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The Role of Phospholipids in the Binding of Ouabain to Sodium- and Potassium-Dependent Adenosine Triphosphatase

KAZUYA TANIGUCHI AND SHOICHI IIDA

Department of Pharmacology, School of Dentistry, Hokkaido University, Sapporo, Japan

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SUMMARY

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The effect of treatment with phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4) on the binding of ouabain to (Na^+ + K^+)-ATPase (ATP phospholydrolase, EC 3.6.1.3) as well as on (Na^+ + K^+)-ATPase activity was studied in the presence of various concentrations of ATP and other ligands. The time courses of [^{3}H]ouabain binding to ATPase in response to phospholipase A were the same in the presence of Mg^{2+} + ATP and of Mg^{2+} + Na^+ + ATP as under control conditions. However, after phospholipase treatment the initial rates of ouabain binding were reduced approximately to 40% of the control in the presence of Mg^{2+} + K^+ + ATP, Mg^{2+} + P_i, or Mg^{2+}. In the presence of both phosphatidylserine and phosphatidylinositol, the ouabain-binding rate increased significantly. The maximum amounts of ouabain binding in the presence of various ligands and 1–250 μM [^{3}H]ouabain were used to estimate binding capacities and dissociation constants for ouabain. The binding capacities of the ouabain-binding site showed no remarkable change as a consequence of phospholipase A treatment. ATPase preparations treated either with or without phospholipase A apparently had two different kinds of ouabain-binding sites in the presence of various physiological ligands. The values for the dissociation constant of the low-affinity site were approximately 10–100 times those of the high-affinity site in the presence of various ligands. The values for the dissociation constant of the high-affinity site in the presence of Mg^{2+} + ATP and Mg^{2+} + Na^+ + ATP were not changed by phospholiase A treatment. The apparent dissociation constant of the ouabain high-affinity site in the presence of Mg^{2+} + K^+ + ATP, Mg^{2+} + P_i, or Mg^{2+} was increased by the phospholipase A treatment. The ligands used reduced the binding affinities of both the high- and low-affinity sites of the control preparation as follows: Mg^{2+} + $P_i \approx Mg^{2+}$ + Na^+ + ATP $\approx Mg^{2+}$ + ATP, Mg^{2+} + Na^+ + K^+ + ATP, Mg^{2+} + K + ATP $\lesssim Mg^{2+}$. The number of high-affinity sites in the presence of Mg^{2+} + P_i appeared to be greater than in the presence of other ligands. The number of low-affinity sites increased 2–3-fold in the presence of K^+ as compared to its absence. The number of sites phosphorylated on the enzyme in the presence of Mg^{2+} + Na^+ + ATP was approximately the same as the number of high-affinity ouabain-binding sites. The V_{max} values with the high and low concentrations of ATP were reduced 70% by phospholipase A treatment. The apparent K_m at low concentrations of ATP was decreased 50%.

These results suggest that the enzyme preparation contains 1 mole each of high- and low-affinity ouabain-binding sites per mole of phosphorylated sites. The reactivity of the high-affinity site with ouabain in the presence of Mg^{2+} + K^+ + ATP, Mg^{2+} + P_i, and Mg^{2+}, but not Mg^{2+} + ATP or Mg^{2+} + Na^+ + ATP, was changed by phospholipase A treatment.

This indicates that there are at least two kinds of ouabain-binding conformations, one induced by $Mg^{2+} + K^+ + ATP$, $Mg^{2+} + P_i$, or Mg^{2+}, in which phospholipids play a role, and another, found in the presence of $Mg^{2+} + ATP$ or of $Mg^{2+} + Na^+ + ATP$, in which phospholipids do not play a role.

INTRODUCTION

Treatment of $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) with phospholipase A (phosphatide acetylhydrolase, EC 3.1.1.4) results in loss of ATPase activity. However, $(Na^+ + K^+)$-dependent, ouabain-sensitivie ATPase activity was restored by the addition of phosphatidylserine and phosphatidylinositol (1). The kinetics of [^{3}H]ouabain binding to $(Na^+ + K^+)$-ATPase in the presence of $Mg^{2+} + Na^+ + K^+ + ATP$ were nearly the same for both the control preparation and that treated with phospholipase A (2). Physiological ligands influenced the sensitivity of $(Na^+ + K^+)$-ATPase to ouabain (3). It was suggested that cardiac glycosides bind to $(Na^+ + K^+)$-ATPase with two different enzyme conformations (3–11). It seemed profitable to study further the effect of phospholipase treatment on the binding of ouabain to $(Na^+ + K^+)$-ATPase in order to clarify not only the mechanism of the ouabain effect but also the effect of phospholipids on $(Na^+ + K^+)$-ATPase. In this report we present a study of the effect of phospholipase A treatment on the initial rates and maximum amounts of ouabain binding to $(Na^+ + K^+)$-ATPase in the presence of various physiological ligands of the enzyme and on ATPase activities in the presence of high and very low concentrations of ATP (12–16).

