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Structure-Activity Relationships of Cardiotonic Steroids for the Inhibition of Sodium- and Potassium-Dependent Adenosine Triphosphatase

IV. Dissociation Rate Constants for Complexes of the Enzyme with Cardiac Oligodigitoxides

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SUMMARY

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The dissociation rate constants (k_d) of (Na^+ + K^+)-ATPase (EC 3.6.1.3) complexes with digitoxigenin or digoxigenin oligodigitoxides were determined by enzymatic assay after dilution. The k_d value of each cardiac glycoside–enzyme complex formed in the Na^+-Mg^{2+}-ATP system (type I complex) was greater than that of the complex formed in the Mg^{2+}-P_i system (type II complex). In type I complexes the k_d value of the digitoxigenin oligodigitoxide complex was greater than that of the digoxigenin oligodigitoxide complex, but this difference was diminished in the type II complex. For a given aglycone and type of complex, the k_d values decreased in the order monodigitoxide > bisdigitoxide > tridigitoxide ≒ tetradigitoxide. The activation energies of these dissociation constants were approximately 30 kcal/mole for the type I complex and 25 kcal/mole for the type II complex and did not change with the number of sugar moieties or the nature of the aglycone. The k_d values of the type II complexes were increased with Na^+ plus ATP in the dilution medium in a manner similar to those of the monoglycosides. On the other hand, K^+ in the dilution medium reduced the k_d values of only the type I complexes containing cardiac oligodigitoxides. These k_d values fell below those of the type II complex, in contrast to the situation with the cardiac monoglycosides. These results suggest that the second and third digitoxose moieties of the cardiac digodigitoxides can bind to the enzyme but that binding by the first digitoxose moiety is predominant.

INTRODUCTION

Many studies have been carried out on the inhibition of (Na^+ + K^+)-ATPase (EC 3.6.1.3) by cardiac glycosides, especially by ouabain. It is well known that inhibition of the enzyme is associated with the formation of stable enzyme–cardiac glycoside complexes in the presence of certain ligands, and that combinations of Mg^{2+} and P_i, or of Na^+, Mg^{2+}, and ATP, are the most

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effective ligand systems in promoting this binding (1–4).

The importance of the interaction between the sugar moiety of cardiac glycosides and this enzyme (5) was recently confirmed by Wallick *et al.* (6). Moreover, we suggested that the 3′-α- and -β-hydroxyl and 5′-α-methyl groups of the sugar moiety in the cardiac monoglycoside are involved in binding to $(Na^+ + K^+)$-ATPase in the presence of Mg^{2+} and P_i (7) and that the 2′-α-hydroxyl group is also involved in the presence of Na^+, Mg^{2+}, and ATP (8). These conclusions were based on studies of the dissociation rate constants (k_d) of various cardiac monoglycoside–enzyme complexes.

As therapeutic drugs, the cardiac tridigitoxides digitoxin and digoxin are more important than the monoglycosides and also form stable complexes with the enzyme. It is therefore important to study the contribution of the second and third sugar moieties to the binding to $(Na^+ + K^+)$-ATPase. We previously compared the association rate constants (k_a) of some oligodigitoxide-enzyme complexes with those of various monoglycoside-enzyme complexes (9). In this paper the dissociation rate constants (k_d) are presented. For convenience we refer to the cardiac glycoside–$(Na^+ + K^+)$-ATPase complex formed in the presence of Na^+, Mg^{2+}, and ATP as the type I complex, and to that formed in the presence of Mg^{2+} and P_i, as the type II complex. This notation is the same as used previously (8).

MATERIALS AND METHODS

As previously described (7–9), NaI-treated microsomes from beef brain (specific activity, 60–80 μmoles of P_i per hour per milligram of protein) were used as the source of the $(Na^+ + K^+)$-ATPase. Digitoxin was a kind gift of Eli Lilly & Company. Digitoxigenin bisdigitoxide, digoxigenin monodigitoxide, digoxigenin bisdigitoxide, digoxin, and digoxigenin tetradigitoxide were purchased from Boehringer/Mannheim. Digitoxigenin monodigitoxide was prepared by partial hydrolysis of digitoxin (10) and purified by silicic acid chromatography as described previously (7).

