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Influence of Certain Ligands on the Dissociation Rate Constants of Cardiac Glycoside Complexes with Sodium- and Potassium-Dependent Adenosine Triphosphatase

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SUMMARY

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The influence of certain ligands on the dissociation rate constants (k_d) of various cardiac monoglycoside–($Na^+ + K^+$)-ATPase complexes was examined. If the complex was formed in the presence of sodium, magnesium, and ATP (type I complex), potassium ion reduced the k_d to the value of the complex formed in the presence of magnesium and phosphate (type II complex). Potassium also changed other dissociation characteristics of the type I complex to those of the type II complex: in the presence of potassium the k_d value became exclusively dependent on the nature of the sugar moiety of the cardiac glycoside. This potassium effect was not reversed by dilution. Sodium could reverse the potassium effect and increase the k_d value of the type I complex. Dissociation of the type II complex was only slightly influenced by potassium, sodium, or ATP alone. Sodium plus ATP increased the k_d value of the type II complex, and the dissociation characteristics of the type II complex were changed to those of the type I complex. This effect of sodium plus ATP was just opposite to the potassium effect, but it was reversible. Since 1,2-cyclohexylenedinitrilotetraacetic acid did not alter the effects of potassium or sodium plus ATP, such changes in the types of cardiac glycoside–enzyme complexes might not involve magnesium. The similarity of these ligand effects on the cardiac glycoside–($Na^+ + K^+$)-ATPase complex to their effects on the native enzyme suggests that the inhibited enzyme complex still retains some portion of the enzyme activity.

INTRODUCTION

It is well known that cardiac glycosides can bind to ($Na^+ + K^+$)-ATPase by a noncovalent bond(s) in the presence of certain ligands (1–5). Combinations of magnesium and phosphate or of sodium, magnesium, and ATP are the most effective ligand systems in promoting this binding. It has also been reported that the number of ouabain-binding sites on the enzyme is the same for the two ligand systems (2, 3, 4, 6) but that the ouabain-enzyme complex formed in the presence of sodium, magnesium, and ATP dissociates faster than the complex formed in the presence of magnesium and phosphate (7–9). These observations suggest that the two complexes are not identical.

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We have examined the dissociation rate constants (k_d) of these two types of cardiac glycoside-(Na^+ + K^+)-ATPase complexes, and have demonstrated that in each complex the dissociation rate constant, the rate-determining step in the dissociation of the complex, and the particular functional groups of the sugar moieties which bind to the enzyme vary with the ligands used in its formation (9, 10).

This paper presents evidence not only that potassium changes the k_d of the complexes formed in the presence of sodium, magnesium, and ATP, but that sodium plus ATP also change the k_d of the complexes formed in the presence of magnesium and phosphate. For convenience, we prefer to the cardiac glycoside-(Na^+ + K^+)-ATPase complex formed in the presence of sodium, magnesium, and ATP as the type I complex, and to that formed in the presence of magnesium and phosphate, as the type II complex. This is the same notation as in our previous paper (9) and that of Van Winkle *et al.* (11).

MATERIALS AND METHODS

The enzyme preparation (NaI-treated microsomes from beef brain) and all cardiac glycosides were the same as reported previously (10).

k_d was determined essentially as reported previously (10). After incubation of the enzyme with a cardiac glycoside in the presence of suitable ligands to form the drug-enzyme to stop the association between enzyme and cardiac glycoside, and the recovery of enzyme activity was measured. The time course of the inhibition of enzyme is first-order, and the k_d of the cardiac glycoside-enzyme complex can be obtained from the slope of this time course.

Unless otherwise indicated, the type I complex was formed by a 5–10 min incubation at 25° with 0.15–0.5 μM cardiac glycoside, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, and 20 mM imidazole HCl buffer (pH 7.3). The incubated preparation was immediately diluted 20-fold with 1 mM Tris-EDTA (pH 7.3), and 0.5-ml aliquots were taken at suitable intervals. Each aliquot was mixed with 0.5 ml of assay medium, and enzyme activity was measured by the linked pyruvate kinase–lactate dehydrogenase spectrophotometric method, which measures formation of ADP by the hydrolysis of ATP. All experiments, formation of the complex, dissociation after dilution, and assay of enzyme activity were done at 25° unless otherwise indicated. The final concentrations during the assay were 100 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP, 1 mM phosphoenolpyruvate, about 0.15 mM NADH, about 50 μg/ml of lactate dehydrogenase, about 30 μg/ml of pyruvate kinase, and 30 mM imidazole HCl buffer (pH 7.3). The hydrolysis of ATP was followed by measuring the decrease in optical density at 340 nm. A Cary 14 spectrophotometer with a slide wire range of 0–0.1 absorbance unit was used. The rapidity of this assay procedure, which needs less than 2 min, is of advantage.

Seven to nine aliquots from one diluted suspension were assayed at different intervals, and the time course of the change in inhibition after dilution was obtained. From the slope of this time course the k_d value was calculated.

