Phosphorylated Intermediates of Na,K-ATPase Proteoliposomes Controlled by Bilayer Cholesterol

INTERACTION WITH CARDIAC STEROID*

Shizuko Yoda and Atsunobu Yoda‡

From the Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706

Fragmental Na,K-ATPase from the electric eel forms three phosphorylated intermediates (EP) with MgATP and Na+: ADP-sensitive K*-insensitive EP (E1P), ADP- and K*-sensitive EP (E2P), and K*-sensitive ADP-insensitive EP (E3P). The EP composition varied with the Na* concentration. In the reconstituted Na,K-ATPase proteoliposomes (PL), the EP composition of the inside-out form was controlled not only by the intravesicular (extracellular) Na* concentration, but also by the temperature and the cholesterol content of the lipid bilayer. When the lipid bilayer of PL contained less than 30 mol % cholesterol, the E3P content did not change significantly while the E2P content increased with an elevation in temperature (3–20 °C). In contrast, when the lipid bilayer contains more than 35 mol % cholesterol, the E1P content increased while the E2P content stayed less than 10% under the same temperature change. These observations suggest that a high cholesterol content in the lipid bilayer interferes with the E3P to E2P conversion. This cholesterol effect was reversed by ionophores (monensin, nigericin, and A23187). Therefore, E1P-rich EP, E2P-rich EP, or E3P-rich EP could be obtained in the PL under a constant Na* concentration by using various concentrations of cholesterol and ionophores. The reaction between the proteolosomal EPs and digitoxigenin (lipid-soluble cardiac steroid) occurred in a single turnover, thereby avoiding unphysiologically high Na* concentrations. The increase in the ADP- and K*-insensitive EP, which indicated formation of the digitoxigenin-Na,K-ATPase complex, was equivalent to the decrease in the E2P under six different sets of conditions, without any significant change in the E1P and E3P contents. This result indicated that E2P is the active intermediate of the Na,K-ATPase for cardiac steroid binding. Although the E2P has been thought to be the active form for binding, it cannot bind with the cardiac steroid in the presence of Na* and in the absence of free Mg2+.

Na,K-ATPase forms phosphorylated intermediates (EP)† by reacting with MgATP and Na* in the absence of K*. Although there are disagreements about the role of these EPs in the presence of Na* and K* (1–3), they are still considered to be the intermediates of the Na,K-ATPase reaction and the Na* transport (4, 5). At least three EPs, an ADP-sensitive K*-insensitive form (E1P), an ADP- and K*-sensitive form (E2P), and a K*-sensitive ADP-insensitive form (E3P), have been recognized in the beef brain (6) and the electric eel enzyme (7). The EP reaction sequence goes as follows: E1P → E2P → E3P, and in the fragmental eel enzyme, E1P was predominant below 60 mM Na*, whereas E2P and E3P were predominant between 60 and 300 mM Na* and above 400 mM Na*, respectively.

The cardiac steroid specifically inhibits the Na,K-ATPase, and it was recently proved that this inhibition mediated the inotropic actions by the low concentrations of ouabain (8). Generally, the Na,K-ATPase inhibition by cardiac steroid has been believed due to some interaction with E2P (9–11). However, the half-maximum Na* concentration for the ouabain binding (20–50 mM) is much higher than that for the E2P formation (less than 5 mM) (12, 13). In the presence of K*, the ATP-dependent ouabain inhibition was decreased as the Na* concentration was increased from 1 to 10 mM (12, 14, 15). These results do not fit the hypothesis of ouabain binding to E3P. Similar discrepancies were also observed in the anthroyl ouabain binding by Lee and Fortes (16). They concluded that the discrepancies indicated the binding of anthroyl ouabain to the third phosphorylated form, which was formed in the presence of rather high Na* concentrations and was different from E1P and E2P. The alternative explanation, however, that the high concentration of Na* accelerated the mutual change of E1P and E2P and/or the association rate of cardiac steroid to the E2P (11) seemed equally valid.

Cholesterol is an important component of the lipid bilayer for the reconstitution of the Na,K-ATPase proteoliposomes (PLs) (17), even though cholesterol contained in the purified kidney enzyme preparation seems to not be essential for Na,K-ATPase activity (18). Recently, Connolly et al. (19, 20) reported the effects of altered bilayer cholesterol contents in the reconstituted sugar transport system. When the eel enzyme is reconstituted into the phospholipid liposomes containing

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‡To whom correspondence should be addressed.

