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INHIBITION OF SODIUM- AND POTASSIUM-DEPENDENT ADENOSINE TRIPHOSPHATASE BY ETHACRYNIC ACID: LIGAND-INDUCED MODIFICATIONS*

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Abstract—Inhibition of phosphorylation and ATP hydrolyzing activity of $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) by ethacrynic acid was facilitated by Na^+ or Mg^{2+} plus inorganic phosphate or Na^+ plus Mg^{2+} and ATP. The inhibition of enzyme activity was antagonized by ATP, ADP and K^+. ATP with or without ouabain did not reverse the acceleration or retardation of the rate of inhibition of enzyme activity by ethacrynic acid in the presence of Na^+ or K^+. Ouabain did not significantly alter the rate of inhibition of ATP hydrolysis and phosphorylation by ethacrynic acid, indicating that the binding sites of ouabain and ethacrynic acid are probably different. Results show ligand-induced conformational changes in $(Na^+ + K^+)$-ATPase and give further support to the allosteric model for cation transport involving the formation of a phosphorylated intermediate.

ETHACRYNIC acid, which is a potent diuretic,[1,2] has been shown to inhibit $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) of the microsomal fraction of kidney cortex.[3–5] It inhibits Na^+ transport across several epithelial tissues such as hamster,[6] rat[7] and rabbit[8] small intestine and isolated toad urinary bladder.[9,10] Ethacrynic acid also inhibits Na^+ efflux from human erythrocytes.[11]

Recently we studied the mechanism of inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid.[12] Two different modes for inhibition of $(Na^+ + K^+)$-ATPase were identified. First, a marked stabilization of the phosphorylated intermediate formed in the presence of Na^+, Mg^{2+} and ATP was observed. This resulted in decreased susceptibility toward K^+-induced dephosphorylation. Second, upon prolonged preincubation of a microsomal preparation with ethacrynic acid or by using a higher concentration of the drug, it was possible to inhibit the formation of the phosphorylated intermediate.[5,12] There was a good parallelism between the inhibition of phosphorylation and ATP hydrolysis when the latter determination was carried out in a medium containing Na^+ and K^+ in a ratio of 4.

The enzyme $(Na^+ + K^+)$-ATPase is thought to exhibit allosteric interaction as derived from kinetic analysis.[13–17] Further supporting evidence was obtained from ouabain binding studies.[18,19] It has been shown that, although Na^+ and ATP alone inhibit ouabain interaction with the enzyme system,[18] a combination of these two

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ligands with Mg^{2+} greatly accelerates [^{3}H]-ouabain binding to ($Na^+ + K^+$)-ATPase.[19–23] Therefore it was concluded that each ligand, either alone or in combination, stabilized different conformations of ($Na^+ + K^+$)-ATPase and thus altered reactivity of the enzyme to a particular inhibitor. Little information is available regarding ligand-induced alteration of the reactivity of ($Na^+ + K^+$)-ATPase to sulfhydryl inhibitors other than *N*-ethylmaleimide (NEM).[24] Skou and Hilberg[24] reported that ATP facilitated NEM-induced inhibition of ($Na^+ + K^+$)-ATPase in the presence of K^+ ion, but that the rate of inhibition was markedly decreased when K^+ ion was replaced by Na^+ ion. These results led Skou[24–26] to propose a model for transport of monovalent cations which rejected the functional significance of the phosphorylated intermediate.

In this communication we have examined the inhibition of phosphorylation of ($Na^+ + K^+$)-ATPase by ethacrynic acid and modification of this inhibition by ligands. Our results are in agreement with the concept of allosteric interactions in ($Na^+ + K^+$)-ATPase. Further, we find support for the existence of two major conformations of the transport enzyme system. Both these forms appear to have high reactivity to ethacrynic acid. Lastly, we present some evidence which makes the proposed model[24–26] for cation transport not involving the phosphorylated intermediate less likely.

MATERIALS AND METHODS

Materials

The microsomal ($Na^+ + K^+$)-ATPase from guinea-pig kidney and [γ-^{32}P]ATP were prepared as described by Post and Sen.[27, 28] [^{32}P]-P_i was obtained from Tracerlab, Waltham, Mass. Ouabain and the disodium salts of ATP and ADP were the products of Sigma Chemical Company. The sodium salts of nucleotides were converted to tris salts by ion-exchange chromatography with Dowex 50-Tris. Ethacrynic acid was a gift from Dr. John E. Baer, Merck Institute for Therapeutic Research, West Point, Pa.

