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QUANTITATIVE ASPECTS OF THE INTERACTION BETWEEN OUABAIN AND $(Na^+ + K^+)$-ACTIVATED ATPASE *IN VITRO**

TAI AKERA

Department of Pharmacology, School of Medicine, Keio University, Tokyo, (Japan) and Department of Pharmacology, Michigan State University, East Lansing, Mich. (U.S.A.)

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SUMMARY

The inhibitory effect of ouabain on $(Na^+ + K^+)$-activated ATPase (Mg^{2+}-dependent, $(Na^+ + K^+)$-activated ATP phosphohydrolase, EC 3.6.1.3) obtained from rat brain microsomal fraction was re-examined using a modified method to estimate the inhibited reaction velocity. This method involves a preincubation of a ouabain–enzyme mixture in the presence of Na^+, Mg^{2+} and ATP to bring the ouabain–enzyme reaction to near equilibrium. The $(Na^+ + K^+)$-activated ATPase reaction was subsequently started by the addition of a KCl solution.

When the ouabain–enzyme reaction was brought to near equilibrium prior to the estimation of the ATPase activity, it was kinetically reversible, although overwhelmingly in favor of the ouabain–enzyme complex.

This method resulted in a significant shift of the log concentration–response curve to the left. The concentration of ouabain to inhibit 50 % of the $(Na^+ + K^+)$-activated ATPase activity was 0.12 μM, whereas it was 0.52 μM with the conventional method for the ATPase assay. Hence, the specific binding of ouabain to the $(Na^+ + K^+)$-activated ATPase molecule was a slow process.

This modified method was not suitable for the study of the effect of *p*-chloromercuribenzoate on $(Na^+ + K^+)$-activated ATPase since the presence of ATP during the preincubation period protected the enzyme from the SH-blocking reagent.

With the modified method, the effect of K^+ to antagonize ouabain inhibition of $(Na^+ + K^+)$-activated ATPase was markedly reduced, indicating that the well-documented effect of K^+ was on the velocity of the ouabain–enzyme complex formation rather than on that of the ouabain-inhibited ATPase reaction.

The ouabain–enzyme reaction was competitive with respect to K^+ at KCl concentrations below 5 mM, although the competition by K^+ was not remarkable. Above this concentration, the reaction was non-competitive with respect to K^+.

Ouabain released from the ouabain–enzyme complex was rebound to the enzyme during an incubation in the presence of Na^+, Mg^{2+} and ATP more easily than ouabain added to the incubation mixture.

Abbreviations: PCMB, *p*-chloromercuribenzoate; S/M ratio, the ratio between the concentration of ouabain in the 100000 × *g* sediment and that in the medium.

* Send reprint request to Dr. T. Akera, Department of Pharmacology, Michigan State University, East Lansing, Mich. 48823, U.S.A.

INTRODUCTION

The effect of ouabain on ($Na^+ + K^+$)-activated ATPase (Mg^{2+}-dependent, ($Na^+ + K^+$)-activated ATP phosphohydrolase, EC 3.6.1.3) has been studied rather extensively during the last decade, confirming the initial finding (see ref. 1) that cardiac glycosides specifically inhibit this enzyme system at low concentrations. In most of the studies, the ouabain–enzyme mixture was preincubated in the presence of Na^+, K^+, Mg^{2+} and an appropriate buffer, and the inhibited reaction velocity was estimated by adding ATP and assaying the amount of released P_i during a 5–30-min incubation period. Under these conditions, however, the formation of the ouabain–enzyme complex is minimal during the preincubation period since it requires ATP in the presence of Na^+ and Mg^{2+} (refs. 2–6).

In a previous paper[7], we have demonstrated that the association of ouabain and the ($Na^+ + K^+$)-activated ATPase is a slow process requiring more than 10 min to be completed even in the absence of K^+. In the presence of K^+, the formation of the ouabain–enzyme complex is slowed down further[3,6,7]. Hence, if ATP is added last to the incubation mixture to start the ATPase reaction, one is essentially measuring the velocity at which the ouabain-enzyme complex is formed rather than the velocity of the inhibited ATPase reaction.

