MOLECULAR PHARMACOLOGY, 10, 509–518

Effect of Potassium on the Conformational State of the Complex of Ouabain with Sodium- and Potassium-Dependent Adenosine Triphosphatase

TAI AKERA, THOMAS TOBIN, ALFRED GATTI, ING-SHIH SHIEH, AND THEODORE M. BRODY

Department of Pharmacology, Michigan State University, East Lansing, Michigan 48824

(Received October 31, 1973)

SUMMARY

AKERA, TAI, TOBIN, THOMAS, GATTI, ALFRED, SHIEH, ING-SHIH, AND BRODY, THEODORE M.: Effect of potassium on the conformational state of the complex of ouabain with sodium- and potassium-dependent adenosine triphosphatase. *Mol. Pharmacol.* **10,** 509–518 (1974).

The effects of K^+ on the ouabain–(Na^+ + K^+)-ATPase [Mg^{2+}-dependent, (Na^+ + K^+)-activated ATP phosphohydrolase, EC 3.6.1.3] complex were studied using partially purified rat brain enzyme preparations. The dissociation rate of the [^{3}H]ouabain-enzyme complex prepared with ATP, Na^+, and Mg^{2+} decreased sharply with temperature between 37° and 22° in the absence of KCl. Potassium stabilized the complex at 37°. The dissociation rate of the [^{3}H]ouabain-enzyme complex in the presence of KCl was temperature-insensitive. Thus the dissociation rates under these two conditions approached each other at low temperatures, and consequently a K^+ effect not observed below 17°. Phlorizin, which has been shown to increase the K^+ affinity of (Na^+ + K^+)-ATPase, enhanced the stabilizing effect of K^+ on the ouabain-enzyme complex. Ouabain-enzyme complexes formed with substrates such as *p*-nitrophenyl phosphate, acetyl phosphate, or carbamyl phosphate, in the presence of Na^+ and Mg^{2+}, were also stabilized by K^+, whereas those formed with Mg^{2+} and P_i weer not. The dissociation rates of [^{3}H]ouabain from the enzyme in the presence of K^+ were similar regardless of the phosphate ligand used to support the binding. The K^+-induced stabilization of the ouabain-enzyme complex formed with ATP, Na^+, and Mg^{2+} was reversible when K^+ was removed. Attempts to convert the ouabain-enzyme complex prepared in the presence of Mg^{2+} and P_i from a K^+-insensitive to a K^+-sensitive conformation were unsuccessful. Deoxycholic acid partially antagonized K^+ effects on the rates of [^{3}H]ouabain binding and dissociation in the presence of ATP, Na^+, and Mg^{2+}. It is concluded that K^+ stabilizes the ouabain-enzyme complex by altering its configuration and that this effect of K^+ is closely related to its effect on the native phospho-enzyme. The stabilization appeared to result from a reduced accessibility of the ouabain binding site on the enzyme. The ouabain-enzyme complex prepared in the presence of ATP, Na^+, and Mg^{2+} and treated with KCl was dissimilar to the ouabain-enzyme complex prepared with Mg^{2+} and P_i.

INTRODUCTION

(Na^+ + K^+)-ATPase [Mg^{2+}-dependent, (Na^+ + K^+)-activated ATP phosphohydrolase, EC 3.6.1.3] has been postulated to undergo a cycle of conformational transitions

This work was supported by Grants NIMH-12783-07 from the National Institute of Mental Health and HL 16052-01 from the National Heart and Lung Institute, and by the Michigan Heart Association. Part of this study was presented in preliminary form [(1973) *Pharmacologist*, 15, 224].

as it hydrolyzes ATP (1). Several schemes have been presented depicting the roles of substrates and activators, such as ATP, Na^+, Mg^{2+}, and K^+, in the induction of such conformational changes in the enzyme (1–7). These schemes assessed the role of K^+ as inducing dephosphorylation of the phospho-enzyme. Following dephosphorylation, it is proposed that the enzyme changes its configuration. However, it seems more reasonable that K^+, acting at an extracellular site, must produce a conformational change in the enzyme to accelerate the intracellular hydrolytic cleavage of the enzyme-phosphate bond. Such a scheme was proposed by Robinson (8), based upon K^+-stimulated phosphatase activity studies. Recently, Charnock *et al.* (9) postulated, but did not provide evidence, that K^+ induces a conformational change of the phospho-enzyme which in turn facilitates the release of P_i from the enzyme.

Nagai *et al.* (10, 11) have demonstrated that Na^+ and K^+ induce changes in the fluorescence of 1-anilino-8-naphthalenesulfonate bound to the enzyme protein. They made no attempt to separate the proposed conformational change from the dephosphorylation. Since it is postulated that both conformational changes and dephosphorylation take place very rapidly after the addition of K^+, it is difficult to study the time sequence of these reactions in native enzyme preparations. Thus whether K^+ acts on the phospho-enzyme to induce a conformational change or to facilitate the dephosphorylation is not known.

We have previously demonstrated that K^+ stabilizes the ouabain-$(Na^+ + K^+)$-ATPase complex formed in the presence of Na^+, Mg^{2+}, and ATP (12). The ouabain-enzyme complex formed in the presence of Mg^{2+} and P_i was stable without K^+, and its stability was unaffected by K^+. Since K^+ also reduced the rate of ouabain binding to $(Na^+ + K^+)$-ATPase, we proposed that K^+ induces a conformational transition of the enzyme in such a manner that the accessibility of ouabain binding sites is reduced. It also follows that the ouabain-enzyme complex formed with Mg^{2+} and P_i is insensitive to K^+ and therefore different from that formed with ATP, Na^+, and Mg^{2+}.

