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IRREVERSIBLE ZINC ION INHIBITION OF (Na^+-K^+)-ADENOSINETRIPHOSPHATASE, Na^+-PHOSPHORYLATION, AND K^+-*p*-NITROPHENYLPHOSPHATASE OF *ELECTROPHORUS ELECTRICUS* ELECTROPLAX[1]

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Abstract—Zinc ion in micromolar concentrations is an irreversible inhibitor of *Electrophorus electricus* electroplax microsomal (Na^+-K^+)-ATPase. The rate of inhibition is dependent on $[ZnCl_2]$ and the extent of inhibition varies with the ratio of $ZnCl_2$ to microsomal protein. The same kinetics are observed for inhibition of K^+-*p*-nitrophenylphosphatase and steady-state levels of Na^+-dependent enzyme phosphorylation. The observations suggest that a Zn^{2+}-sensitive conformational restraint is important to both kinase and phosphatase activities. The fact that inhibition is irreversible has implications for models seeking to relate zinc effects in tissue to inhibition of (Na^+-K^+)-ATPase.

ZINC IS an important constituent of many tissues and is a component of more than 70 metalloenzymes. Zn^{2+} may be an activator or inhibitor of a large number of enzymes (see VALLEE, 1977 for references). Zinc is also present in DNA and RNA, in which it is believed to have a structural role (RIORDAN, 1976). There is little information regarding the effects of Zn^{2+} on cation-stimulated ATP phosphohydrolases. Inhibition of Ca^{2+}-stimulated phosphorylation and Ca^{2+}-stimulated ATPase activity of erythrocyte membranes has been reported (KRUCKEBERG & BREWER, 1977). This effect is believed correlated with reduction in the proportion of irreversibly sickled cells when zinc is used as therapy for sickle cell anemia. In addition, it has been reported that zinc inhibits the (Na^+-K^+)-ATPase (EC 3.6.1.3) of brain (DONALDSON *et al.*, 1971; PRAKASH *et al.*, 1973; HEXUM, 1974). One possible significance of this effect is that zinc when introduced into rat cerebral ventricles produces seizures and other central nervous system disturbances (DONALDSON *et al.*, 1971). We have investigated the kinetics of inhibition and the effects of zinc ion on the partial reactions of (Na^+-K^+)-ATPase from the electric organ of *Electrophorus electricus*. It is found that zinc in micromolar concentrations is an irreversible inhibitor of (Na^+-K^+)-ATPase, Na^+-dependent enzyme phosphorylation, and K^+-NPPase activities. No evidence is observed for interaction between Zn^{2+} and the physiologic ligands.

MATERIALS AND METHODS

Electrophorus electricus was obtained from World Wide Scientific Animals, Ardsley NY. Microsomal fractions of electric organ from *E. electricus* were prepared as previously described (SIEGEL & FOGT, 1977). Measurements of zinc in the whole electroplax and microsomal preparations, generously performed by Dr. K. S. RAJAN, IIT Research Institute, Chicago, showed 911.4 nmol/g dry weight and 6.1 pmol/μg protein, respectively. Tris salts of ATP and of NPP and Tris buffer were obtained from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was obtained from International Chemical and Nuclear Corp. (Irvine, CA). Protein concentrations were determined by the method of LOWRY *et al.* (1951) with bovine serum albumin (Sigma) as the protein standard. Preliminary measurements of enzyme inhibition due to $ZnCl_2$ in imidazole buffer showed that imidazole has a protective effect on the zinc inhibition of the enzyme. Assays were therefore performed in Tris buffer which did not show this effect. Assays were performed in triplicate unless otherwise indicated. All values for percent of inhibition are relative to enzyme activity in control samples which were treated identically with the omission of $ZnCl_2$.

