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Effects of Digoxin and Gitoxin on the Enzymatic Activity and Kinetic Parameters of Na^+/K^+-ATPase

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Inhibition of Na^+/K^+-ATPase activity from human erythrocyte membranes and commercial porcine cerebral cortex by *in vitro* single and simultaneous exposure to digoxin and gitoxin was investigated to elucidate the difference in the mechanism of the enzyme inhibition by structurally different cardiac glycosides. The drugs exerted a biphasic dose-dependent inhibitory effect on the enzyme activity in both tissues, supporting the existence of two sensitive Na^+/K^+-ATPase isoforms. The IC_{50} values for the low and high affinity isoforms were calculated from the inhibition curves using mathematical analysis. The Hill coefficient (n) fulfilled the relationship $1 < n < 3$, suggesting cooperative binding of inhibitors to the enzyme. Kinetic analysis showed that digoxin and gitoxin inhibited Na^+/K^+-ATPase by reducing the maximum enzymatic velocity (V_{max}) and K_m, implying an uncompetitive mode of interaction. Both the isoforms were always more sensitive to gitoxin. The erythrocyte enzyme was more sensitive to the inhibitors in the range of low concentrations but the commercial cerebral cortex enzyme exerted a higher sensitivity in high inhibitors affinity concentration range. By simultaneous exposure of the enzyme to digoxin and gitoxin in combinations a synergistic effect was achieved by low inhibitor concentrations. An antagonistic effect was obtained with erythrocyte membrane enzyme at high inhibitors concentration.

Keywords: Digoxin; Gitoxin; Na^+/K^+-ATPase; Erythrocytes; Porcine cerebral cortex; Inhibition; Kinetics

INTRODUCTION

Na^+/K^+-ATPase is a cell membrane-located enzyme, which plays a key role in the active transport of monovalent cations across membranes.[1,2] The enzyme is composed of an α-subunit, which is the residence of ATP-binding, phosphorylation, Na^+ and K^+ binding and the specific inhibitor ouabain, and the β-subunit, which stabilises the K^+ binding cage.[3] The activity of this enzyme is very sensitive to the action of various bioregulators and is dependent on the lipid state of the membrane.[4,5] Metal ions binding to sulfhydryl groups have often been implicated in both Na^+/K^+-ATPase inhibition[6–9] and physiological effects.[10] Organic compounds of various structures, especially some drugs and pesticides, are also known to alter enzyme activity.[11,12]

One of the most useful groups of drugs in therapeutics, the digitalis glycosides, mainly digitoxin, digoxin and its derivatives (acetyl- and methyldigoxin), are the most frequently used to improve cardiac contractility in the treatment of congestive heart failure.[13] The main pharmacological effect of the cardiac glycosides is Na^+/K^+-ATPase inhibition.[14] When used as therapeutics, the concentrations of digitalis compounds used produce a moderate enzyme inhibition (about 30%), since the concentrations that reach toxic levels inhibit over 60% of the enzyme activity.[13]

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.[15,16] 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.[16,17] The presence of —OH groups at different

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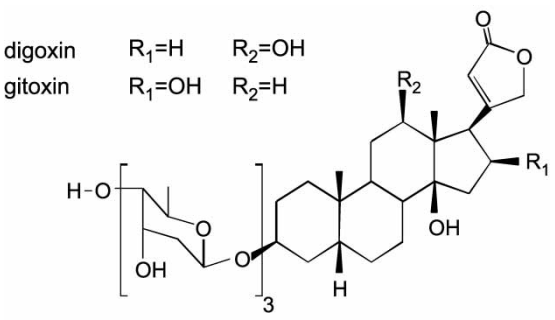

FIGURE 1 Structure of digoxin and gitoxin.

positions of the steroidal skeleton (Figure 1) reduces, in general, the interaction energy, though it depends on the location and spatial disposition of such —OH groups.[18,19]

Digitoxin, one of the most frequently used drugs to improve cardiac contractility, undergoes a complex metabolic degradation generating digitoxigenin, digitoxigenin mono-digitoxoside and gitoxin.[17] 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.[13,20,21]

This work investigates the inhibition and kinetics of interference between digoxin and gitoxin with Na^+/K^+-ATPase activity by single and simultaneous exposure to inhibitors. The aim of the study was to elucidate the difference in the mechanism of their action, as a consequence of the different chemical structures of these drugs. Moreover, since they can be found together in pharmaceuticals from medicinal plants, and also upon degradation of digitoxin, it is worth investigating their simultaneous effects on the activity of their target enzyme, Na^+/K^+-ATPase. We characterized the interactions of digoxin and gitoxin by measuring ATPase activity on human red cell Na^+/K^+-ATPase, and extended the approach by comparing the results with the effects seen on the activity of purified commercial porcine cerebral cortex enzyme.