The k_d values of the various enzyme–cardiac glycoside complexes were determined as previously described (7). Type I complexes were formed by incubating the enzyme with a given cardiac glycoside (0.2–0.7 μM) in the presence of 50 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, and 20 mM imidazole HCl buffer (pH 7.3) for 5–10 min. Binding of the enzyme and drug was stopped by 20-fold dilution with 1 mM Tris-EDTA (pH 7.3). Formation of type II complexes was promoted in the presence of 1 mM $MgCl_2$ and 1 mM Tris-phosphate instead of NaCl, $MgCl_2$, and ATP. The reaction mixture was then treated as described for the type I complex. In all experiments formation of the complex, dissociation after dilution, and assays of enzyme activity were carried out at temperatures indicated in the text below. The time course of recovery of enzyme activity was first-order, and k_d values of the cardiac glycoside–enzyme complex were obtained from the slope of this time course. For further details, see a previous report (7).

RESULTS

The k_d values at various temperatures of each cardiac digitoxide–$(Na^+ + K^+)$-ATPase complex are shown in Figs. 1 and 2. The data suggest that the type I complex with digoxigenin digitoxide is more stable than the complex formed with the corresponding digitoxigenin digitoxide, implying that the 12-hydroxyl group of the steroid moiety stabilizes the type I complex. On the other hand, this effect is smaller in the type II complex; that is, the k_d value of the type II complex is dependent only on the nature of the sugar moiety in the oligosaccharide as well as in the monosaccharide (7).

It is apparent from Figs. 1 and 2 that a greater number of sugar moieties increases the stability of both type I and type II complexes. However, the fourth sugar does not contribute to the stability of either complex. In Fig. 3 the relative change of k_d values as a function of the number of digitoxose moieties at various temperatures is presented. The results suggest that the fourth digitoxose group has almost no effect on the stability of type I

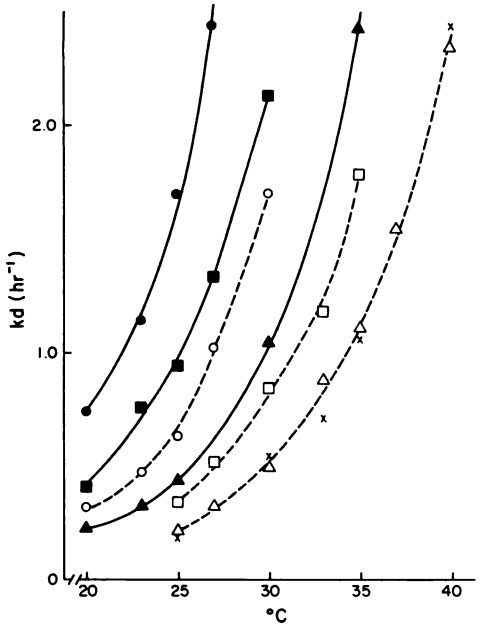

FIG. 1. *Dissociation rate constants (k_d) of type I enzyme complexes with cardiac oligodigitoxides at various temperatures*

●——●, digitoxigenin monodigitoxide; O- - -O, digoxigenin monodigitoxide; ■——■, digitoxigenin bisdigitoxide; □- - -□, digoxigenin bisdigitoxide; ▲——▲, digitoxin; △- - -△, digoxin; X- - -X, digoxigenin tetradigitoxide.

and type II complexes. The contribution of the third digitoxose group to the stability of the drug-enzyme complex is less than that of the second digitoxose in the type II complex, but appears to be the same in the type I complex. Moreover, the relative contribution of the second digitoxose to the stability of the drug-enzyme complex does not appear to be influenced either by the aglycone moiety or by the type of complex.

Arrhenius plots of k_d values (Figs. 4 and 5) give an activation energy of 30 kcal/mole for the type I complex and 25 kcal/mole for the type II complex regardless of the number of digitoxose groups or the nature of the steroid moiety.

It has been reported that K^+ stabilizes the type I enzyme complex with cardiac monoglycosides (11–13). The effect of K^+ on the stability of various $(Na^+ + K^+)$-ATPase–cardiac oligodigitoxide complexes is presented in Tables 1 and 2, which indicate that the k_d value of the type I complex falls below that of the type II complex. In contrast, we previously showed that K^+ stabilized the type I complex formed with cardiac monoglycosides to the same extent as the type II complex when K^+ was added after complex formation (11).