Assay of (Na^+-K^+)-*ATPase.* Microsomes, 4 μg of protein, were exposed in 35 μl of 75 mM Tris–HCl (pH 7.4) to $ZnCl_2$ at 26°C. Specific concentrations of $ZnCl_2$ and exposure times are noted in individual experiments. The pre-treated samples were returned to an ice bath and 5 μl containing Tris–[γ-^{32}P]ATP, $MgCl_2$, NaCl, and KCl were added. Final concentrations in standard assay media were: 3 mM-Tris–[γ-^{32}P]ATP (2×10^3 c.p.m./nmol), 3 mM-$MgCl_2$,

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Abbreviations used: BAL, 2,3-dimercaptopropanol; K^+-NPPase, K^+-stimulated *p*-nitrophenylphosphatase; (Na^+-K^+)-ATPase, (Na^+-K^+)-stimulated ATP phosphohydrolase.

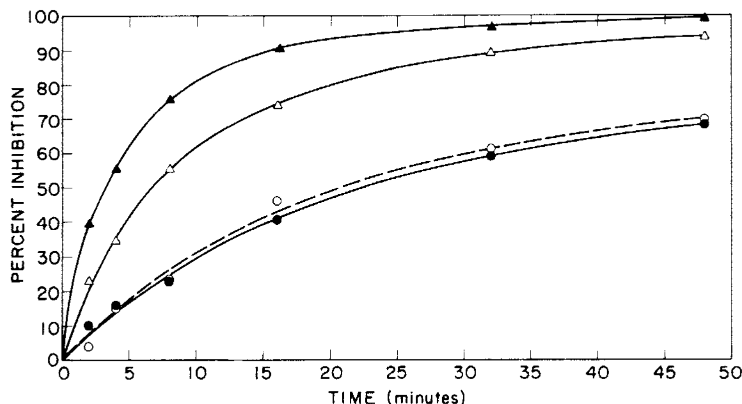


FIG. 1. $ZnCl_2$ inhibition of (Na^+-K^+)-ATPase and of K^+-NPPase. Microsomes were exposed to various amounts of $ZnCl_2$ for various times prior to assay of ATPase or NPPase activity as described in Materials and Methods. The abscissa shows the time of pretreatment of microsomes with $ZnCl_2$ prior to the 5 min assay period. In control samples, (Na^+-K^+)-ATPase activity was 0.914 initially and decreased gradually to 0.783 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ while K^+-NPPase activity was 0.155 initially and decreased gradually to 0.126 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ during the 48 min of pretreatment without $ZnCl_2$. Final concentrations of $ZnCl_2$ in ATPase assays were: ●, 1 μM-$ZnCl_2$; △, 2 μM-$ZnCl_2$; ▲, 3 μM-$ZnCl_2$, or 10, 20, and 30 pmol $ZnCl_2/\mu g$ of microsomal protein, respectively. ○---○, inhibition of NPPase by 1 μM-$ZnCl_2$ (10 pmol/μg protein).

80 mM-NaCl, and 20 mM-KCl. Na^+ and K^+ were omitted for assays of Mg^{2+}-ATPase. Hydrolysis was allowed to proceed for 5 min at 26°C. Then samples were returned to the ice bath and 10 μl of 5% (w/v) ammonium molybdate in 4N-H_2SO_4 were added. Phosphomolybdate was extracted into isobutanol and ^{32}P was measured as described (SIEGEL & ALBERS, 1967).

Assay of steady-state levels of microsomal phosphorylation. Microsomes, 100 μg of protein, were exposed in 40 μl of 75 mM-Tris–HCl (pH 7.4) to $ZnCl_2$ at 26°C for specified intervals. The preincubated microsomes were returned to an ice bath and 10 μl containing Tris-[γ-^{32}P]ATP, $MgCl_2$, and NaCl were added. Final concentrations were: 1 mM-Tris-[γ-^{32}P]ATP (2×10^4 c.p.m./nmol), 3 mM-$MgCl_2$, with or without 80 mM-NaCl. Phosphorylation was allowed to proceed for 15 s at 22°C or 45 s at 2°C before the addition of 50 μl of 10% (w/v) trichloroacetic acid. The acid-stable, non-exchangeable ^{32}P retained in the precipitated protein was measured as described (SIEGEL & ALBERS, 1967). The Na^+-dependent phosphorylation has been shown to involve the catalytic site of (Na^+-K^+)-ATPase (DAHL & HOKIN, 1974).

