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INHIBITION OF OUABAIN-BINDING TO ($Na^+ + K^+$)ATPase BY ANTIBODY AGAINST THE CATALYTIC SUBUNIT BUT NOT BY ANTIBODY AGAINST THE GLYCOPROTEIN SUBUNIT

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Summary

Antibodies against purified ($Na^+ + K^+$)ATPase from the rectal gland of *Squalus acanthias*, as well as against its catalytic subunit, inhibited ouabain binding by as much as 50%. However, antibodies against the glycoprotein subunit did not inhibit ouabain binding. These data suggest that binding of antibody against the catalytic subunit to the enzyme either covers the ouabain binding site or destroys its conformation, while binding of antibody against the glycoprotein has no such effect.

Introduction

It is now generally agreed that the ($Na^+ + K^+$)ATPase (ATP phosphohydrolase, EC 3.6.1.3) consists of a catalytic subunit and a glycoprotein subunit which have molecular weights of approx. 100 000 and 50 000, respectively [1—3]. The stoichiometric ratio of the catalytic subunit to the glycoprotein appears to be 1 : 1 with a quaternary structure of $\alpha_2\beta_2$ [1,4—7]. We previously reported that the ($Na^+ + K^+$)ATPase is inhibited by antibodies against the holoenzyme and against its glycoprotein subunit in an antibody concentration-dependent manner [8]. This indicated that the glycoprotein is an integral component of the ($Na^+ + K^+$)ATPase.

Photoaffinity labeling studies with ethyl diazomalonyl derivative of cymarin

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Abbreviation: SDS, sodium dodecyl sulfate.

[9] and affinity labeling with oxidized ouabain [10] or 2-nitro-5-azidobenzoyl ouabain [11] indicate that the binding site of cardiac glycosides resides on the catalytic subunit. In this communication, we show that ouabain binding to the $(Na^+ + K^+)$ATPase is inhibited by antibodies against the holoenzyme and against the catalytic subunit but not by antibodies against the glycoprotein subunit. These data suggest that binding of antibody against the catalytic subunit to the enzyme either covers the ouabain binding site or destroys its conformation, while binding of antibody against the glycoprotein has no such effect.

Materials and Methods

$(Na^+ + K^+)$ATPase was purified from the rectal gland of *Squalus acanthias*, as described previously [2,12]. The catalytic and glycoprotein subunits were isolated in homogeneous form by preparative SDS-polyacrylamide gel electrophoresis from the enzyme from *S. acanthias* [13]. The immunization procedure and the dialysis procedure for antisera have already been described [8]. Ouabain was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) and randomly labeled [^{3}H]ouabain (13 Ci/mmol) was obtained from New England Nuclear Corporation (Boston, MA). Ouabain binding was measured by a method similar to that of Matsui and Schwartz [14]. Unless otherwise indicated, 80—100 μg $(Na^+ + K^+)$ATPase were incubated with 10^{-5} M [^{3}H]-ouabain (18 Ci/mol) in 2 ml binding medium (5 mM $MgCl_2$, 110 mM NaCl and 50 mM imidazole buffer (pH 7.0)) at 37°C. The ouabain binding reaction was started by the addition of 5 mM ATP at 37°C, and incubation was carried out for 10 min. The reaction was terminated by cooling the incubation vessels in ice-cold water and centrifuging at 100 000 × *g* for 1 h. Nonspecific binding of ouabain was determined in the presence of 10^{-4} M non-labeled ouabain. When nonspecific binding was measured, the specific activity of [^{3}H]ouabain was increased up to 910 Ci/mol, depending on the amount of $(Na^+ + K^+)$ATPase used. Ouabain binding was calculated by subtracting the nonspecific binding from the total binding of ouabain. The effects of various antisera on the binding of ouabain to the $(Na^+ + K^+)$ATPase were studied by substituting 0.7 ml binding medium with 0.7 ml dialyzed antiserum. Preincubation of $(Na^+ + K^+)$ATPase with antisera was carried out at room temperature for 30 min before initiation of the binding reaction with ATP or other ligands. After centrifugation at 100 000 × *g* for 1 h, the pellet was dissolved in 0.2 ml 0.5 M NaOH by heating in a water bath at 80°C. After neutralization by addition of 0.2 ml 0.5 M HCl, an aliquot was counted in 5 ml scintillation counting solution (Packard Instagel).

Results

Dependence of ouabain binding on ATP

Omission of ATP from the complete binding system reduced binding to about 10% of that with the complete system (Table I). Addition of 10^{-4} M non-labeled ouabain also reduced the binding of radioactive ouabain to the level observed in the absence of 5 mM ATP. This indicates that nonspecific

TABLE I

SPECIFIC AND NONSPECIFIC BINDING OF [^{3}H]OUABAIN TO THE ($Na^+ + K^+$)ATPase FROM THE RECTAL GLAND OF THE SPINY DOGFISH SHARK, *SQUALUS ACANTHIAS*

87 μg purified ($Na^+ + K^+$)ATPase from the rectal gland were incubated with 10^{-6} M ouabain ($2.0 \cdot 10^7$ dpm/μmol) in 2 ml binding medium. [^{3}H]Ouabain binding was initiated by the addition of 5 mM ATP followed by incubation at 37°C for 10 min. The reaction was terminated by cooling in ice-chilled water and centrifuging at 100 000 × *g* for 1 h. The pellet was dissolved in 0.2 ml 0.5 M NaOH at 80°C.

