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KINETICS STUDIES ON THE INTERACTION BETWEEN OUABAIN AND (Na^+,K^+)-ATPase

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Summary

The association and dissociation rate constants for the interaction of [^{3}H]-ouabain with partially purified rat brain (Na^+,K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) in vitro were estimated from the time course of the [^{3}H]-ouabain binding observed in the presence of Na^+, Mg^{2+} and ATP by a polynomial approximation-curve-fitting technique. The reduction of the association rate constant by K^+ was greater than its reduction of the dissociation rate constant. Thus, the affinity of (Na^+,K^+)-ATPase for ouabain was reduced by K^+. The binding-site concentration was unaffected by K^+. Consistent with these findings, the addition of KCl to an incubation mixture at the time when [^{3}H]-ouabain binding to (Na^+,K^+)-ATPase is close to equilibrium, caused an immediate decrease in bound ouabain concentration, apparently shifting towards a new, lower equilibrium concentration. Dissociation rate constants which were estimated following the termination of the ouabain-binding reaction were different from those estimated with above methods and may not be useful in predicting the ligand effects on equilibrium of the ouabain-enzyme interaction.

Introduction

The interaction between cardiac glycosides, such as ouabain, and (Na^+,K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) has drawn much attention because such an interaction appears to be involved in the mechanism of the pharmacologic action of this class of compounds (see refs. 1 and 2). Additionally, the glucoside-(Na^+,K^+)-ATPase interaction in vitro has been shown to be useful as a model of the interaction of glycosides with the inotropic receptor occurring in the beating heart, because these two phenomena share many common char-

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acteristics (ref. 3, also see ref. 2). Thus, it is important to know kinetic parameters which determine the equilibrium state of the drug-enzyme interaction in vitro, since such parameters may be useful in predicting the magnitude of drug-receptor interaction in vivo.

Several investigators considered that the binding of ouabain to (Na^+,K^+)-ATPase is a reversible process which follows the law of mass action as follows:

$$E + Ou \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot Ou \qquad (1)$$

where E is enzyme, Ou is ouabain and E · Ou is the ouabain-enzyme complex. Kinetic constants based on this assumption have been determined (ref. 4–8, also see ref. 1). Dissociation rate constant has been determined directly, by terminating the [^{3}H]ouabain binding reaction either by adding excess nonradioactive ouabain or by removing unbound [^{3}H]ouabain by centrifugation and resuspension of the [^{3}H]ouabain-enzyme complex and subsequently monitoring release of bound [^{3}H]ouabain from the complex (for example, refs. 1, 9 and 10).

The concentration of bound ouabain at equilibrium is influenced by the rate constant for association and that for dissociation since the rate of binding equals the rate of release under such a condition. The present study was initiated to investigate whether the dissociation rate constant which affects the magnitude of the ouabain-(Na^+,K^+)-ATPase interaction is the same as the dissociation rate constant which may be observed following the termination of the binding reaction. The results indicate that these two dissociation rate constants are substantially different, suggesting that the ouabain-enzyme interaction is more complex than the above scheme.

Materials and Methods

(Na^+,K^+)-ATPase preparations

Partially purified (Na^+,K^+)-ATPase preparations were obtained from brains of male Sprague-Dawley rats weighing 200–300 g as described previously [11]. In short, brain microsomal fractions were treated with deoxycholic acid and NaI and suspended in a solution containing 0.25 M sucrose, 1 mM EDTA and 5 mM histidine · HCl (pH 7.0). Enzyme preparations were stored at −20°C until use. These enzyme preparations have specific (Na^+,K^+)-ATPase activity of approximately 200 μmol of ATP hydrolyzed per mg protein per h, assayed in the presence of 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris · ATP and 50 mM Tris · HCl buffer (pH 7.5) at 37°C for 10 min. Mg^{2+}-ATPase activity, assayed in the absence of added NaCl and KCl, was less than 5% of the total ATPase activity.

