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Suppression by Sodium, Potassium or Nucleotides of Binding between Cardiac Steroid (Digoxigenin) and Sodium- and Potassium-Dependent Adenosine Triphosphatase Formed in the Presence of Magnesium and Phosphate

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SUMMARY

YODA, ATSUNOBU AND SHIZUKO YODA: Suppression by sodium, potassium or nucleotides of binding between cardiac steroid (digoxigenin) and sodium- and potassium-dependent adenosine triphosphatase formed in the presence of magnesium and phosphate. *Mol. Pharmacol.*, **16:** 120–134, (1979).

The cardiotonic steroid binding to (Na^+ + K^+)-ATPase formed in the presence of the Mg^{2+} and P_i (type II binding) is suppressed by the Na^+, K^+, or nucleotides in different ways. In the ouabain binding at real equilibrium, such effects were difficult to detect because of the high affinity of its sugar moiety to the enzyme. The apparent number of binding sites according to Scatchard plots of digoxigenin binding and the association rate of digoxigenin were reduced by the Na^+ at pH 6.5 and 8.5. This Na^+-effect was delayed in the initial phase at pH 6.5, when the Na^+ was added after the phosphorylation of the enzyme was accomplished by the Mg^{2+} and P_i. Therefore, the Na^+ seems to bind with the nonphosphorylated form of the enzyme, and to reduce level of the phosphorylated enzyme, the active form for digoxigenin binding. In contrast to the Na^+, the K^+ increased the apparent binding constant according to Scatchard plot of digoxigenin binding at pH 6.5 and 8.5; the K^+ seems to bind with the phosphorylated form of enzyme, causing the insensitive complex not to bind with digoxigenin. Unlike either the Na^+ or K^+, triphosphonucleotides suppressed the type II digoxigenin binding only partially. The potencies of ATP and β,γ-methylene ATP were similar; both were most potent among the triphosphonucleotides (its half-maximum concentration ≒ 0.2 mM). GTP and ITP were less potent while AMP was almost ineffective. ADP was as effective as ATP in less than 0.8 mM, but in higher concentrations, ADP increased the inhibition of digoxigenin binding, reducing the increment of the increase, while ATP showed saturation. According to Scatchard plots, ATP decreased only the apparent number of binding sites below pH 8.0, but it increased only the apparent binding constant above pH 8.0. β,γ-Methylene ATP reduced the association rate of digoxigenin at pH 7.0 and 8.3, but did not seem to change the level of phosphorylated protein by the Mg^{2+} and P_i and the dissociation rate of digoxigenin-enzyme complex at both pHs. Therefore, such nucleotide-effect seems to be one of the low-affinity effects of ATP, and seems to include some conformational changes of phosphorylated active form produced by the Mg^{2+} and P_i, reducing its affinity to cardiotonic steroids.

INTRODUCTION

(Na^+ + K^+)-ATPase is integral to the active transport of the Na^+ and K^+ in animal cells, and is inhibited by specific binding with cardiotonic steroids. Most of the studies on this interaction have been per-

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formed using cardiac glycosides, specifically ouabain, although the aglycone moiety is responsible for the physiological effects of glycoside. The ouabain binding is dependent on the presence of certain ligands; the most effective ligand systems are the Na^+-Mg^{2+}-ATP (type I) and the Mg^{2+}-P_i (type II) systems (1–7). Since the type I ligand system is well known as a phosphorylating mixture of the enzyme, and the addition of the K^+ reduces the level of both the phosphorylated protein and the binding of ouabain, it has been suggested that the phosphorylated forms of the enzyme are the active form of the enzyme for the binding of ouabain. Post *et al.* gave evidence that not only the phosphorylated protein formed by the type I system, but also the phosphorylated protein formed by the type II system could bind with ouabain in the absence of magnesium (8).

On the other hand, our kinetic rate studies on the (Na^+ + K^+)-ATPase complexes with various cardiac glycosides (9–11) indicated that cardiac glycosides were bound to the enzyme at two sites, one specific for the steroid moiety and the other for the sugar. The binding of the steroid moiety results in activation of the sugar binding site, with consequent binding of the glycoside portion. The inhibitory action of cardiac glycoside is associated with the steroid moiety, and the sugar moiety stabilizes the enzyme-cardiac glycoside complex. This stabilizing effect is remarkable; in the case of beef brain microsomes, the dissociation rate constant of ouabagenin is about 80 times greater than that of ouabain (ouabagenin rhamnoside) if the drug-enzyme complex is formed in the type II system (12). To eliminate such effects of sugar moiety in the cardiac glycoside, we studied the binding of [12α-^{3}H]digoxigenin instead of [^{3}H]ouabain (13), and the association and dissociation rates of several cardiac aglycones in the type II system (12). Studies of this association rate and the pH effect on the digoxigenin binding (14) show strong correlation of the digoxigenin binding to the phosphorylation of the enzyme, and suggest that this enzyme phosphorylation is essential to the binding of the steroid moiety as Post *et al.* indicated in the case of ouabain (8).

In this report, the reduction of digoxigenin binding in the type II system by the low concentrations of Na^+, K^+ or nucleotides was studied in order to obtain more precise information on the binding mechanism of cardiac steroids and (Na^+ + K^+)-ATPase.

For convenience, we will refer to the phosphorylated protein formed by ATP in the presence of the Na^+ and Mg^{2+} (the type I system) as EP, and another phosphorylated protein formed by the P_i in the presence of the Mg^{2+} (the type II system) as E_rP.[1]

MATERIALS AND METHODS

The (Na^+ + K^+)-ATPase preparation was NaSCN-treated microsomes prepared from frozen beef brain (Pel Freeze Biologicals) according to the method of Klodos *et al.* (15). The specific enzyme activity was 50–70 μmol of P_i/hr/mg protein at 30° (equivalent to 80–110 μmol/hr/mg protein at 37°). The activity of ouabain-insensitive ATPase was less than 2 μmol/hr/mg protein at 30°. When this enzyme preparation was treated with ouabain in the presence of 4 mM Mg^{2+} and 4 mM P_i, the saturated amount of bound ouabain was 270 ± 50 p mol/mg protein.

Digoxigenin and ouabain were purchased from Boehringer-Mannheim, and Sigma, respectively. [^{3}H]ouabain was obtained from New England Nuclear, and [12α-^{3}H] digoxigenin was prepared by the reduction of 12-dehydrodigoxigenin with NaB^3H_4, as reported previously (13). ATP, ITP, ADP, AMP and β,γ-methylene ATP

[1] The abbreviations used are: EP, phosphorylated protein formed by ATP in the presence of Na^+ and Mg^{2+} (type I system); E_rP, phosphorylated protein formed by the P_i in the presence of the Mg^{2+} (the type II system); (Na^+ + K^+)-ATPase, sodium- and potassium-dependent adenosin triphosphatase (EC 3.6.1.3); Type I binding, cardiotonic steroid binding with (Na^+ + K^+)-ATPase in the presence of Na^+, Mg^{2+} and ATP; Type II binding, cardiotonic steroid binding with (Na^+ + K^+)-ATPase in the presence of Mg^{2+} and P_i; AMP PCP, β,γ-methylene ATP; N_a, apparent number of binding sites according to Scatchard plot; K_b, apparent binding constant according to Scatchard plot; k_a, association rate constant; k_d, dissociation rate constant, k'_a, pseudo first-order association rate constant; TCA, trichloroacetic acid.