MATERIAL AND METHODS

Chemicals

All chemicals were of analytical grade. Na^+/K^+-ATPase from porcine cortex brain was purchased from Sigma Chemicals Co. The specific Na^+/K^+-ATPase activity was 2.75 μmol P_i/h/mg protein.

Digoxin and gitoxin were obtained from "Zdravlje", Leskovac. Stock solutions of drug (concentration 0.01 M) were made in water or ethanol with the final concentration of ethanol not $> 2\%$ in the assay.

Preparation of Erythrocyte Membranes

Red cell membranes were prepared according to the method of Post *et al.*[22] with certain modifications. Whole blood, 5 ml, was suspended in 0.5 ml 0.2 M Na_4EDTA and 30 ml of 0.15 M NaCl. The cells were packed by centrifugation at 3000 rpm for 10 min and then washed three times with 0.15 M NaCl. Cell lyses was performed with 5 mM Tris–HCl (pH 7.4) and freezing at −20°C. The haemoglobin-free membranes were sedimented at 3000 rpm for 30 min and washed three times with this buffer. All procedures were carried out at 0–2°C. Protein concentration measurements were made according to the method of Bradford[23] using crystalline bovine serum albumin (BSA) as a standard. The specific Na^+/K^+-ATPase activity[24] was 0.288 μmol P_i/h/mg protein.

ATPase Assay

Total human erythrocyte membrane ATPase activity was assayed in a standard medium containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 2 mM ATP and membrane fragments (100 μg protein) in a final volume of 200 μl. Incubation mixtures were pre-incubated for 10 min at 37°C in the presence of inhibitors (by single exposure and in combination) or distilled water (control). The reaction was started by the addition of ATP, allowed to proceed for 60 min, and interrupted by the addition of ice cold $HClO_4$. The activity obtained in the presence of 2 mM ouabain (without the NaCl and KCl) was attributed to Mg^{2+}-ATPase. Na^+/K^+-ATPase activity was calculated as the difference between the total ATPase and Mg^{2+}-ATPase activity. Commercial porcine cerebral cortex Na^+/K^+-ATPase activity was assayed in the same standard medium but containing 40 μg protein in a final volume of 200 μl. The inorganic orthophosphate (P_i) liberated from the hydrolysis of ATP was measured using a modified spectrophotometric procedure,[7,8] by reading the absorbance at 690 nm. Preliminary studies showed that digoxin and gitoxin or their combinations did not interfere with quantitation of inorganic phosphate.

Data Analysis

Analysis of variance (One way ANOVA, SPSS 10.0 software) was used to compare inhibitions caused by single exposure to investigated inhibitors with inhibitions induced by exposure to digoxin and gitoxin in combination (simultaneously). When a significant F value ($P < 0.05$) was obtained, post hoc test Bonferoni was used.

A synergistic effect was defined as a statistically significant ($P < 0.05$) difference between inhibitions

induced by single and simultaneous exposure, when the mathematical sum of separate digoxin and gitoxin-induced inhibitions was lower than inhibition induced by exposure to the mixture of the investigated inhibitors. An antagonistic effect was defined as above, except that the inhibition induced by simultaneous exposure was less than the mathematical sum of inhibitions caused by single exposure to digoxin and gitoxin.

Kinetic Parameters

Kinetic experiments were carried out according to the slightly modified method of Philips.[25] The initial velocities were measured in the same incubation medium as a function of rising concentrations of $MgATP^{2-}$ (0.1–4.0 mM). The measurements were performed in the absence and presence of inhibitors, while maintaining the concentrations of other ions (Na^+, K^+, Mg^{2+}) constant. The experimental data were fitted to the Michaelis-Menten equation by non-linear regression analysis using EZ FIT.[26] V_{max} and K_m values with standard errors were derived from a Lineweaver–Burk plot.