The purpose of this study was to investigate further the effect of K^+ on the ouabain-enzyme complex.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 200–300 g were used. Enzymes were prepared from brain microsomal fractions following deoxycholic acid and NaI treatment as described previously (13). Protein concentration was assayed by the method of Lowry *et al.* (14). The $(Na^+ + K^+)$-ATPase activity was estimated as described previously, by assaying the amount of P_i released from ATP during a 10-min incubation at 37°. The present enzyme preparations had specific $(Na^+ + K^+)$-ATPase activities of 3.4 μmoles of P_i released from ATP per milligram of protein per minute (mean of six preparations). Mg^{2+}-dependent ATPase activity, assayed in the absence of Na^+ and K^+, was approximately 5% of the total ATPase activity assayed in the presence of Na^+, K^+, and Mg^{2+}. The $(Na^+ + K^+)$-ATPase activity reported here is the difference between the total and Mg^{2+}-dependent ATPase activities and was completely inhibitable by ouabain.

The binding of (^{3}H)-ouabain by enzyme preparations was estimated at 37° by incubating the enzyme preparation (0.1 mg of protein per milliliter) with 0.1 μM [^{3}H]ouabain (New England Nuclear Corporation; specific activity, 13.2 Ci/mmole, diluted with 9 parts of carrier ouabain) in a mixture containing 10 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, and 50 mM Tris-HCl buffer (pH 7.5). The binding reaction was started after a 5-min preliminary incubation period by adding the enzyme preparation. Aliquots were taken at 1, 3, 5, and 10 min and assayed for bound [^{3}H]ouabain. For ligands other than ATP, the complexes were prepared by incubating the enzyme preparation and 0.1 μM [^{3}H]ouabain with 10 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl buffer (pH 7.5) in the presence of 5 mM *p*-nitrophenyl phosphate, acetyl phosphate, or carbamyl phosphate, or with 1 mM $MgCl_2$ and 10 mM

Tris-HCl buffer (pH 7.5) in the presence of 1 mM Tris-phosphate. After a 10-min reaction period at 37°, the binding of [^{3}H]ouabain was stopped by adding 0.1 mM unlabeled ouabain to the mixture.

When the dissociation of [^{3}H]ouabain from the enzyme was monitored, the enzyme concentration was 1 mg of protein per milliliter during the binding reaction. After a 10-min incubation, the mixture was diluted 10-fold with a solution containing nonradioactive ouabain (final concentration of ouabain, 0.1 mM). Since the (ATP, Na^+, and Mg^{2+})-supported binding of ouabain saturates at approximately 1 μM ouabain under these conditions (15), this procedure effectively prevented the further binding of [^{3}H]ouabain. When the dissociation reaction was monitored at temperatures other than 37°, 1.0 ml of the binding mixture was transferred to 9.0 ml of "stopping solution" (10 mM Tris-HCl buffer, pH 7.5, containing 0.11 mM unlabeled ouabain) previously incubated at various temperatures. Subsequent decay of bound [^{3}H]ouabain was monitored by taking 1.0-ml aliquots at 5-min intervals up to 30 min and assaying the amount of bound [^{3}H]ouabain. In some experiments the [^{3}H]ouabain-enzyme complex, free from ATP, Na^+, and Mg^{2+}, was obtained by centrifuging the incubation mixture for two 30-min periods at 100,000 × *g* and 0° after a 10-min binding period. The sediment was resuspended each time with 10 mM Tris-HCl buffer (pH 7.5). Since the ouabain-enzyme complex of rat brain enzyme is stable at 0°, the dissociation of the bound [^{3}H]ouabain was minimal during the centrifugation and resuspension. Dissociation of the complex was subsequently monitored at 37° as above.

Bound and unbound [^{3}H]ouabain were separated by filtering aliquots through Millipore filters (average pore size, 0.8 μm). The filtration of an aliquot and subsequent washing of the filter with 10 ml of 50 mM Tris-HCl buffer took less than 20 sec. The radioactivity trapped on the filter (bound ouabain) was assayed by liquid scintillation counting after dissolving the filter in ethylene glycol monomethyl ether. Counting efficiency (approximately 30%) was monitored by the external standard channel ratio method, calibrated with internal standards. The amount of bound [^{3}H]ouabain observed in the absence of ATP was less than 2% of that observed in the presence of ATP and was subtracted from the latter value to calculate the specific (ATP-dependent) binding reported in this paper.

Data were analyzed for statistical significance by Student's *t*-test. Chemicals were purchased from Sigma Chemical Company. Tris salts of acetyl phosphate and carbamyl phosphate were prepared with Amberlite CG-120.

RESULTS

Effects of phlorizin on binding and release of [^{3}H]ouabain by (Na^+ + K^+)-ATPase. Robinson (16) demonstrated that phlorizin increases the apparent affinity of (Na^+ + K^+)-ATPase toward K^+ and decreases it toward Na^+, and concluded that these actions of phlorizin are due to a heterotropic allosteric modification of the enzyme. In order to demonstrate that the effect of K^+ on the ouabain-enzyme interaction is not unique and is consistent with the effect of K^+ on native enzyme, the influence of phlorizin on K^+-induced changes in the rate of association and dissociation of [^{3}H]ouabain was studied.

