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Inhibition of Na^+/K^+-ATPase and Mg^{2+}-ATPase by metal ions and prevention and recovery of inhibited activities by chelators

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Abstract

Kinetics and inhibition of Na^+/K^+-ATPase and Mg^{2+}-ATPase activity from rat synaptic plasma membrane (SPM), by separate and simultaneous exposure to transition (Cu^{2+}, Zn^{2+}, Fe^{2+} and .Co^{2+}) and heavy metals ($Hg^{2+}$and Pb^{2+}) ions were studied. All investigated metals produced a larger maximum inhibition of Na^+/K^+-ATPase than Mg^{2+}-ATPase activity. The free concentrations of the key species (inhibitor, $MgATP^{2-}$, $MeATP^{2-}$) in the medium assay were calculated and discussed. Simultaneous exposure to the combinations Cu^{2+}/Fe^{2+} or Hg^{2+}/Pb^{2+}caused additive inhibition, while Cu^{2+}/Zn^{2+} or Fe^{2+}/Zn^{2+} inhibited Na^+/K^+-ATPase activity synergistically (i.e., greater than the sum metal-induced inhibition assayed separately). Simultaneous exposure to Cu^{2+}/Fe^{2+} or Cu^{2+}/Zn^{2+} inhibited Mg^{2+}-ATPase activity synergistically, while Hg^{2+}/Pb^{2+} or Fe^{2+}/Zn^{2+} induced antagonistic inhibition of this enzyme. Kinetic analysis showed that all investigated metals inhibited Na^+/K^+-ATPase activity by reducing the maximum velocities (V_{max}) rather than the apparent affinity (K_m) for substrate $MgATP^{2-}$, implying the noncompetitive nature of the inhibition. The incomplete inhibition of Mg^{2+}-ATPase activity by Zn^{2+}, Fe^{2+} and Co^{2+} as well as kinetic analysis indicated two distinct Mg^{2+}-ATPase subtypes activated in the presence of low and high $MgATP^{2-}$ concentration. EDTA, L-cysteine and gluthathione (GSH) prevented metal ion-induced inhibition of Na^+/K^+-ATPase with various potencies. Furthermore, these ligands also reversed Na^+/K^+-ATPase activity inhibited by transition metals in a concentration-dependent manner, but a recovery effect by any ligand on Hg^{2+}-induced inhibition was not obtained.

Keywords: *Transition and heavy metals, Na^+/K^+-ATPase, Mg^{2+}-ATPase, kinetics, inhibition*

Introduction

Na^+/K^+-ATPase and Mg^{2+}-ATPase are membrane enzymes ubiquitous in animal cells that involve adenosine triphosphate (ATP) as a substrate for their functioning [1–3]. The ouabain-sensitive Na^+/K^+-ATPase, or sodium pump, is a membrane bound protein that establishes and maintains the high internal K^+ and low internal Na^+ concentrations, characteristic of most animal cells. The ouabain-insensitive Mg^{2+}-ATPase is much less well characterized, but apparently consists of at least two forms with different molecular weights [4] and sensitivity to metal ions [5,6].

Hydrolysis of ATP catalysed by these ATPases supplies the energy required for many biochemical processes [7] and depends sensitively on pH, temperature, presence of metal ions, such as Na^+, K^+ and Mg^{2+} as well as presence of some modulators. It was reported that highly toxic heavy metals induced inhibition of Na^+/K^+-ATPase and Mg^{2+}-ATPase activities in a concentration-dependent manner [8–11]. The metals of the first transition series (Fe, Co, Cu, Zn) are apparently necessary to some physiological processes, but as previously reported, the higher concentrations alter plasma membrane functioning in a variety of tissues, both *in vitro* and *in vivo* [12–14]. Inhibitory effects of Cu^{2+}, Zn^{2+} and Fe^{2+} on bovine

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cerebral cortex Na^+/K^+-ATPase activity have been obtained and the extent of inhibition seemed to depend on the presence of chelators [15].

Recently, we investigated the *in vitro* influence of some metal ions (Cu^{2+}, Co^{2+}, Hg^{2+}, Cd^{2+}) on Na^+/K^+-ATPase and Mg^{2+}-ATPase activity using rat synaptic plasma membranes (SPM) as a model system [5,6,8,9,16]. The present paper continues our study and investigates the effects of some first transition series elements (Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}) and heavy metals (Hg^{2+} and Pb^{2+}) on the activity of SPM Na^+/K^+-ATPase and Mg^{2+}-ATPase. On the one hand, these effects were induced by separate, and on the other, by simultaneous exposures to investigated metals. It has been shown that some of the investigated ions (Cu^{2+}, Zn^{2+} and Fe^{2+}) form complex entities with ATP. Therefore, our aim was to elucidate whether the inhibition was caused by a decrease in substrate ($MgATP^{2-}$) concentration or was due to interaction with the enzyme. Consequently we followed the influence of the investigated metals on $MgATP^{2-}$ concentration in the enzyme medium. We also investigated the abilities of −SH containing ligands (gluthathione and L-cysteine) and EDTA to prevent and recover the metal ion – induced inhibition since chelators are able to protect against some toxic effects of metal ions [17]. In addition, extensive kinetic studies were undertaken to determine the nature of the enzyme inhibition by the investigated metals.

