na^+ site was clear in these experiments (Fig. 2): $K_{0.5}$ for Na^+ was considerably larger with $MnCl_2$ than with equimolar $MgCl_2$. But the effects on n for Na^+ activation were remarkably similar for both Mn^{2+} and Mg^{2+} (Fig. 2). Thus while the different potencies of Mg^{2+} and Mn^{2+} as competitors with Na^+ for the Na^+ site may be seen in their different alterations in $K_{0.5}$, their nearly identical influence on the cooperativity between the Na^+ sites (as reflected in their effects on n) cannot then be explained in terms of action at the Na^+ site. Instead, the similar effects of Mg^{2+} and Mn^{2+} would seem to represent a distinct action at another site: a regulatory site for divalent cations at which they act, with nearly equal potency, as allosteric modifiers.

These effects, however, might not represent the action of the cations themselves, but instead that of the divalent cation–ATPase complex. To distinguish between these possibilities both ATP and $MgCl_2$ were varied separately (Table I): the n for Na^+ did not follow the rise in Mg–ATP concentration, but instead the rise in free Mg^{2+} concentration (or perhaps the ratio of free to complexed Mg^{2+}), although the nucleotide may modify the response as well.

The concentration of Mg^{2+} also influences the inhibition of the ATPase by oligomycin[5], which too appears to act as a heterotropic modifier affecting, among other kinetic parameters, the cooperativity between Na^+ sites[5]. Since oligomycin antagonized ouabain inhibition[5], it seemed plausible that these two agents bound to different conformers of the ATPase; correspondingly, since Mg^{2+} antagonized oligomycin inhibition, divalent cations should promote ouabain inhibition. Several studies[8–10] have demonstrated the dependence of ouabain binding on Mg^{2+}, and Skou *et al.*[13] recently documented increasing sensitivity with rising concentrations

TABLE I

EFFECT OF $MgCl_2$ AND ATP ON THE KINETIC PARAMETERS FOR Na^+ ACTIVATION

Experiments were performed as in Fig. 1, but with the concentrations of $MgCl_2$ and ATP indicated.

Incubation conditions		*Kinetic parameters for Na^+*	
$MgCl_2$ (mM)	*ATP (mM)*	*n*	*$K_{0.5}$ (mM)*
3	6	1.14 ± 0.02	4.2 ± 0.5
3	3	1.19 ± 0.02	5.0 ± 0.6
6	6	1.24 ± 0.02	4.7 ± 0.5
6	3	1.29 ± 0.03	9.6 ± 0.9

of Mg^{2+}. Again, both $MgCl_2$ and $MnCl_2$, varied from concentrations below to above the optima for ATP hydrolysis, progressively increased the sensitivity to ouabain, as measured by the I_{50}, the concentration of inhibitor to reduce velocity by half (Fig. 3). And, as in the effect on cooperativity between Na^+ sites, the efficacy of Mn^{2+} was strikingly similar to that of Mg^{2+} despite their different potency toward ATP hydrolysis, *i.e.* the change in sensitivity to ouabain did not reflect merely alterations in the turnover rate of the enzyme. Similar data on the equivalent effects of Mg^{2+} and Mn^{2+} on the I_{50} for ouabain were also obtained from experiments in which ATP, NaCl, and ouabain were preincubated for 8 min with the enzyme in the presence of different concentrations of divalent cation, and inhibition then measured after the incubation was begun by adding KCl: the I_{50} for ouabain inhibition fell as the concentration of divalent cation in the preincubation medium was progressively increased. In agreement with the report of Skou *et al.*[13], the influence of the divalent cations correlated better with their free concentration than with that of the cation–ATP complex (data not presented).