In the case of type II complex formation, 1 mM $MgCl_2$ and 1 mM Tris-phosphate were used instead of NaCl, $MgCl_2$, and ATP. Other conditions were the same as for the type I complex.

The 20-fold dilution with Tris-EDTA reduced the concentrations of cardiac glycoside, enzyme, and ligands, and the association rate of cardiac glycoside to the enzyme was decreased more than expected by the lowered concentrations of enzyme and cardiac glycoside. When 50 mM sodium and 0.5 mM ATP were added to the dilution medium, however, the ligand concentrations (with the exception of magnesium) were not appreciably affected by dilution, and the association rate of cardiac glycoside with the enzyme may not have been reduced concomitantly. Inhibition of the enzyme during determination of the dissociation rate constant was 5–15% in the control experiment when dilution was carried out with 1 mM Tris-EDTA alone; it was increased to 10-25% on dilution with Tris-EDTA containing 50 mM NaCl and 0.5 mM ATP.

In some experiments 10 mM CDTA[1] (the

[1] The abbreviation used is: CDTA, 1,2-cyclohexylenedinitrilotetraacetate.

Tris salt adjusted to pH 7.3) was used instead of 1 mM EDTA as the dilution medium. In this case the dilution was 10-fold, and 0.2-ml aliquots of the diluted enzyme suspension were taken for assay. The magnesium concentration of the assay medium was increased to 4.5 mM in order to overcome chelation by CDTA, which was present in the assay medium at a concentration of about 2 mM. As described below, this concentration of CDTA inhibited cardiac glycoside binding and ATP hydrolysis completely (12, 13), and the enzyme in the control experiment was inhibited less than 10%, even if sodium plus ATP were added to the dilution medium.

RESULTS

Effects of ligands on dissociation of type I complex. As shown in Fig. 1, the dissociation rate of the type I complex formed with digitoxigenin monodigitoxide was reduced in the presence of potassium ion. Sodium ion increased the dissociation rate of the type I complex and antagonized the effect of potassium ion. Similar effects of potassium and sodium ions on the dissociation rate were also observed with the type I complex with ouabain (Table 1). The antagonism between sodium and potassium is shown in detail in Fig. 2. The half-maximal concentration of potassium varied with the concentration of sodium, as reported for ATP hydrolysis (14) and ouabain binding (15, 16).

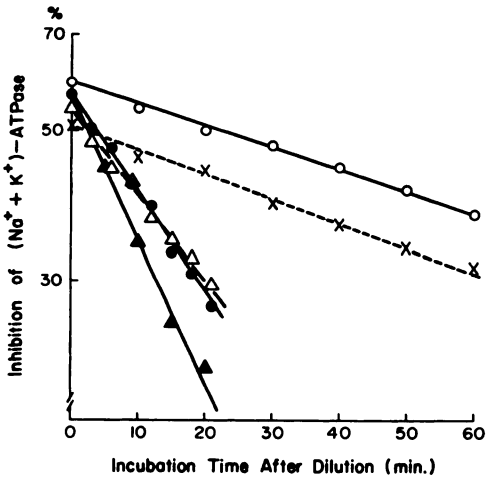

FIG. 1. *Change in dissociation rate of type I digitoxigenin monodigitoxide-(Na^+ + K^+)-ATPase complex by potassium and sodium*

The type I complex was formed as described in the text with 0.2 μM digitoxigenin monodigitoxide and diluted 20-fold with 1 mM Tris-EDTA containing the indicated ligands. Enzyme inhibition was determined at suitable intervals. ●——●, no addition; ○——○, 2 mM KCl; ▲——▲, 50 mM NaCl; △——△, 50 mM NaCl and 2 mM KCl; ×- - -×, type II complex diluted with 1 mM Tris-EDTA.

TABLE 1

Change in dissociation rate constants (k_d) of ouabain-(Na^+ + K^+)-ATPase complex by ligands in dilution medium

Ligand added to dilution medium[a]			k_d at 30°	
2 mM KCl	50 mM NaCl	0.5 mM ATP	Type I	Type II
			hr^{-1}	hr^{-1}
−	−	−	0.87	0.42
+	−	−	0.46	0.39
−	+	−	1.27	0.41
−	−	+		0.43
+	+	−	0.80	
−	+	+		1.43
+	+	+		0.53[b]

[a] Tris-EDTA, 1 mM.

[b] The time course for dissociation was concave upward. The initial rate constant was 0.78 hr^{-1} and decreased to the constant value, 0.53 hr^{-1}.

As reported previously (9), the k_d values of the type I complexes for three digitoxides are different, but those of type II complexes for the same digitoxides are identical. Also the k_d value of the type II complex is lower than that of the type I complex. In the presence of potassium in the dilution medium, the k_d values of the three type I complexes were reduced to identical values at various fixed temperatures, and these values were the same as those of the corresponding type II complexes (Fig. 3). In the case of rhamnosides similar effects of potassium were also observed (Fig. 4).