This problem can be solved by preincubating the ouabain–enzyme mixture in the presence of Na^+, Mg^{2+} and ATP until it reaches equilibrium and then starting the ($Na^+ + K^+$)-activated ATPase reaction by adding K^+ to the mixture, provided that one can obtain an enzyme preparation with low ATPase activity in the presence of Na^+ and Mg^{2+} compared to that in the presence of Na^+, Mg^{2+} and K^+. Using an enzyme preparation with the above characteristic, the inhibition of ($Na^+ + K^+$)-activated ATPase by ouabain was re-examined and compared with that of *p*-chloromercuribenzoate (PCMB), another well-known inhibitor of the enzyme system.

METHODS

Male Wistar rats weighing 200 to 300 g were used. In the present studies, ($Na^+ + K^+$)-activated ATPase preparations obtained from rat brain were used because they had a low Mg^{2+}-dependent ATPase activity. It has been previously shown that rat brain ($Na^+ + K^+$)-activated ATPase was similar to dog heart enzyme, a ouabain-sensitive species, with respect to the time course of the formation and the dissociation of the ouabain–enzyme complex[7], although rat heart ATPase seems to be different[8]. The preparation of the enzyme from the brain microsomal fraction by deoxycholic acid and NaI treatment was performed as described previously[9].

Assays for ($Na^+ + K^+$)-activated ATPase activity were performed with 16 μg of enzyme protein, unless otherwise indicated, in an incubation volume of 1.0 ml with one of the following methods. *Method A*: The enzyme was preincubated with 5.0 mM $MgCl_2$, 50 mM Tris–HCl buffer (pH 7.5) and various concentrations of inhibitors as indicated, with or without 100 mM NaCl and 15 mM KCl. After a 10-min preincubation period at 37°, Tris–ATP was added to a final concentration of 5.0 mM. The incubation was performed for an additional 10-min period at 37°. Mg^{2+}-dependent ATPase activity assayed in the absence of NaCl and KCl was subtracted from the total ATPase activity, assayed in the presence of NaCl and KCl, to calculate the

$(Na^+ + K^+)$-activated ATPase activity. *Method B*: The enzyme was preincubated with 5.0 mM $MgCl_2$, 50 mM Tris–HCl buffer (pH 7.5), various concentrations of inhibitors as indicated, 100 mM NaCl and 5.0 mM Tris–ATP. After a 10-min preincubation period at 37°, KCl or an equivalent volume of water was added. The final concentration of KCl was 15 mM, unless otherwise indicated. The incubation was performed for an additional 10-min period at 37°. Basic ATPase activity, assayed in the absence of KCl, was subtracted from the total ATPase activity, assayed in the presence of KCl, to calculate the K^+-activated portion of the ATPase activity. Since the ATPase activity assayed in the presence of NaCl and $MgCl_2$ did not differ from that assayed in the presence of $MgCl_2$ alone, this K^+-activated portion of the ATPase activity was referred as the $(Na^+ + K^+)$-activated ATPase activity in the text. The assays of P_i released from ATP were performed as described previously[10].

Assays for the binding of [^{3}H]ouabain by $(Na^+ + K^+)$-activated ATPase preparation were performed with 0.2 mg of enzyme protein in an incubation volume of 2.0 ml. The incubation mixture contained 5.0 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris–HCl buffer (pH 7.5) and various concentrations of [^{3}H]-ouabain. After a 5-min preincubation period at 37°, Tris–ATP was added to a final concentration of 5.0 mM. The incubation was performed for an additional 10-min period at 37°. The amount of [^{3}H]ouabain bound to the enzyme was estimated after centrifugation at 100 000 $\times$ *g* for 30 min as described previously[7].

Protein concentration was determined by the method of LOWRY *et al.*[11]. PCMB was freshly dissolved in a 50-mM Tris–HCl buffer (pH 8.5) to a 1-mM concentration and diluted further with water. The experimental results were analyzed for significance by the Student's *t* test.

