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INTERACTION OF CARDENOLIDES OF THE STROPHANTHIDIN SERIES WITH FUNCTIONALLY IMPORTANT SECTIONS OF TRANSPORT Na,K-ATPase

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On the basis of an analysis of the structure–activity interrelationship of cardiac glycosides and their analogs, a four-center model of the binding of the compounds with Na,K-ATPase has been proposed. These centers are represented by the steroid nucleus with a polar group at C-3 and a hydroxy group at C-14, and also by a lactone ring with a double bond. It has been shown that the hydrophobic binding section of the glycoside must be in contact with a hydrophobic region of the enzyme having a fairly large volume.

The directed search for cardiotonic agents with given properties is possible only on the basis of a knowledge of the concrete mechanism of the action of this type of substances at the cell level. This knowledge is important both for those who are engaged in a search for active agents in the plant world and for synthetic chemists working on the complete or partial synthesis of cardiotonic compounds. In the latter case, of course, hope is placed on the production of more active drugs. In view of this, the finding of functionally important structural elements of cardiosteroids and the investigation of features of their interaction with Na,K-ATPase still remains urgent.

According to Barnett [1], these loci in the enzyme are necessary for the binding of a cardiotonic glycoside with Na,K-ATPase. The first binds with the carbohydrate moiety of the glycosides, the second interacts with the hydroxy group in the C-14 position of the steroid molecule, and the third is attached to the lactone ring of the glycoside.

According to a hypothesis due to Yoda and Yoda [2], the interaction of cardiac glycosides with Na,K-ATPase is first effected by a steroid-specific site through which a sugar-binding center is activated, and then the addition of the glycosidic moiety of the inhibitor takes place.

Facts are known which indicate that the glycoside binds to the enzyme through hydrogen bonds [3, 4] or through a stronger chemical interaction [5]. A hypothesis has been put forward that on the binding of cardiosteroids with the receptor site of the transport enzyme, the formation of a covalent bond of the unsaturated lactone ring at C-17 with the nucleophilic center of the enzyme takes place simultaneously with the formation of a hydrogen bond through the hydroxy group at C-14 [6-8]. The combination of these facts leads us to the conclusion that the interaction of cardiac glycosides is effected not with the whole enormous molecule of the enzyme but only with a definite center, the so-called digitalis receptor. No few hypotheses have been expressed on the nature of the binding of cardiosteroids with Na,K-ATPase but hitherto there have been no sufficiently convincing proofs of the mechanism of the interaction of the group of compounds under discussion with the enzyme. The structure–activity relationship has been investigated mainly of cardiac glycosides of the digitalis series, which possess an appreciable capability for cumulation and which exhibit, in the main, a healing effect

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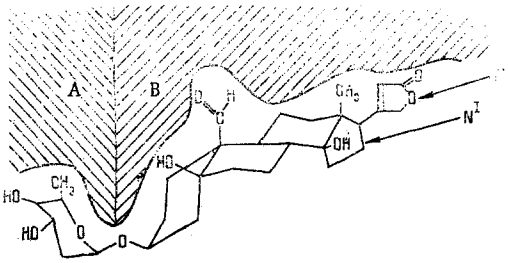

Fig. 1. Model of the binding site for cardiac glycosides in the molecule of Na,K-ATPase and a scheme of its interaction with convallatoxin: A – hydrophilic region; B – hydrophobic region; N – nucleophilic center; N' – binding center for the hydroxyl at C-14.

after definite intervals of time. The cardioactive compounds of the strophanthidin series with less pronounced cumulative properties act faster.

