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MECHANISM OF Na^+/K^+-ATPase AND Mg^{2+}-ATPase INHIBITION BY METAL IONS AND COMPLEXES*

The aim of the study was to give an overview of the mechanism of inhibition of Na^+/K^+–ATPase and Mg^{2+}–ATPase activity, the enzymes playing a key role in the active transport of monovalent cations (Na^+ and K^+) across the cell membrane, induced by the ions of some transition (Cu^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}) and heavy (Hg^{2+} i Cd^{2+}) metals, ammonium decavanadate and noble metals complexes ($[PtCl_2DMSO_2]$, $[AuCl_4]^-$, $[PdCl_4]^{2-}$, $[PdCl(dien)]^+$, $[PdCl(Me_4dien)]^+$). The extensive kinetic analysis was done in order to determine kinetic parameters and the mode of interaction of Na^+/K^+–ATPase and Mg^{2+}–ATPase and the investigated compounds. In addition, the ability of sulphur-donor ligands (L-cysteine and glutathione), as well as EDTA, to prevent metal ions and complexes induced inhibition of Na^+/K^+–ATPase activity and to recover enzymatic activity was investigated. Finally, development of highly sensitive and selective analytical tools on the basis of the immobilized enzyme is discussed in this paper.

Na^+/K^+–ATPase and Mg^{2+}–ATPase (ecto-ATP-ase) are membrane enzymes ubiquitous in animal cells that involve adenosine triphosphate (ATP) as a substrate for their functioning [1–3]. Na^+/K^+–ATPase (EC 3.6.1.3) plays a key role in the active transport of monovalent cations (Na^+ and K^+) across the membrane [2,4]. The enzyme is composed of α-subunit, which contains the adenosinetriphosphate (ATP)–Na^+, K^+– and ouabain–binding sites, as well as the site for phosphorylation and β-subunit, which stabilizes the K^+ binding cage. Na^+/K^+–ATPase acts as a dimer ($\alpha\beta$–$\beta\alpha$). The most widely accepted view related to such a dimmer act is a "flip-flop" model, in which both subunits show complementary conformation:

$$E_1E_2 \leftrightarrows E_2E_1$$

where E is the conformation of each α-subunit. The activity of this enzyme is very sensitive to the presence of some metal ions [5,6] and organic compounds of various structures, especially some drugs and pesticides [7,8]. Beside its transporter function, Na^+/K^+–ATPase acts as the receptor for cardiac glycosides such as ouabain like compounds [9], which is the specific inhibitor of the enzyme. Ouabain binds to the extracellular part of the protein with very high affinity, leading to the inhibition of enzymatic activity. The non specific inhibitors, *e.g.* metal ions and complexes, interfere with the enzyme due to the interactions with sulfhydril groups of cysteine residues. Furthermore, nephrotoxicity, ototoxicity etc., of platinum anticancer drugs, such as cisplatin and chloroplatinic acid, is related to inhibition of Na^+/K^+–ATPase activity. Since Pd(II) complexes have great affinity for binding with –SH containing ligands [10] and react about 10^5 times faster, these complexes represent an excellent model for investigation of the reaction between Na^+/K^+–ATPase and platinum group anticancer drugs. The similar kind of interaction with Na^+/K^+–ATPase was also observed for heavy and transition metal ions [11].

The ouabain insensitive ecto-adenosine triphosphatase (Mg^{2+}–ATPase) which does not belong to the P-type ATPase family, represents an integral membrane protein that, in the presence of divalent cations (Ca^{2+} or Mg^{2+}), hydrolyses extracellular nucleotides because of the outward orientation of its active site [12]. By hydrolysing ATP to ADP, ecto-ATPase represents the major inactivating agent in purine–triphosphate signalling [13]. It is much less well characterized than sodium pump, but apparently consists of at least two forms with different molecular weights [14] and sensitivity to metal ions [5,15].

Our study was undertaken with the aim to make an overview concerning the mechanism of interaction between noble metals complexes, metal ions and Na^+/K^+––ATPase and Mg^{2+}–ATPase. The extensive kinetic analysis was done in order to determine kinetic parameters and type of Na^+/K^+–ATPase and Mg^{2+}–ATPase inhibition. In addition, the ability of sulphur-donor ligands (L--cysteine and glutathione) as well as EDTA to prevent metal ions and complexes induced inhibition of Na^+/K^+–––ATPase and to recover enzymatic activity was investtigated. Finally, development of highly sensitive and selective analytical tools using the immobilized enzyme is discussed in this paper.

ACTIVITY OF Na^+/K^+–ATPase

Na^+/K^+–ATPase activity strongly depends on concentration Na^+, K^+ and Mg^{2+}, as well as on medium acidity. Hydrolysis of ATP to adenosine diphosphate (ADP)

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and adenosine monophosphate (AMP), in which $\{P_3O_{10}^{5-}\}$ is replaced by $\{P_2O_7^{4-}\}$ or $\{PO_4^{3-}\}$ is catalysed by ATPases, and supplies energy required for many biochemical processes [11,16]. The energy changes associated with the hydrolysis depend sensitively on pH, temperature and presence of metal ions. Metal ions alter enzyme activity causing inhibition in a concentration-dependent manner [5,15] that depends on their nature. Because of affinity of many metal ions to bind to adenosine nucleotides, their presence requires also the monitoring the concentration of $MgATP^{2-}$, which is the true substrate for both ATPase activities, and other key species, such as Mg^{2+} and ATP^{4-}.