(AMP PCP) were obtained from P. L. Biochemicals. GTP was purchased from Sigma. All nucleotides were changed to Tris salts by Dowex 50 Tris form. [r-^{32}P]ATP was prepared by the method of Post and Sen (16), but [r-^{32}P]ATP was eluted from the Dowex-1 column by 0.20 N HCl and was neutralized with Tris.

Binding of [^{3}H]digoxigenin or [^{3}H]ouabain was determined, as reported previously (13). The separation of bound digoxigenin was performed by centrifugation (105,000 $g \times 20$ min) at 22°, and bound digoxigenin was calculated by subtracting the amount of [^{3}H]digoxigenin in the supernatant from the total amount of [^{3}H]-digoxigenin. A Scatchard plot was constructed from the results of the 12 different concentrations of added digoxigenin (0.02 μM–0.5 μM), and the apparent number of sites (N_s) and the apparent binding constant (K_b) were obtained. To examine the time for the binding to reach equilibrium, the values of bound digoxigenin were measured for various incubation periods (20 to 90 min) after the addition of 0.02 μM digoxigenin under each ligand condition. The incubation period in each set of experimental conditions was selected as either 35 min or 60 min.[2]

The association and dissociation rates of ouabain were obtained from the changing rate of enzyme activity after the termination of ouabain inhibition by dilution, as reported (9, 10). Those rates of digoxigenin were determined by rapid assay for the active enzyme, as previously reported (12). The assay of EP, which is influenced by the amount of bound digoxigenin, is too rapid to be affected by the change of the enzyme-digoxigenin complex during the assay. However, this method for the association or dissociation rate constants (k_a or k_d) cannot be applied for cases containing the K^+ because of the sensitiveness of EP to the K^+; the method also cannot be applied for ATP because it causes the dilution of [γ-^{32}P]ATP. In these determinations of k_a and k_d of digoxigenin, the assay of EP was performed by filtration and washing with about 70 ml of ice-cold 5% TCA containing unlabeled ATP and the P_i; the use of the semirapid mixing apparatus described by Kanazawa *et al.* (17) improved the accuracy

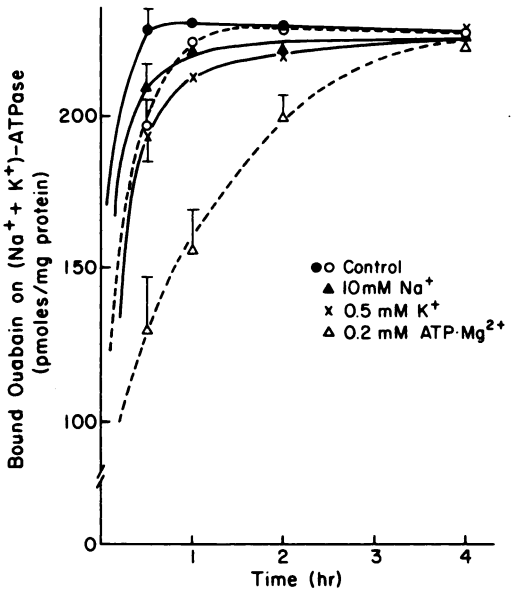


FIG. 1. *Time course of [^{3}H] ouabain binding at 22°*

The incubation mixture containing 1.40 mg/ml (●, ×, ▲) or 0.48 mg/ml (○, △) of the enzyme protein, 50 mM imidazole HCl (pH 6.5), 4 mM Mg^{2+}, 4 mM P_i and 0.4 μM [^{3}H] ouabain was incubated with each ligand as shown. After the time intervals indicated, centrifugation of the mixture was started (105,000 × g for 20 min at 22°). Bound ouabain was calculated from the difference between the radioactivity in the suspended mixture (total ouabain) and that in the supernatant (unbound ouabain). In the experiments of ATP-effect, the enzyme concentrations was reduced to decrease the hydrolysis of ATP by ouabain-insensitive ATPase contaminated in the membrane preparation. After four hour incubation shown in the figure, 40 to 50% ATP was estimated in the supernatant by the Dowex 1 chromatography. The values shown are averages of three sets of experiments; in each experiment, measurements of bound ouabain were duplicated, and the average of experimental error in each point was ± 6 pmol/mg of protein, otherwise shown by bars in the figure.

[2] In each set of experimental conditions, the binding values after 20, 30, 45, and 60 min incubations were duplicated and were examined under the following conditions: (1) the coordinates of those values were graphed horizontally; (2) the deviation from the mean value was less than 3%. If these two conditions were satisfied, the incubation period for that set of experiments was 35 min. If the values after 30, 45, 60, and 90 min incubation periods were satisfied to the above two conditions, the incubation period would be 60 min. In this study, these conditions were satisfactorily met.

of the results. Each coordinate shown in Figs. 7–10, 15, and 16 was the average value from the quadruplicate experiments.

RESULTS

Effect of Na^+, K^+ and ATP on the type II ouabain binding. The interaction of ouabain and (Na^+ + K^+)-ATPase is nearly irreversible; the interaction takes a long period to reach equilibrium. The presence of Na^+, K^+ or ATP (Fig. 1) seemed not to influence the value of bound ouabain in equilibrium, even though the presence of such ligands reduced the association rates of ouabain and lengthened the period to reach equilibrium. Under similar conditions, 10 mM Na^+, 0.5 mM K^+ or 0.2 mM ATP reduced the initial rate of ouabain inhibition to 52%, 46% or 45%, respectively.

In contrast to ouabain binding, digoxigenin binds with (Na^+ + K^+)-ATPase reversibly. As shown in Fig. 2, the type II digoxigenin binding in equilibrium was reduced in the presence of Na^+, K^+ or ATP. Apparently, ATP suppressed the digoxigenin binding only partially compared with Na^+ or K^+.

Effect of Na^+ and K^+ on the digoxigenin binding. When the ligand effect was shown as percentage decrease of the binding, the Na^+-effect was enhanced by the increase of pH from 6.5 to 8.5; but the K^+-effect under the low concentration of K^+ (≤0.5 mM) was not influenced by the pH change (Fig. 3). In the presence of 4 mM K^+, the absolute value of digoxigenin binding decreased and was not influenced by the pH change (Fig. 4).

In order to detail the differences between Na^+-effect and K^+-effect, these ion effects on the digoxigenin binding were examined at pH 6.5 and 8.0 by Scatchard plot (Figs. 5, 6). The K^+ increased the K_b only in lower concentrations, while the Na^+ decreased only the N_s. Similar effects of Na^+ and K^+ were also observed at pH 8.5, using Tris buffer (data not shown).

Effects of Na^+ on the association and dissociation rates of digoxigenin. At pH 6.5, the time course of digoxigenin inhibition showed some lag in the Na^+ effect if the inhibition had been started by the addition of digoxigenin to the mixture of the enzyme with Mg^{2+}, P_i and Na^+, and that lag decreased with the increase of the Na^+ concentration (Fig. 7). At pH 8.0, however, the presence of Na^+ reduced the inhibition rate of digoxigenin without any lag (Fig. 8) and Dixon plots of $1/k'_a$ versus Na^+ concentration were linear.

As shown in Fig. 9, the dissociation rate of the type II digoxigenin-enzyme complex was not changed by the presence of Na^+ in the dilution medium.