RESULTS

Digoxin and Gitoxin Induced Inhibition of Na^+/K^+-ATPase Activity

The activity of Na^+/K^+-ATPase was measured as a function of the separate exposure to digoxin and gitoxin within the concentration range from 1×10^{-10} to 1×10^{-3} M in the incubation mixture. The experimental points that represent the inhibition of erythrocyte Na^+/K^+-ATPase activity, by digoxin and gitoxin, are presented in Figure 2. Results show that the response of the enzymatic activity to the glycosides concentration in the human erythrocyte membrane was biphasic, indicating high and low affinity Na^+/K^+-ATPase isoforms. The inhibition curves of Na^+/K^+-ATPase activity of porcine cerebral cortex are shown in the inset to Figure 2. As can be seen, these curves exhibit a biphasic dose response behavior in a smaller concentration range compared to human blood erythrocytes, which spanned over three orders of magnitude. It is clear from the experimental results that the activity *vs.* digoxin or gitoxin concentration plots in both cases can be represented by the sum of two overlapping sigmoid curves, separated by a plateau.

In the mathematical analysis of the results presented in Figure 2 it was assumed that the mass action principles were fully satisfied[7,27] and that the plot of the total activity represents the line for "two enzymes acting on one substrate". The computer program was set up for the analysis of the data, assuming a two-site model fit. In the first approximation the half maximum inhibition concentrations (IC_{50} values) for the high and low inhibitor affinity isoforms, respectively, were calculated by fitting the experimental results to the sum of two sigmoid curves. The theoretical curves for high and low affinity enzyme isoforms are presented in Figure 3. The activity of the high affinity isoenzyme was obtained by subtracting the calculated low affinity values from the experimental data.

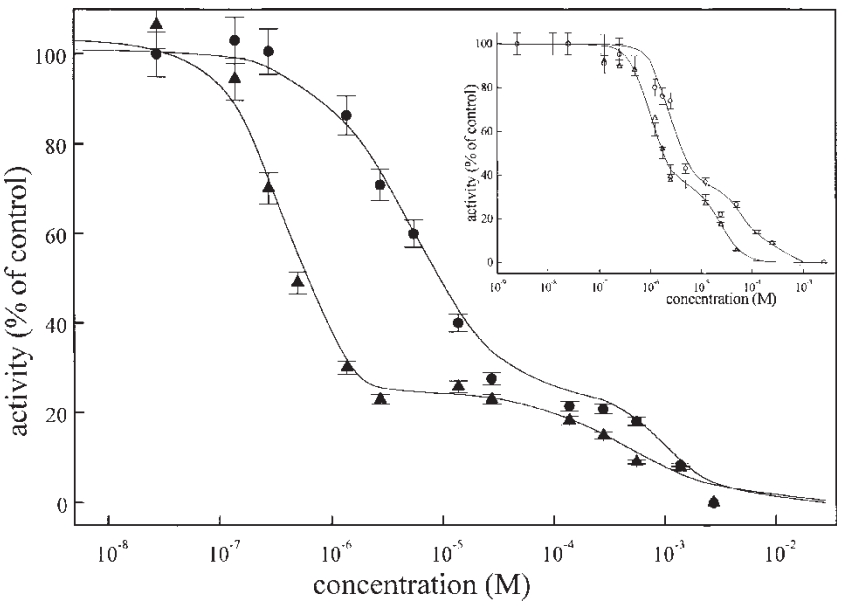

FIGURE 2 Inhibition of Na^+/K^+-ATPase activity by digoxin (circles) and gitoxin (up triangles) in human erythrocyte membrane and commercial porcine cerebral cortex (inset). The experimental conditions are described in Materials and Methods. The symbols represent experimental results. The solid lines represent the theoretical curves assuming two-site model fit, using the data from Table I. The values given are the mean of at least three experiments ± SEM, conducted in duplicate.