Unless otherwise indicated, ethacrynic acid treatment was done as follows: Enzyme with protein concentration between 1·2 and 2·5 mg/ml was treated with a particular concentration of ethacrynic acid, with or without added ligands, for different intervals of time, at 37°. The drug–enzyme interaction was stopped by centrifugation at 45,900 *g* for 5 min at 0°. This was followed by washing the enzyme three times with imidazole glycylglycine buffer, pH 7$\pm$0·1, to remove any unbound drug and ligands. The enzyme was resuspended in imidazole glycylglycine buffer and was used to determine ATP hydrolysis and the incorporation of ^{32}P into the enzyme protein from [γ-^{32}P]ATP.

Phosphorylation with treated and untreated enzyme was carried out on ice as follows: A 1-ml volume of reaction mixture contained 0·4 μmole $MgCl_2$, 16 μmoles NaCl and 0·04 μmole [γ-^{32}P]ATP. The reaction was started with [γ-^{32}P]ATP and was stopped with 5% trichloroacetic acid at 10 sec. The amount of phosphorylated intermediate formed was determined by the previously described methods.[18,29] Total phosphate was estimated by a modification of the method of Bartlett[30] and protein by the method of Lowry *et al.*[31] The Na^+- and K^+-sensitive ATP hydrolyzing activity of the microsomal preparation was determined at 37° by the method of Post and Sen,[27] and this will be referred to as specific activity in the text.

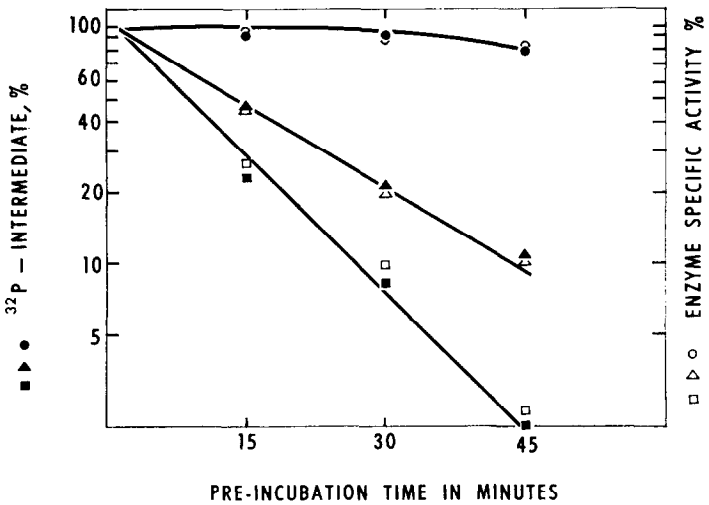

FIG. 1. Effect of sodium ion on inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid. The enzyme was preincubated with 10 mM ethacrynic acid either in the absence (○, ●) or presence of 100 mM (△, ▲) or 300 mM (□, ■) Na^+ at 37° for the time intervals indicated. Each sample was then washed three times and resuspended in buffer as described in Methods. The determination of specific activity and phosphorylation was made by the procedure outlined in Methods. The control enzyme incorporated 202·8 pmoles ^{32}P/mg protein and liberated 112·8 μmoles P_i/mg protein/hr.

RESULTS

Effect of Na^+ upon ethacrynic acid-induced inhibition of $(Na^+ + K^+)$-ATPase

Ethacrynic acid (10 mM) inhibited $(Na^+ + K^+)$-ATPase very slowly (Fig. 1). When 100 mM Na^+ was added to the preincubation medium, it markedly facilitated the rate of inhibition of enzyme activity by ethacrynic acid. There was further acceleration of the rate of inactivation when the concentration of Na^+ was raised to 300 mM (Fig. 1). In each case, the inhibition of phosphorylation matched the inhibition of ATP hydrolysis (Fig. 1). Since maximum phosphorylation[18,29] can be produced by 16 mM Na^+, we tested the effect of 8 mM Na^+ upon the rate of inhibition of phosphorylation and ATP hydrolyzing activity of $(Na^+ + K^+)$-ATPase by ethacrynic acid. Although the acceleration of inhibition of phosphorylation and ATP hydrolysis was much less pronounced, there was a definite increase in the rate of inactivation by ethacrynic acid in presence of 8 mM Na^+ (results not shown). This indicates that the inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid was facilitated in the presence of Na^+.

Reaction of ethacrynic acid to the ouabain-sensitive form of $(Na^+ + K^+)$-ATPase

A phosphorylated intermediate of $(Na^+ + K^+)$-ATPase is obtained in the presence of Na^+, Mg^{2+} and ATP.[29,32,33] Since the binding of ouabain to $(Na^+ + K^+)$-ATPase is markedly increased by phosphorylating the enzyme to E-P,[18–23] we tested the effect of ethacrynic acid upon the phosphorylated form of the enzyme. When ethacrynic acid was preincubated with enzyme, Na^+, Mg^{2+} and ATP, there was a marked increase in the rate of inhibition of phosphorylation and ATP hydrolysis with respect to a control that did not contain Na^+, Mg^{2+} and ATP (Fig. 2). This indicates that, like ouabain, reactivity of $(Na^+ + K^+)$-ATPase to ethacrynic acid was increased by the formation of phosphorylated intermediate.