[^{3}H]ouabain binding

Enzyme preparations (final concentration, 20 μg protein/ml) were incubated with various concentrations of [^{3}H]ouabain (specific activity, 13 Ci/mmol) at 37 ± 0.1°C in the presence of 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris · ATP and 50 mM Tris · HCl buffer (pH 7.5) with or without 5 mM KCl. After a 5-min preincubation without enzyme preparation, the binding reaction was

started by the addition of enzyme to the incubation mixture. At a predetermined time, a 1.0-ml aliquot was removed from the reaction vessel and quickly filtered through a Millipore filter (type AA, pore size 0.45 μm) as described previously [12]. The filter was rinsed twice with 5.0 ml each of the incubation medium which does not contain [^{3}H]ouabain, and dissolved in ethylene glycol monomethyl ether. The radioactivity was estimated by liquid scintillation spectrometry. Counting efficiency (approximately 30%) was monitored with external standard channel ratio method. Non-specific binding of [^{3}H]ouabain, observed in the absence of ATP and approximately 2% of the total binding, was subtracted from the binding observed in the presence of ATP to calculate the ATP-dependent [^{3}H]ouabain binding. It has been shown previously that such an ATP-dependent binding is the stoichiometric binding of ouabain to (Na^+, K^+)-ATPase which results in enzyme inhibition [13,14].

Effects of KCl on the concentration of bound ouabain

Enzyme preparations (final concentration, 20 μg protein/ml) were incubated with 10 nM [^{3}H]ouabain at 37°C in the presence of 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris · ATP and 50 mM Tris · HCl buffer (pH 7.5). The binding reaction was started by the addition of an enzyme preparation to a prewarmed incubation mixture. At 30 min, an equal volume of a pre-warmed mixture containing 10 nM [^{3}H]ouabain, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris · ATP and 50 mM Tris · HCl buffer (pH 7.5) with either no KCl or 10—30 mM KCl was added to the reaction mixture. Aliquots were taken at indicated time intervals and the amount of bound [^{3}H]ouabain was assayed as described above.

The release of [^{3}H]ouabain from (Na^+,K^+)-ATPase

The complex of [^{3}H]ouabain with (Na^+,K^+)-ATPase was prepared by incubating the enzyme preparation (final concentration, 20 μg protein/ml) with 10 nM [^{3}H]ouabain in a total volume of 12 ml under the ligand conditions described as the binding reaction. After a 10-min incubation at 37°C, the binding reaction was terminated by the addition of non-radioactive ouabain (final concentration, 0.1 mM) with a simultaneous 2-fold dilution of radioactive ouabain and enzyme. Subsequent dissociation of the [^{3}H]ouabain-enzyme complex was monitored at 37°C under the same ligand conditions as those in the binding reaction. Aliquots were taken at appropriate time intervals and the amount of [^{3}H]ouabain remaining bound at the time was estimated as described above. Non-specific binding of [^{3}H]ouabain observed in the absence of ATP was subtracted from the binding observed in the presence of ATP to calculate the ATP-dependent [^{3}H]ouabain binding.

Calculations

Binding velocity was calculated by two separate methods. First, the velocity was calculated from the difference in the amount of bound [^{3}H]ouabain observed at two adjacent time points. The velocity calculated from such values is considered as the binding velocity at the time which is the mean of two given time points. The second method of binding velocity estimation utilized a polynomial approximation-curve-fitting technique [15] and computation was performed using a CDC 6500 computer. Using this latter technique, the slopes of a

curve at various times and also the best fitting kinetic parameters were estimated.

The first-order dissociation rate constants following the termination of [^{3}H]-ouabain binding were calculated from the slope of linear regression lines fitted to semilogarithmic plots of the amount of bound ouabain vs. time.

Miscellaneous

Protein concentration was determined by the method of Lowry et al. using bovine serum albumin standard [16]. Statistical analyses were performed using Student's *t*-test. The criterion for statistical significance was a P value of less than 0.05.

Tris · ATP was purchased from Sigma Chemical Company, St. Louis, Mo. [^{3}H]Ouabain was purchased from New England Nuclear, Boston, Mass. Other chemicals were of reagent grade.

Results and calculations

[^{3}H]ouabain studies

ATP-dependent [^{3}H]ouabain binding to partially purified Na^+,K^+-ATPase preparations was a relatively slow process in the absence of KCl (Fig. 1A). The addition of 5 mM KCl further delayed the binding reaction (Fig. 1B). Under the latter condition, it was impossible to reach the steady state of ouabain-binding since approximately 30% of the added ATP would be consumed during