MATERIALS AND METHODS

Enzyme preparation. The methods of partial purification of $(Na^+ + K^+)$-ATPase from ox brain microsomes (1) and phospholipase A treatment of the enzyme have been reported (17). $(Na^+ + K^+)$-dependent, ouabain-sensitive ATPase activities were approximately 13–20 and 60–70 μmoles of ATP per milligram of protein per hour for the treated and control preparations, respectively, under the standard conditions (17). Mg^{2+}-ATPase activities of both preparations were approximately 3 μmoles of ATP per milligram of protein per hour. The concentration of protein was determined by the method of Lowry *et al.* (18).

Determination of [^{3}H]ouabain binding to $(Na^+ + K^+)$-ATPase. In the standard assay procedure, the final concentrations in the incubation mixture were as follows: 5 mM $MgCl_2$, 40 mM Tris-acetate, 0.25 mM EDTA-Tris, 0.19 M sucrose, various concentrations of ouabain (1–250 μM), and 0.5 mg of enzyme protein per milliliter in a final volume of 0.05 ml (pH 6.1) at 37°. The concentrations of the other ligands, when present, were 140 mM sodium acetate, 14 mM KCl, 4 mM Tris-ATP, and 0.5 mM Tris-phosphate. The reaction media less [^{3}H]ouabain were first incubated for 3 min at 37°. The reaction was started by addition of [^{3}H]ouabain at 37°. At various time intervals the reaction was stopped by the addition of 3 ml of ice-cold unlabeled ouabain (pH 6.1 with 40 mM Tris-acetate) at a final concentration of 5 mM, and the reaction mixture was immediately passed through a membrane filter as described previously and counted (2) for ^{3}H. When the reaction mixture contained large amounts of phospholipids, the reaction was stopped by the addition of 6 ml of cold, unlabeled 5 mM ouabain (pH 5.3 with 40 mM Tris-acetate) and centrifuged at 164,000 × *g* for 3 hr at 1°. The precipitates were suspended with 1 ml of 0.1 N NaOH and counted. For each concentration of [^{3}H]ouabain used in the binding experiments, specific binding was measured as the difference between the absence and presence of 5 mM carrier. The background was always less than 5%. The binding capacities and the dissociation constant for ouabain were determined by plotting the maximum amount of ouabain binding in the presence of 1–250 μM [^{3}H] ouabain according to Scatchard (19) as described previously (16). The binding of ouabain to the enzyme in the presence of

some ligands was a slow process, requiring approximately 2 hr of incubation to reach a plateau, as shown in Fig. 2. We regarded the amount bound after 2 hr as maximal. From the values of maximal ouabain binding in each experiment, the absolute amounts of bound ouabain and free ouabain concentrations were calculated and the Scatchard plots were constructed (Fig. 3). High- and low-affinity binding are shown as circles and triangles, respectively. The intersection of the corresponding lines on the abscissae was taken as the number of binding sites, that of the high-affinity sites directly and that of the low-affinity sites as the difference between the two.

Assay of $(Na^+ + K^+)$*-ATPase.* ATPase activity was measured by means of difference spectra and the oxidation of NADH, employing a linked enzyme system in a Hitachi 124 double-beam spectrophotometer equipped with a constant-temperature cell holder at a wavelength of 340 nm. The hydrolysis of ATP was coupled to the oxidation of NADH using pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) as reported previously (16, 17). The advantages of this assay procedure are that ATP hydrolysis can be monitored continuously and inhibitory buildup of ADP is prevented. Both sample and reference cells contained 5 mM $MgCl_2$, 140 mM sodium acetate, 14 mM KCl, various concentrations of MgATP, 0.25 mM EDTA-Tris, 190 mM sucrose, 40 mM Tris-acetate, 1.5 mM phosphoenolpyruvate, 0.2 mM NADH, 0.05 mg/ml of pyruvate kinase, 0.025 mg/ml of lactate dehydrogenase, and ATPase protein and phospholipids as indicated in the figures, with or without 0.17 mM ouabain, in a final volume of 1.55 ml. The final pH was adjusted to 6.1 at 37°. Pyruvate kinase and lactate dehydrogenase activities were always maintained in at least a 1000-fold excess over ATPase activity, as described previously (17). The ATPase reaction was started by the addition of MgATP. After both cuvettes were set in the holder, approximately 10 min were required for equilibration to the indicated temperatures. The temperatures were maintained at 37° ± 0.1° with a Coolnics circulator.