Equilibrium reactions and complexes that strongly influence Na^+/K^+–ATPase mediated ATP hydrolysis in the presence of metal ions are presented in Table 1.

molecules with various and versatile activity [24], recently we investigated the influence of ammonium decavanadate, $(NH_4)_6V_{10}O_{28}\cdot 5H_2O$, on Na^+/K^+–ATPase and ecto-ATPase activity, using rat synaptic plasma membrane as a model system, while the commercial porcine cerebral cortex Na^+/K^+–ATPase served as a reference [25].

The dose-dependent inhibition of all enzymes is obtained (Figure 1). Na^+/K^+–ATPase from both model systems are more sensitive toward decavanadate anion than Mg^{2+}–ATPase. At the concentration of 1×10^{-5} M decavanadate inhibits both SPM and commercial Na^+/K^+––ATPase while the effect of the same concentration of decavanadate on the ecto-ATPase activity is negligible. IC_{50} of the enzyme activity is achieved at $(4.74\pm1.15)\times10^{-7}$ M for SPM Na^+/K^+–ATPase, $(1.30\pm0.10)\times10^{-6}$ M for commercial Na^+/K^+-ATPase while the same effect for ecto-ATPase is observed at several orders of magnitude higher concentration of decavanadate: $(1.05\pm0.10)\times10^{-4}$ M.

Table 1. Equilibrium reactions that influence $MgATP^{2-}$ concentration in the presence of metal ions

Reaction	log (k / dm^3 mol^{-1})	Reaction	log (k / dm^3 mol^{-1})
$ATP^{4-} + H^+ \leftrightarrows HATP^{3-} + H^+$	7.0	$Cd^{2+} + ATP^{4-} \leftrightarrows CdATP^{2-}$	–
$ATP^{3-} + Mg^{2+} \leftrightarrows MgHATP$	–	$Fe^{2+} + ATP^{4-} \leftrightarrows FeATP^{2-}$	–
$Mg^{2+} + ATP^{4-} \leftrightarrows MgATP^{2-}$	4.0	$Hg^{2+} + ATP^{4-} \leftrightarrows HgATP^{2-}$	–
$Mg^{2+} + MgATP^{2-} \leftrightarrows Mg_2ATP^{2-}$	1.6	$Ni^{2+} + ATP^{4-} \leftrightarrows NiATP^{2-}$	5.02
$Cu^{2+} + ATP^{4-} \leftrightarrows CuATP^{2-}$	6.13	$Co^{2+} + ATP^{4-} \leftrightarrows CoATP^{2-}$	4.62
$Zn^{2+} + ATP^{4-} \leftrightarrows ZnATP^{2-}$	4.25	–	–

MODULATION OF Na^+/K^+-ATPase AND Mg^{2+}-ATPase ACTIVITY BY METAL IONS

Great number of biologically important metal ions which are, at trace levels, necessary to support life, at elevated levels become toxic, built up in biological systems, and induce significant health hazards [5,6,11,17––20]. Many investigators have studied the *in vitro* effects of exposure to metal ions on synaptosomes, which have more nonspecific metal binding sites than synaptic plasma membrane (SPM). For instance, Na^+/K^+–ATPase from the kidney contains 36 sulfhydril groups with 34 of them found in the catalytically active α-subunit [21,22]. It can be assumed that inhibition induced by metal ions depends sensitively on their affinity to the ligands containing –SH, $–NH_2$ or –COOH groups, but it is now generally accepted that the most toxic effect of heavy metals is due to their binding to sulfhydril groups of enzymes.

Vanadium as the specific Na^+/K^+–ATPase inhibitor

Na^+/K^+–ATPase belongs to the P-type ATPase family which are distinguished from the other types of cation-translocating ATPase by their strong inhibition by nanomolar concentration of orthovanadate (VO_4^{3-}, a phosphate analog) [16, 23].

Since oligomeric (dimeric – V_2, tetrameric – V_4 and decameric – V_{10}) vanadate species interact with many bio-

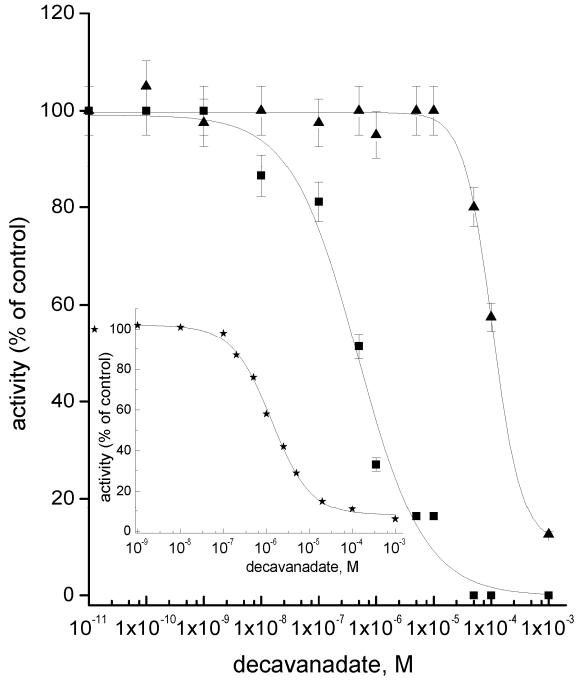

Figure 1. The concentration-dependent inhibition of SPM Na^+/K^+–ATPase (square) and Mg^{2+}–ATPase (triangle) and commercial porcine cerebral cortex Na^+/K^+–ATPase (inset) by ammonium decavanadate.