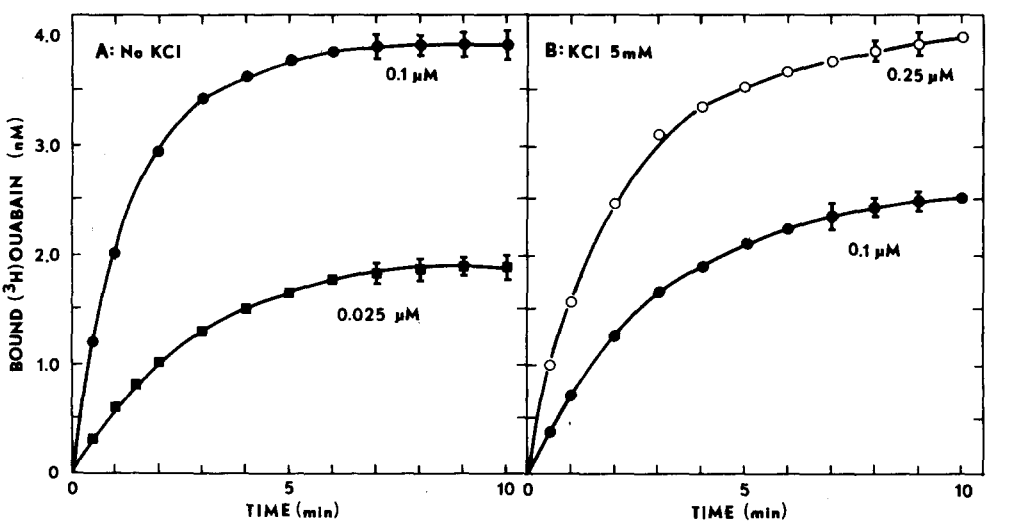

Fig. 1. Time course of [^{3}H]ouabain binding to (Na^+,K^+)-ATPase in the absence and presence of KCl. Partially purified rat brain (Na^+,K^+)-ATPase (final concentration, 20 μg protein/ml) was incubated with various concentrations of [^{3}H]ouabain at 37°C in the presence of 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris · ATP and 50 mM Tris · HCl buffer (pH 7.5), with or without 5 mM KCl. Binding observed in the absence of ATP was subtracted from the binding observed in the presence of ATP to calculate the ATP-dependent ouabain binding. Each point represents the mean value of four enzyme preparations. Vertical line indicates the standard error of the mean of five experiments. Where standard errors are not shown, they are less than 2% of the observed value.

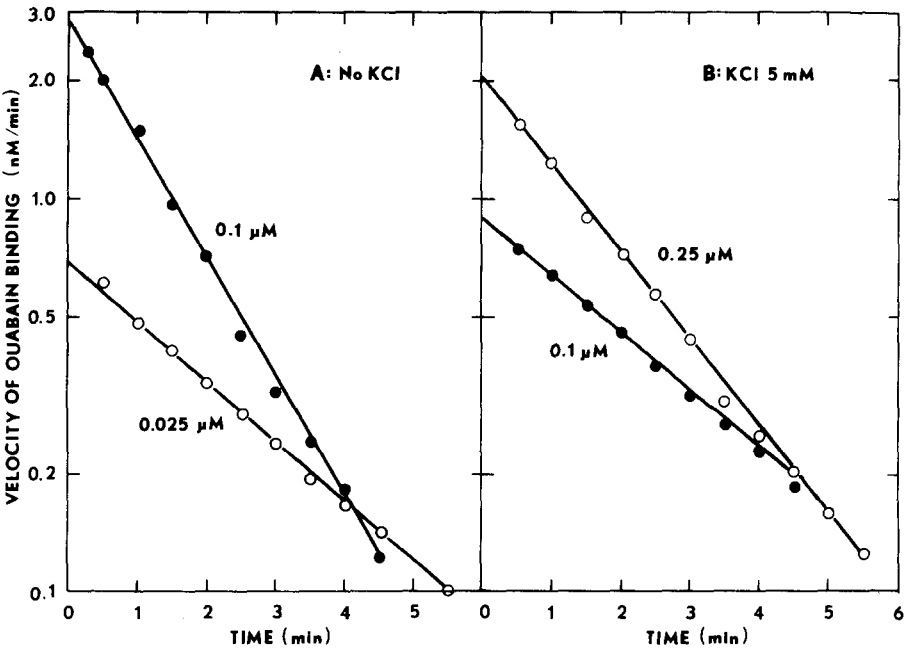

Fig. 2. Semilogarithmic plot of ouabain binding velocity vs. time. Ouabain binding velocity at each time point was calculated from data shown in Fig. 1.

15 min of incubation at 37°C. Additionally, a 15-min incubation of enzyme preparation at 37°C caused a slight reduction in (Na^+,K^+)-ATPase activity ruling out the possibility of a longer incubation time (data not shown).

