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Volume I

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NA^+/K^+-ATPASE - ACTIVITY AND INHIBITION

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Abstract

The aim of the study was to give an overview of the mechanism of inhibition of Na^+/K^+-ATPase activity. For this purpose, the effect of ouabain like compounds (digoxin, gitoxin), platinum group complexes ($[PdCl_4]^{2-}$, $[PdCl(dien)]^+$ and $[PdCl(Me_4dien)]^+$), transition metal ions (Cu^{2+}, $Zn2^+$, Fe^{2+}, Co^{2+}) and heavy metal ions ($Hg^{2+,}$ Pb^{2+}, Cd^{2+}) on the activity of Na^+/K^+-ATPase isolated from rat synaptic plasma membranes (SPM), cortex of pig and human erythrocytes and was investigated.

Introduction

Na^+/K^+-ATPase (EC 3.6.1.37) is a cell membrane located enzyme, which plays a key role in the active transport of monovalent cations (Na^+ and K^+) across the membrane [1, 2]. The enzyme is composed of an **α-subunit**, which contains the adenosinetriphosphte (ATP)-, Na, K^+- and ouabain- binding sites, as well as the site for phosphorylation and a **β-subunit**, which stabilizes the K^+ binding cage . Na^+/K^+-ATPase acts as a dimer (αβ–βα). The most widely accepted view related to such a dimmer acts is a "flip-flop" model, in which both subunits show complementary conformation:

$$E_1E_2 \leftrightarrow E_2E_1$$

where E is the conformation of each a-subunit. The activity of this enzyme is very sensitive to the presence of some metal ions [3,4] and organic compounds of various structures, especially some drugs and pesticides [5,6]. Besides its transporter function, Na^+/K^+-ATPase acts as the receptor for cardiac glycosides such as ouabain. Ouabain binds to the the extracellular part of the protein with very high affinity, leading to the inhibition of enzymatic activity. Furthermore, nephrotoxicity, ototoxicity etc. of platinum anticancer drugs, such as cisplatin and chloroplatinic acid, is related to inhibition of Na^+/K^+-ATPase activity. Since Pt(II) complexes have great affinity for binding with –SH containing ligands [7,8], these complexes interact with Na^+/K^+-ATPase through enzyme sulfhydril groups. This kind of interaction with Na^+/K^+-ATPase was also observed for heavy and transition metal ions [9].

Our study was undertaken with the aim to examine the mechanism of interaction between ouabain-like compounds, platinum group complexes and metal ions with Na^+/K^+-ATPase. The extensive kinetic analysis was done in order to determine kinetic parameters and type of Na^+/K^+-ATPase inhibition. In addition, the ability of sulphur-donor ligands (L-cysteine and glutathione) to prevent metal ions and complexes induced inhibition of Na^+/K^+-ATPase and to recover enzymatic

activity was investigated. Finally, development of highly sensitive and selective analytical tools using the immobilized enzyme is discussed in this paper.

Experimental

Na^+/K^+-ATPase was isolated from rat brain, human blood and the commercial enzyme from porcine cortex brain was purchased by Sigma Chemicals Co. was used. Red cell membranes were prepared according to the method of Post et al. [10] with certain modifications. Synaptosomal plasma membranes (SPM) were isolated from the whole rat brain according to the method of Cohen et al. [11], and were stored at -70^{o}C until use.

The enzyme activity was determined in a standard incubation medium (200 μl), containing 50 mM Tris – HCl (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 2 mM ATP and protein (2 mg/ml) in the presence or absence (control) of the desired concentration of inhibitor. Incubation mixtures were preincubated at 37^o C in the presence of inhibitor or distilled water (control). The reaction was started by the addition of ATP, allowed to proceed. The inorganic orthophosphate (P_i) liberated from the hydrolysis of ATP was measured using modified spectrophotometric procedure based on the stannous chloride method, by reading the absorbance at 690nm.