Measurement of phosphorylated intermediate (EP). The *E*P level at the steady state (5 sec after the start of the reaction) was measured in the presence of 0.5 mg of ATPase protein per milliliter, 5 mM $MgCl_2$, 140 mM NaCl, 190 mM sucrose, 0.25 mM EDTA-Tris, 40 mM Tris-HCl (pH 7.4), and various concentrations of [γ-^{32}P]ATP at 37°. The reaction was started by the addition of [^{32}P]ATP (final volume, 0.1 ml) and terminated by the addition of 3 ml of ice-cold 5% perchloric acid containing 5 mM ATP and 5 mM H_3PO_4. The denatured enzyme suspensions were immediately filtered through a Millipore filter of 0.8-μ pore size and were washed and counted by the methods of Kanazawa *et al.* (15). To measure nonspecific labeling, the reaction mixture was the same as described above except for the omission of 5 mM $MgCl_2$ and 140 mM NaCl and the addition of 10 mM MgATP and 14 mM KCl. [γ-^{32}P]ATP was added to this mixture for 5 sec. The reaction was terminated as described above and counted.

Chemicals. [^{3}H]Ouabain (11.7 Ci/mmole) was obtained from New England Nuclear Corporation. Ouabain was obtained from Tokyo Kasei Company, Ltd., Tokyo. [γ-^{32}P]ATP ammonium salt (more than 10 Ci/mmole) was obtained from the Radiochemical Center, and was converted to the Tris salt by passage through an Amberlite 1R-120 column. The same procedure was used to convert Na_2ATP and K_2HPO_4 to Tris salts. Phosphatidylserine from ox brain and phosphatidylinositol from plants were obtained from Applied Science Laboratories. Phospholipid micelles were prepared as described previously (1). Pyruvate kinase from rabbit muscle and lactate dehydrogenase from pig muscle were obtained from Boehringer/Mannheim Corporation, Tokyo. The other chemicals were of reagent grade.

RESULTS

Effect of phospholipase A treatment on initial rate of ouabain binding. The initial phase of ouabain binding to the ATPase preparations with or without phospholipase A treatment was measured in the presence of various ligands and 1 μM [^{3}H]ouabain

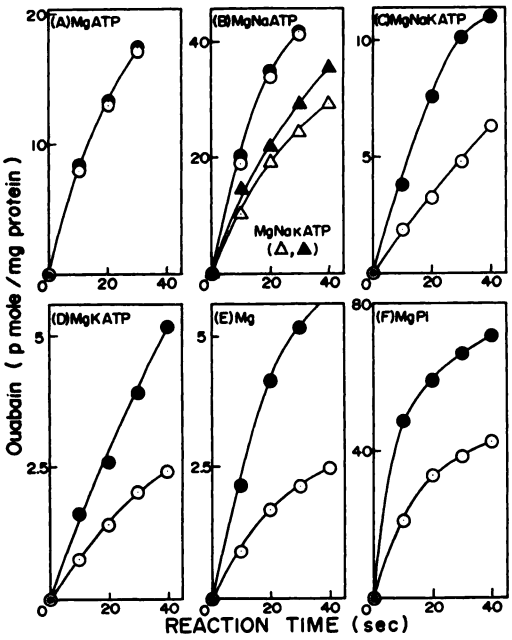

FIG. 1. *Initial phase of [³H]ouabain binding to $(Na^+ + K^+)$-ATPase*

Data were obtained by the Millipore filtration method described in the text. The binding of [³H]ouabain to the preparations (0.5 mg/ml) was measured in 1 μM [³H]ouabain, 5 mM $MgCl_2$, 40 mM Tris-acetate, 0.25 mM EDTA-Tris, and 0.19 M sucrose; the other physiological ligands, when present, were 140 mM sodium acetate, 14 mM KCl, 4 mM ATP, and 0.5 mM P_i at pH 6.1 and 37°. All experiments were performed in duplicate, and the variations between duplicate samples were within ±5%. The results shown are averages. ○ and △, phospholipase A-treated preparation; ● and ▲, control preparation. △ and ▲, the concentration of KCl was 1.4 mM and the concentrations of the other ligands were the same as above.