The reaction velocity was calculated from the slopes of curves shown in Fig. 1. When the binding velocities are plotted against time in semilogarithmic plots (Fig. 2), straight lines are obtained. This finding is consistent with the assumption that the binding reaction follows pseudo-first-order kinetics such as described by Eq. 1. Although experiments were performed with [^{3}H]ouabain concentrations of 0.01, 0.015, 0.025, 0.04, 0.06 and 0.1 μM in the absence of KCl and 0.1, 0.15, 0.25 and 1.0 μM in the presence of 5 mM KCl, only representative lines are shown in Fig. 2. Data representing other concentrations of ouabain also formed straight lines similar to those shown in Fig. 2.

From extrapolation of regression lines in Fig. 2 to the ordinate (0-min values), the initial velocities were estimated and are plotted against [^{3}H]ouabain concentrations in Fig. 3. A linear regression line passing through the point of origin may be fitted to the plot, indicating that the binding reaction is indeed a pseudo-first-order reaction with respect to ouabain concentrations. The slopes of regression lines, 0.027/min and 0.0084/min in the absence and presence of 5 mM KCl, respectively, correspond to the $k_1 \cdot [E]$ value. It should be noted that k_1 is an "apparent" association rate constant. Therefore, the potassium-induced change in $k_1 \cdot [E]$ value does not necessarily indicate a change in the true association rate constant. Alternatively, it may indicate a change in the concentration of the binding form of the enzyme as suggested by Barnett [17]. The true association rate constant should be expressed in $(M \cdot min)^{-1}$ when the con-

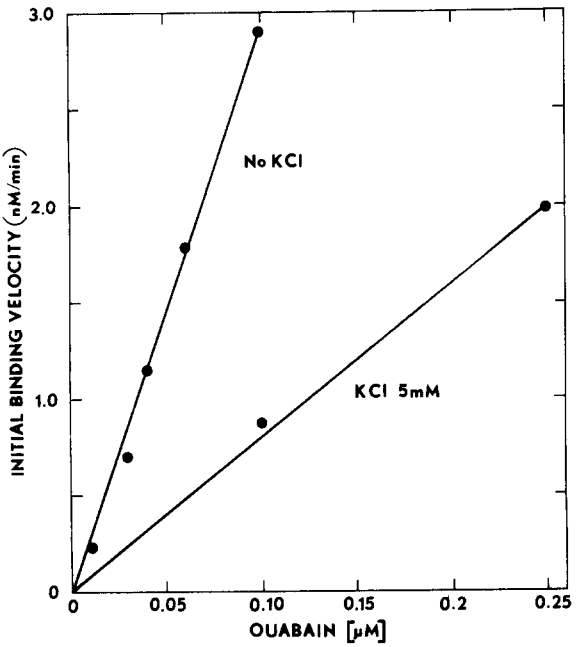

Fig. 3. Relationship between initial binding velocity and ouabain concentrations. Initial velocity of [^{3}H]-ouabain binding in the absence and presence of 5 mM KCl was estimated from the extrapolation of regression lines in Fig. 2 to zero time.

centration of the binding form of the enzyme is expressed in molar concentrations. However, an apparent association rate constant may be expressed in the same unit, $(M \cdot min)^{-1}$, when the concentration of binding sites on the enzyme is expressed in molar concentrations. These findings indicate that 5 mM KCl causes an approximately 70% reduction in ouabain-binding velocity. Additionally, they indicate that there is no cooperative effect for the binding of ouabain to (Na^+,K^+)-ATPase. Under such conditions, it is reasonable to assume that the release of ouabain from the enzyme follows first-order kinetics. Therefore, it appears possible to perform kinetic analysis of the interaction between ouabain and the enzyme based on a reaction scheme such as described by Eq. 1, where k_1 is the apparent association rate constant and k_{-1} is the "overall" dissociation rate constant (k_{-1} may be a sum of several dissociation rate constants which represent various pathways).