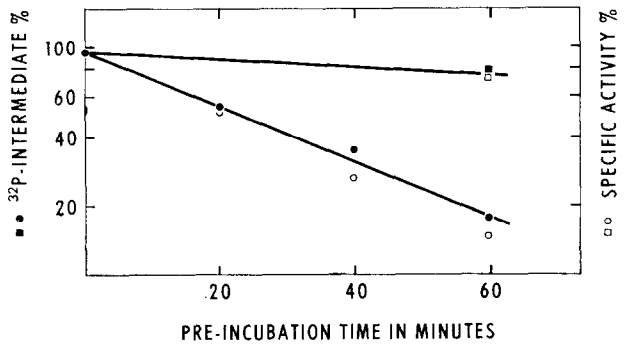

FIG. 2. Effect of formation of the phosphorylated intermediate upon the rate of inhibition of the enzyme by ethacrynic acid. Enzyme was preincubated with 10 mM ethacrynic acid either with 1 mM Mg^{2+}, 32 mM Na^+ and 4 mM ATP (○, ●) or with no added ligand (□, ■) for the indicated time periods. Determination of the amount of [^{32}P]-intermediate formed and specific activity of the washed enzyme was made as described under Methods. The specific activity of the control enzyme before pretreatment was 183·5 μmoles P_i/mg protein/hr and incorporated 253 pmoles ^{32}P/mg protein.

Although ouabain binding to ($Na^+ + K^+$)-ATPase is markedly increased by the formation of E-P, in the presence of Na^+ only, ouabain binding is decreased or completely abolished depending upon the concentration of Na^+ employed and the purity of the microsomal preparation.[18–23] High reactivity of ($Na^+ + K^+$)-ATPase to ethacrynic acid, both in the presence of Na^+ alone or after the formation of E-P, is therefore different from that to ouabain. Since in the presence of Na^+, Mg^{2+} and ATP the enzyme turns over very slowly and splits ATP, even when K^+ is omitted in the preincubation medium, it is possible that the high reactivity of E-P to ethacrynic acid may be due to the transiently formed Na^+-induced conformation. Ouabain binding to ($Na^+ + K^+$)-ATPase is also enhanced by Mg^{2+} and by Mg^{2+} plus inorganic phosphate.[18–23] Under such conditions there is no enzyme turnover. We therefore tested the effect of ethacrynic acid on ($Na^+ + K^+$)-ATPase in the presence of Mg^{2+}, with or without inorganic phosphate (Fig. 3). In the absence of added ligand, the rate of inhibition of phosphorylation and ATP hydrolysis by ethacrynic acid was slow, but was markedly enhanced in the presence of Mg^{2+} (Fig. 3). Mg^{2+} plus inorganic phosphate further increased the rate of inhibition (Fig. 3). Inorganic phosphate alone did not facilitate the rate of inhibition (results not shown). This confirms the high reactivity of the ouabain-sensitive form of ($Na^+ + K^+$)-ATPase to ethacrynic acid.

Reactivity of ethacrynic acid to ouabain-bound ($Na^+ + K^+$)-ATPase

Rapid reaction of ($Na^+ + K^+$)-ATPase to ethacrynic acid when the enzyme was in ouabain-susceptible conformation could mean that the binding sites of these two inhibitors may be the same or in the same region of the enzyme molecule. This possibility was tested by determining the rate of inhibition of the microsomes pre-incubated in the presence of ethacrynic acid, Mg^{2+} and inorganic phosphate, either with or without ouabain. Bound ouabain inhibits both phosphorylation and ATP hydrolysis.[18,29] Therefore, it was necessary to use a microsomal preparation to which ouabain binds reversibly and may be washed off. The reversibility of ouabain binding is controversial.[18,21,23] Tobin and Sen[23] reported that ouabain binding to guinea-