Obtained dose-dependent Na^+/K^+–ATPase inhibition by decavanadate, as well as kinetic analysis implying uncompetitive type of inhibition, is in agreement with previously reported findings that decameric vanadate species block the active site of P-type ATPases and consequently affect phosphorylation step in the enzyme cycle of P-type ATPases [26]. However, this mechanism could not be responsible for obtained Mg^{2+}–ATPase inhibition (a member of E-NTPDases) and probably occurs *via* different mechanism resulting in less sensitivity compared to P-type ATPases.

Inhibition by first transition series elements Fe, Co, Cu, Zn

The metals of the first transition series (Fe, Zn, Co, Cu) take place in many biochemical processes with enzymes and in the great number of them enable their normal function [5,15]. However, the higher concentrations are toxic and cause some diseases, such as Wilson's disease in the case of copper [27]. In the processes of ATP-ases catalyzed hydrolysis, metal ions in a solution compete with enzymes cofactors (Na^+, K^+ and Mg^{2+}) and also form complexes with the enzyme functional groups, such as –SH, $–NH_2$ or –COOH. Thus, understanding the chemical processes between these metals and ATPases, which cause changes in the enzymatic activity, is of great interest.

Recently, we investigated the *in vitro* influence of some metal ions of first transition series elements (Zn^{2+}, Fe^{2+}, Co^{2+} and Cu^{2+}) on Na^+/K^+–ATPase and Mg^{2+}––ATPase activity using rat synaptic plasma membranes (SPM) as a model system [5,11,15,28]. The increasing concentrations of metal ions (within the range from 1×10^{-8} to 1×10^{-2} M) induced inhibition of enzymatic activity in a concentration-dependent manner but with various potencies (Table 2). Sigmoidal inhibition curves for both enzymes were obtained. The half-maximum inhibitory concentrations, IC_{50}, of the investigated ions for both enzymes are summarized in Table 2. 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 2. 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 [11,15,28].

The dependence of $MgATP^{2-}$ concentration on metal concentration in the assay medium assay at pH 7.4 is presented in Figure 2. 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 the concentration of $MgATP^{2-}$, under the experimental conditions, was in excess. By increasing metal ion concentration above 2×10^{-4} M, $MgATP^{2-}$ concentration significantly decreased. Obtained experimental IC_{50} values (Table 2) 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 2). 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 [5,15,28].

The ability of sulphur-donor ligands (L-cysteine and glutathione) as well as EDTA to prevent ATPases inhibition by the transition metal ions was investigated [5,28,29]. The Zn^{2+} induced inhibition curves of Na^+/K^+–––ATPase activity in the presence and absence of chelators are shown in Figure 3, and similar results were obtained for the other metals of the first transition series. 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 recovery of the enzyme inhibited by transition metal ions was investigated by varying the chelator concentration from 1×10^{-6} to 1×10^{-1} M. The effect of chelators was investigated in the presence 1×10^{-4} M of metal

Table 2. Experimental and recalculated "free" IC_{50} values (μM)

Metal ion	Na^+/K^+–ATPase		Mg^{2+}–ATPase	
	Exp.	Calc.	Exp.	Calc.
Fe^{2+}	34	–	170	–
Co^{2+}	168	75	262	136
Cu^{2+}	7.1	0.6	42	33
Zn^{2+}	22	13	108	53
Hg^{2+}	0.7	–	3.6	–
Cd^{2+}	1	–	80	–

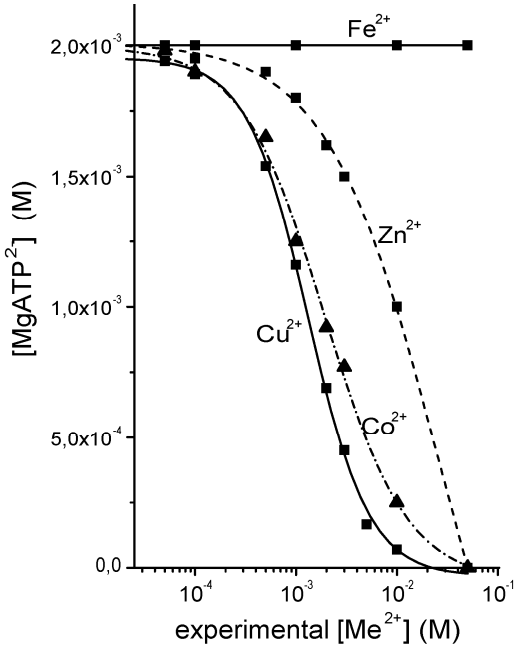

Figure 2. The dependence of $MgATP^{2-}$ concentration in the reaction mixture containing 2mM ATP and 5mM $MgCl_2$ on the experimental Me^{2+} concentration.

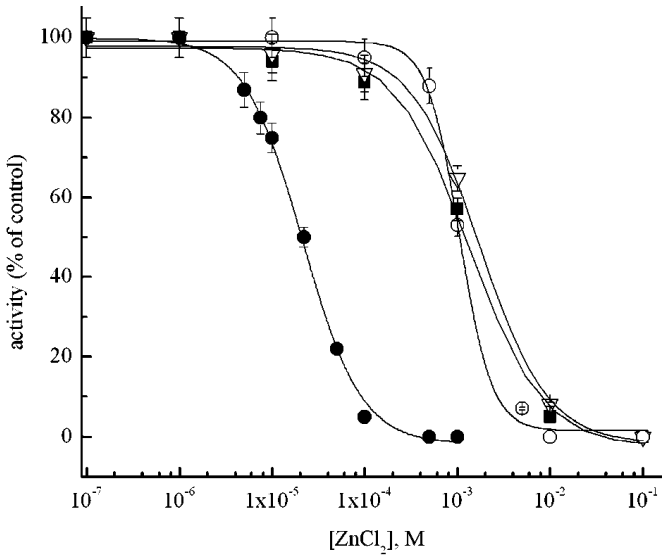

Figure 3. Inhibition of Na^+/K^+–ATPase by Zn^{2+} in the absence (○) and presence of: 10 mM L-cysteine (■), 10 mM glutathione (▲) and 1 mM EDTA (●).