Phlorizin (3 mM) alone failed to alter the rate of [^{3}H]ouabain binding to (Na^+ + K^+)-ATPase at 37° in the presence of ATP, Na^+, and Mg^{2+} (Fig. 1). The rate of [^{3}H]ouabain binding was markedly reduced by 1 mM K^+. Under the latter condition 3 mM phlorizin further reduced the rate of [^{3}H]ouabain binding. Thus phlorizin had no effect on (ATP, Na^+, and Mg^{2+})-supported [^{3}H]ouabain binding by itself, but markedly enhanced the effect of K^+.

In order to study the effect of phlorizin on K^+-induced stabilization of the ouabain-enzyme complex, the [^{3}H]ouabain-enzyme complex was prepared by incubating [^{3}H]ouabain with the enzyme preparation in the presence of ATP, Na^+, and Mg^{2+}. After ATP, Na^+, and Mg^{2+} had been removed by centrifugation and resuspension, the [^{3}H]ouabain-enzyme complex was allowed to dissociate at 37° in 10 mM Tris-HCl buffer (pH 7.5). The decay of the [^{3}H]ouabain-enzyme complex in either the presence or absence of K^+ or

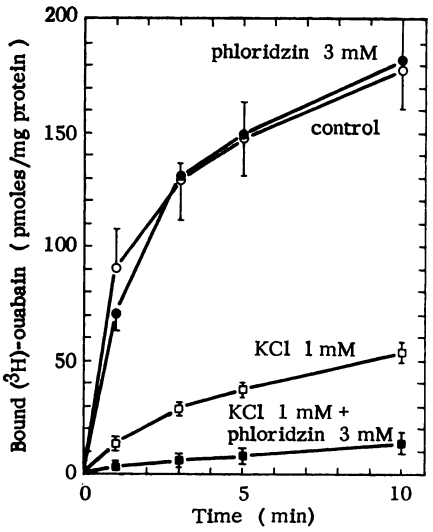

FIG. 1. *Effect of phlorizin on [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase*

Enzyme preparation (1.0 mg of protein in a total volume of 10 ml) and 0.1 μM [^{3}H]ouabain were incubated in the presence of 10 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, and 50 mM Tris-HCl buffer (pH 7.5) at 37° in the absence (circles) or presence (squares) of 1 mM KCl. Open symbols, no phlorizin present; solid symbols, 3 mM phlorizin added. At the indicated times after addition of the enzyme, aliquots were analyzed for bound [^{3}H]-ouabain. Values are means of four determinations with different enzyme preparations; vertical lines indicate standard errors.

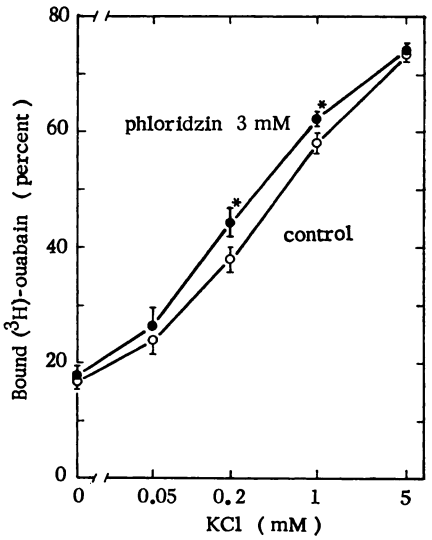

FIG. 2. *Effect of phlorizin on K^+-induced stabilization of ouabain-($Na^+ + K^+$)-ATPase complex*

Enzyme preparation (0.8 mg of protein in a total volume of 40 ml) and 0.01 μM [^{3}H]ouabain (13.2 Ci/mmole) were incubated in the presence of 20 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, and 50 mM Tris-HCl buffer (pH 7.5) at 37° for 10 min. The mixture was centrifuged twice at 100,000 × g for 30 min at 0°, and the resulting sediments were resuspended in 10 mM Tris-HCl buffer (pH 7.5). Dissociation of the [^{3}H]ouabain-enzyme complex (approximately 60 μg of protein in a total volume of 6 ml) was monitored at 37° in the presence of 10 mM Tris-HCl buffer (pH 7.5) and phlorizin or KCl as indicated. The amount of bound [^{3}H]ouabain remaining undissociated after a 10-min incubation was expressed as a percentage of that at the beginning of the incubation. Values are means of five determinations with different enzyme preparations; vertical lines indicate standard errors. Asterisks indicate values significantly different ($p < 0.01$) from corresponding control values by the paired t-test.

phlorizin followed first-order kinetics. Figure 2 shows the percentage of bound [^{3}H]ouabain remaining undissociated after a 10-min incubation period. Potassium (0.05–5 mM) reduced the rates of dissociation in a dose-dependent manner. Phlorizin (3 mM) shifted the curve to the left, indicating that K^+ was more effective in the presence of phlorizin than in its absence. Although the shift of the curve does not appear remarkable on a logarithmic K^+ concentration plot, the concentrations of K^+ needed to reduce dissociation rates 50% in the absence and presence of phlorizin were 0.30 and 0.16 mM, respectively. Thus 3 mM phlorizin significantly increased the affinity for K^+ ($p < 0.01$).