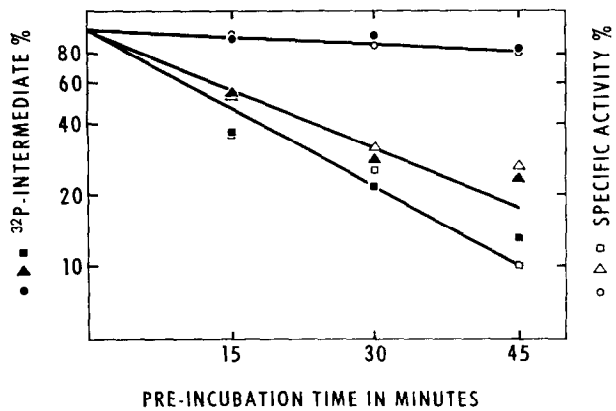

FIG. 3. Stimulation of inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid in the presence of magnesium and magnesium plus inorganic phosphate. Enzyme was preincubated with 15 mM ethacrynic acid either alone (○, ●) or with 4 mM Mg^{2+} (▽, ▼) or 4 mM Mg^{2+} plus 1 mM inorganic phosphate (□, ■) for the time intervals indicated. The rest of the procedure was the same as in Fig. 1. The specific activity of the control enzyme before pretreatment was 174·2 μmoles P_i/mg protein/hr and incorporated 264·8 pmoles ^{32}P/mg protein.

pig kidney $(Na^+ + K^+)$-ATPase was essentially reversible at 37°. Results in Table 1 confirm and extend their finding. By washing the ouabain-treated enzyme three times with buffer, it was possible to recover, almost completely, the original enzyme activity and [^{32}P] incorporation from [γ-^{32}P]ATP (Table 1). The possibility that ouabain may

TABLE 1. REVERSIBLE OUABAIN BINDING TO GUINEA-PIG KIDNEY $(Na^+ + K^+)$-ATPASE*

Enzyme	ATP hydrolysis (μmoles P_i/mg prot./hr)	Phosphorylation (pmoles ^{32}P/mg prot.)
Control	184	209
Ouabain-treated	196	220

* Enzyme containing 1·65 mg protein was preincubated in 2 ml of buffer with 8 μM Mg^{2+}, 2 μM inorganic phosphate and 0·5 μM ouabain at 37° for 45 min. Reaction was stopped by centrifugation at 45,900 *g* for 5 min at 0°. The supernatant was discarded and enzyme was resuspended in 10 ml of imidazole glycylglycine buffer and preincubated at 37° for 10 min to dissociate the enzyme-ouabain complex.[23] It was then centrifuged at 45,900 *g* for 10 min and the enzyme in the form of a pellet was again resuspended in 10 ml buffer. The washing procedure was repeated twice and each time, before centrifugation, the enzyme suspension was pre-incubated at 37° for 10 min. The determinations of specific activity and phosphorylation were carried out as described in Methods.

still remain bound to nonspecific sites after such procedures cannot be precluded. Rates of inhibition of ATP hydrolysis and phosphorylation by ethacrynic acid in the presence of Mg^{2+} plus inorganic phosphate either with or without ouabain treatment were not different (Fig. 4). This indicates that, although the same configuration is necessary, the binding sites of ethacrynic acid and ouabain are different. This confirms

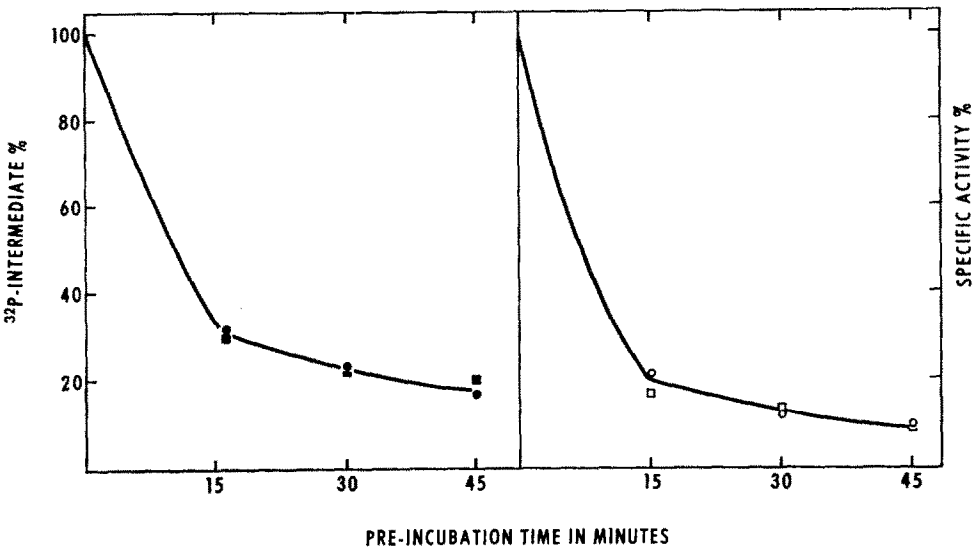

FIG. 4. Effect of ouabain on the facilitation produced by Mg^{2+} plus inorganic phosphate upon ethacrynic acid-induced inhibition of $(Na^+ + K^+)$-ATPase. Enzyme was preincubated with 15 mM ethacrynic acid plus 4 mM Mg^{2+} and 1 mM inorganic phosphate either with (○, ●) or without (□, ■) 0·25 mM ouabain for time intervals indicated. The treated enzymes were washed three times as described in Table 1 to dissociate the ouabain enzyme complex, and their specific activities (right panel) and amount of phosphorylation (left panel) were determined as described in Methods. The specific activity of the control enzyme before pretreatment was 183·5 μmoles P_i/mg protein/hr and incorporated 388 pmoles ^{32}P/mg protein.

