FEATURES OF THE INHIBITION OF TRANSPORT Na^+, K^+-ATPase BY CARDENOLIDE BISGLYCOSIDES

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At the modern stage of the development of our ideas on the mechanism of the action of cardiac glycosides, it may be regarded as indisputably established that the physiological effects characteristic of them are realized through interaction with transport Na^+,K^+-ATPase. This interaction is effected not over the whole of the enormous enzyme molecule, the molecular weight of which, depending on the method of determination, has been calculated to be in the range from 250,000 to 1,000,000 daltons [1], but only with the active section which in the literature has acquired the name of the digitalis receptor.

The whole of the cardiosteroid molecule takes part in the cardiac glycoside–receptor reaction, but even before the discovery of the biochemical basis of the action of cardiotonic glycosides it was clear that the key role in the positive inotropic action is played by the unsaturated five-membered lactone ring in cardenolides and the six-membered ring in the bufadienolides. The unsaturated lactones, which are orientated in a definite manner with respect to the plane of the steroid nucleus, impart to the molecules that specificity of their action on the heart muscle that is absent from other steroid compounds. Consequently, it is natural that after the discovery of the parallelism existing between the inhibition of Na^+,K^+-ATPase and the positive inotropic effect the main attention of investigators should be directed to elucidating the role of the lactone grouping. Starting from the hypothesis that the interrelationship of the cardiosteroids with the enzyme takes place through the lactone ring, a considerable amount of work was carried out on the rearrangement and modification of this necessary element of the structure of the cardiac aglycones. So far as concerns the remainder of the molecule, the necessity for a sterane skeleton and a hydroxy group at C-3 was asserted. Neither the butenolide ring (as in the cardenolides) nor the α-pyrone heterocycle (as in the bufadienolides) possess cardiac activity in themselves.

The role of the sugar component in the mechanism of inhibition is not quite clear. The fact that the activity of the cardiac glycosides decreases or increases in dependence on the structure and length of the monosaccharide units appears to be a factor which is understandable in itself. However, recently it has been observed that the sugars not only change the cardiotonic action inherent in the aglycones but, in individual cases, may eliminate it even if the lactone ring still remains completely uninvolved. Thus, for example, the glycosides of digitoxigenin and of strophanthidin differ little from ouabain and other natural glycosides. Digitoxin 3-D-glucuronoside is approximately 10 times inferior to ouabain in its inotropic action [2]. The replacement in the sugar moiety of a primary hydroxy function by a carboxy function led to a sharp decrease in activity. And this is apparently by no means due to the fact that with the appearance of a carboxy group the molecule has acquired additional hydrophilicity. In the case of the methyl ester of strophanthidin 3-D-glucuronoside the inhibition effect is likewise approximately 10 times less than that of ouabain [3], although the ester groups imparts a certain hydrophobicity to the compound.

In the natural cardiosteroids, as a rule, the sugars are attached to the aglycone through a hydroxy group at C-3. The only exceptions are two bufadienolide glycosides – scilliglaucoside and scillicyanoside – in which the glycosidic residue is attached through a hydroxyl not at C-3 but at C-5 [4]. Unfortunately, the biological action of these compounds had not been determined.

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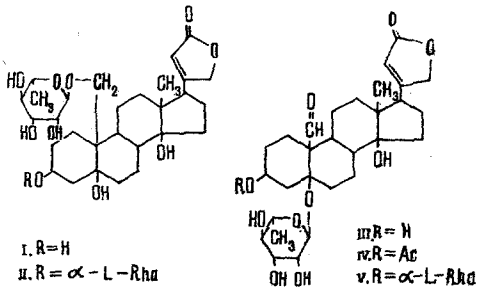