1 The abbreviations used are: EP, phosphorylated intermediate of Na,K-ATPase; E1P, ADP-sensitive K*-insensitive EP; E2P, ADP- and K*-sensitive EP; E3P, K*-sensitive ADP-insensitive EP; E1P, ADP- and K*-insensitive EP; PL, Na,K-ATPase proteoliposome; LCPL, PL containing less than 30 mol % cholesterol in the lipid bilayer; HCPL, PL containing more than 35 mol % cholesterol in the lipid bilayer; CDTA, 1,2-diaminocyclohexanetetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CCCP, carbonyl cyanide m-chlorophenylhydrazine; TEA, triethylamine.
containing cholesterol, the EP of PLs contains more E,P or E*P than that of the fragmental enzyme under the same phosphorylating conditions (7). Furthermore, we have recently found in the PLs that the increase of cholesterol content in the lipid bilayer increased the E*P content and decreased the E,P content under a constant Na+ concentration. In addition, temperature and ionophores also affected the EP composition of PLs under a constant Na+ concentration. These cholesterol and temperature effects on the EP of PLs provide the opportunity to change the EP composition of Na,K-ATPase without changing the Na+ concentration. We have examined the digitoxigenin binding to the PLs, which forms various EP compositions, in the presence of 10 mM Na+ except for one case. From the results obtained, we have concluded that the active phosphorylated intermediate for cardiac stereol binding is E*P and not E,P.

**Materials and Methods**

*Preparation of Na,K-ATPase Proteoliposomes—*The fragmental Na,K-ATPase was prepared from the electric organ of the electric eel, Electrophorus electricus (21), and kept at -80 °C in the presence of 0.25 M sucrose. The specific activity was 20 ± 3 μmol of ADP/min/mg of protein at 37 °C.

In order to remove the potassium cations which interfere with the assays of EP value and Na,K-ATPase from the egg phospholipid (Sigma Type IX-E), the suspension of the lipid (0.25 g/ml) in 20 mM Tris-HCl buffer (pH 7.4) was mixed with a volume of Dowex 50-X8 (Tris form, 20 mesh). After repeating the procedure twice, the lipid was precipitated with 2 volumes of acetone and extracted with CHCl3. The phospholipid was then precipitated with 10 volumes of acetone to remove the neutral lipid. This material was stored at -80 °C as the CHCl3-CH30H (3:1, v/v) solution. The content of potassium cations was checked as follows. The ATPase activity of the fragmental Na,K-ATPase preparation was measured in the Na,K-ATPase assay medium lacking K+ and suspending 1% of the lipid. This ATPase activity should not exceed 5% of the full Na,K-ATPase activity in the optimum assay medium.

From the homogenous mixture of the phospholipid and cholesterol, the translucent liposomes were prepared with sonication. The CHCl3-CH30H (3:1, v/v) solution. The content of potassium cations was checked as follows. The ATPase activity of the fragmental Na,K-ATPase preparation was measured in the Na,K-ATPase assay medium lacking K+ and suspending 1% of the lipid. This ATPase activity should not exceed 5% of the full Na,K-ATPase activity in the optimum assay medium.

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**Digitoxigenin Interaction with EP**—The digitoxigenin binding was examined by two sets of assay systems of EP compositions under various phosphorylating conditions. In one set of the assay system, digitoxigenin (1 final concentration during the reaction with the EP was 0.13 mM) was included in the CDTA solution, while in the other set, digitoxigenin was not. The same batch of PLs was used for these two sets of assays. Each set consisted of both preparations of the EP, ADP-insensitive EP, K+-insensitive EP, ADP-, and K+-insensitive EP, and the control experiment. Average EP values obtained from each triplet assay in the set of experiments can be shown as follows: $E_1$, EP values obtained by the quencher solution without ADP and K+, ADP-insensitive EP-$E_2$, EP values obtained by the quencher solution containing 1.5 mM ADP, K+-insensitive $E_3$, EP values obtained by the addition of the MgCl2 solution, and the same quencher solution as that for the ADP- and K+-insensitive EP at the same time. Calculation is based on the following equations.

$$E_P(\%) = \frac{E_D - E_P}{E_D - E_P - E_P} \times 100$$

$$E_D(\%) = \frac{E_D}{E_D - E_P - E_P} \times 100$$

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Except for the experiments of the digitoxigenin binding, ADP- and K+-insensitive values were used as the control values for the calculation of EP contents as used previously since the EP was less than 5%.