our previous finding that enzymes with 50 per cent or less inhibition of ATP hydrolysis and phosphorylation by ethacrynic acid bind [^{3}H]-ouabain as well as control enzyme not treated with ethacrynic acid.[17]

Effects of nucleotides on the rate of ethacrynic acid-induced inhibition

Skou and Hilberg[24] found that ATP decreases the rate of inhibition of $(Na^+ + K^+)$-ATPase by *N*-ethylmaleimide. Similar results were obtained with ethacrynic acid (Fig. 5). ATP decreased the rate of inhibition of both phosphorylation and ATP hydrolysis. ADP also offers protection against NEM-induced inhibition of enzyme activity, but it is inferior to ATP.[24] In contrast, ADP offered better protection than ATP against ethacrynic acid (Fig. 5). Since ethacrynic acid inhibits phosphorylation, the protective effect of nucleotides may be due to the competition at the active site of the substrate. It is also possible that the decrease in the rate of inhibition of enzyme turnover may be due to the conformational changes produced by the nucleotides. Such an interpretation is supported by the acceleration of ethacrynic acid-induced inhibition of ATP-hydrolyzing activity by AMP (Fig. 6).

Effect of monovalent cation with or without ATP on the rate of inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid

In the presence of Na^+ the rate of inactivation of $(Na^+ + K^+)$-ATPase by NEM is high and this can be effectively reduced by adding ATP in the preincubation medium.[24,25] Since inhibition of $(Na + K^+)$-ATPase by ethacrynic acid is facilitated by

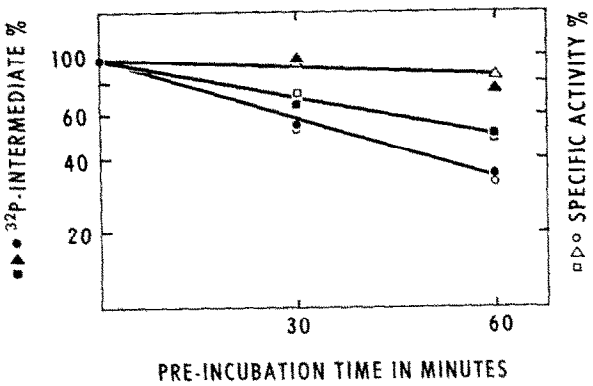

FIG. 5. Protection of ethacrynic acid-induced inhibition of $(Na^+ + K^+)$-ATPase by nucleotides. The preincubation medium contained enzyme, 20 mM ethacrynic acid either without any nucleotide (○, ●) or with 4 mM ATP (□, ■) or 4 mM ADP (△, ▲). Phosphorylation and specific activity of the washed and treated enzyme were determined as described in Methods. The specific activity of the control enzyme before pretreatment was 257 μmoles P_i/mg protein/hr and incorporated 370 pmoles ^{32}P/mg protein.

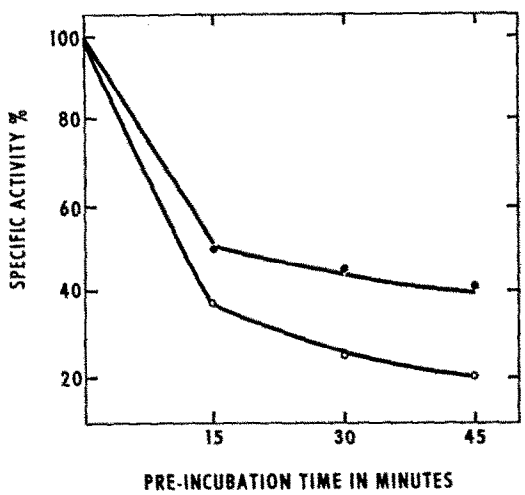

FIG. 6. Effect of AMP upon the inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid. The preincubation mixture contained enzyme plus 25 mM ethacrynic acid with (○) or without (●) 10 mM AMP for the indicated time intervals. The specific activities of the treated and washed enzymes were determined as described in Methods. The specific activity of the control enzyme before pretreatment was 215 μmoles P_i/mg protein/hr.