Assay of K^+*-NPPase.* Microsomes, 4 μg of protein, were exposed in 35 μl of 75 mM-Tris-HCl (pH 7.4) to $ZnCl_2$ at 26°C for specified intervals. The samples were returned to an ice bath and 5 μl containing Tris-NPP, $MgCl_2$, and KCl were added. Final concentrations in the standard assay media were: 10 mM-NPP, 5 mM-$MgCl_2$, and 20 mM-KCl. K^+ was omitted for assays of Mg^{2+}-NPPase. After incubation for 20 min at 26°C, the tubes were returned to the ice bath and 200 μl of 0.2 N-NaOH were added. After sedimentation of $Mg(OH)_2$ at 1800 g for 15 min, the concentrations of p-nitrophenol in the supernatant portions were determined spectrophotometrically at 420 nm. In an experiment designed to compare rates of ATPase and NPPase inhibition (Fig. 1) volumes were tripled and the assay incubation was terminated after 5 min, as for ATPase. NaOH was adjusted accordingly. The K^+-NPPase activity is believed to represent the phosphatase moiety of (Na^+-K^+)-ATPase (DAHL & HOKIN, 1974).

RESULTS

Effects of $ZnCl_2$ *on* (Na^+-K^+)*-ATPase and* K^+*-NPPase activities*

In preliminary experiments, little inhibition of Mg^{2+}-ATPase or of Mg^{2+}-NPPase activities could be demonstrated under conditions in which the Na^+ and K^+ increments were abolished. These activities represented less than 3–6% of total activities in the presence of appropriate concentrations of Na^+ and K^+. In addition, $ZnCl_2$ in the concentrations used did not affect nonenzymatic blank values for either the ATPase or NPPase methods or the absorbancy of p-nitrophenol.

As shown in Fig. 1, exposure of microsomes to $ZnCl_2$ results in progressive inhibition of (Na^+-K^+)-ATPase activity. The percent of inhibition is dependent on the duration of exposure and on the amount of $ZnCl_2$ present in the exposure medium. Eventually almost complete inhibition (to the level of the Mg^{2+} base-line) may be obtained with even 2–3 μM-$ZnCl_2$. In addition, Fig. 1 shows that exposure of microsomes to 1 μM-$ZnCl_2$ produces inhibition of K^+-NPPase activity with a time course identical to that for inhibition of (Na^+-K^+)-ATPase activity. It is clear from these data that the formation of a Zn–enzyme complex leads to inhibition. Since the kinetics are identical, a single Zn^{2+} binding site may be assumed for the inhibition of both ATPase and NPPase activity.

Figure 1 shows that the exposure times necessary to achieve 50% inhibition of (Na^+-K^+)-ATPase activity are 22.5, 6.8, and 3.2 min at 1, 2, and 3 μM-$ZnCl_2$, respectively. If the rate-limiting reaction leading to inhibition were first order with respect to Zn^{2+}, one would not expect the $t_{\frac{1}{2}}$ values to vary

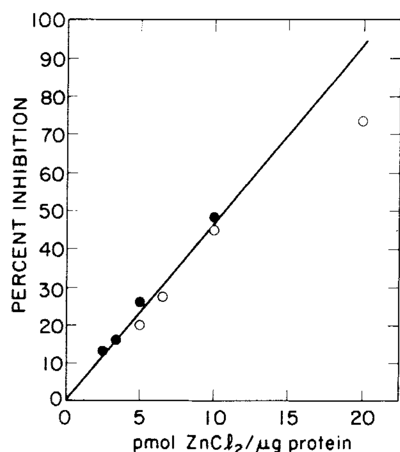


FIG. 2. $ZnCl_2$ inhibition of (Na^+–K^+)-ATPase. Microsomes, 4, 8, 12 and 16 µg protein, were exposed to $ZnCl_2$ for 20 min at 26°C and ATPase assays were performed as described in Materials and Methods. ●, 1 µM-$ZnCl_2$; ○, 2 µM-$ZnCl_2$.