Another remarkable effect of K^+ on the oligodigitoxide complexes is the virtual

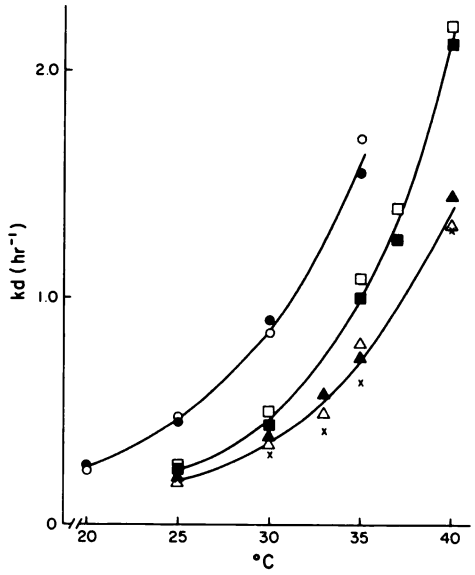

FIG. 2. *Dissociation rate constants (k_d) of type II enzyme complexes with cardiac oligodigitoxides at various temperatures*

●, digitoxigenin monodigitoxide; O, digoxigenin monodigitoxide; ■, digitoxigenin bisdigitoxide; □, digoxigenin monodigitoxide; ▲, digitoxin; △, digoxin; X, digoxigenin tetradigitoxide.

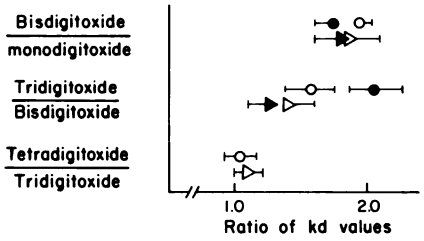

FIG. 3. *Relative change of dissociation rate constant by one digitoxose moiety*

Each point shows the average ratios of the k_d values shown in Figs. 1 and 2 at various temperatures, and the horizontal lines indicate standard errors. ● and O, ratios of type I complexes; ▶ and ▷, those of type II complexes. ● and ▶, ratios of digitoxigenin glycosides; O and ▷, those of digoxigenin glycosides.

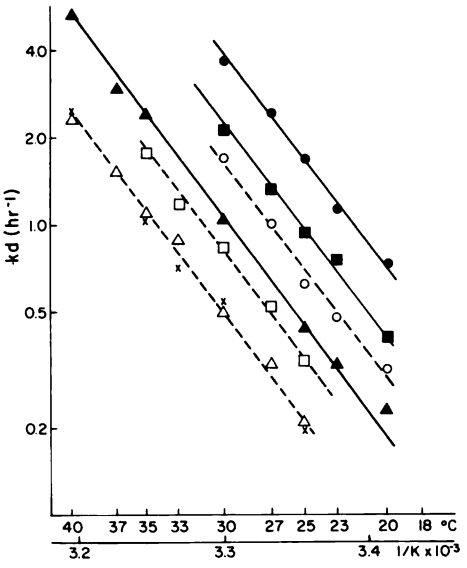

FIG. 4. *Arrhenius plots of dissociation rate constants (k_d) of type I complexes with cardiac oligodigitoxides*

Notation is the same as in Fig. 1.

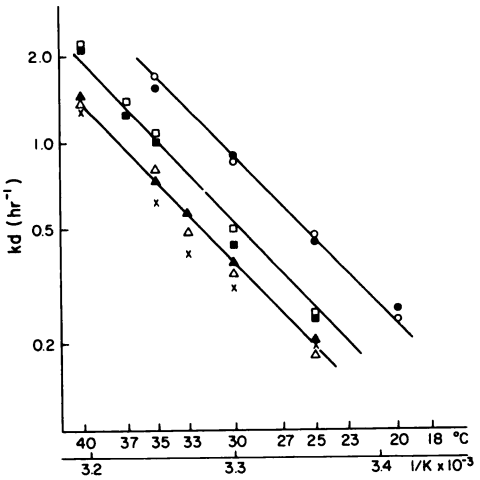

FIG. 5. *Arrhenius plots of dissociation rate constants (k_d) of type II complexes with cardiac oligodigitoxides*

Notation is the same as in Fig. 2.

elimination of the difference among k_d values of the type I complexes with bis-, tri-, and tetradigitoxides.