Results and Discussion

Inhibition of Na^+/K^+-ATPase activity by digitalis

Some widely used digitalis glycosides (synthetic drugs and medical plants isolates) strongly modulate enzyme activity on a concentration dependent manner. In naturally occurring digitalis glycosides the unsaturated γ- and δ-lactones present in 17β-position of the steroidal skeleton are associated with high affinity for the Na^+/K^+-ATPase receptor. Recently, it was confirmed that basicity, i.e. a strong ionic interaction between one of carboxylate residues present in the α- subunit of the Na^+/K^+-ATPase and the cationic form of some digitalis like derivatives is relevant for interference with enzyme activity [12]. The presence of -OH groups at different positions of the steroidal skeleton reduces, in general, the interaction energy, though it depends on the location and spatial disposition of such –OH groups.

Digitoxin, one of the most frequently used drugs to improve cardiac contractility, undergoes a complex metabolic degradation generating digitoxigenin, digitoxigenin mono-digitoxoside and gitoxin. The structural difference between digoxin and its isomer gitoxin, that usually appears as a result of metabolic degradation of digitoxin, is just the hydroxyl (-OH) group close to the C-17β position, which changes the pharmacokinetics and pharmacodynamics of these substances considerably. It has been recognized that digoxin induced inhibition is not only reversible, but also that the enzyme could be reactivated by the specific antidigoxin antibody. The inhibitory effect depends on the protein preparation [13]. However, human blood erythrocyte Na^+/K^+-ATPase was more sensitive to

exposure to gitoxin, compared to that from porcine cerebral cortex. In addition, biphasic inhibitory curves were obtained in both enzyme preparations, indicating the interference of two distinct inhibitor binding sites (Fig.1). The heterogeneity of digoxin sites has been reported in rat brain and beef heart Na^+/K^+-ATPase and has been related to 2 distinct isoform of the α subunit. It is well known that the α3 isoform is especially abundant in the brain and some other vertebrate tissues. Western blot analysis of the Na^+/K^+-ATPase from mature human erythrocyte, purified by ouabain column chromatography, has shown that erythrocytes contains the α1 and α3 isoforms of the α subunit. Our results indicated that the low sensitive α1 isoform was also present.

In our study the IC_{50} values for the high activity isoenzyme in both enzyme preparations were more than two orders of magnitude higher compared to the low affinity isoform. Moreover, the concentration of digoxin that produced 30% inhibition reached the toxic level for gitoxin, displaying the complete inhibition of the high affinity isoenzyme. The high affinity to digoxin and gitoxin can be attributed to the binding to α3 isoform, which is known to be the most sensitive towards the cardiac glycosides.

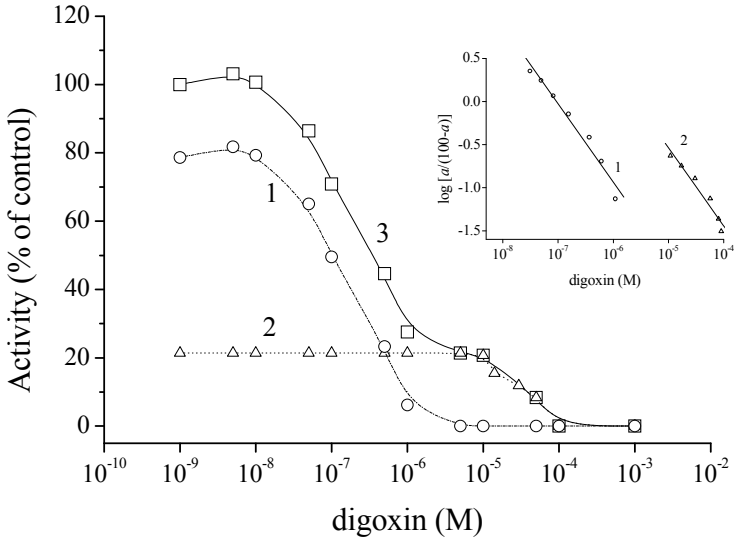

Fig. 1. Inhibition of Na^+,K^+-ATPase activity in human erythrocytes by digoxin. 1 – high activity isoenzyme, 2 – low affinity isoenzyme, 3 - total activity. Inset - Hill analysis of high (1) and low (2) affinity isoenzyme.