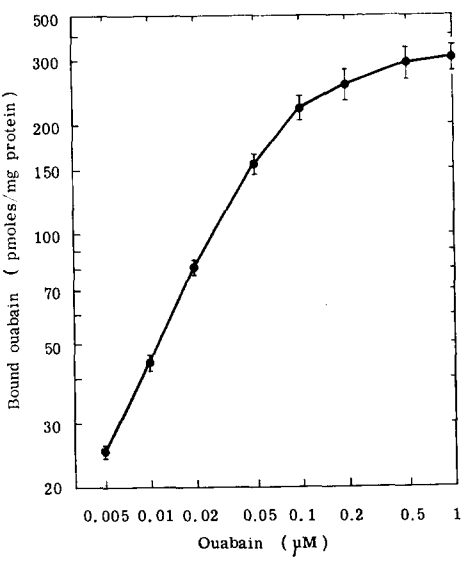

Fig. 1. Binding of [^{3}H]ouabain by $(Na^+ + K^+)$-activated ATPase preparation. [^{3}H]Ouabain was incubated with $(Na^+ + K^+)$-activated ATPase preparation (0.2 mg of protein) in 2.0 ml of a solution containing 100 mM NaCl, 5.0 mM $MgCl_2$ and 5.0 mM Tris–ATP for 30 min at 37°. After centrifugation at 100000 $\times$ *g* for 30 min, the amount of [^{3}H]ouabain in the sediment was assayed. Mean of 4 experiments. Vertical line indicates S.E.

RESULTS

Binding of [^{3}H]*ouabain by* ($Na^+ + K^+$)-*activated ATPase preparation*

The ($Na^+ + K^+$)-activated ATPase preparations used in the present studies had specific activities of approximately 250 μmoles of P_i released from ATP per mg protein per h. ($Na^+ + K^+$)-independent, Mg^{2+}-dependent ATPase activity accounted for 8.9 $\pm$ 0.6% (mean $\pm$ S.E. of 8 preparations) of the total ATPase activity, assayed in the presence of Na^+, K^+ and Mg^{2+} by Method A for ATPase assay described under METHODS.

The binding of [^{3}H]ouabain by these enzyme preparations is shown in Fig. 1. It appears that, under these experimental conditions, one component of the binding mechanism saturated at ouabain concentrations of 0.1 to 0.2 μM. Below these concentrations, non-specific binding was relatively small. For example, in the presence of 0.1 μM ouabain, the amount of ouabain bound to the enzyme after an incubation in the presence of Na^+ and Mg^{2+} but in the absence of ATP was 4.23 $\pm$ 0.04 pmoles/mg protein (mean $\pm$ S.E. of 4 experiments). This value was 1.91% of the value observed after an incubation in the presence of Na^+, Mg^{2+} and ATP. Further increases in ouabain concentration resulted in a slower increase in the amount of bound ouabain.

TABLE I

BINDING OF [^{3}H]OUABAIN BY ($Na^+ + K^+$)-ACTIVATED ATPASE PREPARATION

See legend to Fig. 1. Mean $\pm$ S.E. of 4 experiments. S/M ratio was calculated assuming protein content of the 100000 $\times$ *g* pellet to be 0.172 mg/μl (see text).

[^{3}H]*Ouabain* (μM)	*Sediment* (*pmoles*/0.2 *mg protein*)	*Supernatant* (*pmoles*/2 *ml*)	*S/M ratio*
0.01	8.8 $\pm$ 0.3	11.1 $\pm$ 0.3	1360
0.02	16.3 $\pm$ 0.5	23.8 $\pm$ 0.6	1180

Table I shows the distribution of ouabain between the enzyme preparation and the medium after the incubation. More than 50% of added ouabain remained unbound in the medium at ouabain concentrations approximately one tenth of the saturating concentration for specific binding sites on the ($Na^+ + K^+$)-activated ATPase preparation.