Material and methods

Chemicals

All chemicals for the medium assay were commercially available from Sigma (St. Louis, MO, USA) and were of reagent grade. The metal ion salts: mercury (II) chloride, lead (II) chloride, cobalt (II) chloride, zinc (II) chloride, cooper (II) chloride, iron (II) chloride, NaCl, KCl, stannous chloride, ammonium molybdate, were from Merck (Darmstadt, Germany). All solutions were prepared using de-ionized water.

Animals

Adult male Wistar rats three months, 330–400 g, were housed under standard laboratory conditions in the animal house of the Vinča Institute of Nuclear Sciences (Beograd, Serbia & Montenegro). Animals were kept under controlled illumination (lights on: 5:00 a.m.–5:00 p.m.) and temperature (23 ± 2°C), and had free access to food and water. The "Guiding Principles for the Care and Use of Animals" based upon Helsinki Declaration (1964) and "Protocol of the "Vinča" Institute on care and treatment of laboratory animals" were strictly followed.

Synaptic plasma membrane (SPM) preparation

SPM were isolated from the whole rat brain. After decapitation with a guillotine (Harvard Apparatus), brains were rapidly excised and pooled (6/pool) for immediate preparation of synaptic plasma membranes. The SPM were isolated according to the method of Cohen et al. [18], as modified by Towle and Sze [19]. The mitochondrial contamination and protein content were determined according to the standard procedure [16]. SPM were stored at −70°C until used.

ATPase assay

The standard assay medium for investigation of ATPases activity contained (in mM) 50 Tris–HCl, pH 7.4; 100 NaCl; 20 KCl; 5 $MgCl_2$; 2 ATP; 25 μg SPM proteins. After preincubation for 10 min at 37°C in the absence (control) or in the presence of investigated metals, the reaction was initiated by addition of ATP and stopped after 10 min by adding 22 μl ice cold $HClO_4$ and immediate cooling on ice. The released inorganic orthophosphate (Pi) liberated from the hydrolysis of ATP was determined by a modified spectrophotometric method [6]. The spectrophotometric measurements were performed on a Beckman 5260 UV VIS spectrophotometer. The activity obtained in the presence of Mg^{2+} alone was attributed to Mg^{2+}-ATPase activity. Na^+/K^+ -ATPase activity was calculated by subtracting the Mg^{2+}--ATPase activity from the total ATPase activity in the presence of Na^+, K^+ and Mg^{2+} ions. The results are expressed as mean % enzyme activity compared to the corresponding control value ± S.E.M. of at least three independent experiments done in triplicate.

Since some of the investigated metal ions form complexes with ATP, their presence required the monitoring of the concentration of $MgATP^{2-}$ in the reaction mixture which is the actual substrate for both ATPases. The following reactions in the medium assay were taken into consideration for calculation the concentration of $MgATP^{2-}$, $MeATP^{2-}$ and free, uncomplexed metal ion concentrations:⋆

$$ATP^{4-} + H^+ \rightleftharpoons HATP^{3-} \quad (1)$$

$$HATP^{3-} + Mg^{2+} \rightleftharpoons MgHATP^- \quad (2)$$

$$Mg^{2+} + ATP^{4-} \rightleftharpoons MgATP^{2-} \quad (3)$$

$$Mg^{2+} + MgATP^{2-} \rightleftharpoons Mg_2ATP \quad (4)$$

$$Me^{2+} + ATP^{4-} \rightleftharpoons MeATP^{2-} \quad (Me^{2+} = Cu^{2+}, Zn^{2+} \text{ and } Co^{2+}) \quad (5)$$

Free metal ion, $MeATP^{2-}$ and $MgATP^{2-}$ concentrations were calculated according to the well-known Storer and Cornish-Bowden method [20] taking into

account all the equilibrium reactions involving Mg^{2+}, investigated metal ions and ATP. The stability and equilibrium constants were taken from references [21,22].

The effect of EDTA and −SH containing ligands (L-cysteine and glutathione) on the prevention of the metal ion induced inhibition was measured under the same conditions as described above, with the ligand added to the medium assay before the exposure to metal ions. The recovery of inhibited enzyme activity by investigated ligand was examined by adding ligand in the experimental tube, after 10 min of preincubation in the presence of metal ion at a concentration which induced complete enzyme inhibition. The control tubes contained the corresponding concentration of chelators without metal ions.