Temperature dependence of K^+ effect on dissociation rates of ouabain-enzyme complex. A number of investigators have demonstrated that the ($Na^+ + K^+$)-ATPase reaction is highly temperature-sensitive; hence the concept that the ($Na^+ + K^+$)-ATPase reaction proceeds by different mechanisms above and below the "critical" temperature has been developed (9, 17–19). Charnock *et al.* (9) postulated that below this critical temperature, reported to lie between 18° and 20°, the phospho-enzyme loses its ability to assume a K^+-sensitive conformation. Thus the [^{3}H]ouabain-enzyme complex was prepared at 37° and its dissociation rates were

monitored at various temperatures in an attempt to determine whether a similar critical temperature exists for the stabilizing effect of K^+ on the complex.

In these experiments the binding of [^{3}H]ouabain was stopped by the addition of nonradioactive ouabain to the binding mixture, and the rate of subsequent decay of the [^{3}H]ouabain-enzyme complex was monitored in the presence of 0.1 mM unlabeled ouabain. Under these conditions the decay of the ouabain-enzyme complex also followed first-order kinetics over the temperature range studied (12–37°). In Fig. 3 the first-order rate constants, calculated from the linear regression line fitted to six points obtained at times ranging from 5 to 30 min for each experiment, were plotted against the temperature which prevailed during the dissociation reactioe dissocian. Thtion rate shown in Fig. 3 is slower than that shown in Fig. 2 because of the presence of 1 mM Na^+, which stabilized the complex slightly (12). The rate of dissociation at low temperatures (12° and 17°) was essentially the same in the presence or absence of K^+. The rate of dissociation in the absence of K^+ increased markedly at high temperatures, while in the presence of K^+ it changed far less. Thus K^+ prevented the increase in rate of dissociation at higher temperatures: the conformation induced by K^+ at 37° resembles the conformation at 12° in the presence and absence of K^+.

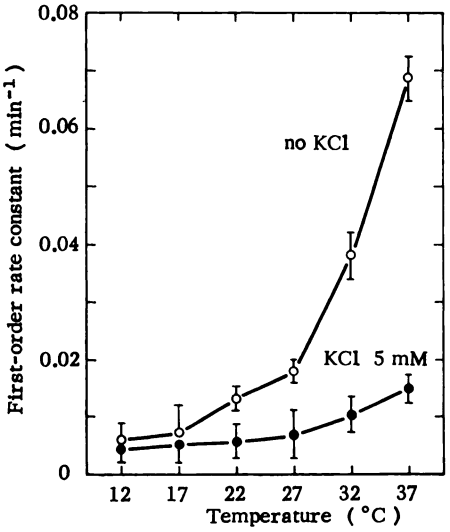

FIG. 3. *Temperature effects on dissociation rate constants of ouabain-ATPase complex prepared in the presence of ATP, Na^+, and Mg^{2+}*

The [^{3}H]ouabain-enzyme complex was prepared by incubating [^{3}H]ouabain with the enzyme preparation in the presence of ATP, Na^+, and Mg^{2+} at 37°. The binding of labeled ouabain was terminated by the addition of unlabeled ouabain, and subsequent decay of the [^{3}H]ouabain-enzyme complex was monitored at various temperatures in the absence (○) or presence (●) of 5 mM KCl. First-order rate constants for the dissociation were calculated for individual experiments. Values are means of five experiments on different enzyme preparations; vertical lines indicate standard errors.

Stability and K^+ sensitivity of [^{3}H]ouabain-enzyme complex formed with different phosphate ligands. It has been reported (12, 20) that the ouabain-enzyme complex formed in the presence of ATP, Na^+, and Mg^{2+} dissociates relatively rapidly at 37° whereas that formed in the presence of Mg^{2+} and P_i dissociates more slowly at the same temperature. The presence of K^+ during the dissociation reaction reduced the dissociation rate of the former complex to that of the latter complex but failed to further reduce the dissociation rate of the ouabain-enzyme complex formed with Mg^{2+} and P_i (12). Since phosphatase substrates, such as *p*-nitrophenyl phosphate, acetyl phosphate, and carbamyl phosphate, also support [^{3}H]-ouabain binding (21), it was of interest to compare the stability and K^+ sensitivity of the ouabain-enzyme complex formed with various phosphate ligands.

The decay of the [^{3}H]ouabain-enzyme complexes formed with the various ligands followed first-order kinetics. Figure 4 shows first-order rate constants for dissociation of the complexes. In the absence of K^+ the ouabain-enzyme complex formed in the presence of ATP, Na^+, and Mg^{2+} had the highest dissociation rate, and that formed in the presence of Mg^{2+} and P_i had the lowest dissociation rate. The ouabain-enzyme complexes formed with *p*-nitrophenyl phosphate, acetyl phosphate, or carbamyl phosphate had intermediate dissociation rates. The higher dissociation rate of complexes prepared with Na^+, Mg^{2+}, and ATP, *p*-nitro-

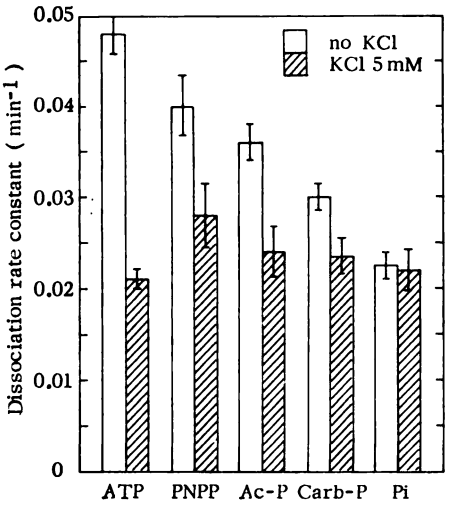

FIG. 4. *Dissociation rate constants of ouabain-ATPase complex prepared with different phosphate ligands*