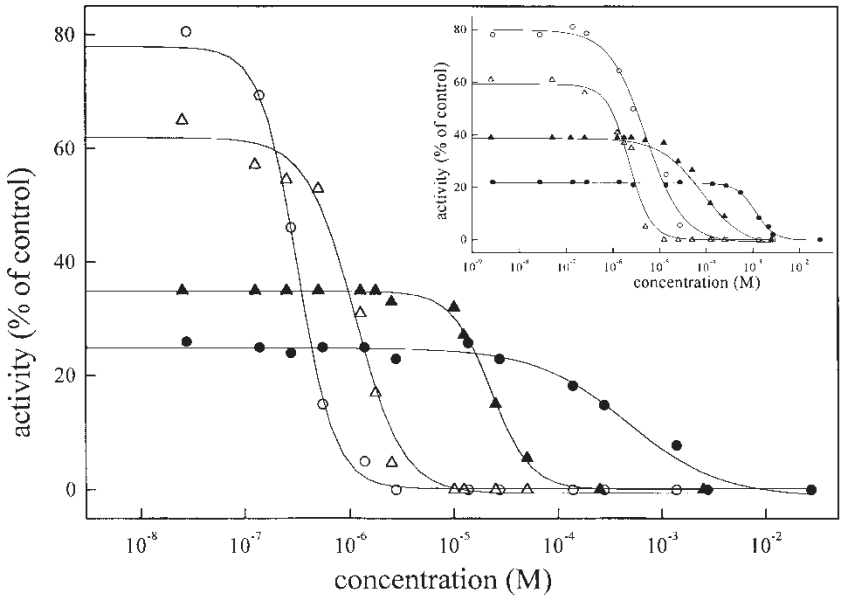

FIGURE 3 The theoretical curves for inhibition of high (open symbols) and low (solid symbols) affinity Na^+/K^+-ATPase isoforms induced by gitoxin and digoxin (inset). Circles—human ghosts membranes; up triangles—commercial porcine cerebral cortex.

Hill analysis was performed on the high and low affinity parts of the inhibition curves. Figure 4 shows the Hill plots for Na^+/K^+-ATPase. The values of the Hill coefficient, n, determined from the inhibition curves by Hill analysis are summarized in Table I, together with the IC_{50} values for the high and low affinity isoforms. As can be seen, the Hill analysis of inhibition always yielded $n > 1$. Using the calculated IC_{50} values, the total activity was recalculated and presented in Figure 2 as a solid curve. The results show that an excellent fit of the experimental points was obtained.

Kinetic Analysis

To evaluate the nature of the Na^+/K^+-ATPase inhibition by digoxin and gitoxin, the kinetic parameters, K_m and V_{max}, were determined by varying the concentration of $MgATP^{2-}$. The kinetic properties of the enzyme were determined in the presence of 1×10^{-6} M of digoxin or gitoxin. This concentration was chosen from the inhibition curves, as the concentration that inhibited the enzyme activity in the high affinity concentration range. The dependence of the initial reaction rate *vs.* substrate concentration in the presence and absence of inhibitors exhibited typical Michaelis–Menten kinetics, and is presented in Figure 5. Kinetic constants were calculated from the experimental data according to the Lineweaver-Burk transformation (Figure 5, inset), and are summarized in Table II. As can bee seen, both digoxin and gitoxin decreased the K_m and V_{max} values of the enzyme to the same extent.

Effects of Simultaneous Exposure to Digoxin and Gitoxin on Na^+/K^+-ATPase Activity

The simultaneous exposure studies were limited to inhibitors concentration that produced inhibition in the "high affinity" concentration range of the inhibition curves. Inhibitions caused by simultaneous exposure to a combined concentration of inhibitors as well as inhibitions induced by single exposure to the same concentrations of digoxin and gitoxin are shown in Table III. As can be seen, digoxin and gitoxin together caused statistically significant synergistic inhibition of porcine cerebral cortex enzyme at all concentrations examined, and erythrocyte enzyme at low inhibitor concentrations. On the contrary, the simultaneous exposure to high concentrations of inhibitors, digoxin(μmol)/gitoxin(μmol) concentrations of 0.1/1.0 and 10/0.1 inhibited erythrocyte Na^+/K^+-ATPase activity antagonistically.