Based on the above assumption, changes in concentrations of bound ouabain with respect to time may be expressed as follows:

$$\frac{d[E \cdot Ou]}{dt} = k_1([E] - [E \cdot Ou])([Ou] - [E \cdot Ou]) - k_{-1}[E \cdot Ou] \quad (2)$$

This equation was fitted to experimental data shown in Fig. 1 with a polynomial approximation-curve-fitting technique using a general non-linear curve-fitting and equation-solving program [15] on a CDC 6500 computer yielding a set of best estimates for k_1, k_{-1} and the binding site concenteation (Table I). Potassium failed to alter the binding site concentration. However, it reduced both the apparent association rate constant and the overall dissociation rate

TABLE I

KINETIC CONSTANTS ESTIMATED USING A POLYNOMIAL APPROXIMATION-CURVE-FITTING TECHNIQUE FROM DATA SHOWN IN FIG. 1

Values are the mean ± S.E. of five experiments.

Ligands	Apparent k_1 $(nM \cdot min)^{-1}$	k_{-1} (min^{-1})	K_d * (nM)	Binding site concentration ** (nM)
Na^+, Mg^{2+}, ATP	0.00486 ± 0.00001	0.254 ± 0.002	52.3	6.00 ± 0.12
K^+, Na^+, Mg^{2+}, ATP	0.00132 ± 0.00001	0.186 ± 0.003	141	5.88 ± 0.10

* Apparent dissociation constant: $K_d = k_{-1}/k_1$.

** Protein concentration = 0.02 mg/ml. Thus, ouabain-binding sites on enzyme are 300 ± 6 and 294 ± 5 pmol/mg protein in the absence and presence of KCl, respectively.

constant. The KCl-induced change in the apparent k_1 value (approximately 73%) was greater than that in the overall k_{-1} value (approximately 27%). Thus, 5 mM KCl caused a 2.7-fold increase in the apparent dissociation constant, indicating that KCl decreases the affinity of (Na^+,K^+)-ATPase for ouabain. These findings predict that KCl reduces the equilibrium concentration of bound ouabain.

Effects of potassium on the concentration of ouabain-(Na^+,K^+)-ATPase complex in vitro

It has been claimed previously that potassium delays the binding of ouabain to (Na^+,K^+)-ATPase but does not affect the ultimate (equilibrium) concentration of bound ouabain formed after a long incubation time [6,17,18]. Present data, however, indicate that the potassium-induced reduction in the association rate constant is of greater magnitude than that in the dissociation rate constant and therefore the concentration of the bound ouabain in the presence of KCl cannot reach the equilibrium concentration of the bound ouabain observed in the absence of KCl, even after a long incubation time. Thus, the effects of KCl on the concentrations of the ouabain-(Na^+,K^+)-ATPase complex were studied in vitro using partially purified rat brain enzyme preparations.

The binding of ouabain to (Na^+,K^+)-ATPase in the presence of Na^+, Mg^{2+} and ATP is a relatively slow process. The addition of KCl to the binding mixture further delays reaction (Fig. 1; also ref. 9). Whether the concentration of bound ouabain in the presence of KCl ultimately reaches the equilibrium concentration of bound ouabain observed in the absence of KCl is difficult to determine because of the differences in the rate of hydrolysis of ATP in the presence and absence of KCl. In the presence of NaCl and KCl, the hydrolysis of ATP and subsequent production of ADP and P_i is markedly greater than those in the absence of KCl. Thus, the effect of KCl on the concentration of the ouabain-(Na^+,K^+)-ATPase complex was studied by adding KCl to the incubation mixture after the drug-enzyme interaction has reached near equilibrium state.

Partially purified enzyme preparations were incubated with 10 nM [^{3}H]-ouabain in the presence of NaCl, $MgCl_2$, Tris · ATP and Tris · HCl buffer (pH 7.5) at 37°C for 30 min (Fig. 4). At this time, a solution containing [^{3}H]-