The [^{3}H]ouabain-enzyme complexes were prepared with various phosphate ligands at 37°. The binding of labeled ouabain was terminated by the addition of nonradioactive ouabain, and subsequent decay of the [^{3}H]ouabain-enzyme complex was monitored at 37° in the absence (open bars) or presence (hatched bars) of 5 mM KCl. Dissociation rate constants were calculated from the slope of a linear regression line fitted to the semilogarithmic plot of 5-30-min values for each experiment. Values represent means of five experiments with different enzyme preparations; vertical lines indicate standard errors. PNPP, *p*-nitrophenyl phosphate; Ac-P, acetyl phosphate; Carb-P, carbamyl phosphate.

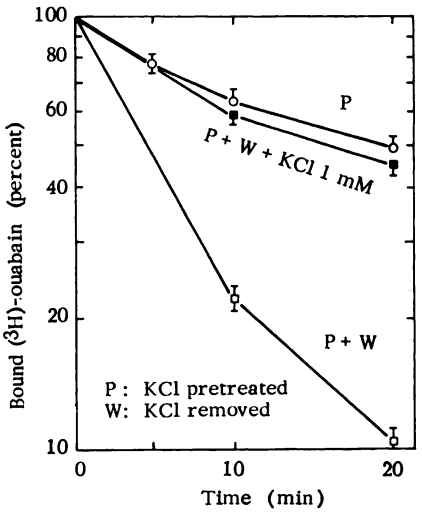

FIG. 5. *Reversal of K^+ effect on ouabain-ATPase complex prepared in the presence of ATP, Na^+, and Mg^{2+}*

The [^{3}H]ouabain-enzyme complex was prepared by incubating [^{3}H]ouabain with the enzyme preparation in the presence of ATP, Na^+, and Mg^{2+} at 37°. After a 10-min incubation period, 1 mM KCl was added to the mixture and incubated for an additional 2 min. ○ (potassium-treated), the dissociation of [^{3}H]ouabain from the enzyme was monitored after terminating labeled ouabain binding by the addition of nonradioactive ouabain, □ (potassium-treated and washed), the ouabain-enzyme complex was centrifuged and resuspended at 0° to remove KCl, and dissociation was monitored at 37° in the presence of 10 mM Tris-HCl buffer (pH 7.5); ■ (potassium-treated, washed, and 1.0 mM KCl added), the washed ouabain-enzyme complex was resuspended with 10 mM Tris-HCl buffer (pH 7.5) containing 1.0 mM KCl, and dissociation was monitored at 37°. Values are means of four experiments with different enzyme preparations; vertical lines indicate standard errors.

phenyl phosphate, acetyl phosphate, or carbamyl phosphate could be slowed by K^+ to the dissociation rate of the complex prepared with Mg^{2+} and P_i. Thus the rate of dissociation of the ouabain-enzyme complexes in the absence of K^+ was variable and dependent on the pathway by which they were formed, whereas that in the presence of K^+ was independent of the pathway.

Reversal of K^+ effect on ouabain-(Na^+ + K^+)-ATPase complex. In order to determine whether the K^+-induced stabilization of the ouabain-enzyme complex was reversible, the ouabain-enzyme complex was prepared, incubated with KCl at 37°, subsequently washed free from KCl by repeated centrifugation and resuspension at 0°, and allowed to dissociate at 37°. Figure 5 shows the results of such studies performed with the ouabain-enzyme complex prepared in the presence of ATP, Na^+, and Mg^{2+}. After the addition of KCl, the ouabain-enzyme complex dissociated at a slow rate. When KCl was removed, however, the KCl-treated complex dissociated rapidly. The half-life of the complex under this condition was approximately 5 min, which is typical of that of a ouabain-enzyme complex prepared with

ATP, Na^+, and Mg^{2+} and dissociating in the absence of K^+ or Na^+ (the rate is faster than that shown in Fig. 3 because of the absence of Na^+). The addition of KCl to the dissociation mixture caused a marked reduction of the dissociation rate of the complex previously treated with KCl and subsequently washed free of KCl. Thus the removal of KCl reversed the stabilizing effect of K^+ on the ouabain-enzyme complex prepared in the presence of ATP, Na^+, and Mg^{2+}. Since the resulting complex was now sensitive to KCl, it appeared that the removal of KCl resulted in a complete reversal of the K^+ effect.

When similar studies were performed with the ouabain-enzyme complex prepared in the presence of Mg^{2+} and P_i, KCl added at the end of the binding reaction failed to stabilize the complex further. Repeated centrifugation and resuspension also failed to produce a complex which was sensitive to KCl (data not shown, but see Table 1 of ref. 12).

Effect of deoxycholic acid on binding and release of [^{3}H]ouabain from $(Na^+ + K^+)$-ATPase. The effect of K^+ in reducing the accessibility of the ouabain binding sites was studied in the presence and absence of deoxycholic acid. The enzyme was exposed to deoxycholic acid (approximately 0.37 mM) during the process of preparation. This treatment at 0–5° increased the enzyme activity. However, at 37° the presence of 0.3 mM deoxycholic acid during the ATPase assay reduced $(Na^+ + K^+)$-ATPase activity 35.2 ± 2.4% (mean ± standard error of four experiments). The presence of 0.3 mM deoxycholic acid also reduced the rate of (ATP, Na^+, and Mg^{2+})-supported [^{3}H]ouabain binding at 37° in the absence of KCl (Fig. 6). KCl (5 mM) also reduced the rate of ouabain binding markedly. In the presence of KCl, however, the initial rate of ouabain binding was greater when 0.3 mM deoxycholic acid was added to the mixture. Thus deoxycholic acid reduced the effect of K^+ on the rate of (ATP, Na^+, and Mg^{2+})-supported [^{3}H]ouabain binding.