As shown previously (9, 10), the k_d values of type I complexes with digitoxigenin monodigitoxide and digitoxigenin 6-deoxyglucoside were almost the same, but the k_d value of the 6-deoxyglucoside type II complex was greater than that of the digitoxide complex. However, in the presence of potassium ion,

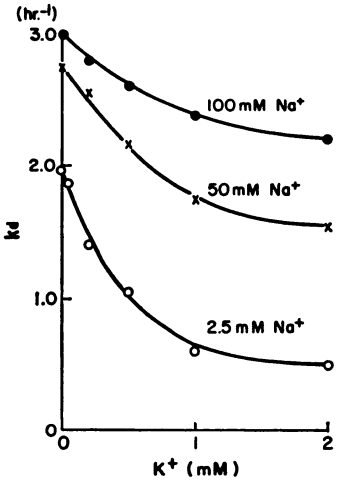

FIG. 2. *Effects of potassium and sodium ions on dissociation rate constants (k_d) of type I digitoxigenin monodigitoxide-(Na^+ + K^+)-ATPase complex at 25°*

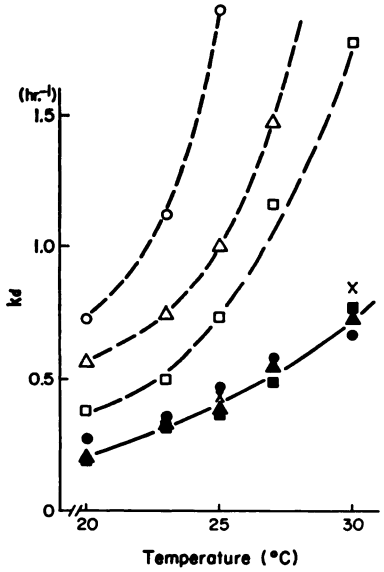

FIG. 3. *Dissociation rate constants (k_d) of type I digitoxide-(Na^+ + K^+)-ATPase complexes at various temperatures*

The type I complex was diluted with 1 mM Tris-EDTA (dashed lines and open symbols) or 2 mM KCl in 1 mM Tris-EDTA (solid lines and closed symbols). Other experimental conditions were the same as in Fig. 1. ○ or ●, digitoxigenin monodigitoxide; △ or ▲, helveticoside (strophanthidin digitoxide); □ or ■, digoxigenin monodigitoxide; ×——×, k_d of type II digitoxide-enzyme complex diluted with 1 mM Tris-EDTA. × at 20° is overlapped by a solid square.

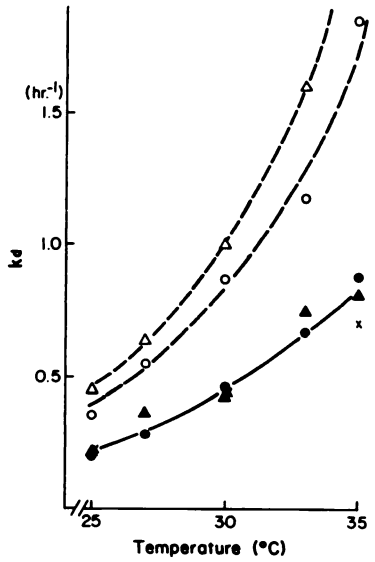

FIG. 4. *Dissociation rate constants (k_d) of type I rhamnoside-(Na^+ + K^+)-ATPase complexes at various temperatures*

The experimental conditions were the same as in Fig. 3. The results of dilution with 1 mM Tris-EDTA are shown by dashed lines and open symbols, and those with 2 mM KCl in 1 mM Tris-EDTA, by solid lines and closed symbols. △ or ▲, convallatoxin (strophanthidin rhamnoside); ○ or ●, ouabain; ×——×, k_d of type II rhamnoside-enzyme complex with 1 mM Tris-EDTA.

the k_d value of the type I digitoxigenin 6-deoxyglucoside–enzyme complex was greater than that of the monodigitoxide complex, as was the case for the type II complex (Table 2). The reversibility of this potassium effect on the type I complex was examined as follows.

After the digitoxigenin monodigitoxide type I complex was formed, it was washed twice with ice-cold 1 mM Tris-EDTA and suspended in the same solution. This suspension was treated with 2 mM potassium chloride at 25°, the mixture was diluted with Tris-EDTA to stop the potassium effect, and the incubation was continued in order to measure the recovery of enzymatic activity. In the case of zero-time treatment with potassium, potassium chloride was mixed after dilution with Tris-EDTA and the complex dissociated rapidly (Fig. 5). When dilutions were made after a 5- or 10-min treatment with potassium, however, the k_d

TABLE 2

Change in dissociation rate constants (k_d) of ($Na^+ + K^+$)-ATPase complexes with digitoxigenin monodigitoxide and digitoxigenin 6-deoxyglucoside by ligands

Ligand added to dilution medium[a]			k_d at 25°					
			Type I			Type II		
2 mM KCl	50 mM NaCl	0.5 mM ATP	Digitoxigenin monodigitoxide	Digitoxigenin 6-deoxyglucoside	r^b	Digitoxigenin monodigitoxide	Digitoxigenin 6-deoxyglucoside	r^b
			hr^{1-}	hr^{-1}		hr^{-1}	hr^{-1}	
−	−	−	1.90	1.71	0.90	0.51	0.80	1.57
+	−	−	0.47	0.72	1.53	0.48		
−	+	−	2.76	2.68	0.97	0.60		
−	−	+				0.70		
−	+	+				2.80	2.64	0.94

[a] Tris-EDTA, 1 mM.