Kinetic analysis

Kinetic analysis was carried out according to a slightly modified method of Phylips [23], by following the initial velocity of the enzymatic reaction in the presence of inhibitor (concentration near IC_{50}) and rising concentrations of ATP (0.25–5.5 mM), while maintaining the concentrations of other ions (Mg^{2+}, Na^+, K^+) constant. The data were analyzed by the softwere package developed [6] and the results were recalculated using EZ FIT [24].

Results

Effects of metal ions on SPM ATPase activities and substrate concentrations

The separate influence of metal ions on Na^+/K^+-ATPase and Mg^{2+}- ATPase activity was investigated in the concentration range 1×10^{-8}–1×10^{-2} M. The increasing concentrations of metal ions induced inhibition of enzymatic activity in a concentration—dependent manner. Sigmoidal inhibition curves for both enzymes were obtained. The half-maximum inhibitory concentrations (IC_{50}) of the investigated ions for both enzymes were determined by Hill analysis of the experimental results and are summarized in Table I. In addition, since it is generally believed that the ionic form of metal ions is responsible for protein interactions, corresponding IC_{50} values of the "uncomplexed", "free" form of metals that form complexes with ATP, were calculated and are presented in Table I. It is clearly apparent that Na^+/K^+ -ATPase is more sensitive to all the investigated metals than Mg^{2+}- ATPase. It is interesting to note that transition metals (except Cu^{2+}) do not inhibit Mg^{2+}-ATPase completely, even when present in concentrations above 1×10^{-3} M. The inhibition of Mg^{2+}-ATPase activity asymptotically approaches 57% for Zn^{2+}, 80% for Fe^{2+} and 78% for Co^{2+} in contrast to 100% for Na^+/K^+-ATPase (results not shown).

Table I. Experimental and recalculated "free" IC_{50} values (μM).

	IC_{50} values (μM)			
	Na^+/K^+-ATPase		Mg^{2+}-ATPase	
Metal ion	Exp.	Calc.	Exp.	Calc.
Fe^{2+}	34	–	170	–
Co^{2+}[a]	168	75	262	136
Cu^{2+}[b]	7.1	0.6	42	33
Zn^{2+}	22	13	108	53
Hg^{2+}	0.7	–	3.6	–
Pb^{2+}	7.5	–	13.6	–

IC_{50} values; [a]ref. 5; [b]ref. 6.

The dependence of $MgATP^{2-}$ concentration on metal concentration in the assay medium assay at pH 7.4 is presented in Figure 1. The results show that at a concentration level below 2×10^{-4} M, metal ions do not have a significant effect on substrate concentration in the enzyme mixture since under the experimental conditions, the concentration of $MgATP^{2-}$ was in excess. By increasing metal ion concentration above 2×10^{-4} M, $MgATP^{2-}$ concentration significantly decreased.

Inhibition of SPM ATPases by simultaneous exposure to metal ions

The effect of simultaneous exposure to combinations of metal ions on the activity of Na^+/K^+-ATPase and Mg^{2+}-ATPase was studied by using the mixtures of Cu/Fe, Cu/Zn, Fe/Zn, and Pb/Hg. The concentrations of metals were 5×10^{-7}–2×10^{-5} M, i.e. limited to producing the inhibition in the concentration range lower than the IC_{50} values.

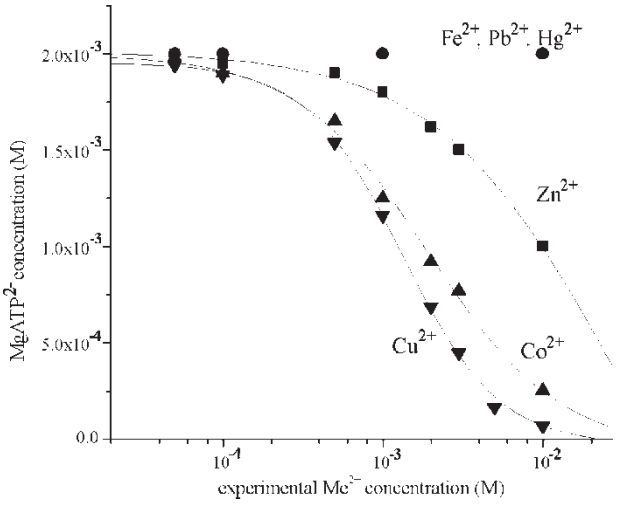

Figure 1. The dependence of $MgATP^{2-}$ concentration in the reaction mixture containing 2 mM ATP and 5 mM $MgCl_2$ on the experimental Me^{2+} concentration: (▼) Cu^{2+}; (▲) Co^{2+}; (■) Zn^{2+}; (●) Fe^{2+}, Hg^{2+}, Pb^{2+}.

The inhibition obtained by exposure to several combinations was compared to the inhibition in the presence of each single metal. The mean values of experimentally determined and mathematically added values are presented in Table II. The mathematically determined means were calculated as the sum of metal ion-induced inhibitions measured separately. Analysis of variance (One way ANOVA) was used to compare the mathematical sum of inhibitions caused by separate exposure to metal ions with inhibitions induced by exposure to two metals in combination (simultaneously). When a significant F value ($P < 0.05$) was obtained, the post hoc test Bonferoni was used to determine the differences.