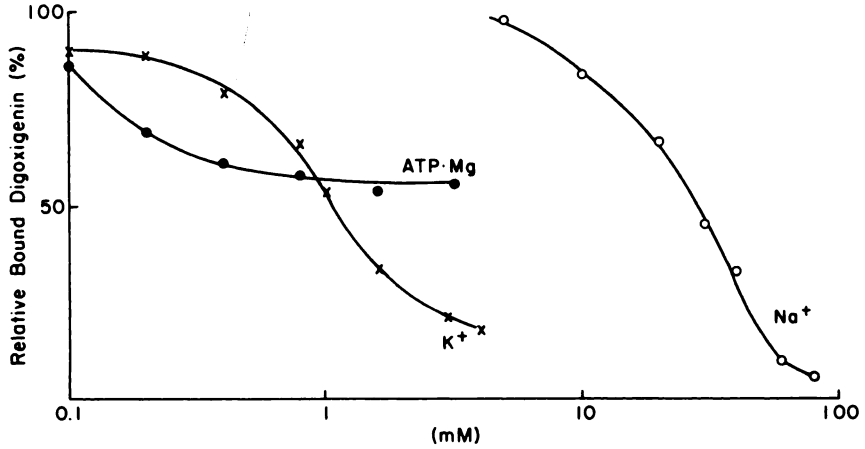


FIG. 2. *Reduction of digoxigenin binding by the Na^+, K^+ or ATP in equilibrium*

The enzyme preparation (0.62 mg/ml) was treated with 0.25 μM [^{3}H]digoxigenin, and various concentrations of the Na^+, K^+ or Mg ATP in the presence of 5 mM Mg^{2+}, 4 mM P_i, and 40 mM imidazole HCl buffer (pH 7.0) at room temperature (22–23°). After 45 min of incubation, the suspension was centrifuged (105,000 g × 20 min at 23°), and the value of bound digoxigenin was calculated as Fig. 1. In absolute terms, 100% corresponds to 144 pmol digoxigenin/mg protein and the average values of triplicated experiments were cited.

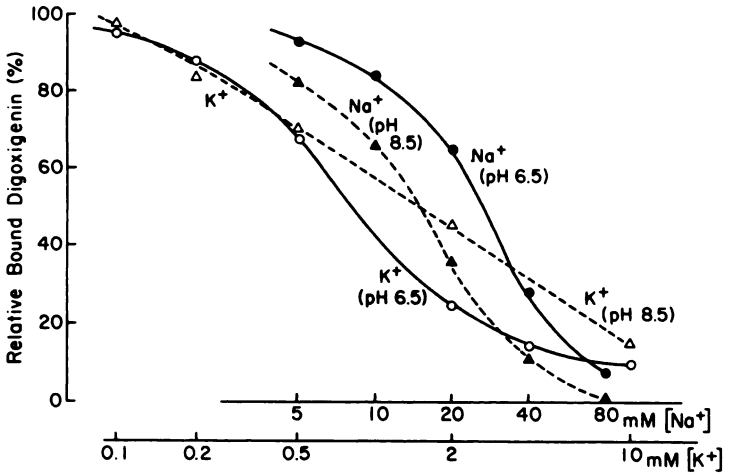


FIG. 3. *Relative reduction of digoxigenin binding by the Na^+ or K^+ at pH 6.5 and pH 8.5*

The enzyme preparation (1.16 mg/ml) was incubated with 0.1 μM [^{3}H]digoxigenin, 4 mM Mg^{2+}, 4 mM P_i and various concentrations of Na^+ or K^+ in the presence of 50 mM imidazole-HCl (pH 6.5) or 50 mM Tris-HCl (pH 8.5) for 45 min at pH 6.5, or 90 min at pH 8.5. Other experimental conditions were the same as those in Fig. 2. In absolute terms, 100% corresponds to 58.2 ± 1.1 pmol/mg protein at pH 6.5 and to 29.0 ± 0.7 pmol/mg at pH 8.5. The values shown are the averages of triplicated experiments.

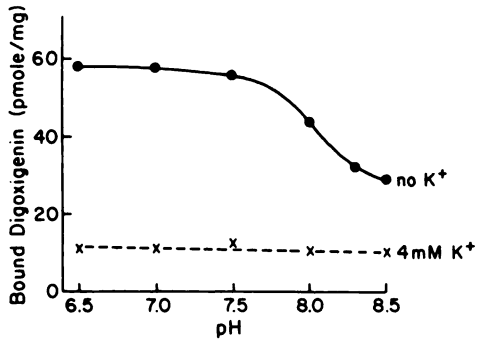


FIG. 4. *Influence of the K^+ on the digoxigenin binding at various pH*

Below pH 8.0, 0.5 ml of the mixture containing enzyme preparation (1.16 mg/ml), 4 mM Mg^{2+}, 4 mM P_i, 0.1 μM [^{3}H]digoxigenin and 50 mM imidazole-HCl buffer, which was adjusted to the pH as cited, was incubated for 45 min at 23° and was centrifuged. Other experimental conditions were the same as in Fig. 1. At pH 8.0 and above that value, 50 mM Tris-HCl buffer was used and was incubated for 60 min. The values are the averages of triplicated measurements.

Since the association rate of digoxigenin at pH 6.5 is about four times higher than that at pH 8.0 (14), the interaction of the Na^+ might seem slow compared with the association rate of digoxigenin at pH 6.5. To examine this idea, the effect of an addition sequence of the Na^+ was studied. In these experiments, the temperature was kept low (10°), to slow down the interaction between ions and the enyzme, and the concentration of digoxigenin was increased to 1.0 μM to keep the high association rate of digoxigenin.

As shown in Fig. 10, line A, the decrease of EP value was biphasic when the Mg^{2+}, P_i and digoxigenin reacted with the enzyme at the same time. The first rapid decrease of EP originated from the phosphorylation of the enzyme by the P_i, specifically the formation of E_1P, and the second slow change originated from the inhibition by digoxigenin (12). The addition of 5 mM Na^+ at zero time with the Mg^{2+} and P_i, slowed down these biphasic changes in both phases (line B). When the enzyme was pretreated with the Mg^{2+} and P_i, and the inhibition was started by the addition of digoxigenin with (line D) or without (line C) the Na^+, this Na^+-effect on the EP change was not observed within 30 sec. On the other hand, if the digoxigenin was added to the enzyme pretreated with the Mg^{2+}, P_i, and the Na^+ (line E), the Na^+-effect was observed with some lag (same as Fig. 7). If the enzyme was pretreated with Na^+, but without the Mg^{2+} and P_i, the Na^+-effect was enhanced

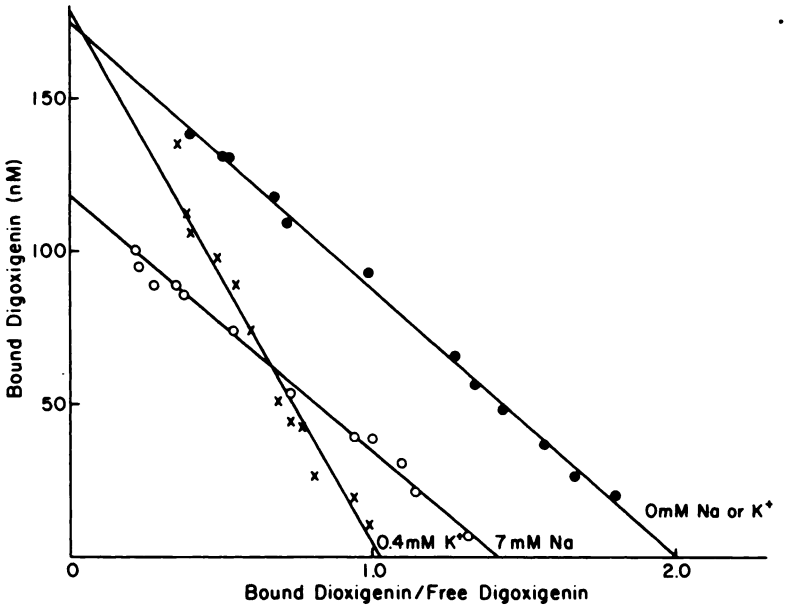


FIG. 5. *Typical Scatchard plots of type II digoxigenin binding with or without the Na^+ or K^+*

A half milliliter of the incubation medium contained 50 mM imidazole-HCl (pH 6.5), 4 mM Mg^{2+}, 4 mM P_i, 20–500 nM [^{3}H]digoxigenin and enzyme preparation (0.40 mg). After 35 min (without Na^+ or K^+) or 60 min (with K^+ or Na^+) incubation at 22–23°, the mixture was centrifuged. Both bound and free digoxigenin were calculated as in Fig. 1.