[b] r = k_d of digitoxigenin 6-deoxyglucoside/k_d of digitoxigenin monodigitoxide.

was reduced to that of the type I complex in 2 mM potassium chloride. This finding indicates that the potassium effect on the type I complex is irreversible.

Effect of ligands on dissociation of type II complex. The k_d value of the type II complex was not influenced by potassium, nor did sodium or ATP alone have much effect; however, sodium plus ATP increased the dissociation rate of the type II complex, as shown in Fig. 6 (digitoxigenin monodigitoxide–enzyme complex) and Table 1 (ouabain-enzyme complex). Potassium antagonized this effect, but the dissociation did not follow first-order kinetics; i.e., the curve for the time course was concave (Fig. 6).

The effects of concentration of sodium and ATP on dissociation of the type II complex are shown in Fig. 7. The half-maximal concentration of ATP was about 60 μM in the presence of 50 mM CaCl, and that of sodium was about 13 mM in the presence of 0.5 mM ATP.

The presence of sodium and ATP in the dilution medium not only influenced the k_d values of type II complexes but also affected their structure-activity relationships (Fig. 8). In the absence of sodium and ATP the k_d values for the type II complexes of three digitoxides or two rhamnosides were the same at various temperatures, but in the presence of sodium and ATP each digitoxide or rhamnoside had a different k_d value, ranked in the same as those of the type I complexes. The k_d value of the type II complex in the presence of sodium and ATP was greater than that of the type I complex, but was almost the same as that of the type I complex in the presence of 50 mM sodium chloride (Tables 1 and 2).

In the absence of sodium and ATP the k_d value of the type II complex with digitoxigenin 6-deoxyglucoside was greater than that with digitoxigenin monodigitoxide, but in the presence of sodium plus ATP the values of the two type II complexes increased and became almost identical (Table 2).

The reversibility of the sodium plus ATP effect on the type II complex was also examined in the same fashion as the potassium effect on the type I complex. A suspension of washed type II complex of digitoxigenin monodigitoxide was treated with 40 mM sodium chloride and 0.4 mM ATP. At various times thereafter the mixture was diluted with 1 mM Tris-EDTA, and the recovery of enzymatic activity was followed. Recovery of enzyme activity in the presence of sodium plus ATP (Fig. 9, solid line) was rapid but was markedly decreased by dilution. This result is remarkedly different from that of the potassium effect on the type I complex, and it may be concluded that the sodium plus ATP effect on the type II complex is reversible. In another experiment a suspension of washed type II complex of digitoxigenin monodigitoxide was treated with 40 mM sodium, 0.4 mM ATP, and 1.0 mM magnesium, and the reversibility of the sodium plus ATP effect in the presence of magnesium

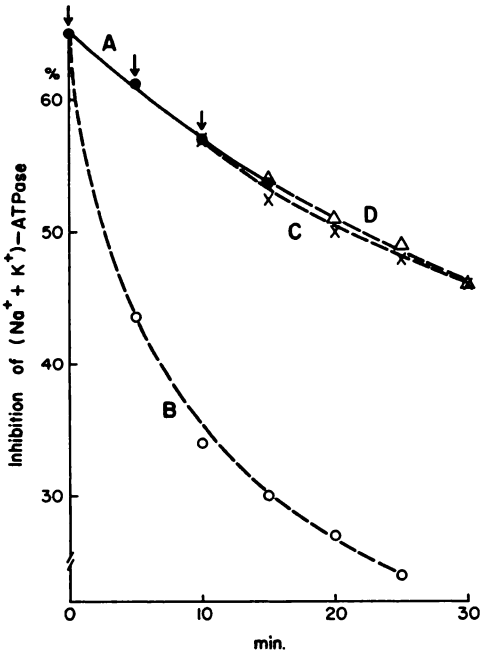

FIG. 5. *Effects of dilution at various times after starting potassium treatment with type I digitoxigenin monodigitoxide-(Na^+ + K^+)-ATPase complex*