I. R = H
II. R = α-L-Rha

III. R = H
IV. R = Ac
V. R = α-L-Rha

We have been able to synthesize cardenolide mono- and bisglycodies with additional monosaccharide residues in the C-5 and C-19 positions [5, 6]. The new compounds have been tested for the inhibition of Na^+,K^+-ATPase under approximately the same conditions as were used previously [3]. In individual cases, to confirm the parallelism between the inhibition and cardiotonic action of the glycosides synthesized we determined their lethal doses on cats (LD_{100}):

Aglycone and glycoside	Concentration causing 50% inhibition, μmole/liter	Lethal dose for cats, mg/kg
Strophanthidol	0,60	0,700
Strophanthidin	0,65	0,324
Strophanthidinic acid	53,0	2,649
Strophanthidin acetate	0.32	0,187
Convallatoxol	Not determined	0,099 [7]
Strophanthidol 19-rhamnoside (I)	Not determined	Inactive [7]
Strophanthidol 3,19-bisrhamnoside (II)	Inactive	Inactive
Convallotoxin	0,22	0,079
Strophanthidin 5-rhamnoside (III)	2,80	1,020
Strophanthidin 3-acetate 5-rhamnoside (IV)	1,29	0,454
Strophanthidin 5,19-bisrhamnoside (V)	1,89	0,643

On comparing the connection between structure and action, the absence of cardiotonic activity in 3β,19-bis-O-α-L-rhamnopyranosylstrophanthidol (II) appears the most surprising [6, 7]. The results of our experiments with this glycoside have something in common with the observations of V. T. Chernobai [7], who found that 19-O-α-L-rhamnopyranosylstrophanthidol (I) possesses no cardiotonic action, while its isomer strophanthidol 3-α-L-rhamnopyranoside (convallatoxol) is characterized by a fairly high biological activity (0.099 mg/kg body weight of the cat). What are the reasons for the loss of the activity of strophanthidol glycosylated by L-rhamnose at the C-19 hydroxyl? The following two reasons are the most likely:

Steric Hindrance. The biological activity of the cardiosteroids is based on their very strict complementarity with the receptor section of the enzyme, which does not permit even minute deviations. Here no small role is played by the angular groups at C-10 and C-13, which project from the plane of the cardiosterane ring. All natural cardiosteroids have a methyl group at C_{13}. The chemical natures of the angular groups at C-10 are different. They may have the form of a methyl group (for example, digitoxigenin, periplogenin), a primary alcohol group (strophanthidol, ouabagenin), or an aldehyde group (strophanthidin, pachygenin). Aglycones with a carboxy group have also been found in nature, but it has not been shown strictly that they are native compounds. It is more likely that the carboxy group is formed during the isolation of the glycosides by the autooxidation of an aldehyde group.

A visual analysis on Stuart models and a rough calculation of internuclear distances (Fig. 1) in a sequence in which the volume of the oxygen-containing functions at C-10 gradually increase and the other elements of the structure of the molecule remain unchanged (periplogenin – strophanthidin – strophanthidol – strophanthidinic acid – strophanthidol 19-rhamnoside) show that the critical dimensions ensuring a high cardiotonic effect are found in strophanthidin and strophanthidol. A further even slight increase in the size of the angular group leads to a serious disturbance of the complementarity between the receptor and the cardiosteroid molecule. While strophanthidinic acid still inhibits Na^+,K^+-ATPase to some extent [3], the 19-rhamnoside (I) and the 3,19-bisrhamnoside (II) of strophanthidol are completely deprived of this capacity. Hence, the conclusion arises that the two last-mentioned compounds possess no cardiotonic activity because the steric volume of the carbohydrate components is too great to enter the groove of the enzyme (Fig. 2).