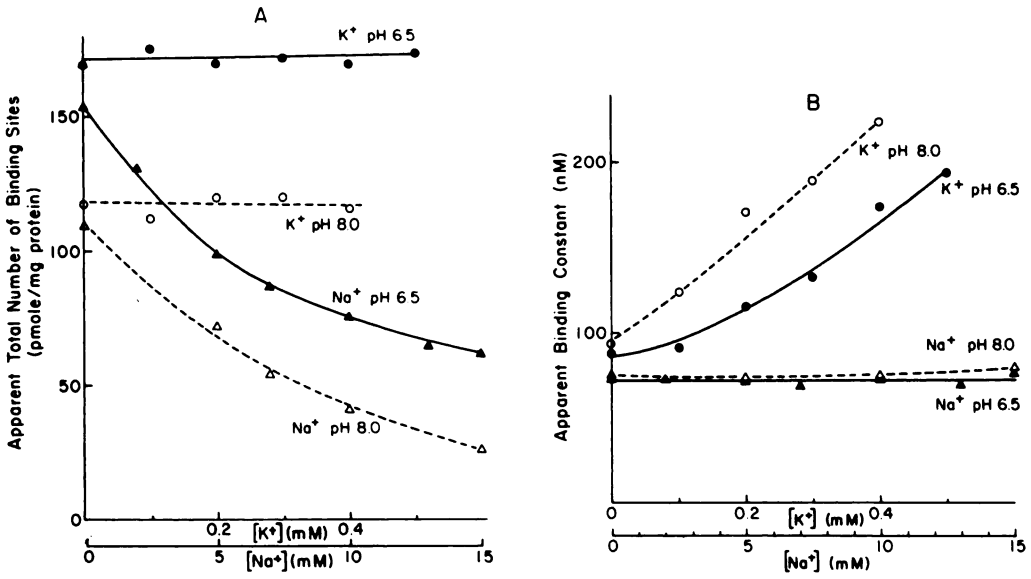


FIG. 6. *Effect of the Na^+ and the K^+ on the type II digoxigenin binding*

Panel A) Change of the apparent total number of binding sites. Panel B) Change of the apparent binding constant. Conditions were the same as in Fig. 5, except that various concentrations of the Na^+ or K^+ were examined at pH 6.5 and 8.0, and at pH 8.0, 50 mM Tris-HCl was used as a buffer instead of imidazole-HCl.

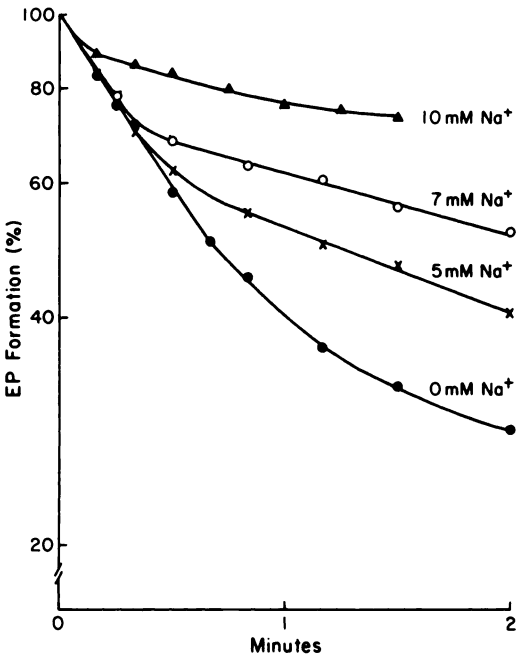


FIG. 7. *Time course of (Na^+ + K^+)-ATPase inhibition by digoxigenin in the presence of Na^+ at pH 6.5*

The inhibition was started at 25° by the addition of 0.1 ml of 2.0 μmol digoxigenin into the 0.9 ml mixture containing the enzyme (about 0.1 mg), 50 μmol imidazole-HCl buffer, 4 μmol Mg^{2+}, and 4 μmol P_i. At each interval, the phosphorylating reagent (0.1 ml) containing about 0.3 mM [γ-^{32}P]ATP, 1 M NaCl, and 2 mM $MgCl_2$, was added; after 3 sec, the enzyme was denatured with 45% TCA (0.5 ml) containing 1 mM P_i and 0.1 mM unlabeled ATP. One ml of the reaction mixture was filtered with a Millipore filter (pore size 0.45 μm), washed with 70 ml of ice-cold 5% TCA solution containing 1 mM P_i and 0.1 mM unlabeled ATP, and finally washed with 10 ml of ice-cold water. The washed precipitate on the filter was made soluble with 1.5 ml of 2-methoxyethanol in a counting vial, and radioactivity was measured with a Packard Tri-Carb liquid scintillation counter, using 5 ml of scintillation medium (5 g of 2.5-diphenyloxazole and 0.3 g of 1.4-bis [2-(5-phenyloxazolyl)]benzene in 1 liter of toluene and 500 ml of Triton X-100.

and its lag disappeared (line F). Furthermore, when the enzyme was pretreated with the Na^+ plus Mg^{2+}, or the Na^+ plus P_i, and the inhibition was started by the addition of digoxigenin with the P_i or Mg^{2+}, respectively, both inhibition curves were the same as line F (data not shown). Assuming that pH and temperature do not change the reaction scheme, it can be concluded from these sequence studies that the Na^+ does not react with E_TP, but does react with the nonphosphorylated enzyme. Na^+-bound enzyme seems to be insensitive to phosphorylation by Mg^{2+} and P_i, and the recoveries of free enzyme from Na^+-bound enzyme or E_TP are slow.