In order to calculate the S/M ratios (the ratio between the concentration of ouabain in the 100 000 $\times$ *g* pellet and that in the medium after the incubation and centrifugation) the protein concentration of the pellet was determined in a separate series of experiments. ($Na^+ + K^+$)-activated ATPase preparation, containing 2.0 mg of protein, was suspended in 10 ml of a 50-mM Tris–HCl buffer (pH 7.5) and centrifuged at 100 000 $\times$ *g* for 30 min. A portion of the resulting pellet was weighed, homogenized and assayed for the protein content. Another portion of the pellet was estimated for specific weight using 10–40% sucrose solutions. The pellet had a specific weight of approximately 1.10. Using this value, protein concentration of the pellet was calculated to be 0.172 $\pm$ 0.036 mg/μl of the pellet (mean $\pm$ S.E. of 4 experiments). Ouabain concentration in the pellet calculated from these values was divided by that

in the supernatant solution to calculate the S/M ratios. Table I shows that more than a 1000-fold concentration of ouabain occurred during the 30-min incubation.

Difference in the observed effect of ouabain and PCMB on $(Na^+ + K^+)$*-activated ATPase activity by different assay procedures*

In Method A for ATPase assay described under METHODS, the formation of the ouabain–enzyme complex is minimal during the ATP-free preincubation period[2–7]. Therefore, the initial velocity of the "inhibited" reaction immediately after the addition of ATP is the same as the uninhibited reaction and the observed velocity of the inhibited reaction is the average of the decreasing reaction velocity. Thus, if the binding of ouabain by $(Na^+ + K^+)$-activated ATPase is a slow process, the observed inhibition of the $(Na^+ + K^+)$-activated ATPase activity should be smaller when assayed by Method A than by Method B. That such is the case is shown in Fig. 2. The inhibition of $(Na^+ + K^+)$-activated ATPase activity by 0.01 to 1.0 μM ouabain was significantly smaller when observed with Method A than with Method B. The concentration of ouabain to inhibit 50 % of the enzyme activity under the present experimental conditions were 0.52 and 0.12 μM by Methods A and B, respectively.

The inhibition of $(Na^+ + K^+)$-activated ATPase by PCMB, however, was greater when observed with Method A than with Method B (Fig. 3). It should be noted that the log concentration–response curves were much steeper with PCMB than with ouabain (Fig. 2). The shape of the curves were also different.