We have performed a systematic investigation of the mechanism of the interaction with transport Na,K-ATPase of various structural analogs of strophanthidin, some isolated from plants of Central Asia and some synthesized in the laboratory of glycoside chemistry of the Institute of the Chemistry of Plant Substances of the Academy of Sciences of the Uzbek SSR, and have compared their effect with that of widely known glycosides of other aglycones. We used purified preparations of Na,K-ATPase from the medullary layer of the porcine kidney (~90% purity in terms of protein). In preceding investigations, using preparations of the Na,K-ATPase of ox brain and pig heart, strophanthidin analogs containing voluminous polar sugar substituents in the C-3, C-5, and C-19 positions were tested for their cardiotonic activity [9-11]. On this basis, a hypothetical model of the binding of cardenolides was put forward which included two essential structural fragments – an area of binding of the polar or hydrophobic section and a nucleophilic center of interaction of the double bond of the lactone ring. At this stage of the work, the structure–activity analysis was supplemented by a number of specially synthesized compounds with hydrophobic substituents in various positions of the steroid nucleus. Table 1 gives a list of the compounds tested, features of which are:

The presence of carbohydrate or, conversely, hydrophobic residues on the carbon atoms of the steroid molecule at C-3, C-5, and C-19;

the α position of the lactone ring at C-17;

the presence of a hydroxy group at C-17; and

a different orientation, or the absence, of the hydroxy group at C-14.

We have previously [9-11] put forward a hypothesis concerning the presence in the active center of Na, K-ATPase of two sites with different functions. These are an "adsorption" center which binds the cardiac glycoside to the receptor and controls and orients the molecule relative to the second, "catalytic" site, where groups interacting with the lactone ring are located. On the basis of the results of a preliminary analysis, it has been suggested that the adsorption of the glycosides must probably take place through a hydrophobic or steric interaction with the corresponding site of the receptor. In the first case, unstable intermediate compounds may be formed for a short time. In the second case the volume parameters of the individual functional groups cause a serious disturbance of complementarity between the receptor and the molecule of the cardiac glycoside. Having compared with one another hydrophibic and hydrophilic derivatives of strophanthidin, we give our preference to a hydrophobic interaction with the corresponding section of the enzyme.

The model given in Fig. 1 on the whole suggests the existence of a barrier only for the polar substituent in position 19. Strongly polar groupings cannot be localized in the hydrophobic region of the protein molecule of the enzyme.

The orientation of the steroid nucleus on the binding of a cardiac glycoside with the enzyme is determined by the position of the polar group in the steroid nucleus. As can be seen from Table 1, the most active are the 3-substituted strophanthidins, which are strongly bound to the enzyme. Regardless of its nature, a carbohydrate component in the C-3 position has a definite role in the interaction with the enzyme, since through the polar groups of the sugars a rapid, complete, and stable binding with the catalytic center of the enzyme takes place, as has been established in experiments with compounds 22-25 and 33, and 34-36 (see Table 1). The same effect is also characteristic for glycosides of other aglycones; for example, compounds 45-51.

TABLE 1. Effect of Inhibitory Activity and Lethal Activity of the Investigated Cardenolids