Effects of ATP and AMP PCP on the type II digoxigenin binding. As shown in Fig. 11 and 12, several nucleotide-Mg^{2+} complexes reduced the digoxigenin binding. All triphosphonucleotides examined here showed some saturation phenomena and did not completely inhibit the binding. ATP was the most potent among them at the half-saturated concentration as well as on the amount of inhibition at the saturated concentration. Every triphosphate increased its inhibitory potency in higher pH (pH 8.3). Magnesium-ADP complex also inhibited type II digoxigenin binding, and its potency was enhanced in the basic con-

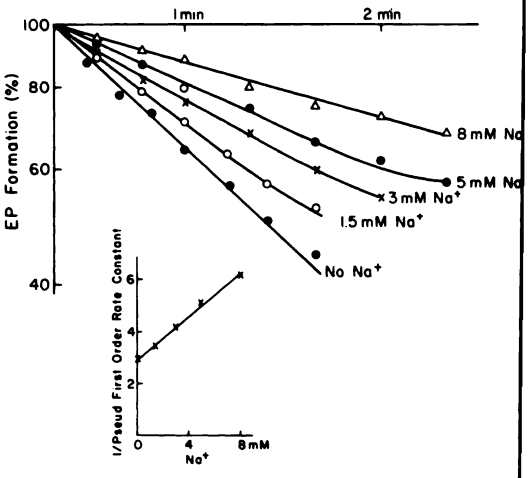


FIG. 8. *Time course of (Na^+ + K^+)-ATPase inhibition by digoxigenin in the presence of Na^+ at pH 8.0*

Conditions were the same as in Fig. 7, except for the higher pH and the following: the inhibition was started by the addition of 4.0 μM digoxigenin instead of 2.0 μM at pH 6.5. Fifty millimolar Tris was used as the buffer instead of imidazole and the concentration of enzyme was 0.23–0.26 mg protein/ml. The inset shows the Dixon plot of Na^+-effect on the pseudo-first-order association rate constant (k'_a) *of digoxigenin.*

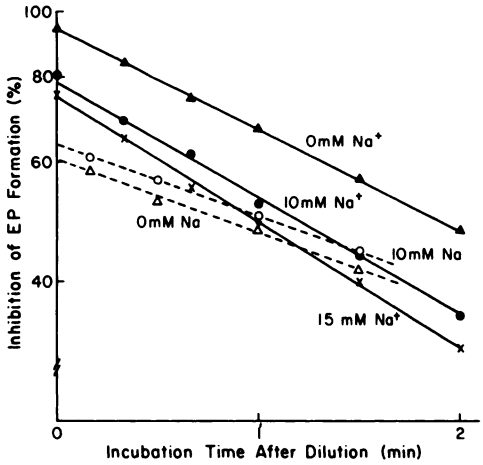


FIG. 9. *Effect of the Na^+ in the dilution medium on the stability of type II digoxigenin enzyme complex at 25°*

The enzyme preparation (1.0–1.1 mg/ml) was incubated at 25° with a mixture of 4 mM Mg^{2+}, 4 mM P_i, 0.8 μM digoxigenin and 50 mM imidazole, adjusted to pH 6.5 (solid line). After 30 to 60 min of incubation, the equilibrated mixture (0.1 ml) was diluted with 1 ml of 10 mM imidazole buffer (pH 6.5) containing 1 mM EDTA and Na^+ as indicated, and was phosphorylated at each interval. For experiments at pH 8.3 (dashed line), 50 mM Tris-HCl buffer and 1.25 μM digoxigenin were used for the inhibition, and 10 mM Tris buffer (pH 8.3) containing 1 mM EDTA with (●, ×, ○) or without (▲, △) Na^+ was used for the dilution. The procedures for phosphorylation and the EP assay were the same as described for Fig. 7.

dition. At low concentration (less than 0.8 mM), the inhibition by ADP was the same as that by ATP, but at the concentration in which ATP showed saturation, ADP increased the inhibition while the increment of the increase was reduced. AMP Mg^{2+} inhibited digoxigenin binding only slightly (Figs. 11, 12).

The ATP effect on type II digoxigenin binding was examined at various pH by the Scatchard plot. At pH 7.0, ATP suppressed digoxigenin binding by the reduction of the N_s, but did not change the K_b (Fig. 13A). Similar reductions of N_s by ATP were also observed at pH 6.5 and 7.5. On the other hand, at pH 8.3, ATP suppressed the digoxigenin binding in a different way (the increase of the K_b), but did not change the N_s. Similar changes by ATP were also observed at pH 8.5. However, at pH 8.0, if ATP were present, the Scatchard plots were not linear (Fig. 13B), suggesting the presence of two types of digoxigenin-enzyme complexes.

For the study of these effects of triphosphonucleotides on k'_a and k_d of the digoxigenin complex, ATP is not a suitable triphosphate because the presence of ATP decreases the radioactivity of EP. In the present case, however, AMP PCP, one of the unhydrolysable ATP homologues, can

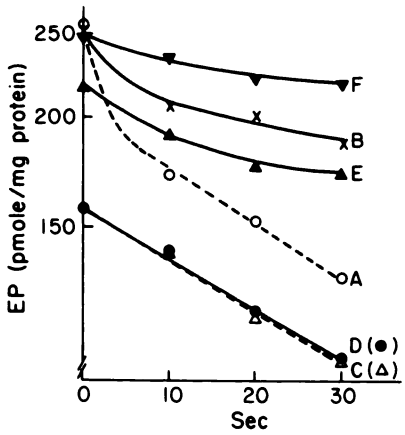


FIG. 10. *Effect of sequence of addition of the Na^+ on the type II inhibition of (Na^+ + K^+)-ATPase by digoxigenin*

The sequence of addition of components to complete the mixture was varied. In 1 ml at 10°, the complete mixtures contained 0.15 mg of the enzyme preparation, 4 μmol $MgCl_2$, 4 μmol P_i, 1.0 nmol digoxigenin, 50 μmol imidazole (pH 6.5) with (——) or without (- - -) 5 μmol NaCl. After 3 min of pretreatment of the enzyme, the mixture was completed at zero time as shown below. At each interval indicated, enzyme inhibition was determined by assaying the formation of EP, as described for Fig. 7. See text for experimental details.

line	Components* during the pretreatment of enzyme	Components added at zero time with digoxigenin
A (○- - -○)	None	Mg^{2+}, P_i
B (×——×)	None	Mg^{2+}, P_i, Na^+
C (△- - -△)	Mg^{2+}, P_i	None
D (●——●)	Mg^{2+}, P_i	Na^+
E (▲——▲)	Mg^{2+}, P_i, Na^+	None
F (▼——▼)	Na^+	Mg^{2+}, P_i

* except imidazole buffer

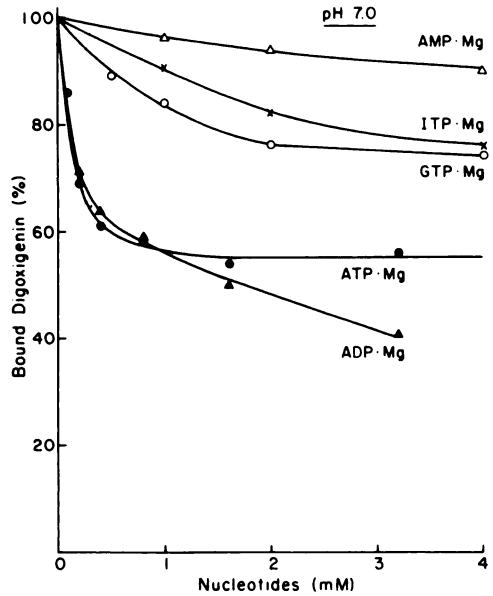


FIG. 11. *Relative reduction of digoxigenin binding by Mg^{2+}-nucleotide complexes at pH 7.0*

The incubation mixture contained the enzyme preparation (0.79 mg/ml), 0.25 μM [^{3}H]digoxigenin, 5 mM Mg^{2+}, 4 mM P_i, 40 mM imidazole-HCl and various concentrations of each Mg^{2+}-nucleotide complex, which was the equimolar mixture of Mg^{2+} and each nucleotide. Its pH was adjusted to 7.0, and then it was incubated for 45 min at 22°. Other experimental procedures were the same as in Fig. 2. In absolute terms, 100% corresponds to 164 pmol/mg protein, and the average values of triplicated experiments were cited.

replace the inhibitory activity of ATP (Fig. 14) and does not interfere the assay of EP.