Cholesterol in the proteoliposomes was determined enzymatically by using the "Test Kit for Cholesterol," obtained from Boehringer Mannheim, and the lipid-P was assayed as reported previously (17). All reagents—ATP and ADP were obtained as a sodium salt from Pharmacia P-L Biochemicals and as a dimocyclohexylammonium salt from Sigma, respectively. 15 [gamma-32P]ATP and [22Na]NaCl were purchased from New England Nuclear and Amersham Corp., respectively. CDP, CDP, monomycin, nigericin, A23187, and histidine were obtained from Behring Diagnostics, egg phospholipid, cholesterol, and oligomycin were obtained from Sigma, and TRA-HCl, CDTA, and sodium isethionate were purchased from Aldrich. The TEA-isethionate solution was prepared by titration of isethionic acid solution, which was obtained by passing the TEA-isethionate solution through a Dowex 50 (H+ form) column, with standardized TEA solution. Digitoxigenin was prepared as previously described by hydrolysis of digitoxin (22).

**Results**

The sidedness effect of Na+ on the EP component of PLs was studied, as shown in Table I. When the intravesicular Na+ concentration was 100 mM in the PLs, the E*P content was higher than the E,P content, whereas the extravesicular Na+ concentration was 10 or 100 mM (Table I). When the intravesicular Na+ concentration was 10 mM, the E,P content was always higher than the E*P content. It is apparent that the intravesicular Na+ concentration controls the predominancy of E*P or E,P in the Na,K-ATPase, although the effect of the intravesicular Na+ concentration on the EP components is not as striking in the PL as in the fragmental enzyme. The present study mainly concerns the change of E*P to E,P, and the Na+ concentrations on both sides of PLs are kept as 10 mM unless otherwise cited. Under such conditions, the E,P would have predominated if the barrier effect were ineffective.
However, it was found that the EP composition of the PLs also varied with the cholesterol content in the lipid bilayer, as shown in Fig. 1. In the PLs containing 14 mol % cholesterol, the EP composition of PLs was similar to that of the original fragmental Na,K-ATPase preparation at 15 °C. With the increase of cholesterol content in the lipid bilayer, the EP content was decreased. Furthermore, an abrupt change was observed around 30–35 mol % cholesterol at 15 °C. The PLs containing more than 35 mol % cholesterol in the lipid bilayer (HCPL) formed the phosphoenzyme containing about 60% E*P and 10% E2P at 15 °C, but the PLs containing about 30 mol % cholesterol (LCPL) formed one containing less than 30% E*P and about 60% E2P.

The temperature effects on the EP composition made these differences between the HCPL and the LCPL clearer (Fig. 2). In the HCPL, although the E*P content increased with the elevation of temperature, E2P content was kept at less than 10%. In contrast, the E*P content of the LCPL did not change significantly, whereas the E2P content increased with the elevation of temperature. From these results, the formation of E*P from E*P was less accelerated in the HCPL than the formation of E*P from E2P by the temperature elevation (3–20 °C). In the LCPL, however, the formation of E*P was more accelerated than that of E*P. This difference indicates that the conversion of E*P to E2P is inhibited by high cholesterol content in the lipid bilayer.

It is known that the increase of cholesterol content in the lipid bilayer causes the decrease of membrane permeability of ions through the lipid bilayer (23). To study further cholesterol effects on the EP composition of HCPL, the effects of anions and ionophores were investigated. As shown in Fig. 3, the replacement of Cl− to the nonpermeable anion, isethionate, did not change the EP composition, but the addition of a lipid-soluble anion, SCN−, increased the E2P content. Thus, SCN− seems to accelerate the conversion of E*P to E2P to some extent.