(Fig. 1). The time courses in the presence of Mg^{2+} + ATP and Mg^{2+} + Na^+ + ATP were approximately the same (Fig. 1A and B). The initial rates of ouabain binding to phospholipase A-treated preparations in the presence of the other ligands were slower than those of the control. Ouabain binding to treated ATPase after 10 sec of incubation was approximately 40% of the control amount in the presence of Mg^{2+} + Na^+ + K^+ + ATP (Fig. 1C), Mg^{2+} + K^+ + ATP (D), Mg^{2+} (E), and Mg^{2+} + H_3PO_4 (F). The difference in the time courses between control and treated preparations in the presence of Mg^{2+}, Na^+, and ATP and a reduced (1.4 mM) concentration of K^+ was less obvious (Fig. 1B, triangles). In our previous report (2) a similar phenomenon was observed in the presence of Mg^{2+}, K^+, ATP, and a higher concentration of Na^+. In the presence of Mg^{2+} + ATP the initial phase of binding was accelerated by Na^+ and inhibited by K^+. The effect of K^+ was antagonized by Na^+. In the absence of ATP inorganic phosphate accelerated the reaction remarkably. The amount of ouabain binding to the control preparation (after 10 sec of incubation with [³H]ouabain) in the presence of Mg^{2+} + H_3PO_4 was approximately twice that in the presence of Mg^{2+} + Na^+ + ATP.

Phospholipase A treatment caused a marked reduction of $(Na^+ + K^+)$-ATPase activity, but in the presence of the phospholipids the activity was increased approximately to the control value, as reported previously (1, 17).

The effects of phosphatidylserine and phosphatidylinositol on the initial rates of ouabain binding to phospholipase A-treated and control preparations in the presence of Mg^{2+}, Mg^{2+} + H_3PO_4, Mg^{2+} + K^+ + ATP, and Mg^{2+} + Na^+ + K^+ + ATP were studied (Table 1). Phospholipase A treatment reduced the initial rates of binding about 50%, but the addition of phospholipids restored the rates to approximately those of the controls in the presence of phospholipids. The control rates also were increased by the addition of phospholipids.

To measure the saturation level of ouabain binding, both ATPase preparations were incubated with 1 μM [³H]ouabain for longer periods of time in the presence of various physiological ligands. These time courses of binding are shown in Fig. 2. Phospholipase A treatment caused no remarkable change in either the rate or maximum amount of ouabain binding in the presence of Mg^{2+} + ATP or Mg^{2+} + Na^+ + ATP. The binding of ouabain to the enzyme in the presence of ligands such as Mg^{2+} + K^+ + ATP or Mg^{2+} proceeded slowly. By 2 hr of incubation, however,

TABLE 1

Effect of phospholipids on initial rate of ouabain binding

The amount of ouabain binding was measured after 10 sec of incubation with 1 μM [^{3}H]ouabain in the presence of 0.5 mg/ml of ATPase protein, 5 mM $MgCl_2$, 40 mM Tris-acetate, 0.19 M sucrose, 0.25 mM EDTA-Tris, and in the presence and absence of phosphatidylinositol (0.75 mg/ml) and phosphatidylserine (2.75 mg/ml) at pH 6.1 and 37°. The concentrations of the other ligands, when present, were 0.5 mM P_i, 14 mM KCl, 140 mM sodium acetate, and 4 mM ATP. All experiments were performed in duplicate, and the variations between duplicate samples were within ±5%. The results shown are averages.

Ligands	Amount of ouabain binding			
	Controls		Phospholipase A-treated	
	No phospholipids	With phospholipids	No phospholipids	With phospholipids
	pmoles/mg		*pmoles/mg*	
Mg^{2+}	2.2	4.5	0.9	4.5
$Mg^{2+} + H_2PO_4$	48.0	65.0	20.8	50.0
$Mg^{2+} + K^+ + ATP$	1.6	3.6	0.6	3.3
$Mg^{2+} + Na^+ + K^+ + ATP$	3.6	6.8	1.7	6.0