The type I complex was prepared by 10 min of incubation at 25° of a 2.5-ml suspension containing 2 mg of enzyme preparation, 1.0 μM digitoxigenin monodigitoxide, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, and 20 mM imidazole HCl buffer (pH 7.3), and precipitated by centrifugation at 20,000 × *g* for 30 min after dilution with 10 ml of ice-cold 1 mM Tris-EDTA (pH 7.3). The pellet was homogenized with the same ice-cold Tris-EDTA and centrifuged again. This washing procedure was repeated twice. The pellet was homogenized with 2 ml of ice-cold 1 mM Tris-EDTA (pH 7.3). Then 0.1 ml of the resulting homogenate was treated with 0.2 ml of 3 mM KCl at 25° and diluted with 5 ml of 1 mM Tris-EDTA at the intervals shown by the arrows. After this dilution the recovery of enzymatic activity was followed at 25°. Inhibition was calculated against the activity at zero time of an enzyme preparation treated in the same way without digitoxigenin monodigitoxide. Curve *A* (●——●), time course of dissociation in 2 mM KCl, obtained by assaying enzyme activity immediately after dilution with 5 ml of 1 mM Tris-EDTA; *B* (○- - -○), time course after zero-time dilution and treatment with potassium, with KCl added immediately after dilution; *C* (×- - -×) and *D* (△- - -△), time courses after dilution 5 and 10 min after treatment with potassium, respectively. The time course 15 min after treatment with potassium overlapped with curves *C* and *D* and is not shown. The concentration of KCl after dilution with Tris-EDTA was less than 0.12 mM and thus was practically ineffective (curve *B*).

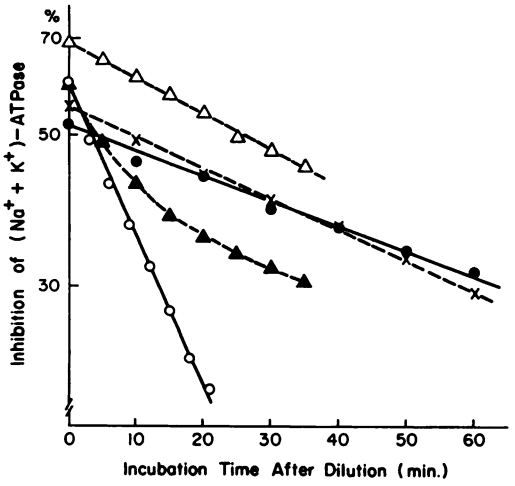

FIG. 6. *Change in dissociation rate of type II digitoxigenin monodigitoxide-(Na^+ + K^+)-ATPase complex by sodium and ATP*

The type II complex was formed as described in the text with 0.15–0.2 μM digitoxigenin monodigitoxide. Other experimental conditions were the same as in Fig. 1. The following ligands were added to the dilution medium (1 mM Tris-EDTA): ●——●, none; ×- - -×, 50 mM NaCl; △- - -△, 0.5 mM ATP; ○——○, 50 mM NaCl + 0.5 mM ATP; ▲- - -▲, 50 mM NaCl + 0.5 mM ATP + 2 mM KCl.

was examined. The results were practically the same without magnesium added (data not shown).

Dilution with 10 mM Tris-CDTA instead of 1 mM Tris-EDTA. In the case of (Na^+ + K^+)-ATPase it has been observed that a trace amount of magnesium is sufficient for phosphorylation of the enzyme (13) or ouabain binding (4, 5). In the present study about 100 μM (type I complex) or 50 μM (type II complex) magnesium was present after dilution. Although most of the magnesium might have been chelated with EDTA, a trace amount of free magnesium was pres-

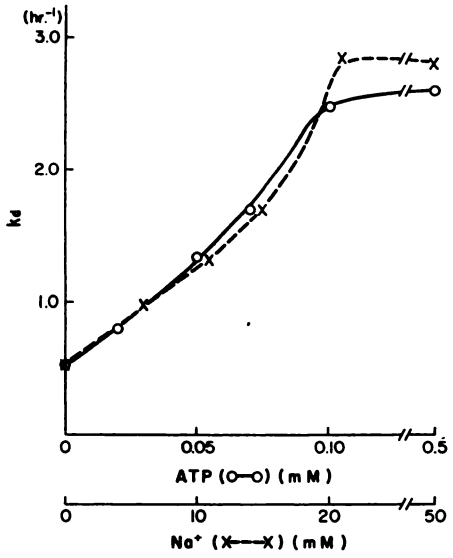

FIG. 7. *Effects of sodium and ATP on dissociation rate constants (k_d) of type II complex*

The type II complex was formed with 0.2 μM digitoxigenin monodigitoxide and diluted 20-fold with 1 mM Tris-EDTA containing various concentrations of sodium and 0.5 mM ATP (X- - -X) or diluted 10-fold with 10 mM Tris-EDTA containing various concentrations of ATP and 50 mM NaCl (O——O), followed by estimation of the k_d values.