inversely with the [Zn^{2+}]. Although the pseudo-first order kinetic plots of these data for each calculated zinc ion concentration are, in fact, linear, the slopes thus obtained are not linearly related to the calculated zinc ion concentrations. It was considered that there might be significant binding of Zn^{2+} to the microsomes which would invalidate the use of calculated [Zn^{2+}] values in determining rate constants. In order to evaluate this possibility, percent inhibition was measured with various amounts of microsomal protein exposed to two different concentrations of $ZnCl_2$. Figure 2 shows that the extent of inhibition of (Na^+–K^+)-ATPase activity is linear with respect to the ratio of total $ZnCl_2$ to microsomal protein at least up to 10 pmol/µg under these conditions. This graph may be extrapolated to 100% inhibition at 20 pmol/µg, which value agrees with the data in Fig. 1 showing that 20 pmol/µg will produce almost complete inhibition. This is an approximate value since it is difficult to obtain true equilibrium measurements and since it is likely that Zn^{2+} binds also to non-enzyme protein. These data show, however, that there is significant binding of Zn^{2+} to microsomes and that increasing the quantity of microsomes decreases the percent inhibition for a given concentration of $ZnCl_2$. Therefore actual rate constants for inhibition could not be determined without measurements of free Zn^{2+} activity. For this microsomal preparation, 50% inhibition is produced by 10 pmol $ZnCl_2$/µg protein in about 20 min at 26°C (Figs. 1 and 2).

We were interested in whether any presumed allosteric changes produced by the physiologic regulatory cations or their competition with Zn^{2+} for binding sites might be detected through an effect on the enzyme reaction with Zn^{2+}. Table 1 shows that the presence of Mg^{2+}, Na^+, and K^+ together with microsomes and $ZnCl_2$ in the exposure mixture had no effect on the inhibition produced by $ZnCl_2$. Time curves obtained as in Fig. 1 with and without exposure to 3 mM-$MgCl_2$ together with $ZnCl_2$ also showed no difference. In a series of other experiments performed as in Materials and Methods but without pre-exposure of microsomes to $ZnCl_2$, there was no significant effect of 7 µM-$ZnCl_2$ added directly to the incubation media on the half-maximal activation concentrations determined for Mg^{2+}, Na^+, or K^+ with ATP as substrate, or K^+ with NPP as substrate. The enzyme activity was inhibited approx 50% by this concentration of $ZnCl_2$ without pretreatment and the level of inhibition in each experiment was fairly constant for the various concentrations of each ligand. These data indicate that Mg^{2+}, Na^+, and K^+ in the incubation media do not alter either the rate of development or extent of Zn^{2+} inhibition. Thus, we found no evidence for an interaction of these ligands with Zn^{2+}.

We were interested in testing the possibility that the formation of a $ZnATP^{2-}$ complex might alter the proportion of inhibition. For this purpose the complete reaction mixture containing ATP was exposed to 7.5 mM-$ZnCl_2$ at 22°C for various times before the addition of microsomes. Hydrolysis was then measured over a 5 min period. The percent inhibition was 35, 30, 24, and 14% in the reaction mixtures exposed to $ZnCl_2$ for 0, 1, 5, 10, and 20 min, respectively. If anything, the prior exposure of ATP to $ZnCl_2$ under these assay conditions decreases the inhibition to a small extent.

Effects on steady-state microsomal phosphorylation

As shown in Table 2, exposure of microsomes to

TABLE 1. $ZnCl_2$ INHIBITION OF (Na^+–K^+)-ATPase

Preincubation (min)	Other cations	Zero $ZnCl_2$	2 µM $ZnCl_2$	7 µM $ZnCl_2$	$ZnCl_2$ Inhibition 2 µM	7 µM
		(nmol/mg/min)			(%)	
0		810	710	614	12	24
15	−	579	144	60	75	90
15	+	664	167	48	75	92

Microsomes were exposed to $ZnCl_2$ either without (−) or with (+) 3 mM-$MgCl_2$, 80 mM-NaCl, and 20 mM-KCl in the exposure medium for 15 min at 26°C.