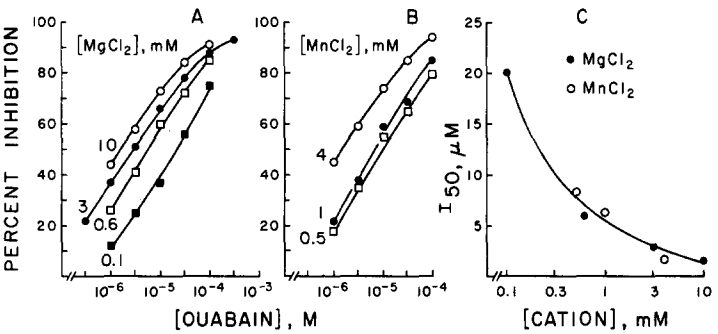
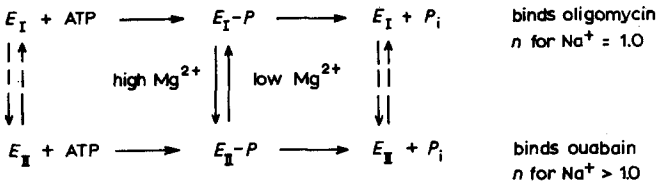


Fig. 3. Effect of divalent cations on ouabain inhibition. The ATPase preparation was incubated in the presence and absence of the ouabain concentrations shown, in the standard medium but with the divalent cation ($MgCl_2$ in Panel A, $MnCl_2$ in Panel B) at the concentrations indicated. The concentration of ouabain for 50% inhibition in each case, the I_{50}, is then plotted against the concentration of divalent cation, $MgCl_2$ (●) or $MnCl_2$ (○), in Panel C.

These observations that the divalent cations modify cooperative responses between the Na^+ sites (Fig. 2) and influence the inhibition by oligomycin[5] and ouabain (Fig. 3) support considerations of Mg^{2+} (and Mn^{2+}) acting at specific sites as heterotropic allosteric modifiers, favoring major conformational state(s) of the ATPase by affecting subunit interactions. On the other hand, conventional formulations of the ATPase reaction scheme[8,10] depict a conformational change in phosphorylated intermediate, from E_1–P to E_2–P, that is sensitive to the Mg^{2+} concentration (E_1–P being demonstrable only in the presence of low concentrations of Mg^{2+} or certain inhibitors). However, when activity is measured at a constant near-optimal Mg^{2+} concentration (as in the ATPase reactions usually studied *in vitro* and presumably as occurring *in vivo*) it is neither clear experimentally whether E_1–P normally exists nor apparent conceptually why it should. Consequently an alternative formulation has been proposed[5,13] where two major forms, which may be designated E_I and E_{II}, and representing the properties of the conventional E_1 and E_2 forms, offer parallel alternative routes, rather than obligatory sequential steps (E_1–P to E_2–P):



Although this scheme may resemble superficially previous formulations, it differs not only in incorporating considerations of allosteric transitions[6], but also in distinguishing between this class of conformational changes (*e.g.* between E_I and E_{II}) involved in allostery and control, and a distinct class of conformational changes, associated with phosphorylation and dephosphorylation of the enzyme, that may be linked to cation translocation[5,14].

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REFERENCES

1 R. F. Squires, *Biochem. Biophys. Res. Commun.*, 19 (1965) 27.
2 J. D. Robinson, *Biochemistry*, 6 (1967) 3250.
3 J. D. Robinson, *Arch. Biochem. Biophys.*, 139 (1970) 17.
4 T. Tobin, S. P. Banerjee and A. K. Sen, *Nature*, 225 (1970) 745.
5 J. D. Robinson, *Mol. Pharmacol.*, 7 (1971) 238.
6 J. Monod, J. Wyman and J.-P. Changeux, *J. Mol. Biol.*, 12 (1965) 88.
7 A. Atkinson, A. D. Gatenby and A. G. Lowe, *Nature New Biol.*, 233 (1971) 145.
8 R. W. Albers, G. J. Koval and G. J. Siegel, *Mol. Pharmacol.*, 4 (1968) 324.
9 A. Schwartz, H. Matsui and A. H. Laughter, *Science*, 160 (1968) 323.
10 R. L. Post, S. Kume, T. Tobin, B. Orcutt and A. K. Sen, *J. Gen. Physiol.*, 54 (1969) 306S.
11 T. Hexum, F. E. Samson, Jr. and R. H. Himes, *Biochim. Biophys. Acta*, 212 (1970) 322.
12 A. Atkinson, S. Hunt and A. G. Lowe, *Biochim. Biophys. Acta*, 167 (1968) 469.
13 J. C. Skou, K. W. Butler and O. Hansen, *Biochim. Biophys. Acta*, 241 (1971) 443.
14 J. D. Robinson, *Nature*, 233 (1971) 419.