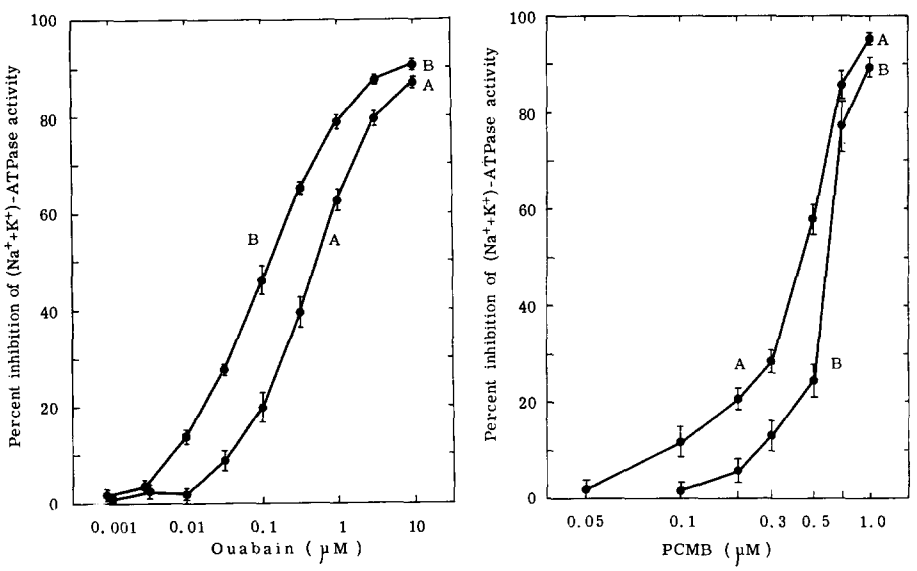

Fig. 2. Difference in the observed effects of ouabain on $(Na^+ + K^+)$-activated ATPase activity by different assay procedures. Log concentration–percent inhibition plot. Curve A, the ouabain–enzyme mixture was preincubated in the presence of NaCl, KCl and $MgCl_2$. ATPase reaction was started by the addition of a Tris–ATP solution (see Method A for ATPase assay under METHODS). Curve B, the ouabain–enzyme mixture was preincubated in the presence of NaCl, $MgCl_2$ and Tris–ATP. $(Na^+ + K^+)$-activated ATPase reaction was started by the addition of a KCl solution (see Method B for ATPase assay). Mean of 4 experiments. Vertical line indicates S.E.

Fig. 3. Difference in the observed effects of PCMB on $(Na^+ + K^+)$-activated ATPase activity by different assay procedures. See legend to Fig. 2. PCMB was substituted for ouabain.

The effect of K^+ *on the ouabain inhibition of* ($Na^+ + K^+$)*-activated ATPase activity*

The effect of K^+ to antagonize ouabain inhibition of ($Na^+ + K^+$)-activated ATPase activity was also markedly influenced by the assay procedures, as can be seen in Fig. 4, where $\alpha/1-\alpha \cdot [I]$ is plotted against the concentration of KCl according to the method of HUNTER AND DOWNS[12]. When the effect of KCl was estimated using Method A for ATPase assay, KCl strongly antagonized the ouabain inhibition. The inhibition of ($Na^+ + K^+$)-activated ATPase activity by 0.3 μM ouabain in the presence of 1 mM KCl was 57.0 $\pm$ 6.0 % (mean $\pm$ S.E. of 6 experiments). This value was significantly different from 22.8 $\pm$ 4.5 %, a value observed in the presence of 15 mM KCl ($P<0.01$). Although a similar pattern, a reduced inhibition of ($Na^+ + K^+$)-activated ATPase activity by ouabain at high KCl concentrations, was observed with Method B for ATPase assay, the effect of KCl was much reduced. With this method, the inhibition of the ($Na^+ + K^+$)-activated ATPase activity by 0.05 μM ouabain in the presence of 1 mM KCl was 48.6 $\pm$ 2.9 % (mean $\pm$ S.E. of 6 experiments), whereas that in the presence of 15 mM KCl was 37.8 $\pm$ 3.9 %. The P value for the difference of these two values was slightly greater than 0.05.

We have previously shown that the antagonism of the ouabain inhibition of the ($Na^+ + K^+$)-activated ATPase by K^+ was a competitive type at low K^+ concentrations[9]. In the Hunter–Downs plot, a competitive inhibition for substrate (KCl) is indicated when the line intercepts both x- and y-axis, whereas a non-competitive inhibition is indicated by a line which intercepts only the y-axis[13]. Therefore it may be assumed in this study that a competitive inhibition of ($Na^+ + K^+$)-