Change in Hydrophobicity. Angular methyl groups impart a definite hydrophobicity to the molecules of the physiologically active steroids. It is not excluded that the active center of ATPase "catches" cardiosteroid molecules by the nonpolar groups, extracting them from aqueous solution. Thanks to the hydrophobic interaction, the methyl groups assist the cardiosteroids to pass out from the polar medium and enter the receptor sec-

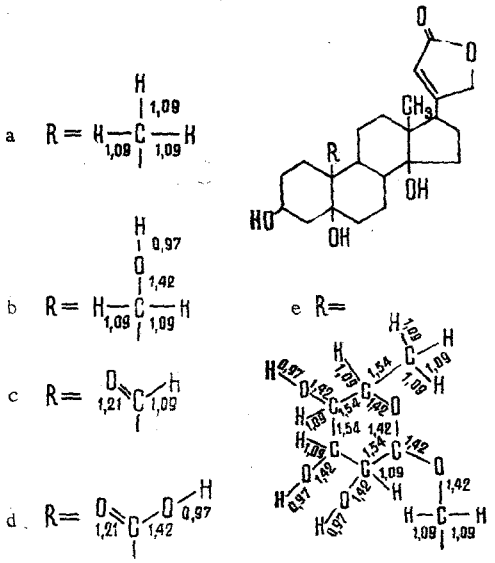

Fig. 1. Approximate internuclear distances (in Å) in the angular groups at C-10 in periplogenin (a), strophanthidol (b), strophanthidin (c), strophanthidinic acid (d), and strophidol 19-rhamnoside (e). The introduction of a rhamnosyl residue in the C-19 position leads to a marked increase in the volume of this part of the molecule. Torsional angles have not been taken into account.

tion of the enzyme. A methyl radical is an electron-donating group and usually increases the basicity of the molecule.

Even in a simple comparison with the aid of thin-layer chromatography [for example, in the chloroform–methanol–water (65 : 35 : 15) system], the five compounds mentioned are separated into two distinct groups according to hydrophobicity. Strophanthidinic acid and strophanthidol dirhamnoside form the group of polar compounds. Periplogenin, strophanthidol, and strophanthidin have similar hydrophobicities and can be regarded as compounds of medium polarity. Thus, the second possible reason for the loss of activity of strophanthidol 3,19-bisrhamnoside is the increased hydrophilicity of the sugar component attached to the hydroxyl at C-19.

It is difficult to state which of these factors exerted the decisive influence on the physiological propertie of strophanthidol 3,19-bisrhamnoside (II) – possibly both. A simple summation of the internuclear distances ir strophanthidol and strophanthidinic acid (without taking torsional angles into account) shows (see Fig. 1) that the difference in volume between the primary alcoholic and the carboxy groups is apparently not so great as t have a marked effect on the cardiotonic activity. A rhamnose residue is very much larger than a primary alcohol group. However, the impossibility of determining the reason for the low activity of strophanthidinic acid does not permit a firm conviction of the decisive significance of steric hindrance, although in the 19-substituted glycosides hindrance is undoubtedly caused by the large dimensions of the sugar component.

There is no doubt that it is impossible to adopt the second hypothesis without objections. The acetylatic of the sugar hydroxyls may impart considerable hydrophobicity to the cardiac glycosides but at the same tim the volume of the molecule actually increases somewhat. For this reason or another, the full acetates of the glycosides are, as a rule, less active than the glycosides.

We tested another group of compounds for cardiotonic activity and inhibiting effect: 5β-O-α-L-rhamn pyranosylstrophanthidin (5-isoconvallatoxin (III)), 5β-O-α-L-rhamnopyranosylstrophanthidin 3-acetate (IV), 3β,5β-bis(O-α-L-rhamnopyranosyl)strophanthidin (V) [5]. A distinguishing feature of the compounds listed i the presence of a sugar molecule at C-5 in them. The introduction of L-rhamnose not at the primary alcoho hydroxyl at C-19 but at the tertiary hydroxyl at C-5 also showed an influence on the activity of the cardioste roids, but not in such a marked form as in the case of strophanthidol 3,19-dirhamnoside (II). Compounds (II V) exhibit an inhibiting influence on Na^+,K^+-ATPase, but to a considerably smaller degree than convallatoxi (Table 1).