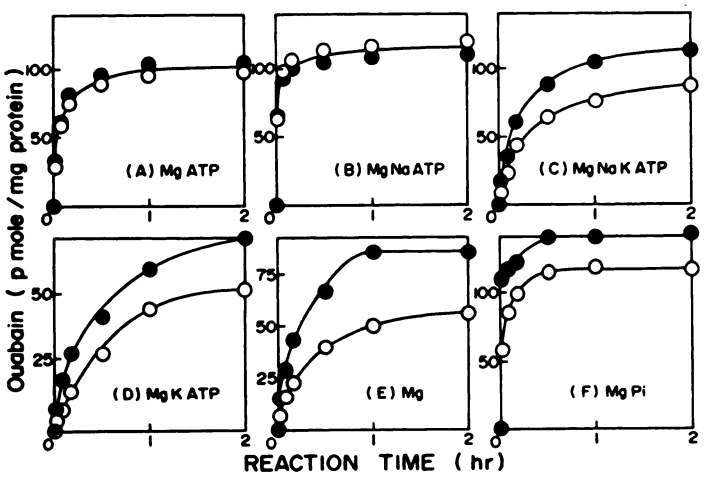

FIG. 2. *Time courses of* [^{3}H]*ouabain binding to* ($Na^+ + K^+$)*-ATPase*
Experimental conditions and symbols are described in the legend to Fig. 1.

binding nearly approached plateau levels. Phospholipase A treatment caused a marked reduction in the initial rate of ouabain binding in the presence of other ligands when K^+ and ATP were simultaneously included or omitted (Fig. 1). Under the same conditions the maximum amount of ouabain binding was less appreciably affected by phospholipase treatment.

Effect of phospholipase treatment on binding capacity and affinity for ouabain. The maximum amount of ouabain binding was studied in the presence of ligands and various concentrations of [^{3}H]ouabain (1–250 μM). The maximum amount of ouabain bound was considered to be attained after a 2-hr incubation with [^{3}H]ouabain. Scatchard plots of phospholipase A-treated and control ATPase are shown in Fig. 3. Since a large range of ouabain concentrations was employed, the plots were divided into two segments, represented as circles and triangles, with different ordinate scales, in order to examine accurately the effects of ligands and phospholipase treatment at all concentrations of ouabain. The difference in the slopes of the two segments was even more obvious when plotted on the same

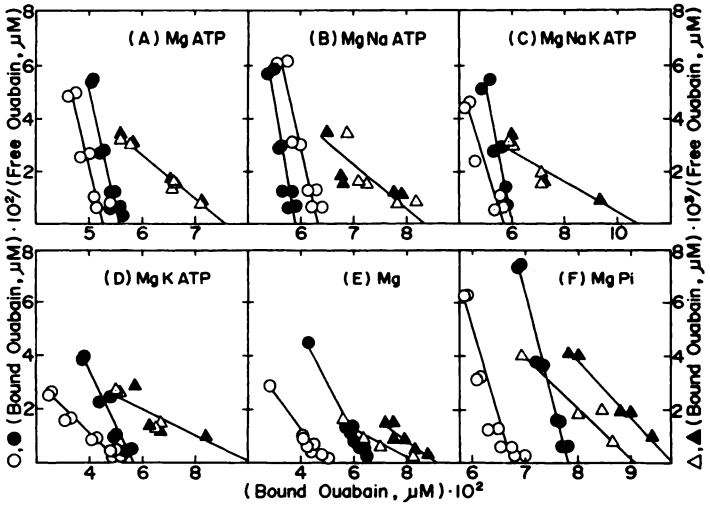

FIG. 3. *Scatchard plots of ouabain binding to (Na^+ + K^+)-ATPase*

The maximum amount of ouabain binding to the enzyme preparations (0.5 mg of protein/milliliter) was measured after 2 hr of incubation in the presence of various concentrations of [^{3}H]ouabain (1–250 μM) and various physiological ligands at pH 6.1 and 37° as described in Fig. 1. The concentration of KCl, when present, was 14 mM. From the values of the maximum amount of ouabain binding in each experiment, the absolute amounts of bound ouabain and free ouabain concentrations were calculated and the Scatchard plots were constructed. ○ and △, phospholipase A-treated preparation; ● and ▲, control preparation. The ordinate scale was expanded 10-fold in the case of △ and ▲. All experiments were performed in duplicate, and the variations between duplicate samples were within ±5%.

scale. From the slopes, the dissociation constants for ouabain were obtained, and from the intercepts on the abscissae, the binding capacities were obtained (Table 2).

Two kinds of ouabain-binding sites were apparent, with high and low affinity for ouabain. For both the high- and low-affinity sites the dissociation constants and binding capacities were compared to see what changes had resulted from phospholipase A treatment or the addition of ligands, or both. Neither dissociation constants nor binding capacities in the presence of Mg^{2+} + ATP or Mg^{2+} + Na + ATP were changed remarkably by phospholipase A treatment. The binding capacities were approximately 110–120 pmoles/mg of protein for the high-affinity sites and 40–50 pmoles/mg of protein for the low-affinity sites in the presence of Mg^{2+} + ATP and Mg^{2+} + Na^+ + ATP, respectively.