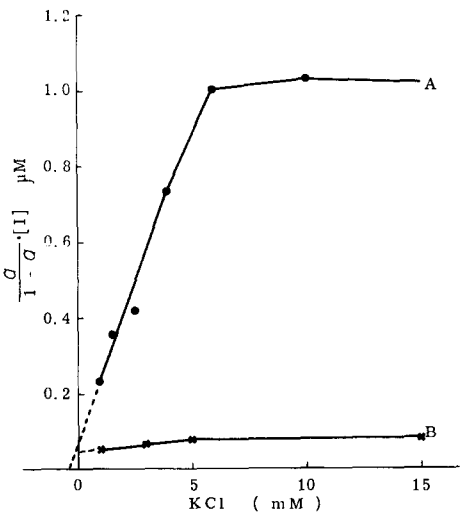

Fig. 4. Difference in the observed effects of K^+ on the ouabain inhibition of ($Na^+ + K^+$)-activated ATPase activity by different assay procedures. Hunter–Downs plot. Curve A, the ouabain–enzyme mixture was preincubated in the presence of NaCl, $MgCl_2$ and various concentrations of KCl. ATPase reaction was started by the addition of a Tris–ATP solution (Method A). Ouabain concentration was 0.3 μM. Curve B, the ouabain–enzyme mixture was preincubated in the presence of NaCl, $MgCl_2$ and Tris–ATP. ($Na^+ + K^+$)-activated ATPase reaction was started by the addition of KCl solutions of various concentrations (Method B). Ouabain concentration was 0.05 μM. Mean of 6 experiments. α: fractional activity of the enzyme preparation inhibited by ouabain. $[I]$: concentration of ouabain in μM.

activated ATPase by ouabain with respect to KCl ensued at KCl concentrations up to approximately 5 mM, and a non-competitive inhibition followed above that concentration.

Apparent K_i, which was estimated from the intercept of the line with the y-axis on the Hunter–Downs plot[13] (Fig. 4, Curve B), was 0.046 μM for the set of experimental data obtained by Method B.

Relationship between the amount of enzyme preparation and the inhibition of $(Na^+ + K^+)$-*activated ATPase activity by ouabain or PCMB*

The inhibitory effect of ouabain on $(Na^+ + K^+)$-activated ATPase activity was estimated in the presence of various amounts of the enzyme preparation in the incubation mixture (Fig. 5). Percent inhibition of the enzyme activity was similar when the amount of the enzyme preparation in the incubation mixture was varied. These results indicate that the ouabain-enzyme interaction is kinetically reversible[13] when the ATPase activity was assayed using Method B.

A similar plot for the effect of PCMB produced a different set of lines (Fig. 6). The line representing the PCMB-inhibited reaction was parallel to that representing the control reaction. This result indicates that the inhibition of $(Na^+ + K^+)$-activated ATPase by PCMB is kinetically irreversible under the present experimental conditions[13].

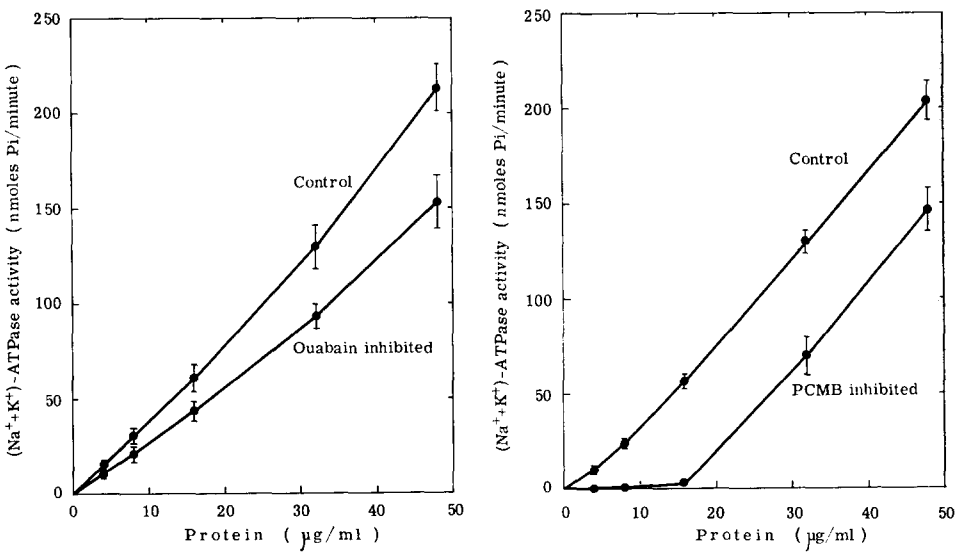

Fig. 5. Enzyme concentrations and the inhibition of $(Na^+ + K^+)$-activated ATPase by 50 nM ouabain. Ouabain was preincubated with various amounts of $(Na^+ + K^+)$-activated ATPase preparation in the presence of NaCl, $MgCl_2$ and Tris–ATP. $(Na^+ + K^+)$-activated ATPase reaction was started by the addition of a KCl solution (Method B for ATPase assay). Incubation time was 5 min (32 and 48 μg protein), 10 min (16 μg protein) or 20 min (4 and 8 μg protein). Mean of 4 experiments. Vertical line indicates the S.E.