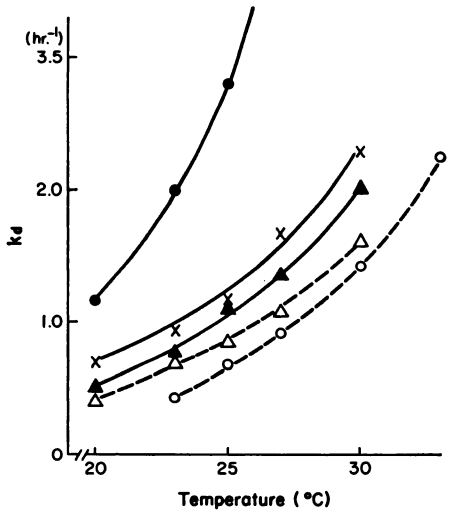

FIG. 8. *Dissociation rate constants (k_d) of type II complexes in mixture of 50 mM NaCl, 0.5 mM ATP, and 1 mM Tris-EDTA at various temperatures*

——, digitoxides: ●, digitoxigenin monodigitoxide; ×, helveticoside; ▲, digoxigenin monodigitoxide. - - -, rhamnosides: △, convallatoxin; ○, ouabain.

ent,[2] and it could have caused slow but progressive inhibition of $(Na^+ + K^+)$-ATPase by the cardiac glycoside when the inhibitor was added after dilution with 1 1 mM Tris-EDTA. As the stability constant of the magnesium-CDTA complex is significantly greater than that of the magnesium-EDTA complex, and since CDTA is 10 times more soluble around pH 7 than EDTA, CDTA can prevent phosphorylation of the enzyme at pH 7.0–7.5 by the chelation of magnesium (12, 13).[3] As shown in Fig. 10, the addition of 10 mM CDTA stopped ouabain binding in the presence of magnesium and phosphate, and the same result was obtained for type I complex formation (data not shown). Therefore the difference between CDTA and EDTA in the dilution medium was examined. As shown in Table 3, no significant differences were observed between 10 mM Tris-CDTA and 1 mM Tris-EDTA in the various dilution media. Therefore magnesium probably is not involved in the dissociation of the various complexes or in the effect of potassium or sodium plus ATP on these complexes.

DISCUSSION

Akera and Brody (7) and Allen *et al.* (8) reported independently that potassium and sodium stabilized the type I ouabain-

[2] The apparent stability constant of the magnesium-EDTA complex is $10^{5.22}$ M^{-1} at pH 7 (17). The stability constants of the magnesium-ATP and the magnesium-phosphate complexes are less than one-tenth of that of the magnesium-EDTA complex (18). Therefore the concentration of free magnesium in the diluted solution was about 0.5 μM when 20-fold 1 mM Tris-EDTA was used to dilute the type I complex, and about 0.25 μM for the type II complex.

[3] The calculated stability constant of the magnesium-CDTA complex is $10^{5.57}$ M^{-1} at pH 7 (19), and the concentration of free magnesium in the diluted solution is about 0.06 μM when 10-fold 10 mM Tris-CDTA is used.

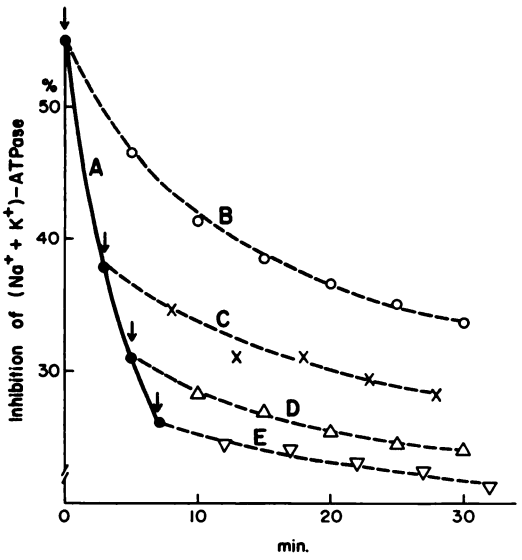

FIG. 9. *Effects of dilution at various times after starting sodium plus ATP treatment with type II digitoxigenin monodigitoxide-(Na^+ + K^+)-ATPase complex*

The type II complex was prepared with 0.5 μM digitoxigenin monodigitoxide, 1 mM $MgCl_2$, and 1 mM Tris-phosphate. After treatment of the homogenate of the washed type II complex with 2 volumes of 60 mM NaCl + 0.3 mM ATP, the suspension was diluted with 1 mM Tris-EDTA and the recovery of enzymatic activity was followed. Other experimental conditions were the same as in Fig. 5. Curve *A* (●——●), time course of dissociation in 40 mM NaCl + 0.2 mM ATP; *B* (○- - -○), *C* (×- - -×), *D* (△- - -△), and *E* (▽- - -▽), time courses after dilution after 0, 3, 5, and 7 min of treatment with sodium plus ATP. After dilution with Tris-EDTA the concentration of sodium was less than 2.4 mM and that of ATP was less than 12 μM. The effect of the diluted sodium and ATP was estimated to be less than 10% of that shown by curve *A* (see Fig. 7).

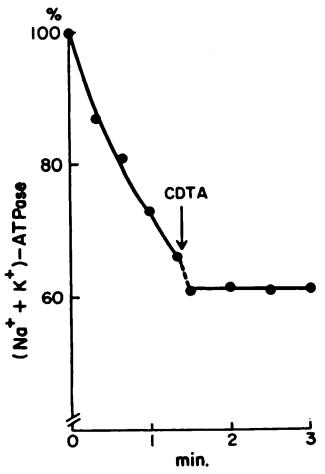

FIG. 10. *Inhibition of ouabain binding with CDTA*

Inhibition was started by the addition of enzyme to a solution containing 1 mM $MgCl_2$, 1 mM Tris-phosphate, and 0.25 μM ouabain at 25°. At suitable intervals aliquots were taken and assayed for enzymatic activity after dilution. After 80 sec of incubation 0.2 volume of 50 mM Tris-CDTA (pH 7.3) was added, and measurement of enzymatic activity was continued.