Na^+ and retarded by ATP, we decided to study the reactivity of the microsomes to ethacrynic acid in the presence of monovalent cations and its modification by the nucleotide ATP. Results shown in Fig. 7 were carried out with the same enzyme. As may be seen, Na^+, Na^+ + ATP, and Na^+ + ATP + ouabain increased the rate of inhibition as compared to ethacrynic acid alone (Fig. 7). In contrast, K^+ and K^+ + ATP + ouabain decreased the rate of inactivation (Fig. 7). Inability of K^+ + ATP to facilitate inhibition cannot be attributed to low concentration of the ion used. When the concentration of K^+ was raised to 20 mM, the rates of inhibition with or

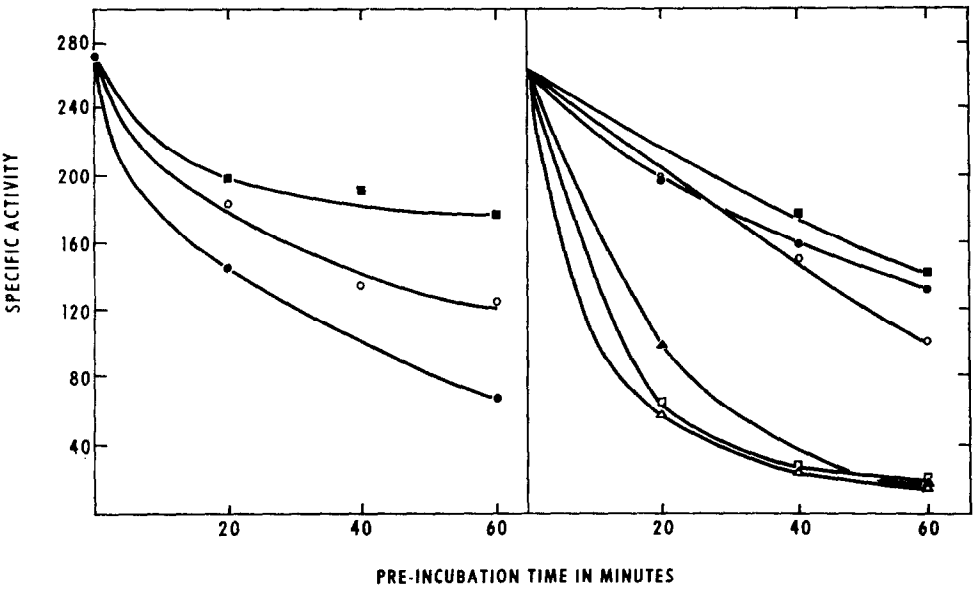

FIG. 7. Modification of ethacrynic acid-induced inhibition of $(Na^+ + K^+)$-ATPase by Na^+ and K^+ in the presence and absence of ATP. In the left panel, enzyme was preincubated with 15 mM ethacrynic acid either alone (●) or with 3 mM ATP (■) or 3 mM ATP plus 0·25 mM ouabain (○) for the indicated time intervals. Each enzyme was then washed three times, resuspended in buffer, and specific activity determined as described in the text. In the right panel, enzyme and 15 mM ethacrynic acid were preincubated with 2 mM K^+ (●) or 20 mM Na^+ (▲) or 2 mM K^+ plus 3 mM ATP (■) or 20 mM Na^+ plus 3 mM ATP (□) or 2 mM K^+, 3 mM ATP plus 0·25 mM ouabain (○) or 20 mM Na^+, 3 mM ATP plus 0·25 mM ouabain (△) for the indicated time periods and the specific activities (expressed as μmoles P_i/mg protein/hr) of the washed enzymes were determined for all the experimental points.

without ATP were essentially similar to that obtained with 2 mM K^+ (results not shown).

Requirement of Na^+ is specific for phosphorylation of $(Na^+ + K^+)$-ATPase.[29] Other monovalent cations like Rb^+, Cs^+ and NH^+ do not support the formation of the phosphorylated intermediate, but facilitate its breakdown.[29] We tested the effects of these monovalent cations upon ethacrynic acid-induced inhibition. All monovalent ions tested decreased the rate of inhibition like K^+ (Fig. 8). Thus only Na^+, which phosphorylates the enzyme, facilitated ethacrynic acid-induced inhibition, while all other monovalent cations tested which dephosphorylate $(Na^+ + K^+)$-ATPase decreased the rate of inhibition.