Fig. 6. Enzyme concentrations and the inhibition of $(Na^+ + K^+)$-activated ATPase by 1 μM PCMB. PCMB was preincubated with various amounts of $(Na^+ + K^+)$-activated ATPase preparation in the presence of $MgCl_2$, NaCl and KCl. ATPase reaction was started by the addition of a Tris–ATP solution (Method A for ATPase assay). Incubation time was 5 to 20 min (see legend to Fig. 5). Mean of 4 experiments. Vertical line indicates S.E.

TABLE II

RE-BINDING OF OUABAIN RELEASED FROM THE OUABAIN–ENZYME COMPLEX

The ouabain–enzyme complex was prepared by incubating the enzyme preparation (1.6 mg of protein) with 25 ml of a solution containing 0.04 μM [^{3}H]ouabain, 80 mM NaCl, 2 mM $MgCl_2$, 2 mM Tris-ATP and 100 mM Tris–HCl buffer (pH 7.5) at 37° for 20 min and subsequently washing twice by centrifugation at 100000 × *g* for 30 min each and resuspension with 10 mM Tris–HCl buffer (pH 7.5) at 0°. The final suspension of the ouabain–enzyme complex containing 0.2 mg of protein in a total volume of 2.0 ml was incubated at 37° for 30 min, in the presence of 100 mM NaCl, 5.0 mM $MgCl_2$ and/or 5.0 mM Tris-ATP. After the incubation, the mixture was centrifuged at 100000 × *g* for 30 min at 0° and the amount of [^{3}H] ouabain in the sediment and supernatant was estimated. Mean ± S.E. of 3 experiments.

Addition to incubation mixture	*Enzyme bound ouabain (pmoles/0.2 mg protein)*
No incubation *	21.2 ± 0.5
Na^+ and Mg^{2+}	12.9 ± 0.3
Na^+, Mg^{2+} and ATP	17.3 ± 0.5

* The ouabain–enzyme mixture was stored at 0° for 30 min and then centrifuged. The dissociation of the ouabain–enzyme complex at 0° was negligible. Thus, this value represents the amount of bound ouabain at the beginning of the incubation.

Re-binding of ouabain released from the ouabain–enzyme complex

Table II shows the release of ouabain from the [^{3}H]ouabain–enzyme complex during a 30-min incubation period at 37°. In the presence of Na^+, Mg^{2+} and ATP, 3.9 pmoles of ouabain were released from the ouabain–enzyme complex containing 0.2 mg of protein. In the absence of ATP, 8.3 pmoles were released under the similar experimental conditions. It is reasonable to assume that the difference between these two values, 4.4 pmoles per 0.2 mg protein, were re-bound to the enzyme.

DISCUSSION

In a previous paper[7], we have demonstrated that the binding of ouabain by ($Na^+ + K^+$)-activated ATPase preparation is a slow process requiring more than 10 min to be completed at 37° in the presence of Na^+, Mg^{2+} and ATP. SIEGEL *et al.*[14], on the contrary, had claimed that a similar reaction was completed within 1 min at 24° and that further incubation failed to increase the amount of enzyme-bound ouabain. SCHWARTZ *et al.*[5] also employed a 3-min incubation period to estimate the ouabain binding, apparently assuming that the binding was a fast reaction. Although the contrasting results could be attributed to the enzyme preparations of different origin, the common finding is that the inhibition of ($Na^+ + K^+$)-activated ATPase activity by ouabain progresses over several minutes[5,15]. Thus, the "specific" binding of ouabain to the ($Na^+ + K^+$)-activated ATPase molecule must be a slow process. In a recent paper, ALLEN *et al.*[16] observed that such binding is in fact a time-dependent, slow process.