A synergistic effect was defined as a statistically significant ($P < 0.05$) difference between inhibitions caused by simultaneous exposure and mathematically calculated mean values of enzyme inhibition for a pair of metals, where the former is larger than the latter. An antagonistic effect was defined as above, except that the inhibition caused by simultaneous exposure is less than the mathematically calculated mean value.

Cu^{2+} and Fe^{2+} together caused an additive inhibition, while simultaneous exposure to Cu^{2+} and Zn^{2+}, or Fe^{2+} and Zn^{2+} induced synergistic inhibition of SPM Na^+/K^+- ATPase activity at all investigated concentrations. Simultaneous exposure of Mg^{2+}-ATPase to Cu^{2+} and Fe^{2+} or Cu^{2+} and Zn^{2+} ions caused synergistic inhibition, while Fe^{2+} and Zn^{2+} together inhibited the enzyme antagonistically. Exposure to the Pb^{2+}/Hg^{2+} combination induced an additive inhibition of Na^+/K^+- ATPase activity as well as antagonistic inhibition of Mg^{2+}-ATPase activity at all concentrations examined.

Effects of chelators on the prevention and recovery of inhibited Na^+/K^+-ATPase activity

The inhibition of Na^+/K^+-ATPase induced by metal ions was investigated in the presence of 1 mM EDTA, 10 mM L-cysteine and 10 mM GSH. The Zn^{2+}-induced inhibition curves of Na^+/K^+-ATPase activity in the presence and absence of chelators are shown in Figure 2, and similar results were obtained for the other metals of the first transition series. It is obvious that the presence of EDTA in the reaction mixture at a 1 mM concentration prevents enzyme inhibition at a metal concentration below 1 mM, since 10 mM L-cysteine and 10 mM GSH induce the same effect. Moreover, in the presence of 1 mM EDTA, or 10 mM sulphur-containing chelators, the effect on the enzyme activity of these investigated metals in the reaction mixture at a total concentration below 1×10^{-4} M was negligible.

The Hg^{2+}-induced inhibition curves of Na^+/K^+-ATPase activity in the presence and absence of chelators are shown in Figure 3. It could be noted that the most protective effect on Na^+/K^+-ATPase activity was achieved in the presence of 10 mM GSH in the reaction mixture, while the ability of EDTA to prevent the Hg^{2+}-induced inhibition was the least. In the presence of 10 mM GSH, the IC_{50} value of $HgCl_2$ was almost five orders of magnitude higher than the IC_{50} value in the absence of any chelator, while the presence of 10 mM L-cysteine caused a four orders of magnitude increase in the IC_{50} value for $HgCl_2$.

Since it was demonstrated that EDTA, L-cysteine and GSH prevent the metal ion-induced inhibition of Na^+/K^+-ATPase, the recovery of the inhibited enzyme activities was investigated by varying the chelator concentration from 1×10^{-6}–1×10^{-1} M. The effect of chelators was investigated in the presence 1×10^{-4} M of metal ions, given the fact that at this

Table II. Inhibition of Na^+/K^+-ATPase and Mg^{2+}-ATPase activity by simultaneous exposure to metal ions in mixtures.

	% Inhibition			
	Na^+/K^+-ATPase		Mg^{2+}-ATPase	
Concentration (μM)	Experimental	Calculated	Experimental	Calculated
0.5 Cu +2 Fe	16.6 ± 2.5	17.2 ± 1.6	7.2 ± 1.3	5.2 ± 1.6
0.5 Cu +20 Fe	26.3 ± 2.8	28.6 ± 3.2	22.5 ± 2.1	3.2 ± 0.8
1 Cu +2 Fe	18.9 ± 1.1	20.4 ± 1.2	7.3 ± 0.9	5.0 ± 0.5
1 Cu +20 Fe	30.7 ± 2.2	32.8 ± 1.3	22.5 ± 2.9	6.4 ± 1.7
0.5 Cu +2 Zn	41.2 ± 2.5	11.6 ± 3.1	12.4 ± 2.4	4.1 ± 1.1
0.5Cu +20 Zn	65.9 ± 4.2	46.2 ± 2.1	17.8 ± 2.4	4.3 ± 0.9
1 Cu +2 Zn	68.4 ± 5.1	13.3 ± 2.3	13.9 ± 3.6	5.2 ± 1.5
1 Cu +20 Zn	100 ± 6.5	49.9 ± 3.6	18.7 ± 1.9	5.5 ± 1.0
2 Fe +2 Zn	20.8 ± 2.2	10.8 ± 2.1	1.2 ± 0.3	3.0 ± 0.2
2 Fe +20 Zn	85.1 ± 3.6	50.4 ± 1.1	3.9 ± 0.7	7.3 ± 1.3
20 Fe +2 Zn	65.1 ± 1.3	40.3 ± 1.4	1.6 ± 0.4	21.1 ± 2.1
20 Fe +20 Zn	100 ± 2.1	61.9 ± 3.3	3.6 ± 0.5	22.3 ± 1.9
1 Pb +0.5 Hg	42.2 ± 2.0	44.5 ± 1.5	14.2 ± 0.3	23.0 ± 1.3
5 Pb +0.5 Hg	75.5 ± 3.3	73.1 ± 1.2	38.7 ± 0.7	53.1 ± 3.3