(Na^+ + K^+)-ATPase complex, by measuring the dissociation of bound radioactive ouabain. The present results confirm their potassium effect, but the sodium effect reported here is opposite to theirs. We found that sodium could reverse the potassium effect and make the type I complex unstable. It is difficult at the moment to explain the reason for this discrepancy. The methods for measuring the dissociation rate were different, and the other authors did not study the sodium effect in detail.

As shown in our previous papers (9, 10), there are characteristic differences between the dissociation rate constants of type I and type II complexes. (a) The k_d values of type I complexes are much greater than those of type II complexes. (b) If the sugar moieties of the cardiac glycosides are the same, the k_d values of type II complexes with these cardiac glycosides, but not of type I complexes, are the same. In other words, the k_d values of type II complexes are dependent on the nature of the sugar moiety, but those of type I complexes are not. This may be due to differences in the rate-determining steps in their dissociations. Also, binding between the sugar moiety of the cardiac glycosides and the sugar-specific site on the enzyme may confer more stability on type II than on type I complexes. (c) In type I complexes the sugar moiety of the glycoside binds to the sugar-specific site on the enzyme with 3′-α and β-hydroxyl groups and 5′-α-methyl and 2′-α-hydroxyl groups, but in type II

TABLE 3

Comparison of CDTA and EDTA as dilution media

All dilution media were adjusted to pH 7.3 with Tris base. When Tris-EDTA was used dilution was 20-fold, and when Tris-CDTA was used dilution was 10-fold. See the text for further details.

Dilution medium	k_d of digitoxigenin monodigitoxide–($Na^+ + K^+$)-ATPase complex at 25°	
	Type I	Type II
	hr^{-1}	hr^{-1}
1 mM Tris-EDTA	1.90	0.44
10 mM Tris-CDTA	1.84	0.46
1 mM Tris-EDTA + 2 mM KCl	0.44	
10 mM Tris-CDTA + 2 mM KCl	0.50	
1 mM Tris-EDTA + 50 mM NaCl + 0.5 mM ATP		2.81
10 mM Tris-CDTA + 50 mM NaCl + 0.5 mM ATP		2.60
20 mM Tris-CDTA + 50 mM NaCl + 0.5 mM ATP		2.93

complexes the sugar moiety binds to the site with 3′-α or β-hydroxyl and 5′-α-methyl groups. Both digitoxigenin 6-deoxyglucoside and digitoxigenin monodigitoxide have the same aglycone moiety, but the former has a 3′-β-hydroxyl and a 2′-α-hydroxyl group, and the latter has a 3′-α-hydroxyl but no 2′-α-hydroxyl. The ratio of k_d values of the digitoxigenin 6-deoxyglucoside and digitoxigenin monodigitoxide complexes (r) is different for type I and type II complexes. As the 3′-α-hydroxyl group has more affinity for the sugar-specific site than the 3′-β-hydroxyl in both types of complexes, the r value is about 1 in the type I complex but is greater than 1.5 in the type II complex. From this r value one can determine whether the cardiac glycoside complex is a type I or type II complex.

The potassium effect on the type I complex (Figs. 3 and 4 and Table 2) suggests that potassium in the dilution medium could change the binding between a cardiac glycoside and the enzyme in the type I complex to resemble that of the type II complex. The sodium plus ATP effects on the type II complex (Fig. 8 and Table 2) suggest that sodium plus ATP in the dilution medium could reverse the binding of the cardiac glycoside–enzyme complex from type II to type I, although the k_d values of type II complexes in the presence of sodium plus ATP are greater than those of type I complexes. This disagreement is explained by the finding that k_d values of type I complexes are increased in the presence of sodium (Fig. 2 and Tables 1 and 2). These results indicate that the conformation of the cardiac glycoside binding site on ($Na^+ + K^+$)-ATPase can be changed by certain ligands even after the cardiac glycoside–enzyme complex is formed. Since the replacement of EDTA by 10 mM CDTA did not alter the ligand effects, magnesium may not be involved in these conformational changes at the binding site. Another important observation is that the potassium effect on the type I complex was not a simple reversible reaction, although the sodium plus ATP effect on the type II complex was reversible. However, it appears that type I and II complexes maintain their binding characteristics after removal of the ligands which promoted complex formation, because their k_d values were not changed by centrifugation and suspension with a new buffer or changing the dilution ratio.