The slow rate of the binding is not due to the progressive depletion of free ouabain in the medium since, in the present study, more than 50% of ouabain was found unbound in the medium at the end of a 30-min incubation even at the lowest ouabain concentration studied.

It is apparent from the present data that in most of the studies concerning ouabain inhibition of the $(Na^+ + K^+)$-activated ATPase, kinetic values were over-estimated. When the ouabain–enzyme complex was formed during a preincubation period in the presence of Na^+, Mg^{2+} and ATP and the velocity of the inhibited reaction was estimated by the addition of KCl, the concentration of ouabain to inhibit 50% of the enzyme activity was 23.7% of that observed with the conventional method.

One of the disadvantages in preincubating the inhibitor–enzyme mixture in the presence of Na^+, Mg^{2+} and ATP is that a significant amount of P_i is released during such preincubation periods. Although it was not possible to employ long preincubation periods, the binding reaction was 96% completed at 10 min[7]. This method for ATPase assay also resulted in a lower inhibition of the enzyme activity by PCMB than the conventional method. This is probably due to the protective effect of ATP against SH-blocking reagents[17]. Thus, one should be careful in selecting the conditions for the inhibition studies involving an allosteric enzyme, especially when the inhibitior may bind only to a particular configuration of the allosteric transitions[3,7,16].

The effect of K^+ to antagonize the inhibition of $(Na^+ + K^+)$-activated ATPase by cardiac glycosides[3,8,18–27] also appears to be over-estimated. The reported effect of K^+ is, in fact, the effect on the average of the binding velocity and the inhibited reaction velocity rather than on the inhibited reaction velocity alone, since the results obtained in those studies are greatly influenced by the velocity of the binding.

K^+, however, was observed to antagonize the ouabain inhibition of $(Na^+ + K^+)$-activated ATPase activity (Fig. 4, Curve B). This finding that K^+ seems to shift the equilibrium of the ouabain–enzyme reaction to some extent is consistent with the observed reversibility of the ouabain–enzyme reaction (Fig. 5), since no such effect may be found in case of an irreversible inhibition. It should be noted that K^+ reduces both the association and dissociation velocities for the ouabain–enzyme complex[7]. Thus, the effect of K^+ on the equilibrium point may be small.

The shape of the log concentration–response curves (Fig. 2) also indicated that the reaction is reversible. A concentration–response curve would be linear for an irreversible inhibitor whereas a log concentration–response curve would be steeper at higher inhibitor concentrations and the range of inhibitor concentration which produces 1 to 100% inhibition should be included in two cycles of logarithm. This result was observed with PCMB (Fig. 3). Ouabain, however, produced a distinctly different pattern (Fig. 2). Furthermore, 1.0 μM ouabain produced only 81% inhibition of the $(Na^+ + K^+)$-activated ATPase activity (Fig. 2, Curve B), whereas apparent saturation of the specific binding sites on the enzyme occurred at 0.1–0.2 μM (Fig. 1). This also indicates the reversible nature of the reaction since some equilibrium exists and the binding sites are not occupied 100% even at apparently saturating concentrations of ouabain.

Thus, although the ouabain–enzyme reaction was a slow reaction and overwhelmingly in favor of the ouabain–enzyme complex (Table I), it was essentially a reversible reaction if the reaction was brought near the equilibrium during the preincubation period. With the conventional method for ATPase assay, irreversible behavior may be observed since the reaction proceeds only in one direction during a relatively short incubation period.

Once the ouabain–enzyme complex has formed, ouabain is not easily dissociated from the complex and only about 39% of the bound glycoside was released in 30 min

even in the absence of ATP in the medium (Table II). In the presence of ATP, Na^+ and Mg^{2+}, more than 50% of the released ouabain appeared to be rebound to the enzyme within 30 min. Since K^+ antagonizes the ouabain inhibition of the ($Na^+ + K^+$)-activated ATPase, the study of the effect of K^+ on the re-binding of the released ouabain would have been relevant. This, however, could not be determined in the present study since K^+ also affects the rate of realese of ouabain from the ouabain–enzyme complex[7].

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