Dissociation constants of the high-affinity sites in the presence of Mg^{2+} + Na^+ + K^+ + ATP and Mg^{2+} + K^+ + ATP were increased approximately 2-fold by phospholipase treatment; this increment was apparently less in the presence of Mg^{2+} and Mg^{2+} + P_i. The number of sites was not changed significantly by the treatment. However, there was a slight increase in the number of sites in the presence of Mg^{2+} + P_i for both the treated and control preparations.

Dissociation constants of the low-affinity sites in the presence of various ligands seemed to be unaffected by the phospholipase A treatment. However, K^+ increased the number of sites to the same as the high-affinity sites in both the treated and control preparations. This interesting effect of K^+ was not observed with the high-affinity sites.

The values for the dissociation constants of the high-affinity sites in the presence of various physiological ligands were one to two orders of magnitude smaller than those of the low-affinity sites. The binding affinities for both sites in the presence of the various ligands followed the order: Mg^{2+} + P_i ≈ Mg^{2+} + Na^+ + ATP ≈ Mg^{2+} + ATP > Mg^{2+} + Na^+ + K^+ + ATP > Mg^{2+} + K^+ + ATP ≈ Mg^{2+}.

Ratio of ouabain-binding capacity to

TABLE 2

Binding capacities of ouabain and dissociation constant (K_D)

The data were obtained from Fig. 3. The numbers of ouabain-binding sites were obtained from intercepts on the abscissae. The number of binding sites with low affinity was corrected for the value for high-affinity sites. Dissociation constants for ouabain were obtained from the slopes of the plots.

Physiological ligands	High-affinity sites				Low-affinity sites			
	Control		Treated		Control		Treated	
	Binding	K_D	Binding	K_D	Binding	K_D	Binding	K_D
	pmoles/mg	*μM*	*pmoles/mg*	*μM*	*pmoles/mg*	*μM*	*pmoles/mg*	*μM*
Mg^{2+} + ATP	112	0.13	106	0.13	40	5.8	42	5.6
Mg^{2+} + Na^+ + ATP	120	0.12	128	0.13	48	6.3	44	5.9
Mg^{2+} + Na^+ + K^+ + ATP	120	0.18	117	0.3	100	16.5	106	16.5
Mg^{2+} + K^+ + ATP	112	0.42	106	1.1	108	23.3	114	23.3
Mg^{2+}	130	0.52	104	0.85	60	18.2	64	18.5
Mg^{2+} + P_i	155	0.11	135	0.15	40	4.8	44	4.8

(Na^+ + K^+)-ATPase phosphorylation capacity. In order to relate ouabain binding to the inhibition of (Na^+ + K^+)-ATPase activity, the amounts of phosphorylated intermediate were measured using the same lot of enzyme preparation employed for the ouabain binding experiments. The amounts of phosphorylated intermediate of the control preparation in the steady state in the presence of Mg^{2+}, Na^+, and 0.025–1.2 mM ATP are shown in Fig. 4. The maximum amount of phosphorylated intermediate was about 118 pmoles/mg, which was approximately the same as that of the high-affinity ouabain-binding sites in the presence of Mg^{2+}, Na^+, and ATP (Table 2). It was previously shown that the amount of steady-state phosphorylated intermediate is approximately the same for both treated and control preparations (1).

Effect of phospholipase treatment on two apparent catalytic sites. Several authors have reported that Lineweaver-Burk plots of (Na^+ + K^+)-ATPase activity gave two interesting straight lines (12–16). Phospholipase A-treated ATPase showed a marked reduction of (Na^+ + K^+)-ATPase activity at relatively high concentration of ATP (1, 2, 17). Therefore it seemed profitable to study the effect of phospholipase treatment on the ATPase activity at relatively low concentrations of ATP. (Na^+ + K^+)-ATPase activities were measured in the presence of various concentrations of ATP,

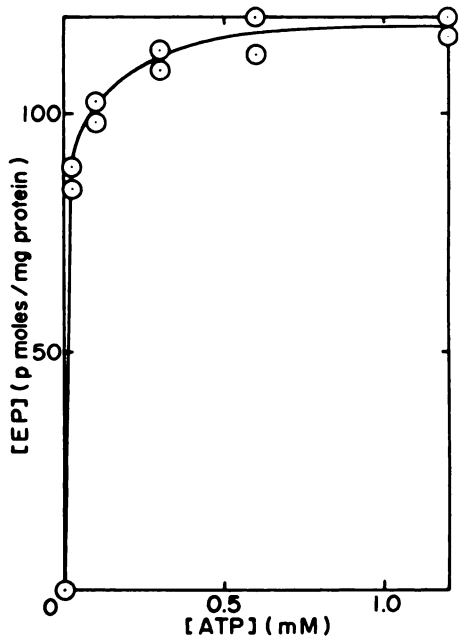

FIG. 4. *Amount of Na^+-dependent phosphorylated intermediate (EP) in the presence of various concentrations of ATP*