The effect of Na^+ plus ATP on the type II complex is also presented in Table 1. These results obtained with the cardiac oligodigitoxides are similar to those previously reported for the monoglycosides (11); i.e., Na^+ plus ATP renders the type II complex unstable.

DISCUSSION

In this report it is shown that cardiac glycosides with a larger number of sugar moieties form more stable complexes with (Na^+ + K^+)-ATPase in the presence of Na^+, Mg^{2+}, and ATP (type I complex) and Mg^{2+} and P_i (type II complex). As reported previously (7, 8), the first sugar moiety greatly increases the stability of the complex with the enzyme, and now it has been observed that the second and third sugar moieties do also. However, the effect of either the second or third sugar moiety is less than that of the first. Since the k_d values of the cardiac aglycone–(Na^+ + K^+)-ATPase complex is suggested to be more than 5 hr^{-1} at 15°, the ratio k_d aglycone/k_d monoglycoside should be more than 10 and much greater than the value shown in Fig. 3. Thus the stabilizing effect on the drug-enzyme complex of the second sugar moiety is much less than that of the first sugar moiety, but not less than that of the third sugar. The fourth digitoxose moiety in digoxigenin tetradigitoxide does not influence the k_d value of the complex. These stabilizing effects on the drug-enzyme complexes by the second and third sugar moieties suggest that they also bind to the enzyme. In the type I complex the contribution of the third digitoxose group to binding to the enzyme appears to be of the same order as that of the second digitoxose group, but in the type II complex the effect of the third sugar is less than that of the second (Fig. 3). The relative contribution of the second digitoxose moiety to the stability of the complex, namely, its affinity for the enzyme, is influenced neither by the type of drug-enzyme complex nor by the nature of the steroid moiety in the oligodigitoxide. A possible explanation is that binding of the second digitoxose moiety to the specific site on the enzyme may not be different for the different types of drug-enzyme complexes; however, studies with acetyl derivatives of digitoxin and digitoxigenin bisdigitoxide (14) do not fit this explanation.

The present study was limited to two sets of oligodigitoxides because other suit-

TABLE 1

Change in dissociation rate constants (K_d) of $(Na^+ + K^+)$-ATPase complexes with digitoxigenin oligodigitoxides by ligands

Ligands added to dilution medium[a]	k_d values at 30°					
	Type I			Type II		
	Monodigitoxide	Bisdigitoxide	Tridigitoxide	Monodigitoxide	Bisdigitoxide	Tridigitoxide
	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}
None		2.13	1.04	0.47[b]	0.44	0.38
	1.90[b]	0.94[b]	0.44[b]			
0.5 mM KCl	0.43[b]	0.26	0.26			
2.0 mM KCl	0.45[b]	0.24	0.25	0.48[b]	0.42	0.35
5.0 mM KCl	0.42[b]	0.23	0.22			
50 mM NaCl + 0.5 mM ATP	2.78[c]	2.23[c]	1.46[c]	2.80[c]	2.34[c]	1.44[c]

[a] Tris-EDTA, 1 mM.

[b] k_d value at 25°.

[c] CDTA (1,2-cyclohexylenedinitrilotetraacetic acid) (10 mM) was used instead of 1 mM Tris-EDTA as dilution medium, and k_d values were measured at 25°.

TABLE 2

Changes in dissociation rate constants (k_d) of $(Na^+ + K^+)$-ATPase complexes with digoxigenin oligodigitoxides by ligands

Ligands added to dilution medium[a]	k_d values at 30°							
	Type I				Type II			
	Monodigitoxide	Bisdigitoxide	Tridigitoxide	Tetradigitoxide	Monodigitoxide	Bisdigitoxide	Tridigitoxide	Tetradigitoxide
	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}
None	0.74[b]	0.84	0.50	0.54	0.43[b]	0.50	0.35	0.31
2 mM KCl	0.40[b]	0.28	0.23	0.24	0.39[b]	0.44	0.35	0.32
50 mM NaCl + 0.5 mM ATP	1.15[b,c]	1.73[c]	0.95[c]	0.90[c]	1.05[b,c]	1.68[c]	0.92[c]	0.95[c]

[a] Tris-EDTA, 1 mM.