As shown in Fig. 14, AMP PCP itself did not change the inhibition of EP level by Mg^{2+} and P_i, which is considered to correspond to the E_rP level (12), but did reduce the association rate of digoxigenin at pH 7.0 and 8.3 (Fig. 15). The dissociation rate of digoxigenin was not influenced with 1 mM AMP PCP in the dilution medium (Fig. 16).

DISCUSSION

The suppression of type II ouabain binding by the Na^+ or K^+ has been studied by several groups (6, 7), and the half-maximal concentrations of these ions have been reported to be different. However, the present results concerning ouabain binding shows that, at real equilibrium, the saturated value of type II ouabain binding seems not to be influenced by the Na^+, the K^+ or ATP, even though a much longer incubation period is needed for it to reach equilibrium in the presence of such ligands (Fig. 1), as has been shown in the case of pH effect (14). This result comes from the high affinity of the sugar moiety on ouabain (a cardiac glycoside) to the sugar specific site on the enzyme (9, 11). Since the binding of the steroid moiety in the cardiac glycoside is a step prior to that of its sugar moiety (10), the binding of digoxigenin (a cardiac aglycone) is a more suitable process to study the influences of Na^+, K^+, or nucleotides than that of ouabain.

In the study of reverse reaction of ATP hydrolysis, Post *et al.* (8) showed that the ($Na^+ + K^+$)-ATPase is phosphorylated into E_rP by P_i in the presence of Mg^{2+}, and that this phosphorylation is suppressed with the Na^+ or K^+. Moreover, Post *et al.* presented evidence that such an E_rP can bind with ouabain in the absence of Mg^{2+}. According

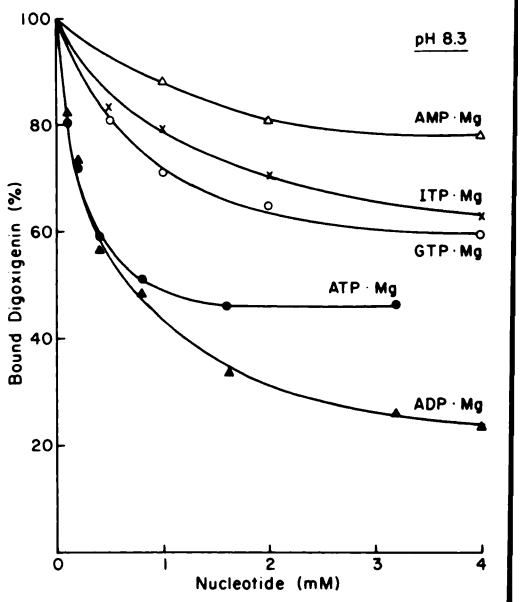


FIG. 12. *Relative reduction of digoxigenin binding by Mg^{2+}-nucleotide complexes at pH 8.3*

Each incubation mixture contained 0.62 mg/ml of the enzyme preparation, 0.20 μM digoxigenin, and 40 mM Tris-HCl buffer (pH 8.3) in the presence of 5 mM Mg^{2+}, 4 mM P_i and various Mg^{2+}-nucleotide complex. The pH of the mixture was 8.3, and the incubation time was 60 min at 22-23°. Other experimental conditions were the same as in Fig. 11. In absolute terms, 100% corresponds to 80.5-82.4 pmol/mg and the average values of triplicated experiments were cited.

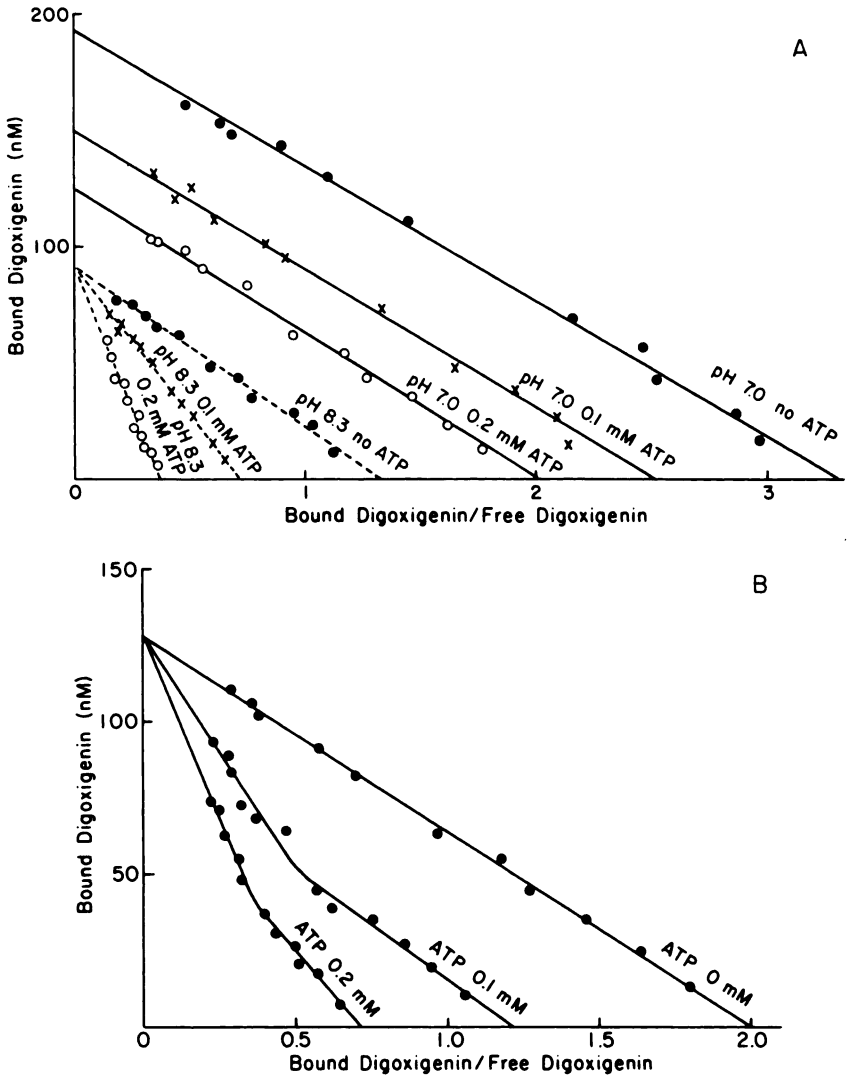


FIG. 13. *Scatchard plot of type II digoxigenin binding in the presence of ATP*

A half ml of the mixture contained 4 mM Mg^{2+}, 4 mM P_i, 20–500 nM [^{3}H]digoxigenin, 40 mM buffer, enzyme preparation and various concentrations of ATP which was added with the equimolecular Mg^{2+}, was incubated at 22–23° for the following durations, and then was centrifuged to separate the unbound digoxigenin:

pH	Buffer	Enzyme protein	Incubation period
7.0	imidazole-HCl	388 mg	35 min
8.0	Tris-HCl	395 mg	60 min
8.3	Tris-HCl	420 mg	60 min

The other conditions were the same as in Fig. 5. The results at pH 7.0 and 8.3 are shown in panel A, and those at pH 8.0 are in panel B.