ions, given the fact that at this concentration, investtigated metals induced complete inhibition of Na^+/K^+–ATPase. The results, presented in Figure 4, show that the chelators have a dose-dependent recovery effect on Na^+/K^+–ATPase activity exposed to all metal ions that belong to the first transition series.

Inhibition by heavy metals

Heavy metal ions, such as Cd^{2+} and Hg^{2+}, exerted a potent inhibitory effect on Na^+/K^+-ATPase and Mg^{2+}-ATPase isolated from different tissues, like rat brain (Table 2) and rat liver, by binding avidly to sulfhydryl groups with similar affinities [5,30,31]. As in the case of transition metal ions, the presence of chelators decreases inhibitor efficiency of investigated heavy metals (Figure 5). The most protective effect on enzyme 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. The obtained 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

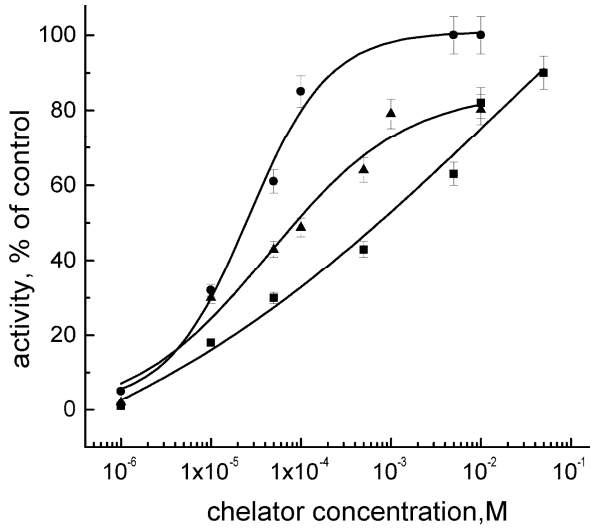

Figure 4. Effects of chelators: EDTA (●), L-cysteine (■) and glutathione (▲) on the recovery of Na^+/K^+–ATPase activity in the presence of 1×10^{-4} M $ZnCl_2$.

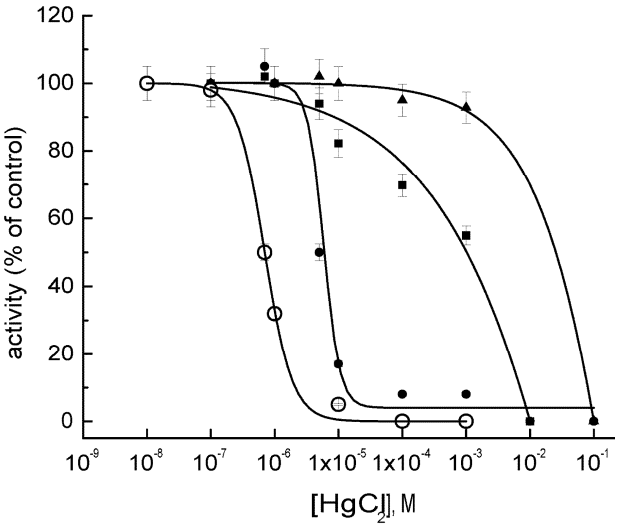

Figure 5. Inhibition of Na^+/K^+–ATPase by Hg^{2+} in the absence (○) and presence of: 10 mM L-cysteine (■), 10 mM glutathione (▲) and 1 mM EDTA (●).

[32,33]. However, recovery of the Hg^{2+}-induced inhibition was not achieved, even when the chelators were present at concentration above 0.01 M.

Sensitivity of enzymes toward Cd^{2+} and Hg^{2+} increased due to its immobilization by adsorption on a nitrocellulose membrane [34] as well as polystyrene support (Figure 6) [6,35]. So, adsorption Mg^{2+}–ATPase on polystyrene support induces 10-fold higher sensitivity toward Cd^{2+}. Obtained biphasic inhibition profile of Mg^{2+}–ATPase activity by Cd^{2+} as well as incomplete inhibition by Zn^{2+}, Fe^{2+} and Co^{2+} indicates the existence of two enzyme subtypes differentiated by their sensitivity to metal ions [31].

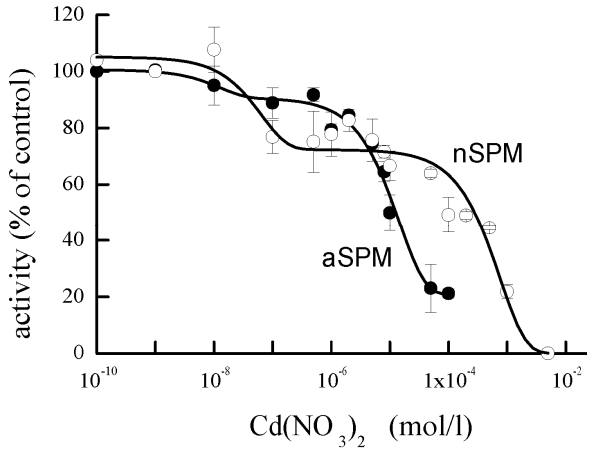

Figure 6. Inhibition by Cd^{2+} of Mg^{2+}–ATPase activity in free (nSPM) and immobilized SPM (aSPM) on polystyrene microtiter plate.

