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STUDIES ON THE REACTION CATALYZED BY TRANSPORT (Na, K) ADENOSINE TRIPHOSPHATASE—I

EFFECTS OF DIVALENT METALS*

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Abstract—The effects of divalent metals (Cu^{2+}, Zn^{2+}, Fe^{2+} and Pb^{2+}) on a microsomal preparation of NaK-ATPase (ATP phosphohydrolase, EC 3.6.1.3) from beef cerebral cortex were studied. These metals are all potent inhibitors of the enzyme with I_{50} values of 1 μM for Cu^{2+} and Zn^{2+}, 3 μM for Fe^{2+} and 20 μM for Pb^{2+}. Kinetic studies examining the effect of low concentrations of divalent metals on K_m and V for MgATP are reported. The results indicate that Fe^{2+} and Pb^{2+} are competitive inhibitors of NaK-ATPase with K_i values of 1·60 μM and 1·90 μM respectively. Cu^{2+} and Zn^{2+} are noncompetitive inhibitors of NaK-ATPase with K_i values of 1·18 μM and 3·48 μM respectively.

THE SODIUM–POTASSIUM-stimulated adenosine triphosphatase [ATP phosphohydrolase, EC 3.6.1.3 (NaK-ATPase)] is an integral part of the sodium pump and is responsible for the transport of Na out of, and K into, animal cells (for recent reviews, see Refs. 1 and 2). This enzyme is involved in many physiological functions, such as excitability of muscles and nerves, control of salt balance by the kidney, enzyme activation and fluid regulation. Several drugs have been shown to modify the activity of NaK-ATPase.[2] Recent studies[3–7] have shown that certain divalent metals of toxicological interest are potent inhibitors of NaK-ATPase. The mechanism by which these divalent metals inhibit NaK-ATPase has not been determined. As a result, it is not known whether all inhibitory divalent metals affect NaK-ATPase through the same mechanism. The primary purpose of this report is to describe the mechanism(s) by which selected divalent metals (Pb^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+}) inhibit NaK-ATPase.

MATERIALS AND METHODS

The chloride salts of all reagents were used except in the case of zinc. The inhibition by the chloride salt of zinc was found to decrease with time after addition to the reaction mixture (cf. Ref. 7). This problem was circumvented with the use of zinc acetate (Fisher Scientific). Sodium, potassium, magnesium, lead, ferrous and cupric chlorides were obtained from Mallinckrodt Chemical Works. ATP, Tris and ouabain were obtained from Sigma Chemical Co. Divalent metal solutions were prepared just

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prior to use. ATP was converted to the Tris salt by passage through a Dowex-50 column (H^+ form) and titrated to pH 7·0 with Tris. Protein was measured according to the method of Lowry *et al.*[8] using bovine serum albumin, Cohn fraction V, as the standard. Water was glass triple distilled and all glassware was cleaned in chromic acid.

NaK-ATPase was prepared from beef cerebral cortex by a modification of the procedure of Schwartz, *et al.*[9] Immediately upon removal from the animal, the brain was placed on crushed ice. In a cold room, the pia mater and clots were removed, the tissue was rinsed in cold distilled water and the grey matter from the cortex was cut off with scissors. To each 60 g of grey matter, 0·32 M sucrose containing 1 mM NaEDTA, pH 7·0, was added to a total volume of 600 ml. This mixture was homogenized in a Waring commercial blender at a speed setting of 5 for 60 sec. Faster or slower speed settings resulted in enzyme inactivation or inadequate homogenization respectively. The homogenate was centrifuged at 600 *g* for 10 min. All centrifugations were carried out using a Sorvall model RC2-B centrifuge using the GSA rotor. The supernatant was carefully decanted (pellet discarded) and centrifuged at 10,000 *g* for 15 min. The supernatant from the second centrifugation was carefully decanted (pellet discarded) and centrifuged at 20,000 *g* for 90 min. The supernatant from the third centrifugation was carefully decanted and discarded. The pellet, corresponding to 60 *g* of starting material, was washed once by suspending it in 450 ml of 0·32 M sucrose containing 1 mM NaEDTA, pH 7·0 using a Waring blender at a speed setting of 5 for 30 sec. This suspension was centrifuged at 20,000 *g* for 90 min. The pellet obtained from this centrifugation was suspended (seven passes at 5000 rev/min) in cold distilled water using a Potter–Elvehjem homogenizer (clearance: 0·006 in.) and used in the following procedure or stored at −70°. This preparation is hereafter referred to as microsomes. The specific activity of a typical preparation of microsomes was 25 μmoles Pi/mg protein/hr.