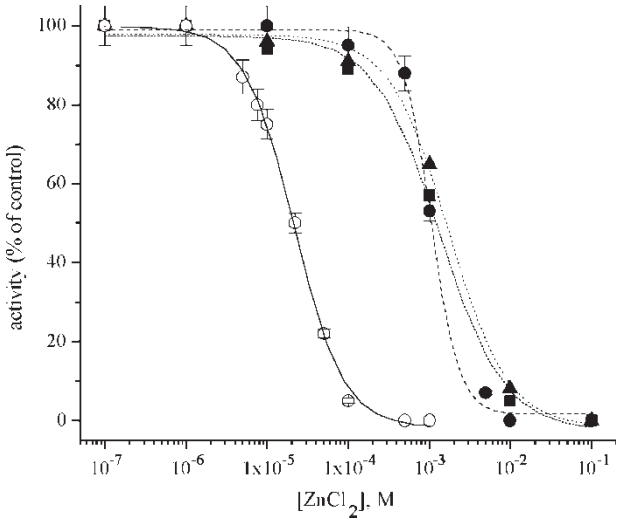

Figure 2. Inhibition of Na^+/K^+-ATPase by Zn^{2+} in the absence (○) and presence of: 10 mM L-Cysteine (■); 10 mM glutathione (▲) and 1 mM EDTA (●).

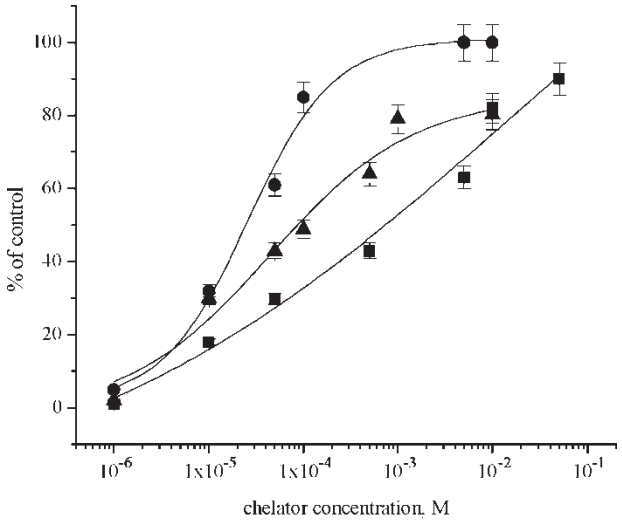

Figure 4. Effects of chelators: EDTA (●); L-cysteine (■) and glutathione (▲) on the recovery of Na^+/K^+-ATPase activity in the presence of 1×10^{-4} M $ZnCl_2$.

concentration, investigated metals induced complete inhibition of Na^+/K^+-ATPase. The results presented in Figure 4 show that the chelators had a dose-dependent recovery effect on Na^+/K^+-ATPase activity exposed to all metal ions that belong to the first transition series. On the contrary, recovery of Hg^{2+}-induced inhibition was not achieved, even with a chelator's concentrations above 0.01 M.

Kinetic analysis

In order to evaluate the nature of metal ion-induced Na^+/K^+-ATPase and Mg^{2+}-ATPase inhibition, kinetic analysis in the presence and absence of inhibitors was performed. The influence of metal ions (concentrations near IC_{50} values) on catalytic activity was investigated by following the initial velocities *vs.* the concentration of ATP (from 0.25–5.50 mM). Dependence of the reaction rate *vs.* $MgATP^{2-}$ concentration for Na^+/K^+-ATPase in the presence and absence of metal ions exibited typical Michaelis–Menten kinetics in all cases. The kinetic parameters (K_m, V_{max}) were estimated from Eadie–Hofstee transformation of the data and are summarized in Table III. It is obvious that all metal ions that belong to the first transition series inhibited enzyme activity noncompetitively by decreasing its V_{max} value, without changing significantly its apparent affinity for ATP. It is worthy to note that the saturating levels of $MgATP^{2-}$ were always present in the assay medium assay at an IC_{50} of metal ions.