It seems reasonable to correlate the metal inhibitory potencies, expressed as IC_{50}, with the stability constants of corresponding metal–cysteine complex (Figure 7). Two parallel linear dependences of the toxicity *vs*. stability constants of the corresponding complexes were obtained for Na^+/K^+–ATPase, as well as Mg^{2+}–ATPase. Moreover, the results confirmed that Mg^{2+}–ATPase is for about two orders of magnitude less sensitive than Na^+/K^+–ATPase toward the inhibition induced by the investigated metal ions.

Kinetic analysis

To evaluate the nature of metal ion induced Na^+/K^+–ATPase and Mg^{2+}–ATPase inhibition, the 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 *versus* the concentration of ATP (from 0.1 to 6.0 mM). $MgATP^{2-}$ concentration was calculated for each composition of the reaction mixture. Dependence of the reaction rate *versus* $MgATP^{2-}$ concentration for Na^+/K^+–ATPase, in the presence and absence of inhibitor, 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 Tables 3 and 4 [5,6,28].

As can bee seen, all metal ions that belong to the first transition serie as well as Hg^{2+} inhibited the enzyme activity by decreasing its V_{max} value without changing significantly its apparent affinity for ATP. Unlike noncompetitive mode of Na^+/K^+–ATPase inhibition by Hg^{2+} and transition metal ions, kinetic parameters obtained in

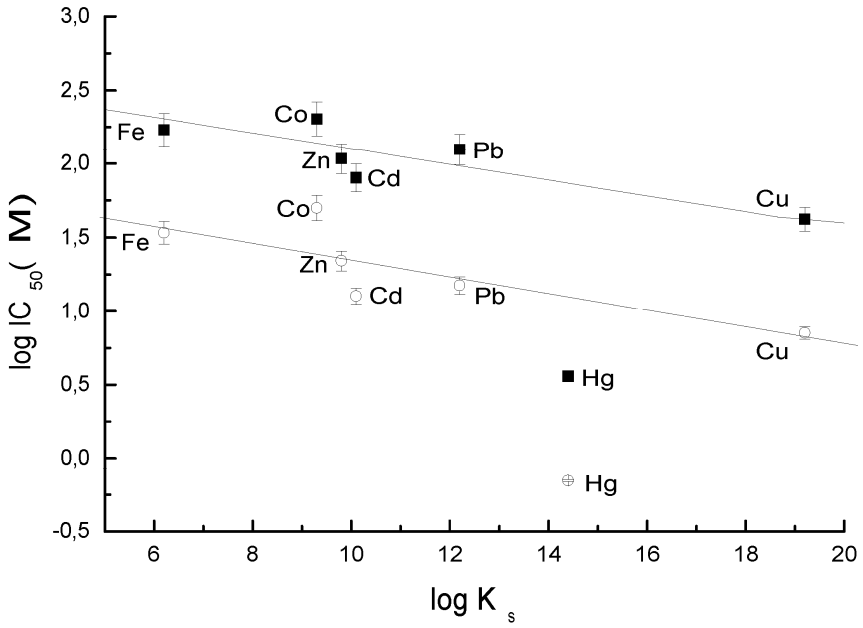

Figure 7. Dependence of IC_{50} values of metal induced inhibition of Na^+/K^+-ATPase (circles) and Mg^{2+}-ATPase (squares) on metal-ion-L-cysteine stability constants.

Table 3. Kinetic analysis of Na^+/K^+–ATPase and Mg^{2+}–ATPase activity in the absence (control) and presence of transition metal ions

Ion	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} (μM P_i/mg/h)	$S_{0.5}$ (mM)	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	25.8 ± 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

Table 4. Kinetic analysis of nSPM (free enzyme) and aSPM (immobilized enzyme) Na^+/K^+–ATPase and Mg^{2+}–ATPase activity in the absence (control) and presence of $Cd(NO_3)_2$ and $Hg(NO_3)_2$

SPM	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} (μM P_i/mg/h)	$S_{0.5}$ (mM)	V_{max} (μM P_i/mg/h)
			Control			
*n*SPM	0.37 ± 0.04	0.53 ± 0.02	0.60 ± 0.08	0.35 ± 0.06	2.5 ± 0.3	0.14 ± 0.01
*a*SPM	0.38 ± 0.05	0.24 ± 0.02	0.22 ± 0.05	0.21 ± 0.02	2.2 ± 0.3	0.14 ± 0.01
			$Cd(NO_3)_2$			
*n*SPM	1.3 ± 0.3	0.51 ± 0.02	0.61 ± 0.09	0.23 ± 0.04	1.9 ± 0.1	0.10 ± 0.01
*a*SPM	0.5 ± 0.1	0.21 ± 0.02	0.23 ± 0.03	0.17 ± 0.01	2.9 ± 0.3	0.10 ± 0.01
			$Hg(NO_3)_2$			
*n*SPM	0.6 ± 0.2	0.32 ± 0.04	0.41 ± 0.05	0.25 ± 0.02	1.5 ± 0.4	0.07 ± 0.01
*a*SPM	0.30 ± 0.09	0.14 ± 0.01	0.12 ± 0.05	0.12 ± 0.03	1.8 ± 0.4	0.08 ± 0.01