Further experiments demonstrated that elevated potassium concentrations were also able to reactivate the enzyme inhibited by digitalis glycosides to a certain extent, and that the reactivation was proportional to the time of exposure to increased K^+ concentration [13]. We have postulated that higher potassium concentrations shift the balance between E_1 and E_2 Na^+/K^+-ATPase conformations

in favor of E_1 and, consequently, enable the continuation of the digoxin impeded enzymatic cycle. Therapeutic effect is achieved with a digoxin concentration that produces a moderate enzyme inhibition (about 30 %), whereas the toxic concentrations inhibit over 60 % of the enzyme activity.

Inhibition 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 of interaction of $[PdCl_4]^{2-}$, $[PdCl(dien)]^{-}$ and $[PdCl(Me_4dien)]^{-}$ complexes with Na^+/K^+-ATPase [7,8]. All these species induced the concentration-dependent inhibition of Na^+/K^+-ATPase activity, and their potency to inhibit Na^+/K^+-ATPase depended on the rate of ligand exchange in the coordinative sphere of the metal ion (Fig.2).

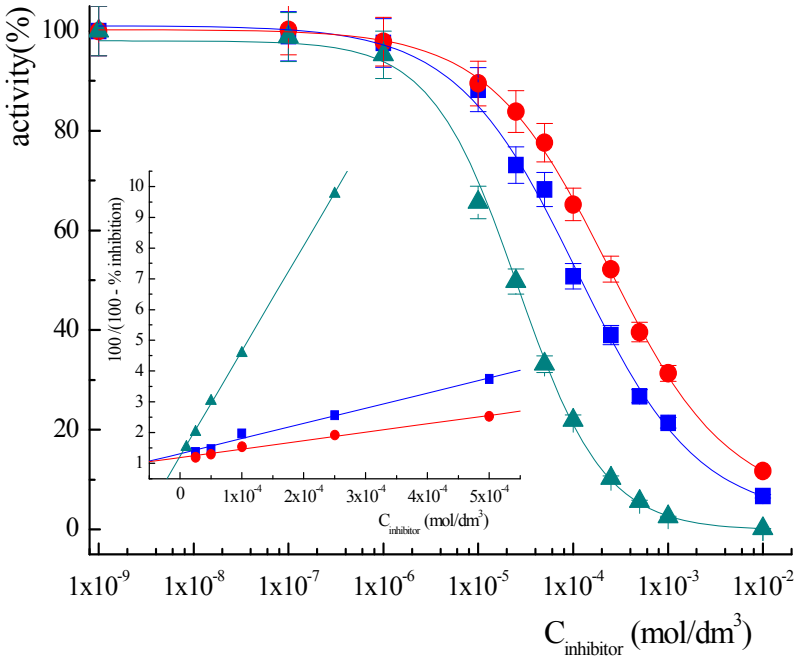

Fig. 2. Inhibition of Na^+/K^+-ATPase activity by $[PdCl_4]^{2-}$ (up triangles), $[PdCl(dien)]^+$(squares) and $[PdCl(Me_4dien)]^+$ (circles). Insert: the Hill plots constructed from the data obtained by inhibition experiments

However, the stability constants of enzyme-Pd(II) complexes were close to the value of the overall binding constant that was reported for the interaction of Na^+/K^+-ATPase with cisplatin, 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 Pd(II) complexes did not affect the binding of the substrate. 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. It was reported that GSH and L-cysteine are 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 [7,8]. However, the affinity of L-cysteine or GSH for this reaction was much higher compared to the Na^+/K^+-ATPase affinity.