to kinetic analysis by Kuriki *et al.* (18), such an E_TP is formed from the ternary complex of ATPase, the P_i and Mg^{2+}. At almost the same time, we indicated from the rate kinetics of cardiac aglycone binding, that E_TP is the intermediate to bind with the cardiotonic steroid and is formed from the complex of enzyme, the Mg^{2+}, and P_i (12). The following reaction scheme between (Na^+ + K^+)-ATPase and cardiac

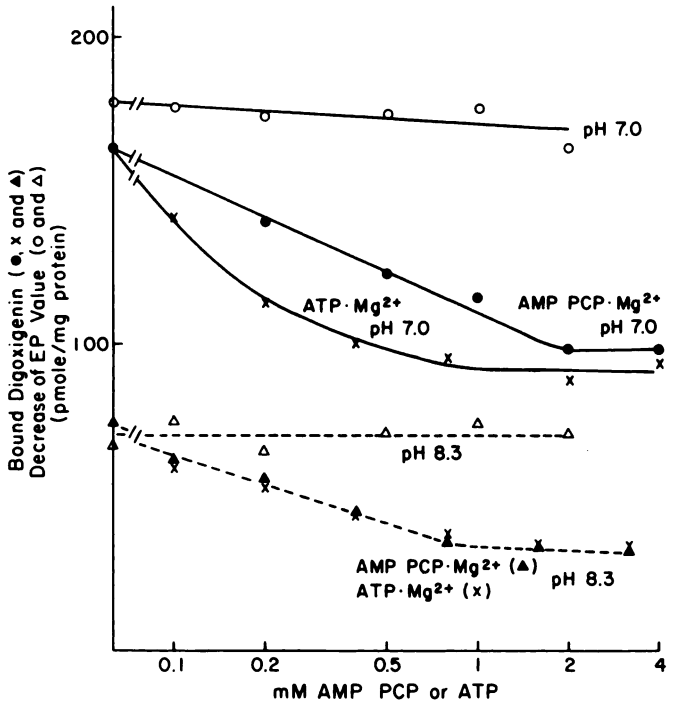


FIG. 14. *Effect of AMP PCP on digoxigenin binding and on the decrease of EP value*

For digoxigenin binding, experimental conditions were the same as in Fig. 11 and 12 except that on the AMP PCP-Mg^{2+} complex was used instead of the various nucleotides. The ATP effects on that binding, which are the same results as in Fig. 11 and 12, are shown also on the absolute scale. These values for binding are the averages of triplicated experiments. For determination of the EP value, 0.5 ml of the reaction mixture, containing the enzyme preparation (240 mg at pH 7.0, or 285 mg at pH 8.3), 50 mM buffer (imidazole-HCl at pH 7.0 or Tris-HCl at pH 8.3), 5 mM Mg^{2+}, 4 mM P_i and various concentrations of AMP PCP were incubated for 2 min at 25°, and then phosphorylated by 0.1 ml of the phosphorylating reagent (the same as in Fig. 7), for 3 sec. The decrease of EP value was calculated from the EP value obtained in the absence of P_i and AMP PCP. The other experimental conditions for EP determination were the same as in Fig. 7. The values for the decrease of the EP value are the averages of quadruplicated experiments.

aglycone in the presence of the Mg^{2+} and P_i was proposed (12) (Fig. 17). From this scheme, the Scatchard plots should represent the following equation, as reported previously:

$$B = \frac{\frac{1}{k_4}+\frac{1}{k_5}}{\frac{1}{k_2}+\frac{1}{k_4}+\frac{1}{k_5}+\frac{1}{k_1[M]}\left(1+\frac{k_{-1}}{k_2}\right)} N - \frac{\frac{1}{k_3}\left(1+\frac{k_{-2}}{k_2}+\frac{k_{-1}k_{-2}}{k_1[M]k_2}\right)}{\frac{1}{k_2}+\frac{1}{k_4}+\frac{1}{k_5}+\frac{1}{k_1[M]}\left(1+\frac{k_{-2}}{k_2}\right)} \frac{B}{F} \quad (1)$$

where $[M] = [Mg^{2+}][P_i]$, B is the total concentration of cardiac aglycone-enzyme complex,

$$\left(B = \left[I \cdot E\begin{matrix}Mg\\ \diagdown\\ \diagup\\ P\end{matrix}\right] + [EI]\right),$$

F is the concentration of the unbound cardiac aglycone ($F = [I]$), and N is the total concentration of the enzyme;

$$N = [E] + [E \cdot Mg^{2+} \cdot P_i] + \left[E\begin{matrix}Mg\\ \diagdown\\ \diagup\\ P\end{matrix}\right] + \left[I \cdot E\begin{matrix}Mg\\ \diagdown\\ \diagup\\ P\end{matrix}\right] + [EI].$$

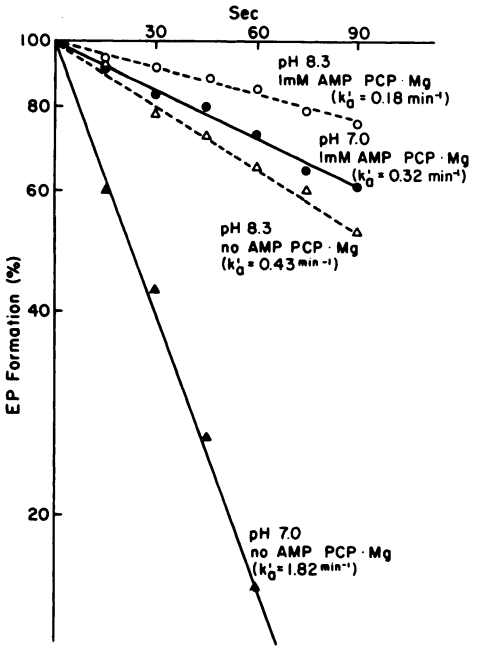


FIG. 15. *Time course of (Na^+ + K^+)-ATPase inhibition by digoxigenin in the presence of AMP PCP-Mg^{2+} complex*

The inhibition was started at 25° by the addition of 0.1 ml of enzyme preparation (145–180 μg protein at pH 7.0, 267 μg protein at pH 8.3), into the 0.4 ml mixture containing 2 μmol Mg^{2+}, 2 μmol P_i, digoxigenin (0.25 nmol at pH 7.0, 1.0 nmol at pH 8.3) and 25 μmol buffer (imidazole-HCl at pH 7.0, Tris-HCl at pH 8.3) with (●, ○) or without (▲, △) 1 mM AMP PCP-Mg^{2+}. Other experimental conditions were the same as in Fig. 7. The pseudo-first-order association rate constant was estimated by least squares.