TABLE 2. $ZnCl_2$ INHIBITION OF Na^+-DEPENDENT PHOSPHORYLATION

$ZnCl_2$ (μM)	$MgCl_2$ only (pmol P/mg protein)	$MgCl_2$ and NaCl (pmol P/mg protein)	Inhibition by $ZnCl_2$ of Δ NaCl (%)
0	39	261	
10		254	10
20	26	232	13
40		134	57
80	39	32	100

Microsomes, 100 μg protein, were exposed to $ZnCl_2$ in 45 μl containing 75 mM-Tris–HCl (pH 7.4) for 15 min at 22°C. Phosphorylation was then measured in the presence of 3 mM-$MgCl_2$ without and with 100 mM-NaCl as described in Materials and Methods. Inhibition of the non Na^+-dependent portion was not significant.

increasing amounts of $ZnCl_2$ resulted in progressively lower levels of Na^+-dependent phosphoenzyme and little or no significant change in the non-specific Mg^{2+}-dependent phosphorylation level. During this 15 min exposure, a ratio of 20 pmol $ZnCl_2$/μg protein (40 μM-$ZnCl_2$) produced 57% inhibition of the Na^+-increment.

To establish the relation of (Na^+–K^+)-ATPase inhibition to inhibition of phosphorylation, microsomes were exposed to $ZnCl_2$ under conditions to produce about 50% inhibition of ATPase activity (10 pmol $ZnCl_2$/μg protein) for 20 min at 26°C. The microsomes were then divided into portions for simultaneous determinations of ATPase activity and steady-state phosphorylation levels. As shown in Table 3, the inhibitions of both activities are similar, 50 and 42%, respectively. These data indicate that the inhibition of enzyme phosphorylation could account for the inhibition of ATP hydrolysis.

Reversibility of zinc inhibition

To determine the reversibility of zinc inhibition of ATPase activity, microsomes were exposed to $ZnCl_2$ and then washed by sedimentation and resuspension. Washed samples were exposed to various metal ion chelating agents, including ATP, EDTA, and BAL. These substances frequently protect the enzyme against denaturation and raise the specific activity. Table 4 shows that although exposure of microsomes to these substances increased the specific enzyme activity in all samples, two such wash treatments did not change the percent inhibition due to added zinc ion. It is not known, however, whether the zinc remained bound to the sedimented microsomes.

Similarly, in other portions removed from these same samples, the zinc ion inhibition of NPPase activity was not reversed by two washings and exposure to EDTA or BAL, as shown in Table 5.

Finally, in a separate experiment, there was no reversal of zinc ion inhibition of steady-state microsomal phosphorylation after a single washing and exposure to BAL, as seen in Table 6.

DISCUSSION

The mechanism of inhibition of (Na^+–K^+)-ATPase by Zn^{2+} is of interest from two viewpoints: (1) the possible implications regarding the enzyme catalytic mechanism and (2) the possible implications regarding biochemical effects of zinc ion in tissue.

(Na^+–K^+)-ATPase reaction model

There is substantial evidence showing that (Na^+–K^+)-ATPase involves kinase, phosphoryl acceptor, and phosphatase functions (ALBERS *et al.*, 1968; DAHL & HOKIN, 1974). For the purpose of this discussion,

TABLE 3. SIMULTANEOUS INHIBITION BY $ZnCl_2$ OF (Na^+–K^+)-ATPase AND MICROSOMAL PHOSPHORYLATION

	ATPase (nmol/mg/min)	Phosphorylation (pmol P/mg protein)
Preincubated with:		
zero $ZnCl_2$	959	181
1 μM-$ZnCl_2$	481	105
	(%)	(%)
Inhibition	50	42