As reported previously, CCCP was as effective as monensin in decreasing the E*P content and in increasing the E2P content at 3 and 15 °C in the LCPL (7). In the present study with the HCPL, CCCP increased the E*P content to some extent at 3 °C but did not affect the EP composition at 15 °C. In contrast, monensin did not affect the EP composition of

<table>
<thead>
<tr>
<th>Na+ concentration (mM)</th>
<th>EP composition</th>
<th>Deviation</th>
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<tbody>
<tr>
<td>Extravesicular side</td>
<td>Intravesicular side</td>
<td>E*P</td>
</tr>
<tr>
<td>10° 10°</td>
<td>6 17 78 ±3</td>
<td></td>
</tr>
<tr>
<td>10 10</td>
<td>15 24 62 ±5</td>
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<tr>
<td>100 100</td>
<td>14 33 53 ±6</td>
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<td>10 100</td>
<td>13 54 33 ±5</td>
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<tr>
<td>100 100</td>
<td>17 48 35 ±10</td>
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<tr>
<td>100° 100°</td>
<td>20 71 9 ±3</td>
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a Fragmental Microsystems.

FIG. 2. Difference of the temperature effect on the EP composition of PLs with the cholesterol contents of the lipid bilayers. A shows the results using the PL which contain 49 mol % cholesterol in the lipid bilayer, and B shows the results using the PLs which contain 28 mol % cholesterol in the lipid bilayer. The experimental procedures used were the same as described in Table 1. The change of the total EP level in each case did not exceed ±5% with the temperature range shown.

FIG. 1. Effect of cholesterol content in the lipid bilayer of PLs on their EP composition. In this experiment, the cholesterol content was assayed after reconstituting the CHAPS-solubilized Na,K-ATPase into the liposomes and represented as the percentage against the mol of cholesterol plus phospholipid. Each point indicates the average of the triplet assays, and the bar indicates the deviation of the obtained value. The solid lines show the results obtained at 15 °C, and the dashed lines show those at 3 °C. The details of the experimental procedures are the same as described in Table I.
the HCPL at 3 °C, whereas it increased the $E_P$ content at 15 °C (Fig. 4). Other ionophores, nigericin, and A23187 increased the $E_P$ content at 15 °C as did monensin (Fig. 5), but this was not the case at 3 °C in the HCPL (data was not shown).

These results are summarized in Table II. We conclude that the temperature elevation and the CCCP addition accelerates the $E_P$ to $E_P$ change of HCPL, whereas the ionophore accelerates the $E_P$ to $E_P$ change of HCPL only at the higher temperatures. This conclusion is also supported by the temperature effect on the EP composition of the HCPL in the presence of A23187 (Fig. 6).

From the obtained results, it became feasible as shown in Table III, to form the $E_P$-rich, $E_P$-rich, or $E_P$-rich $E_P$ without changing Na$^+$ concentration except for one case. Then the interactions of digoxigenin with these $E_P$ were examined. In these experiments, digoxigenin was added with the CDTA solution, which prevents the new formation of $E_P$ by chelating free Mg$^{2+}$, and thus digoxigenin reacted with the already formed $E_P$ in the absence of free Mg$^{2+}$, resulting in the ADP- and K$^+$-insensitive $E_P$ ($E_P$), as in the case of ouabain.$^3$ Therefore, the content of the active $E_P$ for the cardiac steroid binding will be decreased with the digoxigenin interaction, and its decrease will be corresponding to the quencher of the phosphorylation instead of the CDTA solution.

$^3$When 6 mM MgATP (unlabeled) solution was used as the quencher of the phosphorylation instead of the CDTA solution for the assay of $E_P$ components, the decrease in $E_P$ value for 1 s in the absence of ADP and K$^+$ was exceeded by 20% of the total $E_P$ value at 15 °C. Therefore, the estimation of $E_P$ components in the presence of free Mg$^{2+}$ became difficult.
phosphorylation, and the prescribed other ionophores (monensin and A23187) were able to be derived from the increase of EIP. The obtained results clearly indicate the change in E*P composition. The percentages of active Na, K-ATPase Form for ouabain binding were determined by the Na⁺ concentration in the intravesicular medium. In this study, we mainly investigated the effect of the bilayer cholesterol content on the E*P to E2P conversion step. Most of the experiments in this study were performed with 10 mM Na⁺ on both sides of PLs. (Under this condition, the E2P would be predominant if the barrier effects were diminished (7), and the cardiac steroid-EP interaction seems not to be influenced by temperature so much as in the higher Na⁺ concentration (see Fig. 3 in Ref. 11.).)