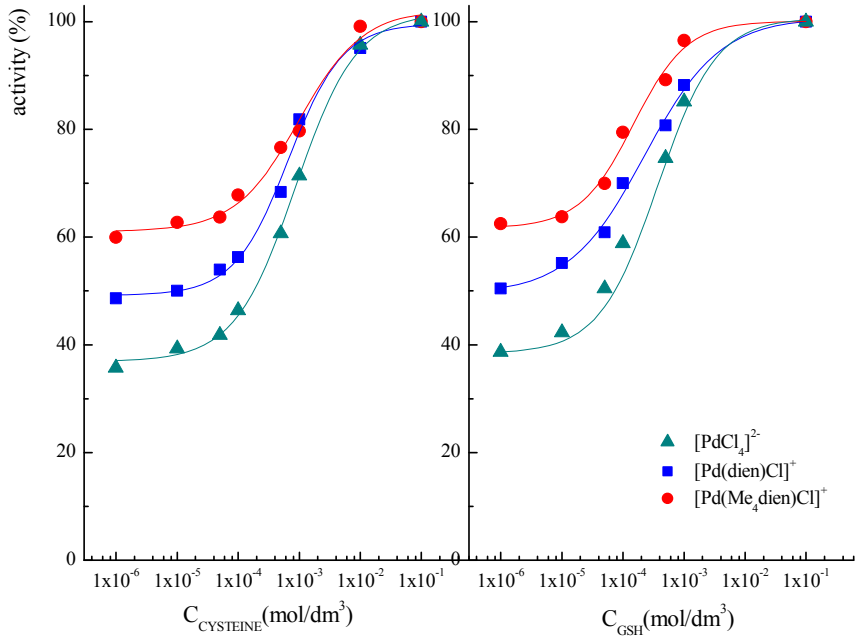

Fig. 3. Recovery effect of L-cysteine and GSH on the Na^+/K^+-ATPase activity inhibited in the presence of $5x10^{-5}$M $[PdCl_4]^{2+}$ (up triangles), $1x10^{-4}$M $[Pd(dien)Cl]^+$ (squares) and $1x10^{-4}$M $[Pd(Me_4dien)Cl]^+$(circles).

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 (Fig.3). 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.

Inhibition by an inorganic salts

The highly toxic heavy metals induced inhibition of Na^+/K^+-ATPase activity in concentration dependent manner. The influence of Pb^{2+}, Cd^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+} and Zn^{2+} on Na^+/K^+-ATPase activity was investigated by single exposure to inhibitors in the concentration range from $1\text{-}10^{-9}$ M to $1\text{-}10^{-3}$ M [3,4]. The IC_{50} values (inhibitor concentration that produced the inhibition of 50% of the enzymatic activity) were ranging from 10^{-7}–10^{-4} M. Heavy metal ions, such as Cd^{2+} and Hg^{2+}, exerted a potent inhibitory effect on Na^+/K^+-ATPase isolated from different tissues, like rat brain and rat liver, by binding avidly to sulfhydryl groups with similar affinities. Sensitivity of enzyme toward Cd^{2+} and Hg^{2+} increased due to its immobilization by adsorption on a nitrocellulose membrane [4,15]. Inhibitory effects of Cu^{2+}, Zn^{2+} and Fe^{2+} on bovine cerebral cortex Na^+/K^+-ATPase activity were obtained and the extent of inhibition seems to depend on the presence of chelators. Kinetics analysis showed that the nature of enzyme inhibition by metals was non competitive. The inhibitory effects of Fe^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+} could be prevented by addition of 1mM strong metal-ion chelator EDTA, 10 mM L-cystein or 10 mM GSH and the effect was dose dependent [3,9]. Recovery of the Hg^{2+}-induced inhibition was not achieved, even when the chelators were present at concentration above 0.01 M.

The synergistic effects with binary combinations of heavy metals on the activity of Na,K-ATPase using the mixtures of Cu/Zn, Cu/Fe, Zn/Fe, Pb/Cd, and Cu/Pb/Zn/Cu ions were obtained in all cases [14]. Moreover, all metal ions in the mixture at concentration levels near IC_{50} values inhibited the enzyme activity completely. The inhibition induced by combination of Pb with Cd was time dependent. Addition of 1mM EDTA in the medium assay recovered 100% of the inhibited enzyme activity.

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+}, 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 qualitative and semi-quantitative test method for selective detection of these analytes in aqueous solutions [14,15]. 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 [3,14]. 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, 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 system 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 digoxin in Lanoxin injection. 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 $(0.31\pm0.02)x10^{-3}$ M was obtained as 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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