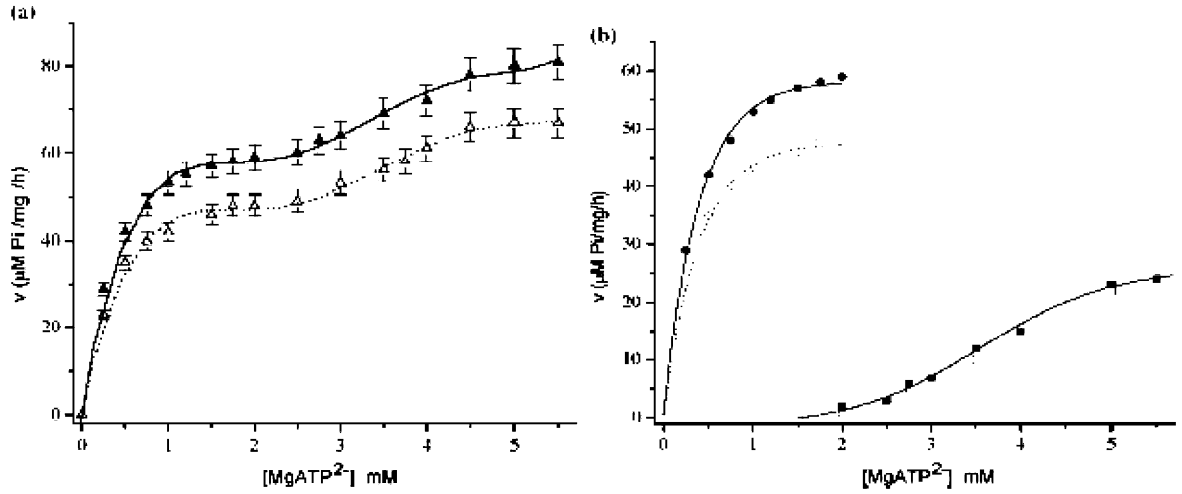

Figure 8. a) The dependence of Mg^{2+}–ATPase activity on $MgATP^{2-}$ in the absence (▲) and in the presence (Δ) of 15 mM $ZnCl_2$. Symbols represent experimental points. b) The Mg^{2+}–ATPase theoretical kinetic curves ($Mg2^+$–ATPase activity vs. *$MgATP^{2-}$ concentration) of: "high affinity "Mg^{2+}–ATPase subtype (circles) 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 $ZnCl_2$ (□).*

the presence of Cd^{2+} suggested competitive type of inhibition.

On the other hand, Mg^{2+}–ATPase activity exhibited biphasic dependence on increasing $MgATP^{2-}$ concentration suggesting existence of two distinct Mg^{2+}–ATPase subtypes (Figure 8a) [5,6,13,28]. The first one, active at a substrate concentration below 1mM, 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 revealed the Michaelis–Menten enzyme kinetics at low and sigmoidal kinetics at high ATP concen-

trations (Figure 8b). These results suggest that Cd^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+} and Cu^{2+} are noncompetitive inhibitors of "high affinity" Mg^{2+}–ATPase, while effects of Zn^{2+}, Fe^{2+} and Co^{2+} on the kinetic parameters of the "low affinity" Mg^{2+}–ATPase was negligible (Tables 3 and 4).

Inhibition of sodium pump by noble metals complexes

The modification of cysteine residues in proteins due to its ability to strongly coordinate complex metal ions is one of the arguments of critical importance for the design of novel types of pharmacological agents, based on the Pt(II), Au(III) and Pd(II) complexes. Our study was undertaken with the aim to examine the mechanism their interaction with Na^+/K^+–ATPase [10,36,37]. All investigated species induced the concentration-dependent inhibition of Na^+/K^+–ATPase activity in the concentration range from 10^{-9} to 10^{-3} M, and their potency to inhibit Na^+/K^+–ATPase dependent on the rate of ligand exchange in the coordinative sphere of the metal ion. As an example, Figure 9 represents the inhibition curves for $[PtCl_2DMSO_2]$ and $[AuCl_4]^-$ induced inhibition of Na^+/K^+–ATPase activity. It is worthy to notice (data not shown) that L-cysteine partially prevented the inhibition induced by $[PtCl_2DMSO_2]$. The Hill plots for the investigated complexes were used to calculate IC_{50} values. IC_{50} values and kinetic parameters are given in Table 5.

However, the stability constants of complexes between the enzyme and platinum group compounds were close to the value of the overall binding constant that was reported for the interaction of Na^+/K^+–ATPase with cisplatin [38], but were also two orders of magnitude lower compared to the aqua complexes of heavy and transition metals. Kinetic analysis indicated a noncompetitive type of inhibition and suggested that the complexes did not affect the binding of the substrate. As an example, the Michaelis–Menten dependence of initial reaction rate against substrate ($MgATP^{2-}$) concentration, in absence and presence of 5×10^{-5} and 1×10^{-4} M $[PdCl(dien)]^+$, is presented in Figure 10. Furthermore, kinetic analysis showed that $[PdCl(dien)]^+$ inhibited Na^+/K^+–ATPase by reducing enzyme ability to convert substrate to product, V_{max}, rather than apparent affinity for substrate, K_m, implying the noncompetitive nature of enzyme inhibition. The obtained results suggested that the inhibitor interfered with an inhibitory site on the enzyme that was remoted from the active site for the substrate ($MgATP^{2-}$). The similar behavior was noticed by other complexes.