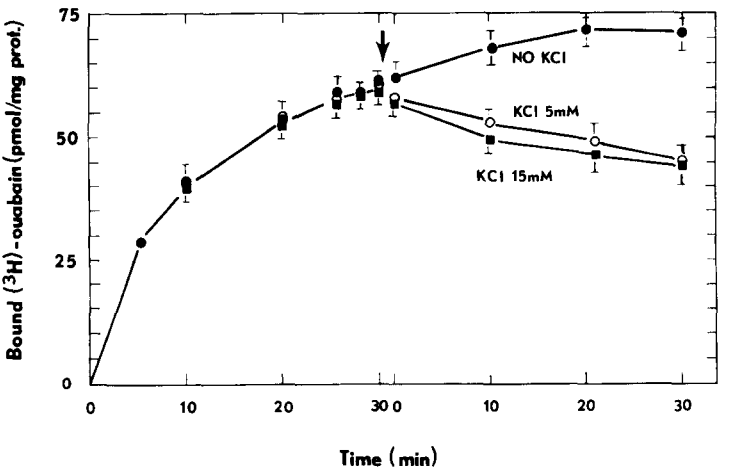

Fig. 4. Effect of potassium on bound [^{3}H]ouabain concentrations. Partially purified rat brain enzyme preparation (20 μg protein/ml) was incubated with 10 nM [^{3}H]ouabain in the presence of 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris · ATP and 50 mM Tris · HCl buffer (pH 7.5) at 37°C. At the time indicated by the arrow, an equal volume of a solution containing 10 nM [^{3}H]ouabain, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris · ATP and 50 mM Tris · HCl buffer (pH 7.5), either with or without 10 or 30 mM KCl, was added to the incubation mixture. Each point represent the mean value of four enzyme preparations. Vertical line indicates the standard error of the mean.

ouabain, NaCl, $MgCl_2$, Tris · ATP and Tris · HCl buffer (pH 7.5), but without enzyme preparation, was added in control experiments to the incubation mixture causing a 2-fold dilution of the enzyme protein (expressed as mg/ml). This procedure would not alter the equilibrium concentration of bound ouabain (expressed as pmol/mg protein) since it reduces the concentrations (expressed in nM) of the enzyme and the ouabain-enzyme complex to the same extent. Under these conditions, the concentration of the ouabain-enzyme complex continuously increased with time after the addition (Fig. 4). It should be noted that 10 nM [^{3}H]ouabain causes approximately 20% occupation of the binding sites at 30 min (total binding sites, approximately 300 pmol/mg protein; see Table I) and thus the equilibrium concentration of the ouabain-enzyme complex is sensitive to alterations in association or dissociation rate constants. The addition of a similar solution containing KCl, at 30 min, caused an immediate decrease in the concentration of ouabain-enzyme complex (Fig. 4). These data indicate that the equilibrium concentration of the ouabain-(Na^+,K^+)-ATPase complex in the presence of KCl is significantly lower than that in the absence of KCl, and thus confirm the results of above kinetic calculations.

Dissociation rate constants following the termination of the binding reaction

In this series of experiments, [^{3}H]ouabain-binding reaction was terminated by the addition of excess non-radioactive ouabain, and subsequent release of [^{3}H]ouabain from its binding site on (Na^+,K^+)-ATPase was monitored. Non-radioactive ouabain and dissolved in a mixture which has the same composition as the binding mixture but lacking [^{3}H]ouabain and enzyme preparation so that the dissociation reaction can be observed under the same ligand condition as the binding reaction. Semilogarithmic plots of bound ouabain vs. time yielded

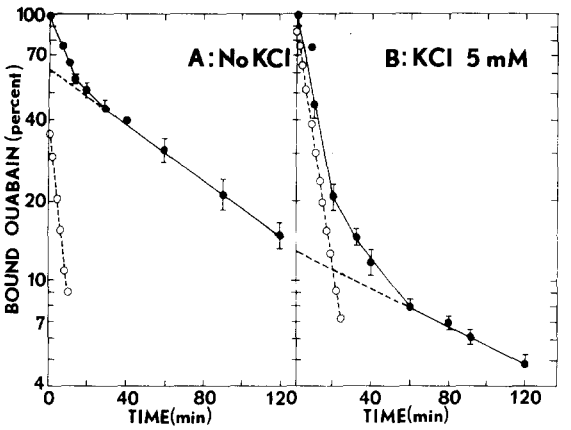

Fig. 5. Time course of the release of [^{3}H]ouabain from (Na^+,K^+)-ATPase and the effect of KCl. The [^{3}H]-ouabain-(Na^+,K^+)-ATPase complex was prepared by incubating the partially purified rat brain enzyme preparations with 10 nM [^{3}H]ouabain in the presence of 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris · ATP and 50 mM Tris · HCl buffer (pH 7.5) at 37°C for 10 min in the absence and presence of 5 mM KCl. Subsequently, nonradiolabelled ouabain (final concentration, 0.1 mM) was added to terminate the [^{3}H]-ouabain binding and subsequent release of labelled ouabain from (Na^+,K^+)-ATPase was monitored under the same ligand condition as the binding reaction. Values of [^{3}H]ouabain-binding observed in the absence of ATP (approximately 2% at time zero) were subtracted. Filled circles represent mean values of four enzyme preparations. Vertical line indicates the standard error of the mean. For open circles and broken lines, see text.