The EP composition of PL was changed by changing the phosphorylation temperature and the cholesterol content in the lipid bilayer. As shown in Fig. 2, the E*P content in the PLs was decreased with the temperature elevation whether the cholesterol content was high or low. The barrier effect of the lipid bilayer on the E2P to E*P change is not specific to the cholesterol content in the lipid bilayer and disappeared at high temperatures. Since this E2P to E*P change at 3°C was accelerated with CCCP in the HCPL (Fig. 4), as well as in the LCPL (7), it is suggested that this change might involve the proton movement across the lipid bilayer.

The cholesterol effects on the EP composition of PL were more marked at 15°C than at 3°C. Especially, the remarkable change of the EP composition was observed between 30 and 35 mol % cholesterol in the lipid bilayer at 15°C (Fig. 2). In the LCPL containing 28 mol% cholesterol, the E*P to E2P change and the E2P to E*P change both proceeded with the elevation of temperature, but in the HCPL containing 49 mol% cholesterol, only the E2P to E*P change proceeded (Fig. 2). Therefore, the interference on the EP composition seems to be specific in the lipid bilayer containing high cholesterol, compared to the interference with the E*P to E2P conversion.

Although cholesterol is known to be one of the major lipid components of the purified kidney Na,K-ATPase preparation, it seems to be nonessential for the Na,K-ATPase activity in the fragmental enzyme (18). On the other hand, it is known that the increase of the cholesterol content in the lipid bilayer reduces the permeability of ions and neutral substances through the bilayer. This cholesterol effect on the permeability is remarkable in the bilayers of phospholipids containing one saturated and one unsaturated fatty acid chain (23, 24).

In the present study, the main lipid component of the PLs is egg phosphatidylcholine primarily containing one saturated and one unsaturated fatty acid (25). As a result, the present cholesterol effect on the PLs is considered, at least partially, to be derived from the increase of the barrier effect in the lipid bilayer and not from the direct interaction between cholesterol and the Na,K-ATPase molecule.

This hypothesis is supported by the results that the addition of ionophores and a lipid soluble anion (SCN⁻) caused an increase in E*P content and a decrease in E2P content. Since the efficiencies among various ionophores carrying different cations could not be differentiated in the HCPL at 15°C, it is unlikely that the movement of any special cation across the lipid bilayer controls the E2P to E*P conversion. Perhaps the charge movement across the bilayer mediated by the cation-
proton exchange reaction of the ionophore reduces the barrier effect which controls the change of E*P to E,P, although direct experimental evidence to support such a hypothesis is not present.

Many results related to the cholesterol effects on PLs can be explained by the permeability change of the lipid bilayer with cholesterol content as described. However, the abrupt change of the EP composition which occurs between 30 and 35 mol% in the cholesterol content may not be explained by such a simple permeability change because the permeability change of the lipid bilayer is considered to be continuous with the cholesterol content except around the phase transition point. Recently, Melchior's group has reported a similar abrupt change of the sugar transport activity with the change of cholesterol content in the lipid bilayer (19, 20). They insisted that the bilayer fluidity was not a major determinant of the transport activity change and speculated that the potential controlled the membrane transport protein activity.

In the present study, monensin was not effective at 3°C in the HCPL, although it was effective at the same low temperature in the LCPL as reported previously (7). Unlike monensin, CCCP at 3°C effectively counteracted the barrier effect on the E,P to E*P change in the HCPL. This difference between CCCP and monensin seems to be due to an increase in the rigidity of HCPL at 3°C, which reduces the carrier action of monensin which has a larger molecular mass than CCCP. However, this does not explain the inability of CCCP to counteract the barrier effect of high cholesterol on the E*P to E,P conversion. As described in the following paper (26), this E*P to E,P conversion is thought to mediate the release of two Na+ to the intravesicular medium, while the E,P to E*P conversion releases only one Na+. Therefore, a more efficient compensation of the charge difference may be necessary in order to promote the E*P to E,P conversion. The transport of protons by CCCP may be less efficient than that of Na+ by the ionophores and may be insufficient to promote the E*P to E,P conversion.