The present study shows that the Na^+ reduces the N_s in equilibrium, but does not change the K_b (Fig. 5–7). Such Na^+-effects are unexpected from the reaction scheme of ouabain binding representing a reversible reaction (7), but they are similar to those which occur in concentration changes of Mg^{2+} and/or P_i, as reported previously (13). Also, Na^+ seems to reduce the level of [E·Mg^{2+}·P_i] in the scheme. This concept is supported by the results obtain from experiments for the effect of sequence of the Na^+ addition at pH 6.5 (Fig. 10). From these experiments, we may conclude that the Na^+ may react with free enzyme, E, to form the complex NaE as shown in the scheme. All of the enzymes pretreated with the Na^+ alone, the Na^+ plus Mg^{2+} or the Na^+ plus P_i were inhibited with digoxigenin at the same initial rate (line F, in Fig. 10); this rate was significantly lower than that of the enzyme pretreated with the Na^+ plus Mg^{2+} plus P_i (line E). This conclusion about the interaction of the Na^+ and free enzyme also agrees with the results for the phosphorylation of (Na^+ + K^+)-ATPase by the Mg^{2+} and P_i, which was reported by Post *et al.* They reported that, in the presence of 16

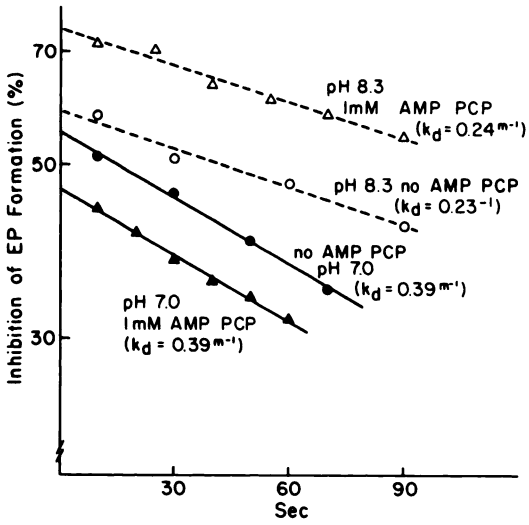


FIG. 16. *Effect of AMP PCP in the dilution medium on the stability of type II digoxigenin complex at 25°*

The enzyme preparation (1.1–1.8 mg/ml) was incubated at 25° in a mixture of 4 mM Mg^{2+}, 4 mM P_i, digoxigenin (0.3 or 0.25 μM at pH 7.0, 2.5, μM at pH 8.3) and 50 mM buffer (imidazole-HCl at pH 7.0, Tris-HCl at pH 8.3). After 30 to 60 min of incubation, the equilibrated mixture (0.1 ml) was diluted with 1 ml of 10 mM buffer (imidazole-HCl at pH 7.0, Tris-HCl at pH 8.3) containing 1 mM EDTA with (▲, △) or without (●, ○) 1 mM AMP PCP, and was phosphorylated at each interval. Other experimental conditions were the same as in Fig. 9. The dissociation rate constants were estimated by least squares.

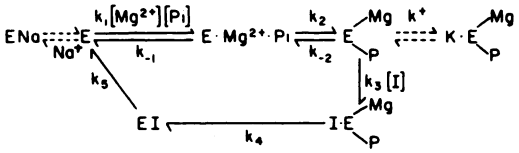
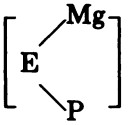


FIG. 17. *Type II binding scheme of (Na^+ + K^+)-ATPase and cardiac aglycone*

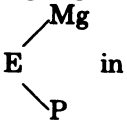
E: free (Na^+ + K^+)-ATPase which is not bound with any ligands. I: cardiac aglycone. The parts shown with solid lines were reported previously (12).

mM Na^+, the phosphorylation by the Mg^{2+} and P_i is abolished (8), but the amount of phosphorylated protein formed by Mg^{2+} and P_i in the absence of the Na^+ decreased gradually after addition of the Na^+ (19).

In contrast to the Na^+, the K^+ changes the apparent binding constant of digoxigenin binding in the Scatchard plots, but does not change the apparent number of binding sites. Unfortunately, a suitable method to estimate the association or dissociation rates of digoxigenin to the enzyme in the presence of the K^+ is not available at the moment, and there is disagreement about the K^+-effect on E_rP formation. Kuriki *et al.* reported that the K^+ enhanced the level of E_rP (18), in contrast to the results reported by Post *et al.* (8). However, the reaction mechanism shown in the scheme may explain the binding results. On equation 1 derived from the scheme, the K_b according to the Scatchard plot is the coefficient of B/F and the N_s is the whole term of N including its coefficient. Therefore, such K^+-effect means that the K^+ changes only the coefficient of B/F, that is, the K^+ changes the k_3 and/or k_{-2}, and such changes of k_3 or k_{-2} may occur if the K^+ interacts with E_rP,  as shown in the scheme. The result reported by Post *et al.* that the phosphorylated enzyme formed by the Mg^{2+} and P_i in the presence of the K^+ cannot bind with ouabain (8) is favorable to this formulary interpretation.

The presence of two different ATP sites on the (Na^+ + K^+)-ATPase is well known. The high affinity site (K_m = 0.2 μM) is for ATP binding and EP formation, and acts as a phosphate donor. Although another low affinity site (K_m = 0.1 mM) is suggested to act as an activator for ATP hydrolysis, there is some disagreement about the function of this site. Post *et al.* (20) reported that ATP activates the dissociation of the K^+ from the enzyme ("E_2 form")-K^+ complex and produces "E_1 form." Simons' study of the K^+-K^+ exchange at red cells supported this idea and indicated that no hydrolysis of phosphate bond occurred at the low affinity site (21). Tonomura's group has presented kinetical evidence that high concentration of ATP increased the EP level and accelerated the "E_1 ATP" to "E_2 ATP" in their scheme (17).

In the present study, the relationship of nucleotide structure to this nucleotide-effect is different from that of the nucleosidase activity (compare Table II in reference 22). Specifically, AMP PCP, the unhydrolyzable ATP homologue, is as active as ATP, so that this nucleotide-effect does not include the splitting of γ-phosphate group in the nucleotide. Moreover, the half-maximum concentration of ATP in this effect, 0.2 mM, is similar to that of ATPase and apparently different from the values of ATP binding or EP formation. Thus, this nucleotide effects seems to be one of the low-affinity effects of ATP and seems to relate to the conformational change of the enzyme similar to the case of the K^+-K^+ exchange at red cells (21).

From the experiments using AMP PCP, inhibition of the type II digoxigenin binding seems to originate from the decrease of the association rate of digoxigenin (Fig. 15) and not from the change of the dissociation rate of the digoxigenin-enzyme complex (Fig. 16), as in the cases caused by the Na^+. However, the level of the E_rP seems not to be changed in the presence of AMP PCP (Fig. 14), in contrast to the cases of the Na^+ reported (8, 18). Another important difference of the nucleotide-effect from the effects of alkali ions is that no triphosphonucleotide can overwhelm the digoxigenin binding perfectly. Such saturation phenomena of the triphosphonucleotide-effects and the decrease of association rate may be explained by assuming the formation of another conformation of phosphorylated protein, which can bind with digoxigenin at a lower association rate than  in the reaction scheme shown in Fig. 17. However, more studies are needed to explain other features of this effect by this scheme.

Although ADP is not as effective as ATP in the K^+-K^+ exchange (23), ADP inhibited the type II digoxigenin binding, in a way similar to that of ATP. If the concentration

is higher than 1 mM, ADP increases the inhibition, even though its increment is reduced, as opposed to the ATP, which shows saturation in this range. The reasons for such differences between ADP and ATP, which may explain the differences between such nucleotide-effect and the change from "E_2 form" to "E_1 form" proposed by Post (20) and Simons (21), remain undiscovered.

In conclusion, the Na^+, K^+, and ATP, the activators of (Na^+ + K^+)-ATPase for ATP hydrolysis, suppress type II digoxigenin binding in different ways. The Na^+ reduces the E_rP level by binding to the free form of the enzyme, while the K^+ binds with the E_rP and suppresses the level of the active form of the E_rP. On the other hand, ATP does not seem to reduce the total amount of E_rP, but changes the conformation of active E_rP, reducing its affinity to a cardiac aglycone.

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