an upward concave curve either in the absence or presence of 5 mM KCl (Fig. 5, solid lines). This finding is in contrast to previous reports by several investigators [5,7], and suggests that there may be more than two different complexes formed under the conditions of present binding studies. A log-linear relation against time was observed after 60 min of incubation in the absence of KCl (Fig. 5A). A regression line fitted to this part of the curve was extrapolated to zero time, and the difference between observed values and those on the extrapolated regression line was replotted yielding a second straight line (Fig. 5A, open circles). This finding indicates that observed dissociation curve of the ouabain-enzyme complex can be accounted for by two independent exponential components each having different rate constant for dissociation. Similar results were observed for the dissociation reaction of the ouabain-enzyme com-

TABLE II

DISSOCIATION RATE CONSTANTS OBSERVED FOLLOWING THE TERMINATION OF THE [^{3}H]-OUABAIN-BINDING REACTION

The rate constant and relative abundance of each component were calculated from the slope and intercept of each regression line shown in Fig. 5.

Ligands	Phase	k_{-1} (min^{-1})	Relative abundance of component (%)
Na^+, Mg^{2+}, ATP	1	0.126	36
	2	0.0123	64
K^+, Na^+, Mg^{2+}, ATP	1	0.101	87
	2	0.0084	13

plex formed and dissociating in the presence of 5 mM KCl (Fig. 5B). Dissociation rate constant and relative abundance of each component were calculated from slopes and ordinate intercepts of regression lines (Table II). It appears from Fig. 5 that the dissociation rate is faster in the presence of KCl than in its absence. However, calculated dissociation rate constants are smaller in the presence of KCl than in its absence (Table II). Seemingly faster dissociation in the presence of KCl is due to the relative abundance of the relatively unstable component formed under this condition. Additionally, it should be noted that dissociation rate constants calculated from observation of the release of bound [^{3}H]ouabain following the termination of the binding reaction (Table II) are lower than those shown in Table I.

Discussion

The time course of the drug binding in vitro is affected by the association and dissociation rate constants whereas the initial velocity of the binding reaction is primarily determined by the association rate constant. Thus, it should be possible to analyze the time course and initial velocity of drug binding in vitro and to calculate the association and dissociation rate constant even though the equilibration state of the binding is not reached during the observation period. Present studies indicate that it is possible to analyze the time course of the drug binding by a polynomial approximation-curve-fitting technique [15], and to calculate the kinetic constants under such conditions. The present results confirm previous reports [5,6,19] that the binding reaction between ouabain and (Na^+,K^+)-ATPase follows a pseudo-first-order reaction with respect to ouabain concentrations.

The dissociation rate constant which affects the equilibrium concentration of bound ouabain is that constant which is calculated from the time course of the ouabain binding reaction. Since the magnitude of the potassium-induced changes in association rate constants was greater than that of the potassium-induced changes in dissociation rate constant, it may be predicted that KCl will reduce the equilibrium concentration of bound ouabain. Such results were observed when KCl was added to the incubation mixture containing (Na^+,K^+)-ATPase preparation, [^{3}H]ouabain, NaCl, $MgCl_2$ and Tris · ATP. These results indicate that the above calculations are correct and that the ultimate magnitude of the ouabain-enzyme interaction in the presence of KCl does not reach that in the absence of KCl, in contrast to previous reports [6,17,18]. The cause of the apparent discrepancy is not known. However, differences in results may be due to differences in the sources of enzyme preparations or differences in experimental design. For example, when the ouabain-binding reaction is initiated in the incubation mixture containing NaCl, $MgCl_2$ and ATP, the concentrations of ATP, ADP and Pi in the presence and absence of KCl are markedly different at the time of equilibrium for the ouabain-enzyme interaction, whereas those concentrations are relatively similar under the present experimental conditions. Alternatively, the concentration of bound ouabain is insensitive to changes in association and dissociation rate constants when the ouabain binding is close to the saturation of binding sites in the presence of high concentrations of ouabain such as those employed in some of the previous studies. Under such

circumstances, the concentration of bound ouabain is primarily determined by the concentration of the enzyme and is insensitive to alterations in kinetic constants.