DISCUSSION

The relationship between diuresis and inhibition of $(Na^+ + K^+)$-ATPase by thiol-reacting diuretics is not established.[34] At least two objections have been raised: (1) after administration of chlormerodrin, enzyme isolated from a diuresing kidney of a dog was not found to be inhibited.[34] (2) Upon administration *in vivo*, concentration of ethacrynic acid in the microsomal fraction of a dog kidney was much lower than that required for 50 per cent inhibition of enzyme activity *in vitro*.[34]

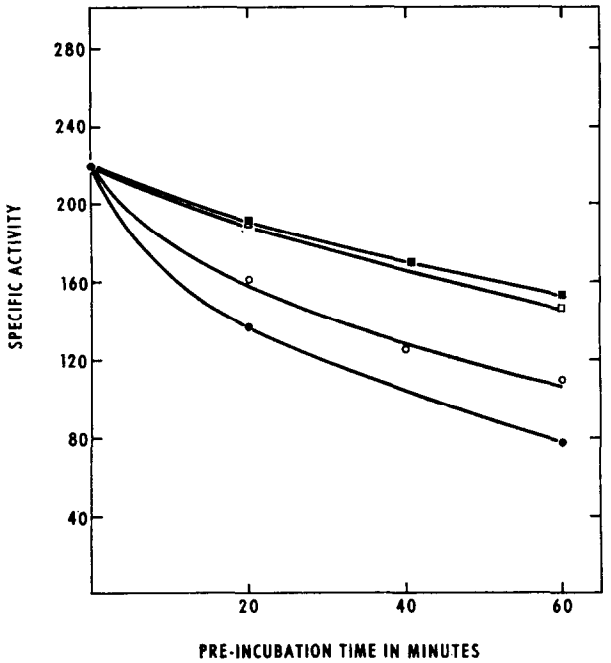

FIG. 8. Effect of monovalent cations on inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid. Enzyme was preincubated with 15 mM ethacrynic acid either alone (●) or with 5 mM NH_4^+ (■) or 8 mM Cs^+ (□) or 2 mM Rb^+ (○) for the indicated time intervals. The rest of the procedure was the same as in Fig. 7. The ordinate represents rate of ATP hydrolysis expressed as μmoles P_i/mg protein/hr.

Ethacrynic acid has been shown to stabilize[12] the phosphorylated intermediate of $(Na^+ + K^+)$-ATPase, which results in decreased apparent affinity for K^+. Since the concentration of K^+ is high in the assay medium *in vitro*, mild inhibition of $(Na^+ + K^+)$-ATPase as a result of stabilization of the phosphorylated intermediate may not be observed.[12] From the present study, comparison of the concentration of ethacrynic acid for 50 per cent inhibition of enzyme activity *in vitro* and of that reached in the microsomal fraction of a natriuretic kidney appears to be unwarranted. First, the amount of protein in a microsomal enzyme preparation determines the dose of ethacrynic acid required to inhibit $(Na^+ + K^+)$-ATPase.[12] Second, the effects of ligands have previously been ignored. Ethacrynic acid-induced inhibition was increased by Na^+ (Fig. 1), Mg^{2+} (Fig. 3), AMP (Fig. 7) and $Na^+ + Mg^{2+}$ and ATP (Fig. 2). Since all these ligands are present in the body, a smaller concentration of ethacrynic acid may be a more effective inhibitor of $(Na^+ + K^+)$-ATPase *in vivo* than what is required for 50 per cent inhibition of enzyme activity in a ligand-free system *in vitro*.

The enzyme $(Na^+ + K^+)$-ATPase has been claimed to exhibit allosteric interaction.[13–20] Two major conformations of the enzyme have been described.[21] Na^+ stabilizes an inward facing form of E_1 and ATP and Mg^{2+} are required in the presence of Na^+ to transform this conformation of the enzyme to the outward form. Under such conditions, the enzyme is phosphorylated to E_1-P, which subsequently changes to E_2-P, which is believed to be in communication with the outside of the cell.[21] In the absence of Na^+, the E_2 form may also be obtained with Mg^{2+} alone.[18,21,32]

These conclusions have been derived from studies of ouabain binding and trans-phosphorylation of NEM-treated and untreated enzymes.[18–21,32,35–38] The proposed model first described by Albers *et al.*[36] is as follows:

$$E_1 \underset{}{\overset{Na^+ + Mg^{2+} + ATP}{\rightleftharpoons}} E_1\text{-}P + ADP$$

$$E_1\text{-}P \xrightarrow{Mg^{2+}} E_2\text{-}P$$

$$E_2\text{-}P \xrightarrow{K^+} E_2 + P_i$$

$$E_2 \underset{+ Mg^{2+}}{\overset{-Mg^{2+}}{\rightleftharpoons}} E_1$$

Ethacrynic acid appears to have high reactivity to both these forms, since Na^+, Mg^{2+} and the phosphorylated intermediate facilitated ethacrynic acid-induced inhibition (Figs. 1–4). The reaction of $(Na^+ + K^+)$-ATPase to ethacrynic acid was slow in the presence of either ATP or ADP (Fig. 6) or in the absence of any added ligand (Figs. 1–3). A third major conformation, E′, has recently been suggested to be induced by ouabain.[32] Ethacrynic acid reacts to E′ as promptly as to E_2 (Fig. 4). This indicates that the sites of binding of ouabain and of ethacrynic acid are different.