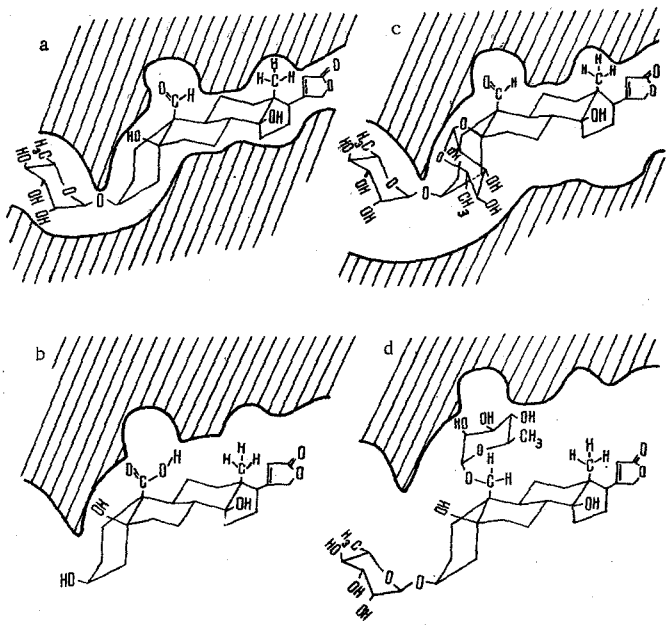

Fig. 2. Schematic models of the adsorption center of the reception section of Na^+,K^+-ATPase. Only the convallotoxin molecule (a) is completely complementary to the receptor. Strophanthidinic acid (b) possesses a small activity, since the carboxy group is in weak contact with the enzyme groove for the angular group. In strophanthidin 3,5-bisrhamnoside (c), the sugar radical at C-5 and the C-19 aldehyde group are located above the plane of the steroid nucleus (Stuart models). This compound disturbs the steric forms of the enzyme slot and therefore is only slightly active. In the case of strophanthidol 3,19-bisrhamnoside (d), the sugar residue at C-19 is so large that contact with the surface of the receptor is impossible.

Why do cardiosteroids of different structures possess different activities sometimes very remote from one another?

Structural features of the cardiac glycosides are more specific than those of other steroid compounds that are customary for the animal organism. Although a multiplicity of synthetic analogs having activities greater than those of the natural sex hormones and corticosteroids has been created, in not one case has it been possible to synthesize compounds even approximating in biological activity to the cardiosteroids most frequently used in medicine. Substitutes for cardiac glycosides are unknown. There is no doubt that we are dealing with a unique creation of living nature. The interaction of the cardiosteroids with transport ATPase is not fortuitous. In plants producing cardiac glycosides it has apparently been placed in deep concealment or has been transformed in the process of evolution.

The specificity of the action of cardiosteroids is closely connected with the specificity of the structure of the receptor section of Na^+,K^+-ATPase. The strict orientation of the peptide chains predetermines the distribution of the polar and nonpolar groups, and therefore only those molecules interact with the enzyme that, in size, steric interrelationship of the individual rings and functional groups of the steroid nucleus, degree of polarization, lipophilicity, etc., come into contact with the active center sufficiently closely. If complete complementarity in the structures of the cardiosteroid and the enzyme is not achieved, the effect of the biological action is weakened, and this to a greater degree the greater the deviation from the optimum position.

In our view, the active center of Na^+,K^+-ATPase consists, as it were, of two sections differing in functions. One of them, which we shall call the adsorption section, binds the cardiosteroids with the receptor and concentrates and orients the molecule relative to the second, catalytic, section where the groups entering into direct interaction with the lactone ring are concentrated. It is most likely that the adsorption takes place with the aid of the angular groups and, in the first place, through their hydrophobic interaction with the corresponding section of the receptor. Under these conditions, in a short time unstable intermediate compounds may arise. It is possible that the binding of the cardiosteroids with the active center liberates some additional energy that stimulates the work of the other section of the enzyme that is responsible for reaction with the

participation of the lactone grouping. The easier the cardiosteroid molecule penetrates into the enzyme "slot," the more strongly is it bound with the sorption center and the more energetically does interaction take place at the "outlet," where the primary role is played by the lactone ring. And, conversely, if complete complementarity is not achieved at the sorption center, the effect of the biological action will be weakened, and this to a greater degree the greater the deviations from the optimum position.