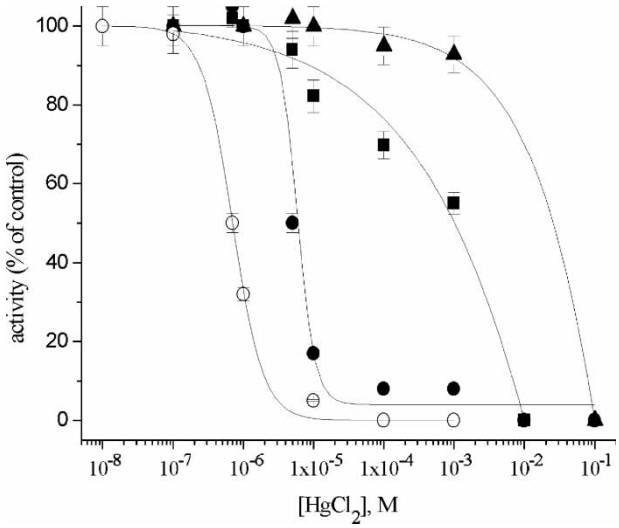

Figure 3. Inhibition of Na^+/K^+-ATPase by Hg^{2+} in the absence (○) and presence of: 1 mM EDTA (●); 10 mM L-cysteine (■) and 10 mM glutathione (▲).

Mg^{2+}-ATPase activity exhibited biphasic dependence on increasing $MgATP^{2-}$ concentration in the absence and presence of all investigated metal ions. The Mg^{2+}-ATPase activity as a function of $MgATP^{2-}$ concentration, in the presence and absence of $ZnCl_2$ is presented in Figure 5(a). The obtained functions are not purely of the Michaelis–Menten type, and could be approximated by two overlapping curves separated by a plateau. The results indicate that the enzyme activity at each ATP concentration represent the sum of the activity of two distinct Mg^{2+}-ATPase subtypes i.e. the plot of the total activity represents the line for "two enzymes acting on one substrate". The first one, active at a substrate concentration below 1 mM, is denoted as "high affinity", while the second, active at $MgATP^{2-}$ concentration above 2 mM, is denoted as "low affinity" Mg^{2+}-ATPase. Analysis of the data by a PC software package [20] revealed the Michaelis–Menten enzyme kinetics at low and sigmoidal kinetics at high ATP concentrations. The theoretical curves for "high" and "low affinity" Mg^{2+}-ATPase subtypes in

Table III. Kinetic analysis of Na^+/K^+-ATPase and Mg^{2+}-ATPase activity in the absence (control) and presence of metal ions.

	Na^+/K^+-ATPase		Mg^{2+}-ATPase			
			High affinity		Low affinity	
	K_m (mM)	V_{max} (μM P_i/mg/h)	K_m (mM)	V_{max} (μMP_i/mg/h)	$S_{0.5}$	V_{max} (μM P_i/mg/h)
Control	0.69 ± 0.05	46.10 ± 2.20	0.32 ± 0.05	68.18 ± 1.20	3.79 ± 0.06	19.80 ± 0.90
Fe^{2+}	0.72 ± 0.09	25.80 ± 0.03	0.32 ± 0.04	57.80 ± 0.05	3.70 ± 0.04	19.80 ± 0.20
Co^{2+}	0.75 ± 0.09	33.20 ± 0.12	0.31 ± 0.03	55.20 ± 0.09	3.72 ± 0.06	18.90 ± 0.90
Cu^{2+}	0.74 ± 0.05	31.80 ± 0.80	0.33 ± 0.02	54.00 ± 1.20	3.64 ± 0.10	15.60 ± 1.00
Zn^{2+}	0.70 ± 0.09	19.60 ± 0.20	0.33 ± 0.01	56.70 ± 0.30	3.73 ± 0.03	19.73 ± 0.90

Experimental concentration of metal ions: $Zn^{2+} = 1.5 \times 10^{-5}$ M; $Cu^{2+} = 7.5 \times 10^{-6}$ M; $Fe^{2+} = 3.4 \times 10^{-5}$ M; $Co^{2+} = 1 \times 10^{-4}$ M.