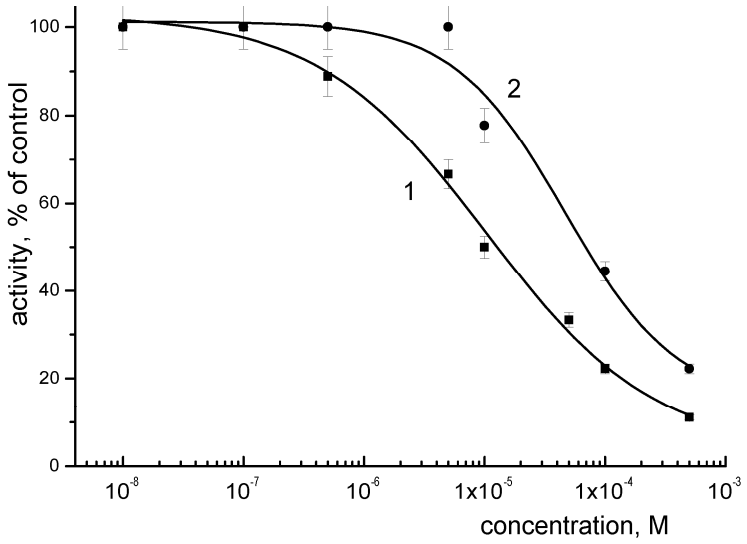

Figure 9. Dependence of the Na^+/K^+–ATPase activity on the $[PtCl_2DMSO_2]$ (1) and $[AuCl_4]^-$ (2). Results are expressed as the % of the activity of the enzyme.

Table 5. IC_{50} values and kinetic parameters for Na^+/K^+–ATPase inhibition by Au(III), Pt(II) and Pd(II) complexes obtained for human erythrocytes

Complex	IC_{50} (M)	K_i (M)	V_{max} (μmol P_i/h/mg)	K_m (M)
Control	–	–	2.71 ± 0.03	0.29 ± 0.01
$[PtCl_2DMSO_2]$	$(1.20 \pm 0.14)\times10^{-5}$	2.33×10^5	–	–
$[AuCl_4]^-$	$(7.15 \pm 0.19)\times10^{-5}$	1.90×10^4	–	–
$[PdCl_4]^{2-}$	$(2.25 \pm 0.21)\times10^{-5}$	3.97×10^5	1.17 ± 0.03	0.29 ± 0.02
$[PdCl(dien)]^+$	$(1.21 \pm 0.13)\times10^{-4}$	1.04×10^4	1.39 ± 0.02	0.29 ± 0.01
$[PdCl(Me_4dien)]^+$	$(2.36 \pm 0.30)\times10^{-4}$	4.20×10^4	1.94 ± 0.03	0.29 ± 0.01

Considering the fact that Pd(II) complexes are model compounds for their Pt(II) analogs, which showed similar inhibitory effects on Na^+/K^+–ATPase, the conclusion can be drawn that the same kind of enzyme–inhibitor interaction can be expected for the widely-used platinum anticancer drugs. Our earlier studies showed that these complexes strongly interact with sulphur containing ligands, such as L-cysteine, glutathione and methionine [39−41] and are able to act as potent enzyme reactivators. The reactivation is usually due to the formation of complex between thiols and the metal ion bonded to the –SH groups of the enzyme. More likely, the mechanism of interaction of enzyme, L-cysteine and GSH with Pd(II) complexes was similar, since it involved substitution of the Cl^- ligand by SH-donor ligands [10]. However, the affinity of L-cysteine or GSH for this reaction was much higher compared to the Na^+/K^+––ATPase affinity.

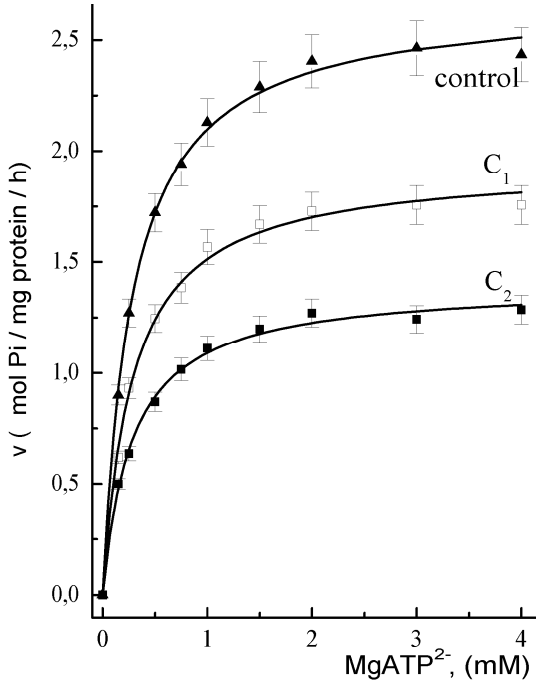

Figure 10. Michaelis–Menten dependence of initial reaction rate against substrate ($MgATP^{2-}$) concentration in absence (▲) and presence of $[PdCl(dien)]^+$ ($c_1 = 5\times10^{-5}$ M (□), $c_2 = 1\times10^{-4}$ M (■)). The experimental points are the mean of at least three experiments ±SEM, done in duplicate.

The inhibitory effects of Pd(II) complexes were prevented and recovered by the addition of L-cysteine or GSH, which showed high potency to extrude and substitute the enzyme from the Pd(II) complex (Figure 11). Is seems that prevention and recovery of Pd(II) complexes-induced Na^+/K^+–ATPase inhibition were realized due to the competition between the SH functional groups of protein and thiols (L-cysteine or GSH) for substitution of the Cl^- ligand in the coordination sphere. These results are consistent with the kinetic analysis, suggesting that these complexes are reversible noncompetitive inhibitors of this enzyme. Since platinum anticancer drugs react in the same manner as their palladium analogs, it was also suggested that L-cysteine or GSH might have the ability for detoxification after chemotherapy.