When the same [^{3}H]ouabain-ATPase complex was prepared in the absence of deoxycholic acid, and the rate of [^{3}H]ouabain dissociation was monitored at 37° in the absence of KCl, 0.3 mM deoxycholic acid failed to influence the dissociation rate (Fig. 7; open vs. solid circles). In the presence of 5 mM KCl, 0.3 mM deoxycholic acid enhanced the rate of [^{3}H]ouabain dissociation (Fig. 7; open vs. solid squares). Although the magnitude of the effect of deoxycholic acid in the presence of KCl was small, the difference was statistically significant ($p < 0.01$).

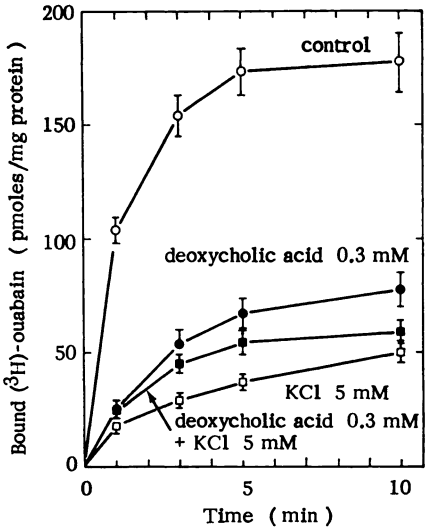

FIG. 6. *Effects of deoxycholic acid on (ATP, Na^+, and Mg^{2+})-supported binding of [^{3}H]ouabain to $(Na^+ + K^+)$-ATPase*

The ATP-dependent binding of [^{3}H]ouabain was monitored at 37°. KCl (5 mM) or deoxycholic acid (0.3 mM) was added to the reaction mixture as indicated. Values are means of four experiments with different enzyme preparations; vertical lines indicate standard errors.

Thus deoxycholic acid had a minimal effect on binding and dissociation of [^{3}H]ouabain in the absence of KCl. In the presence of KCl, however, deoxycholic acid increased the initial rate of (ATP, Na^+, and Mg^{2+})-supported [^{3}H]ouabain binding and also increased the rate of dissociation of the ouabain-enzyme complex, or partially antagonized the K^+ effect on ouabain binding and dissociation.

DISCUSSION

Several investigators (9, 22) have demonstrated that an Arrhenius plot of $(Na^+ +$

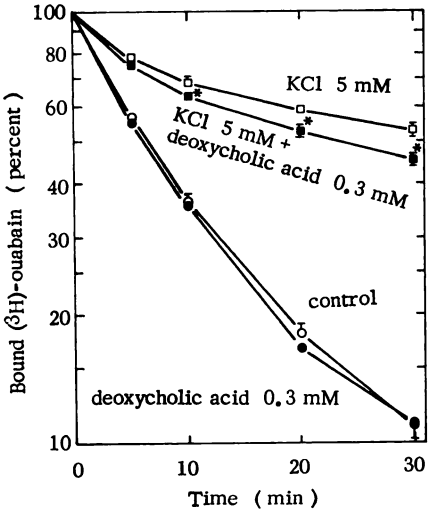

FIG. 7. *Effects of deoxycholic acid on dissociation rates of [³H]ouabain-ATPase complex prepared in the presence of ATP, Na^+, and Mg^{2+}*

The [³H]ouabain-ATPase complex was prepared by incubating [³H]-ouabain with the enzyme preparation in the presence of ATP, Na^+, and Mg^{2+} at 37°. Binding of labeled ouabain was terminated by the addition of nonradioactive ouabain, and subsequent decay of the [³H]ouabain-enzyme complex was monitored at 37° in the absence (circles) or presence (squares) of 5 mM KCl. Open symbols, no deoxycholic acid present; solid symbols, 0.3 mM deoxycholic acid present during the dissociation. Values are means of four experiments with different enzyme preparations; vertical lines indicate standard errors. Asterisks indicate values significantly different from those in the absence of deoxycholic acid ($p < 0.01$).

K^+)-ATPase activity reveals a break point between 18° and 20°. Charnock *et al.* (9) suggested that the enzyme cannot assume the K^+-sensitive conformation (E_2-P, Fig. 8) below this critical temperature. The present data indicate that a similar critical temperature exists for K^+ to stabilize the ouabain-ATPase complex prepared in the presence of ATP, Na^+, and Mg^{2+}. Since the dissociation rate of such a ouabain-enzyme complex was the same in the presence and absence of K^+ below 17° and the dissociation rate in the presence of K^+ was relatively unaffected by temperature change, it would appear that the ouabain-enzyme complex is in a K^+-insensitive form with respect to ouabain dissociation (OuE_x, Fig. 8) at low temperatures.