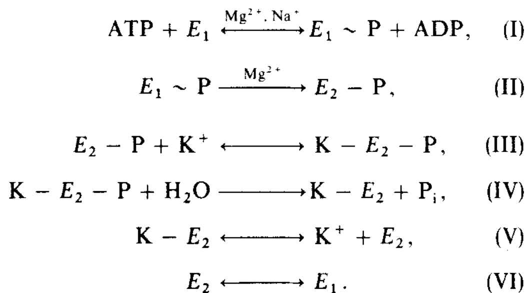
Microsomes, 100 μg protein, were exposed in 700 μl of 25 mM-Tris–HCl (pH 7.4) without or with 1.13 μM-$ZnCl_2$ (10 pmol Zn^{2+}/μg protein) for 20 min at 26°C. A portion of 35 μl containing 4 μg protein from each sample was transferred immediately to 5 μl of reaction mixture for assay of (Na^+–K^+)-ATPase activity for 5 min as described in Materials and Methods. To the remainders, 100 μl of ice cold phosphorylation reaction mixture was immediately added. Final concentrations were described in Materials and Methods except that ATP was 0.1 mM (3×10^7 c.p.m./μmol). Phosphorylation was carried out for 45 s at 2°C before the addition of 100 μl of 50% trichloroacetic acid. Protein was sedimented at 1800 *g* for 30 min and then treated as described in Materials and Methods. The percent inhibition developing during the 5 min hydrolysis assay is not subtracted.

TABLE 4. IRREVERSIBILITY OF $ZnCl_2$ INHIBITION OF (Na^+-K^+)-ATPase

	Assay condition	Control	Exposed to 80 μM-$ZnCl_2$	Inhibition (%)
		(nmol/mg/min)		
Unwashed		1309	252	81
1st wash		1335	370	72
	ATP	1906	372	81
	EDTA	2113	710	66
	BAL	2210	529	76
2nd wash		360	77	79
	ATP	483	65	87
	EDTA	699	131	81
	BAL	783	132	83

Microsomes, 500 μg protein, were exposed in 500 μl of 75 mM-Tris–HCl (pH 7.4) with and without 80 μM-$ZnCl_2$ (80 pmol Zn^{2+}/μg protein) for 20 min at 26°C. Duplicate portions of 2 μl were removed for assays of ATPase activity under conditions described in Materials and Methods except that protein was 2 μg and incubations were for 10 min. Portions of 4 μl were removed for NPPase assays (Table 5). The remainders were diluted to 5.3 ml with 75 mM-Tris–HCl (pH 7.4) and centrifuged at 50,000 g for 30 min at 2°C. Pellets were resuspended in 475 μl of 75 mM-Tris–HCl (pH 7.4) and duplicate portions of 2 μl were removed for ATPase assays. The remainders were again diluted to 5.3 ml and centrifuged as before. The pellets were finally resuspended in 400 μl of 75 mM-Tris–HCl (pH 7.4) and duplicate portions of 2 μl were removed for ATPase assays. Protein was measured in all resuspended pellets. Samples from resuspended pellets for ATPase assays were preincubated for 15 min in 35 μl of 75 mM-Tris–HCl (pH 7.4) alone or with 1 mM-EDTA, 1 mM-BAL, or 3 mM-ATP before the addition of 5 μl of reaction mixture.

the proposed reaction sequence is briefly summarized (SIEGEL & GOODWIN, 1972):



$$ATP + E_1 \xleftrightarrow{Mg^{2+}, Na^+} E_1 \sim P + ADP, \quad (I)$$

$$E_1 \sim P \xrightarrow{Mg^{2+}} E_2 - P, \quad (II)$$

$$E_2 - P + K^+ \longleftrightarrow K - E_2 - P, \quad (III)$$

$$K - E_2 - P + H_2O \longrightarrow K - E_2 + P_i, \quad (IV)$$

$$K - E_2 \longleftrightarrow K^+ + E_2, \quad (V)$$

$$E_2 \longleftrightarrow E_1. \quad (VI)$$

It is believed that NPP substitutes for the enzyme phosphate in the K^+-stimulated reaction with E_2 (IV). This model assumes that the difference between E_1 and E_2 is in a conformational restraint that is reversible during the cycle for complete hydrolysis of ATP (FAHN *et al.*, 1966). There may be, however, multiple conformational restraints important to catalytic function and certain of these may be common to both E_1 and E_2 (SIEGEL & FOGT, 1977). The model does not indicate, nor is it known, whether E_2 can function as a phosphatase independently of another E_1 unit or without exhibiting some E_1 character even in the absence of Na^+ and ATP.