[b] k_d value at 25°.

[c] CDTA (1,2-cyclohexylenedinitrilotetraacetic acid) (10 mM) was used instead of 1 mM Tris-EDTA as the dilution medium.

able sets of cardiac oligosaccharides were not available. Thus no information could be obtained as to which functional groups of the second and third sugar moieties can bind with specific site(s) of the enzyme.

Although the second and third sugar groups may bind to specific sites of the enzyme, the following characteristic differences in k_d values between type I and type II complexes shown in the case of the cardiac monoglycosides (7, 8) were also observed in complexes with the oligodigitoxides. (a) The type I enzyme complexes with digitoxigenin bisdigitoxide or with digitoxin are less stable than the complexes with digoxigenin bisdigitoxide or with digoxin, respectively, but dependence on the nature of the aglycone is not seen in the type II complex; i.e., the k_d values of the type II complexes are exclusively dependent on the nature of the sugar moiety. (b) In each bis- and tridigitoxide the type I complex is less stable than the type II complex at various given temperatures. These differences between type I and type II complexes of the cardiac oligodigitoxides may be explained by assuming that the binding of the first sugar moiety with the enzyme

is predominant over that of the other sugar moieties.

The effects of ligands on the stability of various ($Na^+ + K^+$)-ATPase–cardiac oligodigitoxide complexes were also studied. The Na^+ plus ATP effect is the same as in the cardiac monoglycosides. However, the K^+ effect on the type I complex with cardiac oligosaccharides is significantly different from that with the monoglycosides. As reported previously by us (11) and others (12, 13), potassium reduced the k_d value of the type I complex with cardiac monoglycoside to the same value as that of the type II complex. However, potassium also reduced the k_d value of the type I enzyme complexes with various cardiac oligodigitoxides ($2.13 \sim 0.54\ hr^{-1}$) to the same low values ($0.25 \pm 0.03\ hr^{-1}$). Not only did potassium diminish the differences in k_d values attributable to the aglycone moiety, as was the case for the type I cardiac monoglycoside–enzyme complexes, but it also decreased the k_d differences due to the number of sugar moieties. The resulting low k_d values were smaller than those of the type II complexes. On the other hand, potassium did not change the k_d value of the type II complex with either cardiac monoglycoside or oligodigitoxide. This difference in effects on the type I complexes between monoglycosides and oligosaccharides might be explained as a change in the affinity of the specific binding site on the enzyme for the second sugar moiety; i.e., the affinity for the second sugar moiety of the type I complex might be greater than that of the type II complex in the presence of potassium. Therefore the k_d value of the type I complex in the presence of potassium appeared to be lower than that of the type II complex. Moreover, the potassium-induced increase in affinity for the second sugar moiety may have diminished the difference in k_d values among bis-, tri-, and tetradigitoxides, since the contribution of the digitoxose moiety to the k_d value of the type II complex is much less than that of either the first or second sugar moiety.

The Arrhenius activation energies of dissociation of both types of complexes are the same in the case of the cardiac monoglycosides (7, 8). Therefore, the rate-determining step for the dissociation of each type of cardiac oligosaccharide–enzyme complex may be identical with that of the corresponding monoglycoside-enzyme complex. Dissociation of binding between the third or second digitoxose moiety and the enzyme therefore must precede the rate-determining step, probably stepwise from dissociation at the terminal sugar to the first sugar moiety, followed by a conformational change of the first sugar-specific site of the enzyme into the inactive form, and, finally, dissociation of the steroid from its binding site.

From the present study it may be concluded that in both type I and II complexes binding of the first sugar moiety to ($Na^+ + K^+$)-ATPase predominates over that of the others, but that the second and third sugar moieties also bind to specific sites on the enzyme and significantly increase the stability of the drug-enzyme complex.

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