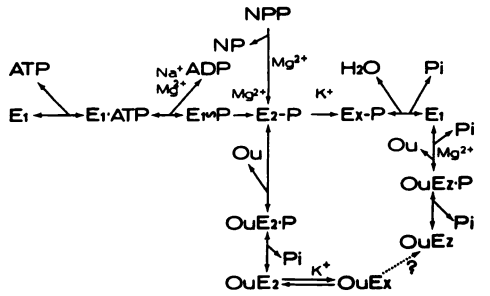

FIG. 8. *Reaction sequence for (Na^+ + K^+)-activated ATPase and its inhibition by ouabain*

E_1, native configuration of the enzyme; $E_1 \sim P$, transient high-energy form associated with E_1; E_1-P, phosphorylated configuration of the enzyme; E_x-P, transient potassium-phospho-enzyme complex; NPP, *p*-nitrophenyl phosphate; NP, *p*-nitrophenol; Ou, ouabain. Note that intermediary steps may exist.

Furthermore, phlorizin enhanced the effect of K^+ on both binding and dissociation of [³H]ouabain in the presence of Na^+, Mg^{2+}, and ATP. It has been demonstrated that in the presence of ATP, Na^+, and Mg^{2+} [³H]-ouabain binding occurs to the K^+-sensitive form (E_2-P, Fig. 8) of the phospho-enzyme (23, 24), and the effect of K^+ on the rate of ouabain binding is due essentially to its effect in decreasing the concentration of this particular form of the phospho-enzyme (5, 23). Additionally, like its effect on the phospho-enzyme, a K^+ effect on the ouabain-enzyme complex may be demonstrated by substituting either NH_4^+, Rb^+, Cs^+, or Tl^+ for K^+, but not Na^+ or Li (12). Thus it appears that the effect of K^+ on the ouabain-enzyme complex prepared in the presence of ATP, Na^+, and Mg^{2+} (OuE_2 → OuE_x, Fig. 8) is related to that of K^+ on the native phospho-enzyme.

The present data and the results reported previously by us (12, 24) and other workers (5, 6, 25, 26) are consistent with the scheme presented in Fig. 8 for the effect of K^+ on (Na^+ + K^+)-ATPase in the presence of ouabain. Since the extracellular K^+ concentration (5.6 mM) is significantly higher than the half-maximal concentration of K^+ required to induce a conformational change (1 mM) (12), OuE_x may be the predominant form of the inhibited enzyme *in vivo*. The re-

lationship between E_x and E_1 is not yet clear, although they may represent two different forms of the enzyme, as proposed by Robinson (8).

The [^{3}H]ouabain-enzyme complexes prepared with different phosphate ligands had different dissociation rates in the absence of K^+. In the presence of K^+, however, all the ouabain-enzyme complexes had the same slow dissociation rate regardless of the phosphate ligand used to support ouabain binding. Thus it appears that the ouabain-enzyme complex prepared with *p*-nitrophenyl phosphate, acetyl phosphate, or carbamyl phosphate is a mixture of at least two distinct forms of ouabain-enzyme complexes: a K^+-sensitive form (OuE_2), which has a high dissociation rate, and a K^+-insensitive form (OuE_x or OuE_z), which has a low dissociation rate. The proportion of each form of the ouabain-enzyme complex depends upon the particular phosphate ligand used to support ouabain binding. It is unlikely that the formation of a K^+-insensitive complex is supported by P_i released from various phosphate ligands during the incubation at 37° or contained as an impurity. No relationship between K^+ sensitivity and the concentration of P_i was observed.

Post *et al.* (23) reported that ouabain causes the accumulation of a phospho-enzyme which is insensitive to K^+ with respect to dephosphorylation. Nevertheless the ouabain-enzyme complex prepared with ATP, Na^+, and Mg^{2+} was sensitive to K^+ with respect to [^{3}H]ouabain dissociation rates. Furthermore, the half-life of the phospho-enzyme in the presence of ouabain is relatively short (23), whereas the ouabain-enzyme complex retains K^+ sensitivity with respect to the rate of ouabain dissociation even after several hours of centrifugation and resuspension at 0°. Thus it would appear that dephosphorylation and conformational changes are independent phenomena, although the conformational change may accelerate the rate of dephosphorylation in the absence of ouabain. These results strongly suggest that the binding of K^+ to $(Na^+ + K^+)$-ATPase causes a conformational transition independent of dephosphorylation.

The centrifugation and resuspension which removed K^+ from the ouabain-enzyme complex prepared in the presence of ATP, Na^+, and Mg^{2+} and treated with K^+ resulted in a reversal of the K^+-induced stabilization ($OuE_x \rightarrow OuE_2$, Fig. 8). Centrifugation and resuspension of the ouabain-enzyme complex prepared in the presence of Mg^{2+} and P_i, however, failed to produce a complex which rapidly dissociated or was K^+-sensitive. Thus it would appear that the ouabain-enzyme complex prepared with ATP, Na^+, and Mg^{2+} and treated with KCl (OuE_x) is not identical with the ouabain-enzyme complex prepared with Mg^{2+} and P_i (OuE_z), although these two forms of the ouabain-enzyme complex have similar dissociation rate constants. These two forms may represent two different conformational states of the enzyme. Alternatively, the ouabain binding sites on the enzyme may be different. Two different ouabain binding sites on each enzyme molecule have been proposed recently (27).