The microsomes were subsequently treated with sodium iodide to remove MgATPase, as described by Nakao *et al.*[10] with the following modifications: (1) magnesium chloride was omitted from the sodium iodide solution, and (2) the pellet obtained after treatment with sodium iodide was washed twice with 5 mM NaEDTA, pH 7·0, followed by two washes with 5 mM NaCl (Chelex resin treated). The magnesium chloride was omitted to reduce the likelihood of Mg^{2+} carryover with the enzyme. The NaEDTA wash was included to remove extraneous divalent metals present in the sodium iodide solution. Washes with 5 mM NaCl were included to remove traces of NaEDTA. The final washed pellet was resuspended in distilled water and referred to as NaI-enzyme. The specific activity of the NaI-enzyme was usually 55–65 μmoles Pi/mg protein/hr. Treatment with sodium iodide effectively reduced MgATPase activity to a level which was not detectable in the kinetic experiments.

Contamination of assay reagents by divalent metals could give rise to spurious results in the kinetic studies. Removal of contaminating divalent metals from the sodium, potassium and Tris solutions was accomplished using Chelex resin. Chelex 100 resin was prepared for use as described by Willard *et al.*[11] where the resin is first swelled in 7 N NH_4OH and then washed with 0·1 N HCl. Solutions to be treated with Chelex were made up at a concentration ten times that required for a given procedure. The concentrated solution was added to the Chelex resin from which most of the water had been removed by filtration over a suction flask. Then, (a) the mixture

was stirred for 10 min, (b) the filtrate was removed by suction, (c) the resin was washed several times, and (d) the washes were combined with the original filtrate until a 10-fold dilution of the original filtrate had resulted. Chelex-treated reagents were always compared to untreated reagents at the same concentration and found to consistently yield higher specific activities (T. D. Hexum, unpublished observation).

NaK-ATPase activity was determined by measuring the amount of P_i produced. Each reaction tube contained in a final volume of 1·0 ml: 30 mM Tris–HCl, pH 7·5; 130 mM NaCl; 20 mM KCl; 30–40 μg NaI-enzyme; 6 mM $MgCl_2$ and 4 mM Tris–ATP. In the kinetic experiments, divalent metal was added as indicated in the figures; $MgCl_2$ and Tris–ATP were varied at a constant ratio of 1·5 (Mg/ATP). Ouabain-sensitive (NaK-ATPase) activity was determined by subtracting the amount of P_i produced in the presence of 10^{-4} M ouabain from the amount of P_i produced in the absence of ouabain. MgATPase was determined from the amount of P_i released in the presence of ouabain after correction for endogenous P_i. All determinations were made in duplicate.

After all additions except ATP were made, the reaction tubes were incubated at 37° for 3 min in a shaking bath. The reaction was initiated with ATP unless otherwise indicated, and terminated after 5 min by the addition of 1 ml of cold 10% trichloroacetic acid. Tubes were centrifuged to remove precipitated protein and aliquots taken and analyzed for P_i using a modified Fiske–Subbarow method.[12] Data from the kinetic studies are presented as Lineweaver–Burk plots with slopes and intercepts being obtained by the method of least squares. Elements of a normal equation for least squares fit were generated using program code 15.01 for an Olivetti Underwood Programma 101 desk top computer.