By analogy with other enzymes it is not excluded that the Na^+,K^+-ATPase entering into interaction with the cardiosteroids in accordance with Koshland's well-known hypothesis itself undergoes some conformational changes. But, again, only a cardiosteroid molecule that comes into contact with the sorption center better is capable of changing the spatial arrangement of the chemical groups forming the receptor section.

The two-center model well explains why cardiac glycosides having five- and six-membered lactone rings but differing from one another by the structural elements of the steroid nucleus or the sugar component possess dissimilar cardiotonic effects. In particular, the low activity of cardenolide glycosides of the 5α series (glycosides of corotoxigenin, uzarigenin, etc.) becomes clear. Obviously, the conformational changes connected with the trans linkage of rings A/B impart to the cardiosteroid molecule a steric form that does not favor contact with the sorption center. The A/B-cis configuration proves to be preferable. The cardenolide bisglycosides with an unchanged butenolide ring but with sugar components in unusual positions (at C-19 and C-5) that we have considered apparently fit equally poorly into the enzyme slot and disturb its usual steric forms (see Fig. 2).

It was stated above that a definite sequence of polar and nonpolar groups is characteristic for the receptor section of Na^+,K^+-ATPase. Consequently, while the steroid part of the cardiac glycoside molecule is bound with the sorption center by hydrophobic interaction, the sugar part must enter into interaction with the digitalis receptor with the aid of polar groups. This position is separate to some degree from the point of contact of the steroid part and is more capable of binding a polar sugar molecule which, in the natural glycoside, is usually attached to the cardiosteroids through a hydroxy group at C-3. The absence of a sugar component has an adverse effect on the inotropic action – as a rule, aglycones are less active than glycosides. Only an acetate ardical can, to some extent, replace a missing sugar component (strophanthidin 3-O-acetate is more active than strophanthidin but less active than the usual strophanthidin monoglycosides – cymarin, erysimin, convallatoxin, etc.). A further increase in weight in the homologous series, i.e., the replacement of acetate by propionate or butyrate leads to the practically complete loss of activity.

So far as concerns the mechanism of the action on the catalytic section of the receptor, as early as 1963 Repke [8] put forward the hypothesis that the lactone ring is connected with the active center by a hydrogen bond the strength of which is proportional to the fractional negative charge on the carbonyl group of the lactone.

All the experience accumulated up to the present time on the comparison of the activity of cardiac glycosides with their structure shows that a lactone ring alone, even if it is suitably oriented in relation to the rest of the molecule, is insufficient. Another obligatory condition for cardiac activity is a hydroxy group at C-14. In 1942-1944, several cardenolides were synthesized that differed from the natural aglycones only by the absence of a 14β-hydroxyl – in particular, 14-deoxydigitoxigenin [9] and 5,6-dehydro-14-deoxydigitoxigenin [10]. The compounds synthesized did not exhibit cardiac activity.

Therefore, it is better to assume that in the binding of the cardiosteroids with the receptor section of the transport enzyme an irreversible (at least for the cardiac aglycone) interaction of the 17β-lactone ring with the β-oriented hydroxyl at C-14, putting into effect the whole mechanism of extra- and intracellular structures the final result of which at the level of the target organ – the heart – is a positive inotropic effect. It is possible that the interaction of the obligatory functional groups of the cardiosteroids is similar to the alkaline isomerization reaction of the cardenolides anb bufadienolides, which takes place with the formation of inactive products. However, this similarity has yet to be shown.