Compound	Cardenolide	Concentration of the cardenolide (μM) causing 50% inhibition of kidney Na,K-ATPase (~90% purity as protein)	Lethal dose (CAU*), μmole/kg (according to the literature)
	Strophantidin and its derivatives		
1	Strophanthidin	0,90	0,80 [12]
2	Strophanthidol	6,50	1,72
3	Strophanthidinic acid	61,00	6,06
4	Strophanthidin 3-acetate	1,32	0,42 [12]
5	Strophanthidin 3-benzene	0,75	0,17 [13]
6	Strophanthidin 3-acetate 19-benzoate	Inactive	
7	Strophanthidol 3,19-dibenzoate	"	
8	Isostrophanthidin	81,00	Inactive
9	17α-Hydroxystrophanthidin [14]	21,00	"
10	17α-Hydroxystrophanthidin 3-acetate [14]	18,00	2,20
11	17α-Pseudostrophanthidol [15]	Inactive	
12	17α-Pseudostrophanthidol 3-acetate	"	
13	14α-Pseudostrophanthidol	"	
14	14α-Pseudostrophanthidol 3-acetate	"	
15	3,19-Epoxy-5,6-anhydrostrophanthidin ethylal [12]	"	
16	Dihydrostrophanthidin	"	
17	Dihydrostrophanthidin 3-acetate	"	
	Derivatives of strophanthidin with uronic acids		
18	Strophanthidin 3-β-D-glucopyranosiduronic acid; methyl ester	7,80	0,33 (FAU)
19	Strophanthidin 3-β-D-glucopyranosiduronic acid; methyl ester triacetate	8,70	
20	Strophanthidin 3-β-D-glucopyranosiduronic acid; amide	5,50	0,86 (FAU)
21	Strophanthidin 3-β-D-galactopyranosiduronic acid; methyl ester triacetate	5,20	
	Monoglycosides of strophanthidin and its derivatives		
22	Corchoroside A (strophanthidin 3-bovinoside)	0,78	,17
23	Erysimin (strophanthidin 3-digitoxide)	0,48	0,15 [12]
24	Strophanthidin 3-ribopyranoside	0,80	0,37
25	Strophanthidin 3-rhamnopyranoside (convallatoxin)	1,05	0,14 [12]
26	Strophanthidin 3-rhamnopyranoside; triacetate	8,40	1,01
27	Strophanthidin 3-glucopyranoside, tetraacetate	7,52	1,01
28	Strophanthidin 5-rhamnopyranoside	10,00	,52
29	Strophanthidin 3-acetate 5-rhamnopyranoside	1,80	,76
30	Strophanthidol 19-rhamnopyranoside	Inactive	
31	17α-Pseudostrophanthidol 3-rhamnopyranoside [15]	25,20	
32	17α-Pseudostrophanthidol 3-rhamnopyranoside; triacetate [15]	Inactive	
33	Strophanthidin 3-cymaropyranoside (cymarin)	0,54	

The cardiotonic effect of the glycosides is retained when they are glycosylated with uronic acid in the C-3 position, but it is expressed somewhat more feebly (compounds 18-21 in Table 1). The introduction of L-rhamnose at C-5 (compounds 28, 29, and 42) also lowers the effect.

Particular attention is merited by a group of compounds acylated at the C-3 hydroxyl (compounds 4-7). In these, the polar groups forming the ester bond are localized between two hydrophobic fragments – the radical of the acid and the steroid residue. While strophanthidin 3-acetate and strophanthidin 3-benzoate are only slightly inferior to strophanthidin and its natural glycosides in activity, strophanthidol 3-acetate 19-benzoate and strophanthidol 3,19-dibenzoate proved to be completely inactive.

By comparing compounds 11, 31, and 32, it is possible to evaluate the role of a polar substituent in the C-3 position as a parameter determining the orientation of the inhibitor on the enzyme. With a considerable change in the structure of the strophanthidin molecule as, for example, in the case of 17α-pseudostrophanthidol (compound 11), the capacity for inhibiting Na,K-ATPase practically disappears. However, the glycosylation of this compound at C-3 leads to a partial restoration of its activity (compound 31). In its turn, the complete acetylation of the sugar residue (compound 32), as a result of which the hydrophilicity of the substituent in the C-3 position falls, leads to a partial or complete loss of activity by the compounds formed. Thus, the acetylation of the sugar residue is unfavorable for the manifestation of inhibitory activity. Such a loss of activity is also observed on the acetylation of hydroxyls of sugar residues at C-3, C-19, and C-5 (compounds 26,

TABLE 1 (continued)