Apart from the question as to how high cholesterol content controls the E*P to E,P conversion, the barrier effect provides opportunities to invariably produce the E,P-rich, the E*P-rich, or the E*P-rich EP under the same Na+ concentration, while avoiding the presence of the unphysiologically high Na+ concentration.

Sen et al. reported in 1969 (9) that the dephosphorylation rate of EP decreased with ouabain. This EP stabilization by ouabain was not observed in the presence of K+ and the EP from the N-ethylmaleimide-treated Na,K-ATPase preparation was not stabilized by ouabain. Since E,P and E*P were believed to be the main forms of EP and the E*P form was not recognized at that time, the E,P was considered as the active intermediate for ouabain binding. It was later observed that the half-saturated concentration of Na+ for the ouabain association was much higher than that for the phosphorylation (13, 14) and that the electric eel enzyme bound with ouabain efficiently in the presence of 100 mM Na+ wherein the ADP-sensitive EP was predominant (11). But it was concluded that the conversion of E,P to E,P by ouabain would be accelerated by the presence of high concentration of Na+ (100 mM Na+). After Nerby et al. (6) proposed the presence of the third phosphorylated intermediate between E,P and E*P in 1983, Lee and Fortes (16) suggested the possible presence of the similar intermediate between E,P and E*P. They demonstrated that the third intermediate has higher affinity to anthroyl ouabain than E,P or E*P from the anthroyl ouabain binding to the kidney Na,K-ATPase in the presence of various concentrations of Na+. In order to support such a conclusion, they proved that the Na-ATPase activity curve and the anthroyl ouabain-association rate curve against the Na+ concentration were identical. They also showed that the optimal Na+ concentration for these reactions was 0.4 M different from the optimum Na+ concentration for the E,P formation (2 M).

The present experiment investigating the digitoxigenin interaction takes a different approach. In contrast to their experiments using anthroyl ouabain, it is a single turnover experiment. Using a 10 mM Na+ solution on both sides of the PLs in most experiments can eliminate the complexity of the effect of Na+ concentration. Even under the condition of the single turnover, it would be problematic if two or more intermediates were to have similar activities. Fortunately, the results obtained under various conditions (Fig. 7) indicate that E*P is the single active intermediate in the absence of free Mg2+ (10). In the present experiment, digitoxigenin, a lipid-soluble cardiac steroid, must be used instead of ouabain because only the inside-out form of the PL is phosphorylated, and the binding site of the cardiac steroid is located on the intravesicular (extracellular) side. Since our previous studies indicated that the cardiac glycoside (ouabain, etc.) bound to the Na,K-ATPase with its steroid moiety at first, resulting in the binding with the sugar moiety (13), the active intermediate of Na,K-ATPase for the cardiac glycoside binding is considered to be the same as that for the cardiac steroid binding, namely the E*P.

Cardiac glycosides and steroids bind to the Na,K-ATPase in the Mg-, system with highest rates as well as in the Na-Mg-ATP system. In the presence of Mg2+, P binds to the enzyme with an affinity about 0.1–0.6 mM (27, 28), which is the same for phosphorylation (29). This phosphorylated product of Na,K-ATPase has been speculated to be E,P and the active intermediate for the ouabain binding in the Mg2+-P system. In the ouabain binding, this affinity of P, for phosphorylation increased very much to about 6 μM, but sodium inhibited this ouaiba binding (the half-maximum concentration of Na+ is about 4 mM (26)). Since the two types of the ouabain-Na,K-ATPase complexes can be distinguished by their dissociation rates (13, 21), the active intermediate for the ouabain binding in the Mg-, system and the second type of the ouabain-enzyme complex as in the Mg-P, system may be different from that in the Mg-Mg-ATP system, E*P. However, it still remains unresolved whether the E,P can bind with ouabain in the presence of Na+ and form the same ouabain-enzyme complex as in the Mg-, system because the E,P has not been obtained in the presence of Na+ at the present.

Furthermore, the conclusion that E*P is the only active cardiac steroid binding form in the Na-Mg-ATP system provides solid evidence that the E*P is different from the E,P or E,P.

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Active Na,K-ATPase Form for Ouabain Binding
Active Na,K-ATPase Form for Ouabain Binding