RESULTS AND DISCUSSION

Conventionally, the NaK-ATPase reaction is started with the addition of enzyme. However, the degree of inhibition occurring at a given concentration of Cu^{2+} depended on the time between addition of inhibitor and the initiation of the reaction with enzyme. Table 1 shows the effect of preincubating Cu^{2+} with the assay mixture containing ATP on inhibition of NaK-ATPase. The enzyme was added to the reaction mixture at various times after the addition of Cu^{2+}. The data in Table 1 show that decreased inhibition of NaK-ATPase by Cu^{2+} occurs as the interaction time between Cu^{2+} and the reaction mixture increases. The reaction constituent with

TABLE 1. EFFECT OF TIME OF ADDITION OF Cu^{2+} TO REACTION MIXTURE (PLUS ATP) FOR NaK-ATPASE

Time* (min)	NaK-ATPase activity† (μmoles/mg/hr)
No Cu^{2+}	66·7
9	17·4
6	14·1
3	8·1
0·25	2·0

* At various times, 10 μM Cu^{2+} was added to the tubes as indicated. The tubes were allowed to stand for the appropriate times and then the reaction was started by the addition of beef brain NaI-enzyme. Time is the period between addition of Cu^{2+} and initiation of the reaction.

† Tubes contained: 130 mM Na^+; 20 mM K^+; 6 mM Mg^{2+}; 30 mM Tris–HCl, pH 7·5; 3 mM NaATP; and ±0·1 mM ouabain.

TABLE 2. EFFECT OF TIME OF ADDITION OF Cu^{2+} TO REACTION MIXTURE (MINUS ATP) FOR NaK-ATPASE

Time* (min)	NaK-ATPase activity† (μmoles/mg/hr)
No Cu^{2+}	82·8
9	4·0
6	6·7
3	4·0
0·25	6·7

* At various times, 10 μM Cu^{2+} was added to the tubes as indicated. The tubes were allowed to stand for the appropriate times and then the reaction was started by the addition of 3 mM NaATP. Time is the period between addition of Cu^{2+} and initiation of the reaction.

† Tubes contained: 130 mM Na^+; 20 mM K^+; 6 mM Mg^{2+}; 30 mM Tris–HCl, pH 7·5; 30–40 μg NaI-enzyme; and ±0·1 mM ouabain.

which Cu^{2+} interacts appears to be ATP, since the variability in the amount of inhibition produced by Cu^{2+} could be eliminated if the reaction were started with ATP, i.e. if Cu^{2+} and ATP are not preincubated together. Table 2 shows the effect of preincubating the reaction components (minus ATP), Cu^{2+} and enzyme for various periods of time. The reaction was then initiated by adding ATP. The results show that the inhibition by Cu^{2+} was no longer time-dependent. Similar results were obtained for Zn^{2+}, Fe^{2+} and Pb^{2+}.

All experiments were carried out by starting the reaction with ATP to minimize the possibility that the inhibitory action of divalent metal might be reduced through the time-dependent formation of an inactive complex. Figure 1 shows the effect of increasing concentrations of various divalent metals on NaK-ATPase. The I_{50} (concentration required for 50 per cent inhibition) for Zn^{2+} and Cu^{2+} is 1 μM. The I_{50} for Fe^{2+} is 3 μM, while that for Pb^{2+} is 20-fold higher than for Zn^{2+} and Cu^{2+}, being 20 μM. The NaK-ATPase was inhibited 90 per cent or more at concentrations of any divalent ion exceeding 0·1 mM.

Kinetic studies (Figs. 2–5) in which MgATP was varied at several concentrations of various reversible inhibitory metals suggest a mechanism by which Pb^{2+}, Fe^{2+},

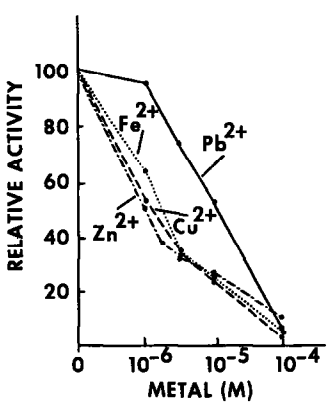

FIG. 1. Inhibition of NaK-ATPase by divalent metals. The conditions and components are as described under the experimental procedure. Relative activity is the specific activity of the enzyme at a particular concentration of metal divided by the specific activity of the control.