The dissociation rate constant which affects the magnitude of the ouabain-(Na^+,K^+)-ATPase interaction should be determined from the time course of the binding reaction rather than from that of the dissociation reaction observed after the termination of ouabain binding. The present paper is the first report of such a value and it demonstrates that the dissociation rate constants determined by these two methods are substantially different. While the latter method yields more information such as the presence of two separate components of the ouabain-(Na^+,K^+)-ATPase complexes which have different rate constants for dissociation, the dissociation rate constants determined from the time course of the binding reaction are more important in the prediction of ligand effects on the magnitudes of drug-enzyme interaction in vitro.

The apparent dissociation rate constants were different during the binding reaction and after termination of the binding reaction. This observation indicates that the interaction between ouabain and (Na^+,K^+)-ATPase does not proceed as a reaction scheme shown in Eq. 1. One of the possible explanations is that there are several separate sequential and parallel pathways for the dissociation of the ouabain-enzyme complex under the experimental condition of Fig. 1. Thus, the k_{-1} value in Eq. 1 and Table I is the "overall" dissociation rate constant which is possibly a sum of several independent dissociation rate constants, each value representing a separate pathway. After the termination of the binding reaction, some of the intermediate complexes may disappear rapidly and the "overall" dissociation rate constant observed under such a condition is now a sum of those for the remaining pathways. Other explanations may also be possible. The present study indicates that the dissociation rate constant estimated from the time course of the binding reaction has a predictive value for the magnitude of the drug-enzyme interaction in vitro. The binding of cardiac glycosides to myocardial (Na^+,K^+)-ATPase, and the dissociation of the glycosides, have been shown to be intimately related to the magnitude of the inotropic action of these agents (ref. 20, also see ref. 2). Whether the dissociation rate constant estimated from the time course of the binding reaction has a predictive value for the magnitude of drug effects in vivo is to be determined.

In summary, association and dissociation rate constants may be calculated from the time course of the ouabain binding reaction. The dissociation rate constants calculated from data obtained after the termination of the binding reaction are different from those obtained by the former method and may not be relevant to the prediction of the ligand effects on the equilibrium concentration of the ouabain-(Na^+,K^+)-ATPase complex.

Acknowledgments

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References

1 Schwartz, A., Lindenmayer, G.E. and Allen, J.C. (1975) Pharmacol. Rev. 27, 3—134
2 Akera, T. and Brody, T.M. (1976) Life Sci. 18, 135—142
3 Akera, T., Ku, D., Tobin, T. and Brody, T.M. (1976) Mol. Pharmacol. 12, 101—114
4 Hansen, O. (1971) Biochim. Biophys. Acta 233, 122—132
5 Erdmann, E. and Schoner, W. (1973) Biochim. Biophys. Acta 307, 386—398
6 Lindenmayer, G.E. and Schwartz, A. (1973) J. Biol. Chem. 248, 1291—1300
7 Lane, L.K., Copenhaver, Jr., J.H., Lindenmayer, G.E. and Schwartz, A. (1973) J. Biol. Chem. 248, 7197—7200
8 Inagaki, C., Lindenmayer, G.E. and Schwartz, A. (1974) J. Biol. Chem. 249, 5135—5140
9 Akera, T. and Brody, T.M. (1971) J. Pharmacol. Exp. Ther. 176, 545—557
10 Allen, J.C., Harris, R.A. and Schwartz, A. (1971) J. Mol. Cell. Cardiol. 3, 297—300
11 Akera, T. and Brody, T.M. (1969) Mol. Pharmacol. 5, 605—614
12 Akera, T., Tobin, T., Gatti, A., Shieh, I.-S. and Brody, T.M. (1974) Mol. Pharmacol. 10, 509—518
13 Hansen, O., Jensen, J. and Norby, J.G. (1971) Nat. New Biol. 234, 122—124
14 Allen, J.C., Martinez-Maldonado, M., Eknoyan, G., Suki, W.N. and Schwartz, A. (1971) Biochem. Pharmacol. 20, 73—80.
15 Dye, J.L. and Nicely, V.A. (1971) J. Chem. Educ. 48, 443—448
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Barnett, R.E. (1970) Biochemistry 9, 4644—4648
18 Allen, J.C. and Schwartz, A. (1970) J. Mol. Cell. Cardiol. 1, 39—45
19 Lindenmayer, G.E. and Schwartz, A. (1970) Arch. Biochem. Biophys. 140, 371—378
20 Ku, D., Akera, T., Pew, C.L. and Brody, T.M. (1974) Naunyn-Schmiedeberg's Arch. Pharmacol. 285, 185—200