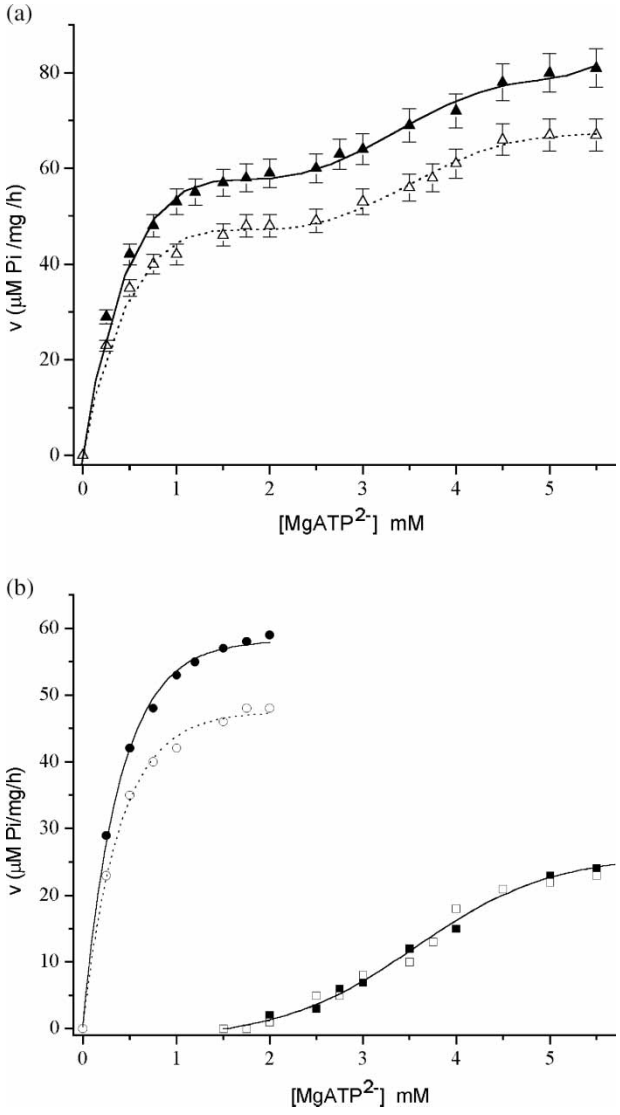

Figure 5. (a) The dependence of Mg^{2+}-ATPase activity on $MgATP^{2-}$ in the absence (▲) and in the presence (Δ) of 15 μM $ZnCl_2$. Symbols represent experimental points. (b) The Mg^{2+}-ATPase theoretical kinetic curves (Mg^{2+}-ATPase activity *vs* $MgATP^{2-}$ concentration) of: "high affinity "Mg^{2+}-ATPase subtype (○) in the absence of $ZnCl_2$ (●) and in the presence of $ZnCl_2$ (○); and "low affinity" Mg^{2+}-ATPase subtype (squares) in the absence of $ZnCl_2$ (■) and in the presence of $ZnCl_2$ (□ or □).

the presence and absence of Zn^{2+} are presented in Figure 5(b). The kinetic parameters for "high affinity" subtype, in the presence and absence of metal ions, were calculated by the Eadie–Hofstee transformation and are summarized in Table III. These results suggest that Zn^{2+}, Fe^{2+}, $Co^{2+}$and Cu^{2+} are noncompetitive inhibitors of this enzyme, because they significantly decrease its V_{max} value without changing significantly its apparent affinity for ATP. The activity of the "low affinity" subtype was reconstructed by subtracting the calculated "high affinity" values at each ATP concentration from the experimentally measured total activity. From the obtained results, initial velocity was recalculated. The iterative procedure was repeated until the best fit of the experimental results was achieved. The kinetic constants of the "low affinity" Mg^{2+}-ATPase were calculated by Hill analysis and are presented in Table III. It is notable that the effects of Zn^{2+}, $Fe^{2+}$and Co^{2+} on the kinetic parameters of the "low affinity" Mg^{2+}-ATPase was negligible.

Discussion

Results of our study showed that the examined metal ions, as previously reported [5,6,9,25], inhibited rat brain Na^+/K^+-ATPase and Mg^{2+}-ATPase activities and had various potencies (Table I). It is reasonable to assume that this potency depends on the nature of the metal ion as well as the nature of the enzyme. Obtained IC_{50} values of Zn^{2+} and Fe^{2+} for Na^+/K^+-ATPase are of the same order of magnitude as the IC_{50} values reported for bovine cerebral cortex Na^+/K^+-ATPase [15]. Experimental IC_{50} values (Table I) of all investigated ions that form inactive $MeATP^{2-}$ complex are below 2×10^{-4} M for both enzymes, while the concentration of substrate, $MgATP^{2-}$, remains practically unchanged (Figure 1).These results indicate that inhibition of the enzyme activities was due to the metal ion binding on the enzyme binding sites rather than the influence on the formation of an inactive $MeATP^{2-}$ complex.

Furthermore, incomplete inhibition of Mg^{2+}-ATPase by Zn^{2+}, Fe^{2+} and Co^{2+} is in accordance with kinetic analysis that confirms the existence of at least two Mg^{2+}-ATPase subtypes. One of them,

denoted as "low affinity" Mg^{2+}-ATPase, being insensitive towards the inhibition induced by Zn^{2+}, Fe^{2+} and Co^{2+} and their presence, had no effect on kinetic parameters. Incomplete inhibition of Mg^{2+}-ATPase activity supports the results of earlier studies of rat brain ATPases which suggested that there are two different Mg-ATPases differentiated by their sensitivity to metal ions [25] and ehacrinic acid [4]. Two distinct Mg^{2+}-ATPase activities differentiated by their sensitivity to Ca^{2+}, La^{3+} and F^-, were isolated from Triton-X-100 solubilized human erythrocyte membranes [26]. On the contrary, as previously reported [6,9], Cu^{2+} and heavy metals (Pb^{2+}, Cd^{2+} and Hg^{2+}) inhibit Mg^{2+}-ATPase activity completely, while significantly changing the kinetic parameters for both Mg^{2+}-ATPases. These results are also in accordance with synergistic inhibition caused by simultaneous exposure to Cu^{2+} and Zn^{2+} or Cu^{2+} and Fe^{2+} suggesting different modes of interaction of Cu^{2+} and Zn^{2+} (as well as Cu^{2+} and Fe^{2+}) with Mg^{2+}-ATPases.