Like NEM, ethacrynic acid is a sulfhydryl inhibitor and its inhibition of $(Na^+ + K^+)$-ATPase is increased by Na^+ (Fig 1) and decreased by ATP (Fig. 6). Therefore we expected that, as with NEM,[24,26] it would be possible to observe changes in affinity of cations induced by ATP by determining the rate of inhibition of enzyme activity by ethacrynic acid. Results showed that, with or without ATP, the rate of inhibition by Na^+ was always faster than with K^+ or $K^+ + ATP$. K^+ as well as $K^+ + ATP$ appeared to exhibit protection (Fig. 7). These results are not compatible with the model,[25,26] where, in the absence of phosphorylation, ATP may change the conformation of the cation transport system from an inside-facing to an outside-facing form.

Since the amount of Na^+-dependent [^{32}P]-incorporation is decreased markedly while $(Na^+ + K^+)$-ATPase activity remains unaffected by the addition of K^+ to a microsomal preparation at 0°, Skou[26] argued that the [^{32}P]-incorporation cannot be attributed to an intermediate in the $Na^+ + K^+$-sensitive ATPase reaction. From kinetic studies, Kanazawa *et al.*[39] concluded that $(Na^+ + K^+)$-ATPase can hydrolyze ATP by two routes: simple hydrolysis through the Michaelis complex, and hydrolysis via phosphorylated enzyme. They further suggested that the former is the main pathway for ATP hydrolysis at 0°, while the latter pathway predominantly operates in the presence of $Na^+ + K^+$ when the incubation temperature is raised to 37°. These conclusions were made on the basis of an assumption that the phosphorylation step is rate limiting in ATP hydrolysis and, since Kanazawa *et al.*[39] found that at 0° the rate of $(Na^+ + K^+)$-ATPase activity was always lower than the initial rate of [^{32}P]-incorporation, they concluded that the Na^+-dependent [^{32}P]-incorporation

observed at 0° could not be due to a functional intermediate in the enzyme reaction. However, in the present study, it was found that inhibition of Na^+-dependent phosphorylation by ethacrynic acid ran parallel with the inhibition of ATP hydrolysis. In our experiments, phosphorylation was carried out at $2\pm1°$, while ATP hydrolysis was determined at 37°. This indicates that Na^+-dependent [^{32}P]-incorporation in the kidney microsomes is due to a functional intermediate in the enzyme reaction, even at 0°. The apparent contradiction between the present finding and the kinetic analysis of Kanazawa *et al.*[39] is probably due to the assumption made by the latter group of workers regarding the rate-limiting step of ATP hydrolysis. More recently, Stone[40] determined the rate constants of different steps involved in the hydrolysis of ATP by $(Na^+ + K^+)$-ATPase. According to his calculations and those of others,[41] the rate-limiting step is not the Na^+-dependent phosphorylation but the transformation of E_1-P into E_2-P. If conversion of E_1-P to E_2-P is temperature-dependent and is also the rate-limiting step for the hydrolysis of ATP, then it is possible to explain both the inability of K^+ to stimulate $(Na^+ + K^+)$-ATPase activity at 0° and the disparity between the rate of Na^+-dependent ATPase activity at the steady state level and Na^+-dependent [^{32}P]-incorporation.

In conclusion, facilitation of the inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid in the presence of some ligands, either individually or in combination, may partly explain the disparity in the concentration of ethacrynic acid found in the microsomes of a diuresing kidney and that required for 50 per cent inhibition of enzyme activity *in vitro*. This strengthens the possibility that the natriuresis by ethacrynic acid may, at least in part, be mediated by the inhibition of $(Na^+ + K^+)$-ATPase. Furthermore, ample evidence is given to show ligand-induced alterations in the reactivity of the transport enzyme system to ethacrynic acid, implicating allosteric interactions of $(Na^+ + K^+)$-ATPase. Results provide some additional support to the hypothesis[18,21,32,35–38] that the Na^+ pump may exist in two major conformational states and that incorporation of ^{32}P into the microsomal protein from [γ-^{32}P]ATP occurs as a possible intermediate step in the translocation of Na^+.

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