Compound	Cardenolide	Concentration of the cardenolide (μM) causing 50% inhibition of kidney Na,K-ATPase (~ 90% purity as protein)	Lethal dose (CAU*), μmole/kg (according to the literature)
	Strophanthidin diglycosides		
34	k-Strophanthin-β (strophanthidin 3-strophanthobioside)	1,15	0,17
35	Olitoriside (strophanthidin 3-olitoribioside)	1,50	0,16
36	Erysimoside (strophanthidin 3-digilanidobioside)	0,30	0,13
37	Erysimoside pentaacetate (strophanthidin 3-digilanidobioside; pentaacetate)	15,50	1,10
38	Strophanthidol 3,19-bismaltopyranoside	Inactive	
39	Strophanthidol 3,19-bismaltopyranoside; tetradecaacetate		
40	Strophanthidol 3,19-bisrhamnopyranoside		
41	Strophanthidin 3,19-bisrhamnopyranoside; tetradecaacetate		
42	Strophanthidin 3,5-bisrhamnopyranoside	2,00	0,26
43	Strophanthidin 3,5-bisrhamnopyranoside; hexaacetate	Inactive	
	Glycosides of other aglycones		
44	Digitoxigenin	6,20	1,23 (12)
45	Digitoxigenin 3-ribopyranoside	0,10	
46	Evomonoside (digitoxigenin 3-rhamnopyranoside)	1,51	0,53
47	Ouabain (ouabagenin 3-rhamnopyranoside)	1,00	0,19
48	Cuspidoside (bipindigenin 3-rhamnopyranoside) (lokundjoside)	0,55	0,19
49	Periplogenin 3-rhamnopyranoside	1,32	0,57 [16]
50	Periplogenin 3-fucopyranoside (ledienoside)	0,70	0,26 [17]
51	Apobioside (cannogenin 3-strophanthobioside)	0,70	0,21

*CAU – dose of the substance under trial calculated to 1 kg body weight of the animal causing the stoppage of the heart of a cat; FAU – the same for a frog. The determination of the activity of the glycosides on cats is presented according to results in the literature; the others were determined on cats and frogs in the pharmacology laboratory of the Institute of the Chemistry of Plant Substances of the Academy of Sciences of the Uzbek SSR.

27, 32, 37, 39, 41, 43). This leads to a considerable increase in the hydrophobicity and the total volume of the cardiac glycoside molecule. For this reason or another, the complete acetates of the glycosides are, as a rule, less active than the glycosides themselves. In this connection, it is interesting to note a high activity of spin-labeled strophanthidin derivatives having a hydrophobic substituent of large volume in the C-19 position [18].

The same effect was detected by Rogers and Lazdunski [19] in the photoaffinity labeling of strophanthidin in the C-19 position. In contrast to the highly polar substituents in compounds 30, 38, and 40, this residue cannot substantially change the orientation of the hydrophobic nucleus of the inhibitor in the enzyme. The introduction into the C-19 position of an oxyethylol group (compound 15) or a carboxy group (compound 3), imparting a certain hydrophilicity to the molecules of these substances, likewise led to the loss of inhibitory activity. In the formation of cardiotonic activity and the capacity for suppressing Na, K-ATPase that correlates with it, no small role is played by a hydroxy group at C-14. As is already well known, isostrophanthidin (compound 8) does not possess cardiotonic activity because of the fixation of the lactone ring and the appearance of a bond between carbon atom 14 and the lactone ring. A comparison of compounds 11-14, 31, and 32 shows that the shift of the hydroxyl from the C-14 to the C-8 position likewise has a considerable effect on the activity of the cardenolide compound, up to its complete loss.

Compounds 16 and 17 with saturated lactone rings have a low activity.

The facts given above indicate the absence of an inhibiting activity for the 17α-isomers of strophanthidin (compounds 11, 12, 31, and 32). Only the 17α-isomers are active. In all natural compounds, there is a hydrogen in the 17α position. The presence of a hydroxy group at C-17 apparently has a spatial influence on the lactone ring at the same carbon atom, which leads to a decrease or loss of cardiotonic activity (compounds 9 and 10).

Thus, at least four functionally important fragments of its molecule participate in the binding of a cardiac glycoside with the enzyme. These are the steroid nucleus, the hydroxy group at C-14, the polar group in the C-3 position, and the electrophilic center in the lactone ring.

The model given in Fig. 1 suggests the binding and the relatively rigid orientation of the steroid nucleus and of the polar substituent of a cardiac glycoside at the boundary of separation of polar and nonpolar regions of the enzyme. With such an orientation of the inhibitor in the enzyme, the spatial approach of the electrophilic lactone ring and of the nucleophilic center of the enzyme (N) is ensured. The hydroxy group at C-14 possibly forms a hydrogen bond with the center (N'). Furthermore, the hydrophobic binding sites of the cardiac glycoside must be in contact with a hydrophilic region of the enzyme of fairly large volume.