The amount of *E*P at steady state was measured after 5 sec of incubation with [^{32}P]ATP in the presence of 0.5 mg of ATPase protein per milliliter, 5 mM $MgCl_2$, 140 mM sodium acetate, 0.19 M sucrose, 0.25 mM EDTA-Tris, 40 mM Tris-HCl (pH 7.4), and various concentrations of [^{32}P]ATP at 37°. The amount of *E*P was corrected for background values obtained from the nonspecific labeling.

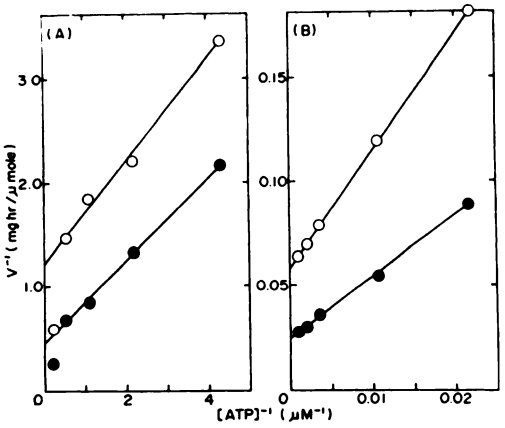

FIG. 5. *Lineweaver-Burk plots of* $(Na^+ + K^+)$-*ATPase activity*

$(Na^+ + K^+)$-ATPase activities were measured in the presence of 0.014 mg of protein per milliliter, 5 mM $MgCl_2$, 140 mM sodium acetate, 14 mM KCl, 0.19 M sucrose, 0.15 mM EDTA-Tris, 40 mM Tris-acetate, and various concentrations of MgATP at pH 6.1 and 37°. Other additions for the maintenance of constant ATP concentration during the assay are described in the text. ○, phospholipase A-treated preparation; ●, control preparation. A. Low concentrations of ATP. B. High concentrations of ATP.

and Lineweaver-Burk plots were constructed. A typical example is shown in Fig. 5. The plot was divided into two segments, A and B, to accommodate the low and high ranges of ATP concentrations used. From the slopes and intercepts of the vertical line the apparent K_m and V_{max} values were obtained. V_{max} values for both controls were 43.6 ± 4.5 (n = 3) and 2.32 ± 0.20 (n = 3) μmoles of ATP per milligram of protein per hour in the presence of high and low concentrations of ATP, respectively. Those values were decreased to 13.1 ± 1.5 (n = 3) and 0.76 ± 0.09 (n = 3) μmoles of ATP per milligram of protein per hour, respectively, by phospholipase A treatment. K_m values for both controls were 124 ± 8 (n = 3) and 0.86 ± 0.08 (n = 3) μM in the presence of high and low concentrations of ATP, respectively. The corresponding values for the treated preparations were 104 ± 10 (n = 3) and 0.44 ± 0.06 (n = 3) μM. V_{max} values for both phospholipase A-treated preparations in the presence of high and low concentrations of ATP were reduced to approximately 30% of the controls, and the K_m value of the high-affinity site for ATP was reduced to approximately 50% of the control. In the presence of phosphatidylserine and phosphatidylinositol those values were increased to nearly the control value.[1]