Deoxycholic acid partially antagonized the effect of K^+ on the rates of both (ATP, Na^+, and Mg^{2+})-supported [^{3}H]ouabain binding and the decay of the resulting ouabain-enzyme complex. These data are in agreement with the observations of Taniguchi and Iida (27) that phospholipase A treatment of the enzyme failed to alter the apparent dissociation constant of [^{3}H]ouabain in the presence of Na^+, Mg^{2+}, and ATP but increased it in the presence of Mg^{2+}, K^+, and ATP. However, these authors reported that phospholipase A treatment decreased the rate of [^{3}H]ouabain binding in the presence of Mg^{2+}, K^+, and ATP, whereas the present data indicate an increase in the rate of [^{3}H]ouabain binding with deoxycholic acid treatment in the presence of Na^+, K^+, Mg^{2+}, and ATP. Whether the discrepancy is due to the differences in treatment or to the presence or absence of Na^+ is not known. Our data suggest that a lipidic barrier regulates the accessibility of the ouabain binding sites on the $(Na^+ + K^+)$-ATPase or, alternatively, that a lipid moiety controls the affinity of K^+ for the enzyme.

CONCLUSIONS

From the above studies it is concluded that potassium stabilizes the ouabain-$(Na^+ + K^+)$-ATPase complex prepared with ATP, Na^+, and Mg^{2+} above 22° but fails to do so below 17°. The dissociation rate of the ouabain-enzyme complex in the presence of K^+ is minimally affected by temperature change; phlorizin enhances the stabilizing effect of K^+ on the ouabain-enzyme complex. The effect of K^+ on the ouabain-enzyme complex appears to be related to its effect on the native phospho-enzyme and to be due to a conformational change in the enzyme rather than to dephosphorylation. The conformational change in the ouabain-enzyme complex induced by K^+ or low temperature is reversible upon removal of K^+ or upon rewarming.

p-Nitrophenyl phosphate, acetyl phosphate, and carbamyl phosphate can induce K^+-sensitive forms of the ouabain-enzyme complex. In the presence of K^+, ouabain-enzyme complexes have the same slow dissociation rates regardless of the phosphate ligand used to support the ouabain binding. Finally, the ouabain-enzyme complex prepared with ATP, Na^+, and Mg^{2+} and treated with K^+ is not identical with the ouabain-enzyme complex prepared with P_i and Mg^{2+}.

ACKNOWLEDGMENT

The authors thank Mr. Roxy H.-M. So for expert technical assistance.

REFERENCES

1. Albers, R. W., Koval, G. J. & Siegel, G. J. (1968) *Mol. Pharmacol.*, **4**, 324–336.
2. Charnock, J. S., Rosenthal, A. S. & Post, R. L. (1963) *Aust. J. Exp. Biol. Med. Sci.*, **41**, 675–686.
3. Post, R. L., Sen, A. K. & Rosenthal, A. S. (1965) *J. Biol. Chem.* **240**, 1437–1445.
4. Hoffman, J. F. (1966) *Am. J. Med.*, **41**, 666–680.
5. Matsui, H. & Schwartz, A. (1968) *Biochim. Biophys. Acta*, **151**, 655–663.
6. Sen, A. K., Tobin, T. & Post, R. L. (1969) *J. Biol. Chem.* **244**, 6596–6604.
7. Formby, B. & Clausen, J. (1969) *Hoppe-Seyler's Z. Physiol. Chem.*, **350**, 973–980.
8. Robinson, J. D. (1970) *Arch. Biochem. Biophys.*, **139**, 164–171.
9. Charnock, J. S., Cook, D. A. & Opit, L. J. (1971) *Nat. New Biol.*, **233**, 171–192.
10. Nagai, K., Lindenmayer, G. E. & Schwartz, A. (1970) *Arch. Biochem. Biophys.*, **139**, 252–254.
11. Nagai, K., Lindenmayer, G. E. & Schwartz, A. (1972) *Arch. Biochem. Biophys.*, **152**, 329–338.
12. Akera, T. & Brody, T. M. (1971) *J. Pharmacol. Exp. Ther.*, **176**, 545–557.
13. Akera, T. & Brody, T. M. (1969) *Mol. Pharmacol.*, **5**, 605–614.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
15. Akera, T. (1971) *Biochim. Biophys. Acta*, **249**, 53–62.
16. Robinson, J. D. (1969) *Mol. Pharmacol.*, **5**, 584–592.
17. Ahmed, K. & Judah, J. D. (1965) *Can. J. Biochem.*, **43**, 877–880.
18. Swanson, P. D. (1966) *J. Neurochem.*, **13**, 229–236.
19. Samson, F. E., Jr. & Quinn, D. J. (1967) *J. Neurochem.*, **14**, 421–427.
20. Allen, J. C., Harris, R. A. & Schwartz, A. (1971) *J. Mol. Cell. Cardiol.*, **3**, 297–300.
21. Siegel, G. J. & Josephson, J. (1972) *Eur. J. Biochem.*, **25**, 323–335.
22. Taniguchi, K. & Iida, S. (1972) *Biochim. Biophys. Acta*, **274**, 536–541.
23. Post, R. L., Kume, S., Tobin, T., Orcutt, B. & Sen, A. K. (1969) *J. Gen. Physiol.*, **54**, 306S–326S.
24. Tobin, T., Akera, T., Baskin, S. I. & Brody, T. M. (1973) *Mol. Pharmacol.*, **9**, 336–349.
25. Schwartz, A., Matsui, H. & Laughter, A. H. (1968) *Science*, **160**, 323–325.
26. Tobin, T. & Sen, A. K. (1970) *Biochim. Biophys. Acta*, **198**, 120–131.
27. Taniguchi, K. & Iida, S. (1973) *Mol. Pharmacol.*, **9**, 350–359.