DISCUSSION

It is generally considered that the digitalis cardiac glycosides interact with Na^+/K^+-ATPase receptor due to the electron withdrawing forces of the partially positive electron poor β-carbon atom with an anionic site and the partially negative electron rich carbonyl oxygen of the 17β-lactone substituent with a hydrogen donor sites.[14,16] The hydroxyl group in position-12 (digoxin) and -16 (gitoxin) of the steroidal skeleton have a considerably influence on the distribution of the electron charge of the unsaturated γ-lactone.[16,18,19]

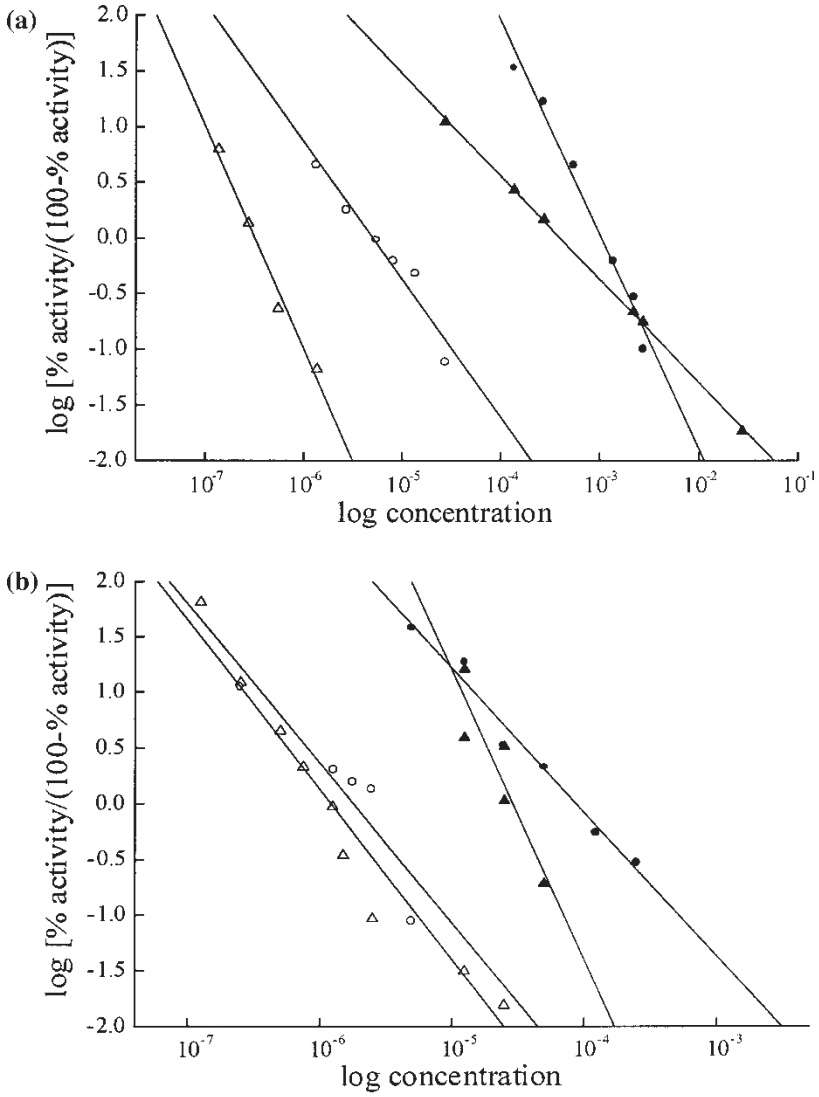

FIGURE 4 Hill plots of digoxin (circles) and gitoxin (up triangles) inhibition of Na^+/K^+-ATPase at low (solid symbols) and high (open symbols) affinity sites for the enzyme contained in human erythrocyte membrane (a) and commercial cerebral cortex enzyme (b). Regression lines were calculated by means of the least squares method. The R values were in the range 0.9196–0.9997.

In this work, the effects of digoxin and gitoxin were tested by their ability to alter the activity of Na^+/K^+-ATPase from human blood erythrocyte and from synaptosomal porcine cerebral cortex membrane, as diverse model systems.