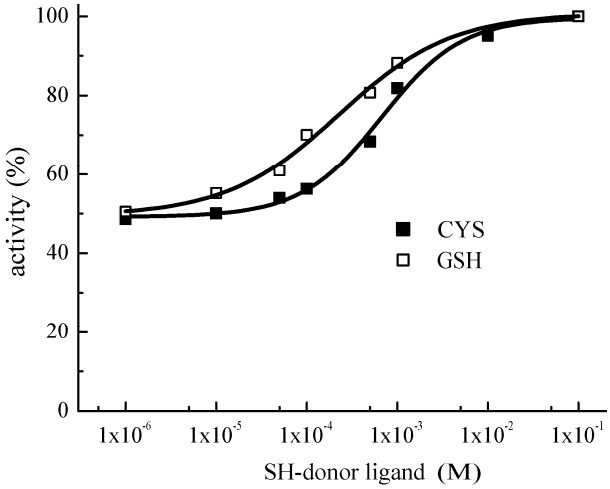

Figure 11. Recovery effect of L-cysteine and GSH on the Na^+/K^+–ATPase activity inhibited in the presence of 1×10^{-4}M $[Pd(dien)Cl]^+$.

Na^+/K^+–ATPase AS AN ANALYTICAL TOOL

In summary, the study of the mechanism of enzyme inhibition by various compounds is of great importance for elucidation of their potential toxicity. Moreover, development of highly sensitive and selective analytical tools using the immobilized enzyme is currently among the topic interest in the bioanalytics.

The effect of certain metal ions (Pb^{2+}, Cd^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+}) and various organic compounds (cardiotonic drugs, organic solvents, pesticides) on Na^+/K^+–ATPase activity, as described in the previous part, offers the possibility to develop a simple quailtative and semi-quantitative test method for selective detection of these analytes in aqueous solutions [17,42––44]. The method is based on the spectrophotometric determination of inorganic ortho-phosphate (Pi), liberated from ATP in the Na^+/K^+–ATPase-catalysed reaction, that serves as a measure of the enzymatic activity [5,17]. The concentration of Pi liberated in the reaction medium from ATP after exposure of the enzyme to analytes was dose dependent on the analyte concentration. Heavy metals (Pb, Cd, Hg, Cu, Fe and Zn), toxic organic compounds (pyridine, urea) and some pesticides (malathion and the products of its chemical and photochemical transformations, chlorpyrifos, permethrin) showed diverse effects, either the inhibition or stimulation of the enzyme activity. The potency of using ATPase system as a biological component for semi--quantitative and qualitative multi-response sensing sys-

tem for detection of different compounds is based on the level of change of enzyme activity in the presence of analyte. By varying the medium assay composition, some organic compounds and heavy metal salts can be simultaneously detected using the reaction of ATPases catalysed ATP hydrolysis. However, the test based on the Na^+/K^+–ATPase for detection of analytes is simple and useful for quick measurements.

This proposed method was already applied to the quality control of photodegradation of digoxin, and its concentration in Lanoxin injection [8,17]. The tested sample of Lanoxin injection was added to the standard medium assay (0.2 ml final value), containing 1 mM EDTA. The activity was measured as described, and compared to the control value. The same result, $(0.31\pm \pm 0.02)\times 10^{-3}$ M, was obtained using standard method (HPLC) with the mean standard deviation of 6.45%. This finally confirms applicability of sensing system based on the Na^+/K^+–ATPase in the product quality control.

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IZVOD

MEHANIZAM INHIBICIJE Na^+/K^+–ATPaze I Mg^{2+}–ATPaze METALNIM JONIMA I KOMPLEKSIMA

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(Pregledni rad)

U ovom radu je dat pregled mehanizma interakcije Na^+/K^+–ATPaze i Mg^{2+}–ATPaze, enzima koji igraju ključnu ulogu u aktivnom transportu katjona kroz ćelijsku membranu, i jona prelaznih metala (Cu^{2+}, Zn^{2+}, Fe^{2+} i Co^{2+}), jona teških metala (Hg^{2+} i Cd^{2+}), amonijum-dekavanadata i kompleksa plemenitih metala ([$PtCl_2DMSO_2$], [$AuCl_4]^-$, [$PdCl_4]^{2-}$, [$PdCl(dien)]^+$, [$PdCl(Me_4dien)]^+$). Kinetička analiza je urađena u cilju određivanja kinetičkih parametara i tipa inhibicije enzima ovim jedinjenjima. Takođe je ispitivana sposobnost L-cisteina i glutationa (koji sadrže sumpor) da spreče inhibiciju Na^+/K^+–ATPaze matalnim jonima i kompleksima plemenitih metala i reaktiviraju aktivnost inhibiranog enzima. Na kraju, razvoj osetljivih i selektivnih analitičkih oruđa na bazi imobilizovane Na^+/K^+––ATPaze je razmotren.

Ključne reči: Na^+/K^+–ATPaza • Mg^{2+}–ATPaza • Prelazni i teški metali • Kompleksi plemenitih metala • Dekavanadat • Inhibicija • Kinetička analiza

Key words: Na^+/K^+–ATPase • Mg^{2+}––ATPase • Transition and heavy metals • Noble metal complexes • Decavanadate • Inhibition • Kinetic analysis