TABLE 5. IRREVERSIBILITY OF $ZnCl_2$ INHIBITION OF K^+-NPPase

	Assay condition	Control	Exposed to 80 μM-$ZnCl_2$	$ZnCl_2$ Inhibition (%)
		(nmol/mg/min)		
Unwashed		85	5	94
1st wash		62	5	92
	EDTA	96	24	75
	BAL	111	23	79
2nd wash		29	5	83
	EDTA	59	9	85
	BAL	71	21	70

This experiment was performed as described in Table 4. Portions of 4 μl were removed at each step for assays of NPPase activity as described in Materials and Methods except that incubations were for 30 min.

TABLE 6. IRREVERSIBILITY OF $ZnCl_2$ INHIBITION OF PHOSPHORYLATION

Assay condition	Control (pmol P/mg protein)	Exposed to 400 μM-$ZnCl_2$ (pmol P/mg protein)	Inhibition (%)
Unwashed	274	27	90
Washed	423	0	100
BAL	658	79	88

Microsomes, 2 mg protein, were exposed in 400 μl of 75 mM-Tris–HCl (pH 7.4) with and without 400 μM-$ZnCl_2$ (80 pmol Zn^{2+}/μg protein) for 20 min at 26°C. Duplicate portions of 20 μl were removed for assays of steady-state phosphorylation as described in Materials and Methods except that incubations were for 15 s at 22°C. The remainders were diluted to 5.3 ml with 75 mM-Tris–HCl (pH 7.4), and centrifuged at 50,000 *g* for 30 min at 2°C. Pellets were resuspended in 280 μl of 75 mM-Tris–HCl (pH 7.4) and duplicate portions of 20 μl were removed for phosphorylation assays. Protein was measured in the resuspended pellets. Samples from resuspended pellets were preincubated for 15 min at 2°C in 40 μl of 75 mM-Tris–HCl (pH 7.4) plus and minus 1 mM-BAL before the addition of 10 μl of reaction mixutre.

It is found that Zn^{2+} in micromolar concentrations is an irreversible inhibitor of (Na^+–K^+)-ATPase, K^+-NPPase, and Na^+-dependent enzyme phosphorylation activities. The kinetics of inhibition are similar for the three measured enzyme activities, thus allowing the parsimonious assumption that a single binding site for Zn^{2+} is involved.

Since enzyme phosphorylation is inhibited by Zn^{2+}, an earlier suggestion (HEXUM, 1974) that Zn^{2+} inhibits conversion of $E_1 \sim P$ to $E_2 - P$ (II) is not tenable. Inhibition of the kinase or an action of Zn^{2+} at the phosphoryl acceptor site of E_1 preventing enzyme phosphorylation (I) could easily account for inhibition of ATP hydrolysis. It is more difficult to understand, however, how inhibition of the kinase activity could lead to inhibition of K^+-NPPase activity in terms of this model. One possibility is that NPP hydrolysis also proceeds through the same phosphoenzyme formation as does ATP hydrolysis. This possibility, however, is not supported by available evidence. Phosphorylation of native brain enzyme preparations by [^{32}P]NPP could not be demonstrated except at low pH (ROBINSON, 1971; INTURRISI & TITUS, 1970). Moreover, ouabain, which inhibits NPPase as well as ATPase and phosphorylation by [γ-^{32}P]ATP (ALBERS *et al.*, 1968), was found to stimulate phosphorylation by [^{32}P]NPP, at least part of which could be contributed by $^{32}P_i$ present in the samples (INTURRISI & TITUS, 1970). If NPP itself phosphorylates the ouabain–enzyme complex, it is not through the ATP dependent path (I), which is inhibited by ouabain.