The results of the study show, that digoxin and gitoxin inhibit Na^+/K^+-ATPase in both preparations in a concentration-dependent manner, but with diverse potency. However, gitoxin exerted the highest inhibitory potency on the enzyme in both preparations. The structural difference between digoxin and gitoxin is responsible for their diverse potency to inhibit Na^+/K^+-ATPase activity, although they both have the same chemical formula. It seems that the hydroxyl group in position-16 of the steroidal skeleton of gitoxin increases the partial

TABLE I IC_{50} values and Hill coefficients (n) for high and low affinity isoenzymes as calculated from experimental data by Hill analysis

	High affinity		Low affinity	
	IC_{50} (M)	n	IC_{50} (M)	n
human erythrocytes				
digoxin	$(4.64 \pm 0.07) \times 10^{-6}$	1.24 ± 0.15	$(1.04 \pm 0.05) \times 10^{-3}$	1.92 ± 0.13
gitoxin	$(2.98 \pm 0.10) \times 10^{-7}$ (s)	2.01 ± 0.19	$(4.10 \pm 0.90) \times 10^{-4}$	1.08 ± 0.22
porcine cerebral cortex				
digoxin	$(2.18 \pm 0.20) \times 10^{-6}$	1.43 ± 35	$(8.67 \pm 1.08) \times 10^{-5}$	1.29 ± 0.09
gitoxin	$(1.18 \pm 0.10) \times 10^{-6}$	1.52 ± 0.09	$(2.85 \pm 0.08) \times 10^{-5}$	2.59 ± 0.66

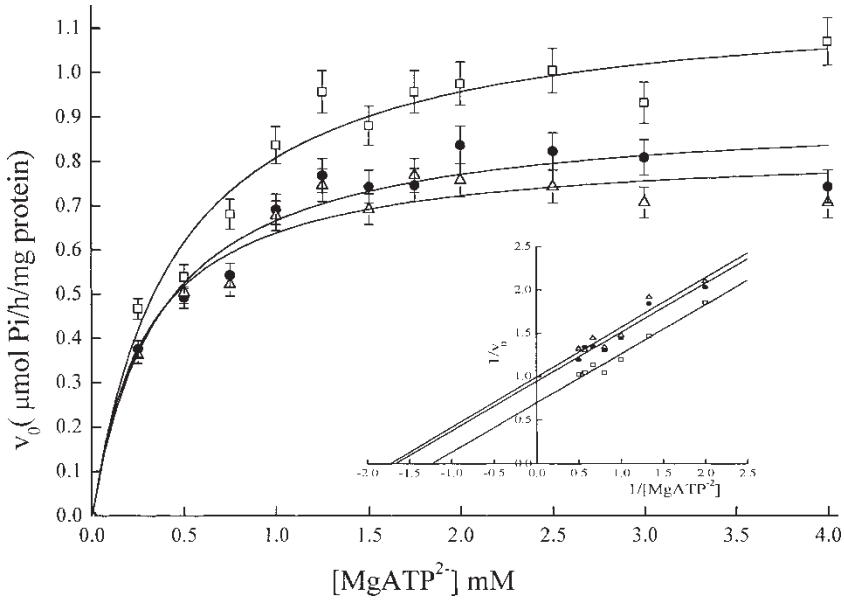

FIGURE 5 Porcine cerebral cortex Na^+,K^+-ATPase activity dependence of ($MgATP^{2-}$) in the absence (squares) and presence of 1×10^{-6} M digoxin (circles) and gitoxin (up triangles). The experimental conditions are described in Materials and Methods. The values given are the mean of at least three experiments ± SEM, conducted in duplicate. The Lineweaver–Burk transformation of the data is shown in the inset.

positive charge on the electron poor β-carbon, causing an increase in the affinity for ionic interactions between gitoxin and the carboxylate residues of the enzyme.[18,19]

The inhibitory effect, as shown in our study, depends also on the protein preparation. However, human blood erythrocyte Na^+/K^+-ATPase is more sensitive to exposure to gitoxin, compared 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.The heterogeneity of digoxin sites has been reported in rat brain and beef heart Na^+/K^+-ATPase and related to 2 distinct isoform of the α subunit.[27–31] 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.[18,19]