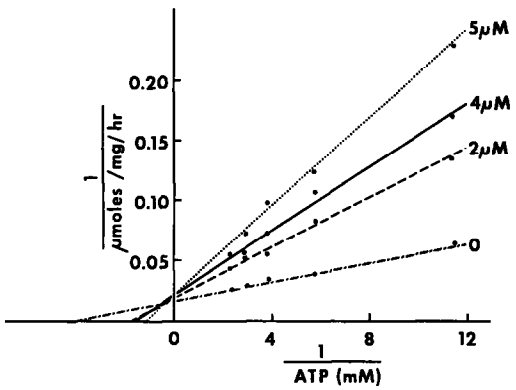

FIG. 2. Lineweaver–Burk plot of the inhibition of NaK-ATPase by Pb^{2+}. The conditions and components are as described under the experimental procedure. The reaction was started by the addition of ATP.

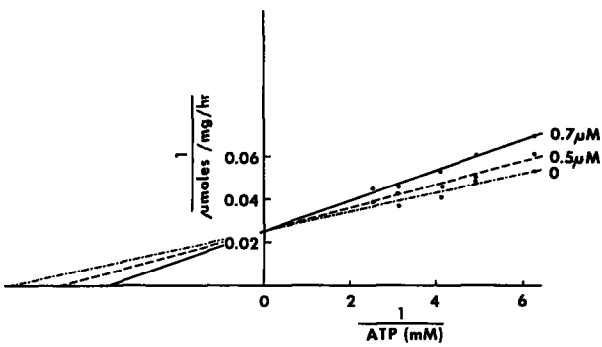

FIG. 3. Lineweaver–Burk plot of the inhibition of NaK-ATPase by Fe^{2+}. The conditions and components are as described under the experimental procedure. The reaction was started by the addition of ATP.

Zn^{2+} and Cu^{2+} inhibit NaK-ATPase. The results are indicative of two types of interaction between enzyme and inhibitor. As shown in Figs. 2 and 3, Pb^{2+} and Fe^{2+} are competitive inhibitors of NaK-ATPase with respect to MgATP, the true reaction substrate.[13] The inhibition occurs either as a result of these metals binding to the substrate site of the enzyme and thus preventing access of MgATP to the enzyme, or alternatively, Fe^{2+} and Pb^{2+} form complexes with ATP which bind to the enzyme and likewise prevent the access of MgATP to the enzyme. The K_i values for Pb^{2+} and Fe^{2+} are shown in Table 3. The K_m for MgATP is 0·2 mM.

TABLE 3. COMPARISON OF MECHANISM OF INHIBITION AND K_i

Metal	Type of inhibitor	K_i (M × 10^{-6})*
Pb^{2+}	Competitive	1·90 ± ·28
Fe^{2+}	Competitive	1·60 ± ·46
Zn^{2+}	Noncompetitive	3·48 ± ·78
Cu^{2+}	Noncompetitive	1·18 ± ·18

* The values are the means and standard errors for the K_i values as obtained from Figs. 2–5 for the respective metals.

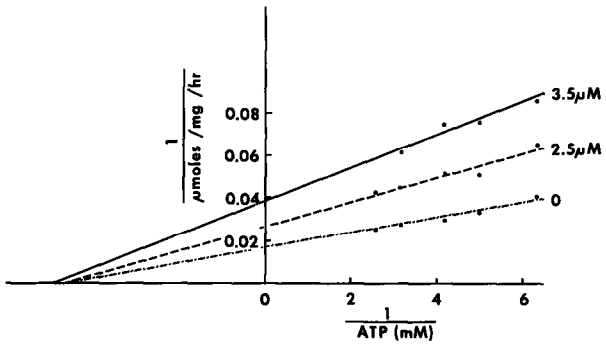

FIG. 4. Lineweaver–Burk plot of the inhibition of NaK-ATPase by Zn^{2+}. The conditions and components are as described under the experimental procedure. The reaction was started by the addition of ATP.