The additive inhibition of SPM Na^+/ K^+-ATPase activity obtained by simultaneous exposure to Cu^{2+} and Fe^{2+} (as well as Hg^{2+} and Pb^{2+}) suggests that both metal ions are competing for the same inhibitor binding sites and there is an excess of inhibitor binding sites within the concentration ranges examined. Competition between Hg^{2+} and Pb^{2+} is in agreement with the chemical similarities of these ions [21,22], as well as with the previously reported fact that both ions are noncompetitive inhibitors of the investigated enzyme [9]. The results that Cu^{2+} in combination with Zn^{2+}, as well as Fe^{2+} in combination with Zn^{2+}, inhibit Na^+/K^+-ATPase activity synergistically, but additivelly with each other (Cu^{2+}/Fe^{2+}), suggest that Zn^{2+} binds at different binding sites, which are linked to different but associated mechanisms of inhibition. The Zn^{2+}-induced inhibition may include, as suggested earlier [15], Zn^{2+} binding to the Mg^{2+}-binding site, which directly affects the phosphorylation step in the enzyme cycle [15], while Cu^{2+}- and Fe^{2+}-induced inhibition involve interactions with other functional groups on the enzyme. However, Zn^{2+} binding to the Mg^{2+}-binding site could not be responsible for Mg^{2+}-ATPase inhibition, since this enzyme appears not to belong to the P-type ATPases although synergistic inhibition obtained by simultaneous exposure to Cu^{2+} and Zn^{2+} (as well as Cu^{2+} and Fe^{2+}) suggest that these pairs of metals bind to different types of binding site. The antagonistic inhibition of Mg^{2+}-ATPase activity with Fe^{2+} and Zn^{2+} at all concentrations examined suggest that the two metals are competing for a limited number of binding sites or the binding of one metal decreases the binding of second through a conformational change in the enzyme.

Our study clearly showed that the presence of 1 mM EDTA, 10 mM L-cysteine and 10 mM GSH prevents enzyme inhibition induced by Zn^{2+}, Fe^{2+}, Co^{2+} and Cu^{2+}. These results are in agreement with the strong chelating effects of the investigated ligands which significantly decrease the ionic form of metals able to interact with enzymes. Strong protective effects of $-$SH containing-ligands on Hg^{2+}-induced inhibition are a sufficient basis for a plausible assumption that Hg^{2+} is a potent reagent for thiol groups. Previous studies have shown that Hg^{2+} inhibition is due to its binding to sulphydryl groups of Na^+/K^+-ATPase [27,28], which means that the lowest protective effect of EDTA can be explained by supposing that Hg^{2+} binds more efficiently to the $-$SH groups of enzyme in comparison to this ligand.

Moreover, the recovery of transition metal-induced inhibition of Na^+/ K^+ -ATPase is probably due to competition between the functional groups of protein and ligands for complex formation with these metal ions. These results are consistent with the kinetic analysis suggesting that these ions are reversible, noncompetitive inhibitors of this enzyme. In spite of the fact that the kinetic analysis showed that Hg^{2+}—induced inhibition was reversible [9], the attempt to reactivate the enzyme clearly failed. Hence, we may assume that the Hg binding affinity for the enzyme far exceeds its affinity towards either of the investigated chelators.

To summarize, our results confirm that the inhibitory effects of metal ions may be attributed to their ionic forms and that the induced inhibition is likely due to direct interaction between metal ions and enzymes instead of between ions and ATP. Kinetic analysis showed that the nature of Na^+/ K^+—ATPase inhibition by the investigated metals is non-competitive. The incomplete inhibition by Zn^{2+}, Fe^{2+} and Co^{2+}, as well as kinetic analysis indicated two distinct SPM Mg^{2+}-ATPase subtypes with different sensitivity to metal ions. The inhibitory effects can be prevented by addition of strong metal—ion chelators, such as EDTA, L-cysteine and GSH. Finally, the effect of these chelators on the recovery of transition metal-induced enzyme inhibition is dose-dependent, but recovery of the Hg^{2+}-induced inhibition was not achieved, even when the chelators were present at concentrations above 0.01 M.

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Note

*Hg^{2+}, Fe^{2+} and Pb^{2+} don't form complexes with ATP.

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