The low affinity isoform is also more sensitive to gitoxin, but both digitalis compounds exert a very strong interaction with the receptor in both preparations. It is clear from the results presented in Figure 3 that in human erythrocyte membranes the high affinity isoenzyme accounts for about 79% and the low affinity isoenzyme for 21% of the specific Na^+/K^+-ATPase activity. The similar ratio between high and low affinity isoenzyme was also obtained in porcine cerebral cortex (Figure 2, inset) and rat brain microsomes.[27] The high affinity to digoxin and gitoxin can be attributed to the α_3 isoform, which is known to be the most sensitive towards the cardiac glycosides.[32] It is well known that the α_3 isoform is especially abundant in the brain and some other vertebrate tissues.[13] Western analysis of the Na^+/K^+-ATPase from mature human erythrocyte, purified by ouabain column chromatography, has shown that erythrocytes contains the α_1 and α_3 isoforms of the α subunit.[33] Our results indicated that the low sensitive α_1 isoform was also present

TABLE II Kinetic analysis of Na^+/K^+-ATPase from porcine cerebral cortex activity in the absence and presence of inhibitor (4×10^{-6} M)

	K_m (mM)	V_{max} (μM P_i/h/mg)	K_m/Vmax
Control	0.81 ± 0.05	1.43 ± 0.06	0.566
Digoxin	0.60 ± 0.07	1.06 ± 0.08	0.566
Gitoxin	0.58 ± 0.08	1.01 ± 0.09	0.574

TABLE III Percent inhibition of Na^+/K^+-ATPase by single and simultaneous exposure to digoxin and gitoxin

Digoxin		Gitoxin		
Conc (μM)	% Inhibition	Conc (μM)	% Inhibition	Digoxin+gitoxin % Inhibition
porcine cerebral cortex				
0.1	1.0	0.2	2.2	12.0
0.1	1.0	0.5	11.1	32.4
0.1	1.0	1	31.8	39.3
0.2	1.9	0.1	2.0	15.8
1	7.8	0.1	2.0	24.2
10	63.0	0.1	2.0	78.7
human erythrocytes				
0.1	1.0	0.2	18.2	24.4
0.1	1.0	0.5	45.4	58.8
0.1	1.0	1	63.8	59.3
0.2	2.0	0.1	7.7	27.3
1	12.3	0.1	7.7	41.8
10	50.8	0.1	7.7	50.0

and inhibited in both preparations with similar affinity towards the investigated digitalis compounds. It is obvious from the inhibition curves, that the same ratio of isoforms was obtained in both preparations.

Hill analysis always yielded a Hill coefficient $n > 1$, which suggests cooperative binding of digitalis. Based on the Hill coefficient the conclusion can be drawn that there is a positive cooperative interaction between a minimum of two binding sites on the enzyme. In addition, gitoxin exerts the higher value of *n* in both preparations.

Kinetic analysis of the results showed that digoxin and gitoxin behaved as uncompetitive inhibitors of Na^+/K^+-ATPase activity. The inhibitors interfered with the enzyme by binding to the enzyme–substrate complex causing structural distortion of the active site. The results showed that the both inhibitors decreased K_m and V_{max} values of the enzyme to the same extent. Moreover, parallel straight lines were obtained from Lineweaver–Burk analysis of the kinetic data in the absence and presence of both inhibitors (Figure 5, inset), i.e. the constant ratio $K_m/V_{max} = 0.570$ confirmed the uncompetitive mode of interaction.

The synergistic effect by simultaneous exposure to digoxin and gitoxin of Na^+/K^+-ATPase was obtained for both investigated preparations using low inhibitor concentrations. This result was expected, taking into account the positive cooperative interaction between inhibitors and protein. The antagonistic inhibition of erythrocyte Na^+/K^+-ATPase in the presence high concentrations of inhibitors in the combination, suggests that both inhibitors are competing for a limited number of inhibitor binding sites on the enzyme. This is in concordance with the fact that the erythrocyte membranes were less purified than the commercial porcine cerebral cortex (electrophoretic profile not shown).

In conclusion, good agreement of the data was obtained, indicating similarity in the response of ATPases to inhibitors in both biological systems. However, the human erythrocyte membranes showed the higher sensitivity towards the inhibitors. Since the erythrocytes from human blood can be easily prepared for measurement of ATPases activity, the results confirmed that they could be recommended as a better model system for *in vitro* investigations of mechanism, kinetics and structure–activity relationship of pharmaceuticals on target enzymes.

Acknowledgements

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