The alternative hypothesis regarding the action of Zn^{2+} is that some conformational property of the enzyme which is critical in common to both kinase (E_1) and phosphatase (E_2) catalytic mechanisms is susceptible to modification by Zn^{2+}. Since the inhibition by Zn^{2+} is irreversible, some understanding of this property and of the enzyme group reactive with zinc might be obtained through studies of zinc binding to the purified enzyme.

It is of further interest that several inhibitory divalent cations have qualitatively different effects on phosphorylation. While Zn^{2+} inhibits phosphorylation, Pb^{2+} stimulates phosphorylation in the absence of Na^+ (SIEGEL & FOGT, 1977), and Ca^{2+} stimulates Na^+-dependent phosphorylation (TOBIN *et al.*, 1975). The kinase, presumably, depends on a number of different conformational restraints which can be selectively modified by these cations.

Zn^{2+} *Effects in tissue*

Several of the observations regarding Zn^{2+} inhibition of (Na^+–K^+)-ATPase *in vitro* are of possible significance regarding zinc ion effects in tissues. The electroplax microsomal preparation employed in this study contained 6.1 pmol of zinc/μg protein, which is similar to quantities of zinc found in portions of brain (DONALDSON *et al.*, 1973; RAJAN *et al.*, 1976). These quantities are close to values of added zinc required for electroplax enzyme inhibition *in vitro* (10 pmol/μg for 50% inhibition). The potency of Zn^{2+} as an inhibitor of brain (Na^+–K^+)-ATPase *in vitro* is also within the same order as shown by the available data (DONALDSON *et al.*, 1971; HEXUM, 1974). The question arises as to whether a significant proportion of the (Na^+–K^+)-ATPase *in vivo* exists in a state of inhibition due to endogenous zinc.

Much of the tissue zinc may be presumed to be tightly bound and not available to produce inhibition of the enzyme. Protective effects of membranes and proteins are shown, for examples, by the present data in which increasing quantities of microsomes decreases the proportion of inhibition due to zinc (Fig. 2), and in spermatozoa by the protective effect of albumin against zinc toxicity (LINDHOLMER, 1974). Nevertheless, different subcellular fractions exhibit various proportions of tightly bound zinc (RAJAN *et al.*, 1976*a*)

and the amount of free Zn^{2+} with access to the enzyme *in vivo* is not known.

The present study shows that zinc is an irreversible inhibitor. This observation is supported by and helps to explain the failure of catechol amines to reverse Zn^{2+} inhibition (HEXUM, 1977) despite the high affinity of these amines for binding Zn^{2+} (RAJAN *et al.*, 1976*b*) and the residual inhibition of enzyme activity in extracted cerebral microsomes after *in vivo* administration of Zn^{2+} (DONALDSON *et al.*, 1971). We must suppose, therefore, that any free Zn^{2+} that does gain access to enzyme sites *in vivo* produces permanent inactivation.

It has been suggested that certain physiologic effects of catechol amines might be mediated through disinhibition of (Na^+-K^+)-ATPase by their chelation of divalent cation inhibitors of this enzyme, possibly including Cu^{2+}, Zn^{2+}, Ca^{2+}, and Fe^{2+} (HEXUM, 1977). The observations described above indicate, however, that if applied to Zn^{2+}, this model obligatorily would refer the protective effect of chelators to newly synthesized enzyme and would not account for rapid effects of the chelator.

If the production of seizures by intraventricularly administered zinc in rats is due to the inhibition of (Na^+-K^+)-ATPase (DONALDSON *et al.*, 1971), then one must consider the possibility that spontaneous decreases in tissue zinc binding components locally within the cortex under pathologic conditions could lead to a focus of hyperexcitability through allowing sufficient Zn^{2+} to inhibit the (Na^+-K^+)-ATPase locally.

Finally, the facts that Zn^{2+} inhibition is relatively slow to develop, irreversible, and dependent on the concentration of Zn^{2+} as well as on the ratio of Zn^{2+} to microsomal protein are important factors in designing experiments to compare different effects of Zn^{2+} to inhibition of (Na^+-K^+)-ATPase.

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