To elucidate the correlation between the efficiency of the inhibition of Na,K-ATPase and the cardiotonic action of the glycosides investigated, which was evaluated from their capacity for stopping the cat heart, we used literature figures for the determination of the CAU [12, 13, 16, 17] and experimental results obtained on cats or frogs in the pharmacology laboratory of the Institute of the Chemistry of Plant Substances of the Academy of Sciences of the Uzbek SSR (see Table 1).

A comparison of the cardiotonic activities of these substances with the efficacy of their influence on Na,K-ATPase revealed the existence of a characteristic logarithmic dependence. This dependence has been made the basis of a method of the enzymatic testing of cardiac glycosides which can be used in the initial selection of new drugs with potentially cardiotonic action.

EXPERIMENTAL

The microsomal fraction of porcine kidney medullary layer was isolated by Skou's method [20]. To obtain purified Na,K-ATPase from porcine kidney medullary layer we used Jorgensen's method [21] in A. B. Chetverin's modification [22]. The purified Na,K-ATPase preparations with a high specific activity were free from Mg-ATPase background.

The medium for the determination of the Na,K-ATPase activity contained 3 mM $MgCl_2$, 135 mM NaCl, 5 mM KCl, 1 mM EDTA, and 30 mM Tris-HCl (pH 7.4). The final volume of the reaction mixture was 0.5 ml. After incubation at 37°C for 15 min with various concentrations of the substances under investigation in the absence of ATP, the reaction was begun by the addition of ATP to a final concentration of 2 mM. After incubation for 10 min at 37°C, the reaction was stopped by the addition of 3% perchloric acid (final concentration 1.7%). Inorganic phosphorus was determined by Panusz's method [23] and protein by a modified Lowry method [24].

The concentration causing 50% inhibition (I_{50}) was determined from the logarithmic dependence of the percentage inhibition of the logarithm of the concentrations of the substances tested.

SUMMARY

On the basis of the results of an analysis of the structure–activity interrelationship of cardiac glycosides and their analogs, a four-center model of the binding of the compounds with Na,K-ATPase has been put forward. These centers are represented by the steroid nucleus with a polar group at C-3 and a hydroxy group at C-14 and also a lactone ring with a double bond. It has been shown that the hydrophobic binding site of the glycoside must be in contact with the hydrophobic region of the enzyme of fairly large volume.

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FRAGMENTATION OF THE UNSUBSTITUTED CARBOHYDRATE UNITS OF CARDENOLIDE MONOSIDES

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In the mass spectra of unsubstituted monosides of cardenolide nature are observed the M^+ peaks, and also, in each case, three characteristic processes of the fragmentation of the carbohydrate unit. The formation of the ions $AglOCH{=}OH^+$ is the most universal property of these compounds. The stability of ions of the type under consideration depends on the nature and position of attachment of the sugar residue and on the number of polar groups in the aglycone. The laws of the fragmentation of the carbohydrate unit are extended to the spectra of glycosides of other classes.

Mass spectrometry is widely used to detect new carbohydrate-containing plant compounds. In this process, the wider use of "mild" methods of ionization, with the aid of which the peaks of the molecular ions of unsubstituted glycosides are obtained with incomparably greater intensity than on the use of electron impact (EI), is of no little importance. importance.

This was first demonstrated for the case of cardenolides by Brown et al. [1], who compared the field ionization (FI) and EI spectra of the aglycones digitoxigenin and strophanthidin and their mono-, bi-, and trioside and also individual monosaccharides. Furthermore, it has been found that in the FI spectra the fragments formed by the sequenation of the carbohydrate chain are more stable.

The fragmentation of glycosides under the action of EI takes place less selectively, but the spectra obtained by this method contain the peaks of the ions arising on the cleavage of the bonds of the carbohydrate chain. This feature of the spectra is not discussed by Brown et al. [1]. Nevertheless, it permits useful conclusion to be drawn in a comparison of the spectra of glycosides with different sugar residues or with different aglycones.

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