DISCUSSION

In our previous report (16) the existence of two different kinds of ouabain-binding sites was demonstrated in the presence of Mg^{2+} + Na^+ + K^+ + ATP. Ouabain bound to the high-affinity site caused a marked reduction in $(Na^+ + K^+)$-ATPase activity in the presence of high and low concentrations of ATP (16). The present experiments show that both phospholipase A-treated and control ATPase preparations have two different kinds of ouabain-binding sites, with high and low affinities, in the presence of various physiological ligands of $(Na^+ + K^+)$-ATPase (Fig. 3 and Table 2). The number of phosphorylated sites on the enzyme was approximately the same as the number of ouabain-binding sites with high affinity in the presence of Mg^{2+} + Na^+ + ATP (Table 2 and Fig. 4). There were approximately the same number of high- and low-affinity sites in the presence of Mg^{2+} + Na^+ + K^+ + ATP (Table 2). These observations suggest that 2 moles of ouabain are able to bind per catalytic site. The values for the dissociation constants of the low-affinity sites for ouabain were 10–100 times those for the high-affinity sites in the presence of various physiological ligands. The dissociation constants for both sites were influenced by ligands in the same manner. In the presence of ATP the dissociation constants for both sites were increased by K^+, and this increase was antagonized by Na^+. In the absence of ATP the dissociation constant was decreased by inorganic phosphate. K^+ increased not only the dissociation constant of the enzyme-ouabain complex, as described above, but also the dissociation constant of the enzyme-ATP complex, as reported Nørby and Jensen (20) and Hegyvary and Post (21). The ouabain-binding capacity of the high-

[1] Unpublished observations.

affinity site was approximately constant in the presence of ATP, but K^+ increased the capacity of the low-affinity site approximately 2–3-fold. Hegyvary and Post (21) also reported that the number of ATP-binding sites appeared to be 2-fold greater in the presence of K^+ than in its absence. Our results may reflect some regulatory mechanism in $(Na^+ + K^+)$-ATPase by K^+, as suggested by Hegyvary and Post (21).

Many workers have suggested that cardiac glycosides bind to $(Na^+ + K^+)$-ATPase during two different conformational states of the enzyme (3–11). The present study also indicates the existence of at least two different enzyme conformations. Approximately 70% of the phospholipids in the ATPase preparation were changed to lyso compounds by phospholipase A treatment (1). Hydrophobicity in the vicinity of active site of the enzyme may be diminished by such treatment. The partial loss of hydrophobicity in the vicinity of the active site might not only reduce the initial rate of ouabain binding to some conformations of the enzyme but also increase the dissociation constant for ouabain (Fig. 1C–F and Table 2). However, phospholipase treatment did not cause any remarkable changes either in the rate of binding or in the dissociation constant of other conformations (Fig. 1A and B and Table 2).

The ouabain-binding capacity of the enzyme was not changed significantly by phospholipase A treatment in spite of a marked reduction in $(Na^+ + K^+)$-ATPase activity. The amount of Na^+-dependent phosphorylated intermediate in the steady state was not changed significantly, as reported previously (1). These findings support our hypothesis that phospholipid molecules provide both a negative charge and hydrophobicity in the vicinity of the active site rather than being components of the active site itself (1, 2). Independently of the above observations on the role of phospholipids, we have recently suggested that the phase transition of phospholipid molecules may be related to some regulatory mechanism of Na^+ and K^+ transport across the cell membrane (17). The changes in $(Na^+ + K^+)$-ATPase activity (1, 2) and ouabain binding reactivities effected by phospholipase A treatment, as shown in the present study, may be related to an absence of the phase transition which is essential for $(Na^+ + K^+)$-ATPase activity.

Phospholipase A treatment caused a marked reduction of $(Na^+ + K^+)$-ATPase activities in the presence of high (1, 2, 17) and low concentrations of ATP, as described above. These observations suggest that both catalytic sites (12–16) with high and low affinity for ATP require phospholipids and are in accordance with our conclusion (16), deduced from ouabain binding experiments, that both enzymatic sites are related to the $(Na^+ + K^+)$-ATPase reaction (16).

Several authors have suggested that maximum amounts of cardiac glycoside binding were obtained in the presence of $Mg^{2+} + Na^+ + ATP$ or $Mg^{2+} + P_i$ (4, 5, 8). However, Scatchard plots of our ouabain binding data showed that the maximum amount of ouabain binding was independent of the ligands used except for $Mg^{2+} + P_i$, which slightly increased it. This contradiction may easily be explained by the fact that the other experiments were performed at fixed concentrations of cardiac glycoside and with relatively short incubation times (4, 5, 8). Under those conditions the amount of binding should be regarded as rate of binding.

Both phospholipase A-treated and -untreated enzyme preparations contained an Mg^{2+}-ATPase activity of 3–3.5 μmoles of ATP per milligram per hour at pH 7.4. During the longer incubation in the presence of ATP, such as that shown in Fig. 2, considerable ADP and P_i should also have been produced. Several authors (4, 5) have reported that Mg^{2+} + ADP can support binding of the cardiac glycoside to the enzyme. We cannot exclude the effect of ADP and P_i on the maximum amount of ouabain binding (Fig. 2A and B). Although this does not change our conclusion, further study is required to clarify the effect of ATP, ADP, and P_i on the ouabain-binding mechanism.

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