EXPERIMENTAL

The source of ATPase was the gray matter of rat and cattle brains. The microsomal fraction of the cells of the cerebral cortex was isolated by a modification of Skou's method [11]. The brain was suspended in 10 volumes of an iced solution containing 25 mmole of Tris-HCl, pH 7.4, 0.25 mole of sucrose, 5 mmole of Na_2EDTA and 0.1% of sodium deoxycholate (DOC). The homogenate was centrifuged at 12,000g for 30 min and then at 40,000g for 60 min. The material obtained was washed with the same buffer but without the DOC. The microsomal fraction was dissolved in a 1 mM solution of Tris-Edta (pH 7.1) at the rate of 6 mg of protein per 1 ml, and was stored at 2-4°C.

The ATPase activity was determined from the increase in the amount of inorganic phosphate per mg of protein per hour. The membrane preparation (10 μg) was incubated at 37°C with 15-min preincubation with the cardiotonic substances in a medium containing 2 mM $MgCl_2$, 135 mM NaCl, 5 mM KCl, 30 mM Tris-HCl (pH 7.4), and 2 mM ATP. The reaction was stopped by the addition of trichloroacetic acid (final concentration 5%). The level of Na^+,K^+-activated ATPase was calculated from the difference in the enzymatic activities determined in the incubation medium with a complete set of ions ($Mg^{2+}+Na^++K^+$) and on the addition of $1\cdot10^{-4}$ M of olitoriside to the medium. The inorganic phosphorus was determined by a method based on the absorption in UV light of unreduced phosphomolybdic acid in the modification of Ya. Kh. Turakulov et al. [12]. Protein was determined by Lowry's method [13].

SUMMARY

Bisglycosides of strophanthidol and strophanthidin obtained by partial synthesis behave differently with respect to Na^+,K^+-ATPase. Strophanthidol 3,19-bisrhamnoside does not possess the biological activity characteristic for cardiac glycosides. Strophanthidin 3,5-bisrhamnoside and compounds similar to it that are glycosylated at the C-5 hydroxyl possess a reduced capacity for inhibition.

A two-center model of the digitalis receptor is put forward which explains the cause of the different activities of cardenolide glycosides differing from one another by structural elements in the steroid part of the molecule or of the sugar component.

LITERATURE CITED

1. R. Thomas, J. Boutagy, and A. Gelbert, J. Pharm. Sci., 11, 1649 (1974).
2. R. Petersen, H. Flasch, and N. Heinz, Arzneim.-Forsch./Drug Res., 27 (I), 642 (1977).
3. N. M. Mirsalikhova, F. T. Umarova, N. Sh. Pal'yants, and N. K. Abubakirov, Khim. Prirodn. Soedin., 188 (1976).
4. A. Jaunin, H. P. Weber, and A. Wartburg, Helv. Chim. Acta, 56, 2117 (1973).
5. N. Sh. Pal'yants, M. B. Gorovits, and N. K. Abubakirov, Khim. Prirodn. Soedin., 765 (1976).
6. N. Sh. Pal'yants and N. K. Abubakirov, Khim. Prirodn. Soedin., 125 (1977).
7. V. T. Chernobai, Zh. Obshch. Khim., 34, 1690, 3852 (1964).
8. K. Repke, Proceedings of the Second International Pharmacological Meeting, 1963, Vol. 4, Prague, Czechoslovakia (1965), p. 65; Pharmazie, 27, 693 (1972).
9. J. Fried, R. G. Linville, and R. C. Elderfield, J. Org. Chem., 7, 362 (1942).
10. L. Ruzicka, T. Reichstein, and A. Fürst, Helv. Chim. Acta., 24, 76 (1941).
11. J. C. Skou, Biochem. Biophys. Acta, 58, 314 (1962).
12. Ya. Kh. Turakulov, L. I. Kurgul'tseva, and A. I. Gagel'gans, Biokhimiya, 32, 106 (1967).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. I. Randall, J. Biol. Chem., 193, 265 (1951).