Therefore the new complex formed from the type II complex by sodium plus ATP is not identical with the type I complex itself, although the former might have the same glycoside-binding site as the type I complex. This effect of sodium plus ATP appeared to be related to a reaction step(s) of the native enzyme. It has been demonstrated that in the absence of magnesium the enzyme binds ATP with or without sodium (12, 20). The dissociation constant of this binding is 0.22 μM, less than 1 % of the half-maximal concentration of ATP (60 μM) required to produce the sodium plus ATP effect. This comparison might not justify excluding a correlation between ATP binding to the native enzyme and the sodium plus ATP effect on the type II complex, because this ATP binding is inhibited in the type II complex (12). The concentration of sodium producing a half-

maximal response to the sodium plus ATP effect on the type II complex is 13 mM, and this value is similar to those reported for the formation of the type I complex, i.e., 17–20 mM (15), 13.7 mM (16), and 10 mM (21). The half-maximal concentration of sodium needed in the phosphorylation of (Na^+ + K^+)-ATPase by sodium, magnesium, and ATP is 1.6 mM in the enzyme preparation from beef kidney (22) and 4 mM in that from the electric organ of electric eel (23). Therefore it is likely that the sodium plus ATP effect may be related to at least one intermediate step in type I complex formation.

On the other hand, all the data concerning the potassium effect on the type I complex support the interpretation that the type I complex is changed to type II by potassium ion, because both the dissociation characteristics and stability of the altered complex are the same as the respective parameters of the type II complex formed from the enzyme, cardiac glycoside, magnesium, and phosphate. It may also be possible that this potassium effect is the same as one of the reaction steps in the enzymatic hydrolysis of ATP, which has been shown to be related to potassium (19, 24).

As described above, the sodium plus ATP effect on the type II complex and the potassium effect on the type I complex indicate that some portions of the (Na^+ + K^+)-ATPase reaction are retained even in inhibited type I or type II cardiac glycoside–enzyme complexes, and those steps which are retained might involve a conformational change of the sugar-specific site on the enzyme. The data are not sufficient to indicate which reaction step corresponds to each ligand effect.

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REFERENCES

1. Matsui, H. & Schwartz, A. (1968). *Biochim. Biophys. Acta*, 151, 655–663.
2. Schwartz, A., Matsui, H. & Laughter, A. H. (1968) *Science*, 160, 323–325.
3. Albers, R. W., Koval, G. J. & Siegel, G. J. (1970) *Mol. Pharmacol.*, 4, 324–336.
4. Tobin, T. & Sen, A. K. (1970) *Biochim. Biophys. Acta*, 198, 120–131.
5. Skou, J. C., Butler, K. W. & Hansen, O. (1971) *Biochim. Biophys. Acta*, 241, 443–461.
6. Hansen, O. & Skou, J. C. (1973) *Biochim. Biophys. Acta*, 311, 51–66.
7. Akera, T. & Brody, T. M. (1971) *J. Pharmacol. Exp. Ther.*, 176, 545–557.
8. Allen, J. C., Harris, R. A. & Schwartz, A. (1971) *Biochem. Biophys. Res. Commun.*, 42, 366–370.
9. Yoda, A. & Yoda, S. (1974) *Mol. Pharmacol.*, 10, 494–500.
10. Yoda, A. (1973) *Mol. Pharmacol.*, 9, 51–60.
11. Van Winkle, W. B., Allen, J. C. & Schwartz, A. (1972) *Arch. Biochem. Biophys.*, 151, 85–92.
12. Hegyvary, C. & Post, R. L. (1971) *J. Biol. Chem.*, 246, 5234–5240.
13. Post, R. L., Kume, S., Tobin, T., Orcutt, B. & Sen, A. K. (1969) *J. Gen. Physiol.*, 54, 306S–326S.
14. Robinson, J. D. (1970) *Arch. Biochem. Biophys.*, 139, 17–27.
15. Siegel, G. J. & Josephson, L. (1972) *Eur. J. Biochem.*, 25, 323–335.
16. Lindenmayer, G. E. & Schwartz, A. (1973) *J. Biol. Chem.*, 248, 1291–1300.
17. Ringbom, A. (1959) in *Treatise on Analytical Chemistry* (Kolthoff, I. M. & Elving, P. J., eds.), Vol. I, pp. 543–628, Wiley-Interscience, New York.
18. Bock, R. M. (1960) in *The Enzymes* (Boyer, P. D., Lardy, H. & Myrbäck, K., eds.), Vol. 2, Ed. 2, pp. 1–38, Academic Press, New York.
19. Post, R. L., Hegyvary, C. & Kume, S. (1972) *J. Biol. Chem.*, 247, 6530–6540.
20. Jensen, J. & Norby, J. G. (1971) *Biochim. Biophys. Acta*, 233, 395–403.
21. Yoda, A., Yoda, S. & Sarrif, A. M. (1973) *Mol. Pharmacol.*, 9, 766–773.
22. Post, R. L., Sen, A. K. & Rosenthal, A. S. (1965) *J. Biol. Chem.*, 240, 1437–1445.
23. Siegel, G. J. & Albers, R. W. (1967) *J. Biol. Chem.*, 242, 4972–4979.
24. Kanazawa, T., Saito, M. & Tonomura, Y. (1970) *J. Biochem. (Tokyo)*, 67, 693–711.