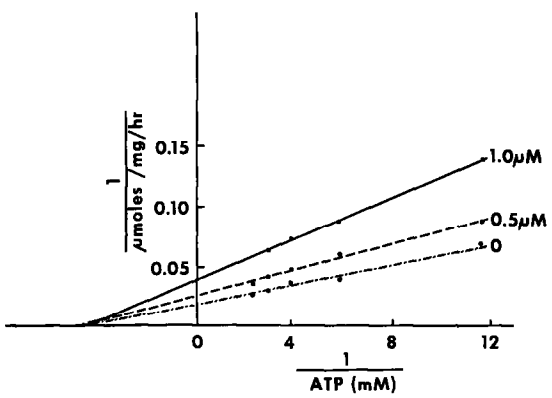

FIG. 5. Lineweaver–Burk plot of the inhibition of NaK-ATPase by Cu^{2+}. The conditions and components are as described under the experimental procedure. The reaction was started by the addition of ATP.

Initial velocity studies where ATP was varied against Zn^{2+} or Cu^{2+} show these metals to be noncompetitive inhibitors toward MgATP (Figs. 4 and 5). The K_i values for Zn^{2+} and Cu^{2+} are shown in Table 3. Both Zn^{2+} and Cu^{2+} form complexes with ATP.[14] Log K for Zn ATP^{2-} and Cu ATP^{2-} is 4·80 and 5·77 respectively. However, as described above, the inhibitory effect of Cu^{2+} is reduced by preincubation in media containing ATP but not enzyme. Since neither of these metals is a competitive inhibitor of NaK-ATPase, CuATP and ZnATP are not likely to be the active inhibitory species of the NaK-ATPase reaction. Rather the divalent metal itself is the active species and probably interacts at a position distinct from the substrate site. The location may well be the site at which Mg^{2+} brings about the purported conversion of enzyme from one phosphorylated form, E_1-P, to another, E_2-P, i.e.

$$E_1\text{-P} \xrightarrow{Me^{2+}} E_2\text{-P}$$

where Me^{2+} is divalent metal.[15,16] Competition of Cu^{2+} and Zn^{2+} with Mg^{2+} could stabilize the enzyme in the E_1-P configuration and prevent hydrolysis of ATP.

The noncompetitive inhibition by Cu^{2+} as shown in Fig. 5 is not in agreement with the findings of Ting-Beall *et al.*,[5] who reported that Cu^{2+} was uncompetitive

with respect to ATP. The results reported here indicate that as a noncompetitive inhibitor, Cu^{2+} can interact not only after the EMgATP complex has formed but with free enzyme as well. The discrepancy may be explained by the fact that Ting-Beall *et al.*[5] did not take any steps to remove contaminating metals from their enzyme preparation or assay reagents (cf. Ref. 4). Therefore, the possibility exists that a second inhibitory metal was present and that a combination of inhibitory effects was being observed.

This paper has reported on investigations into the mechanism by which certain divalent metals (Pb^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+}) inhibit the reaction catalyzed by NaK-ATPase. The results show that all divalent metals examined do not inhibit NaK-ATPase by the same mechanism. Pb^{2+} and Fe^{2+} are competitive inhibitors, whereas Zn^{2+} and Cu^{2+} are noncompetitive inhibitors. One interpretation of the kinetic data is that Zn^{2+} and Cu^{2+} inhibit the conversion of E_1-P to E_2-P. Therefore, these metals may be useful to determine further information about the NaK-ATPase reaction mechanism.

Finally, the potent inhibitory nature of these divalent metals on NaK-ATPase may explain their toxic neurological effects in certain conditions such as lead encephalopathy and Wilson's disease. In addition, it has been suggested[4] that certain seizure phenomena are a result of the toxic effects of metals. Therefore, the possibility exists that the neurological impairment seen in these conditions may, in part, be a result of NaK-ATPase inhibition by divalent metals.

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