Conditions	[^{3}H]Ouabain bound (pmol)
Complete system	225 ± 0.65 *
$+10^{-4}$ M ouabain	24.6 ± 0.63
—5 mM ATP	21.6 ± 0.69
—($Na^+ + K^+$)ATPase	31.6 ± 0.71

* Mean ±S.E. Each value is the average of at least three determinations.

binding of ouabain was quite small. When the entire procedure was carried out in the absence of added ($Na^+ + K^+$)ATPase, the radioactivity was the same as that observed in the experiments in which no ATP was added. It should be pointed out that under the conditions of our experiments, 10^{-6} M ouabain produced about half-saturation of the ouabain binding sites. Except for the experiments of Table I, 10^{-5} M ouabain was used for binding studies.

Effect of various antisera on ouabain binding

Table II summarizes the effects of various antisera on ouabain binding in the presence and absence of 10^{-4} M nonradioactive ouabain. The maximum binding of ouabain in the complete binding system with 10^{-5} M ouabain under control experimental conditions was approx. 4.4 nmol ouabain/mg ($Na^+ + K^+$)ATPase protein. Nonspecific binding of ouabain measured in the presence of 10^{-4} M non-labeled ouabain was usually less than 0.5 nmol/mg protein. Addition of preimmune serum did not affect the binding of ouabain to the ($Na^+ + K^+$)-ATPase in the presence or in the absence of 10^{-4} M nonradioactive ouabain. Antiserum against the shark ($Na^+ + K^+$)ATPase holoenzyme inhibited specific

TABLE II

EFFECTS OF VARIOUS ANTIBODIES ON MAXIMUM BINDING OF [^{3}H]OUABAIN TO RECTAL GLAND ($Na^+ + K^+$)ATPase

0.7 ml various antisera was preincubated with 10 μg rectal gland ($Na^+ + K^+$)ATPase in 2 ml ouabain binding medium at room temperature for 30 min. The binding reaction was initiated by addition of ouabain and 5 mM ATP, and incubation was carried out at 37°C for 10 min.

Additions	[^{3}H]Ouabain bound (pmol/mg protein)	
	without 10^{-4} M ouabain	with 10^{-4} M ouabain
None	4460 ± 61 *	357 ± 37
Preimmune serum	4380 ± 46	413 ± 31
Antibodies against holoenzyme	1840 ± 46	382 ± 20
Antibodies against catalytic subunit	2850 ± 62	357 ± 10
Antibodies against glycoprotein	4280 ± 62	397 ± 15

* Mean ±S.E. Each value is an average of at least three determinations.

ouabain binding to the enzyme by 50%. Ouabain binding to the $(Na^+ + K^+)$-ATPase was also inhibited significantly by antibodies against the catalytic subunit. However, antibodies against the glycoprotein did not inhibit ouabain binding. The fact that antiserum directed against the catalytic subunit inhibited ouabain binding, but antiserum directed against the glycoprotein did not, provides independent support for the photoaffinity studies which suggest that the cardiac glycoside binding site resides on the catalytic subunit.

Discussion

The salient observation of the present study is that antibodies against the catalytic subunit, but not antibodies against the glycoprotein, inhibit ouabain binding to the purified $(Na^+ + K^+)$ATPase. This supports the photoaffinity studies which show that the ouabain binding site resides on the catalytic subunit. Earlier studies showed that antibodies against the catalytic subunit or the glycoprotein formed strong precipitation bands with the enzyme and inhibited $(Na^+ + K^+)$ATPase activity (Ref. 8 and unpublished data). The inhibition of ouabain binding could be due to steric hindrance [15] by the bound antibody or possibly due to a conformational change in the $(Na^+ + K^+)$ATPase induced by antibody which markedly reduces the affinity of the ouabain binding site [16].

It is of interest that ouabain binding was inhibited only 50% by antibody. This is similar to the inhibition of catalytic activity [8]. It is possible that there are two classes of ouabain binding sites and that only one class is accessible to antibody. This may relate to the data of Charnock et al. [17] which suggested that the ouabain binding site may exist in the membrane in at least two environments.

After this work was completed, Jean and Albers [18] reported in a preliminary note that antibody against the glycoprotein, but not that against the catalytic subunit, inhibited ouabain binding. Conversely, antibody against the catalytic subunit, but not that against the glycoprotein, inhibited phosphorylation of the enzyme by ATP in the presence of Mg^{2+} and Na^+. Our data are more consonant with photoaffinity labeling with cardiac glycoside derivatives [9,10] which indicate that the ouabain binding site is on the catalytic subunit. The discrepancy between the observations of Jean and Albers [18] and ourselves may be due to the fact that the former authors used Lubrol extracts of electroplax of *Electrophorus electricus* which had specific activities of only 5—10% of our purified enzyme used here. It is of interest, however, that they also found only approximately half-inhibition of ouabain binding by antibody.

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