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Covalent Labeling of the Digitalis-Binding Component of Plasma Membranes

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SUMMARY

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Oxidized [^{3}H]ouabain was prepared and its binding to renal plasma membranes was studied. More than 90% of the oxidized ouabain was bound by the (Na^+ + K^+)-ATPase (EC 3.6.1.3) component of the membranes. This binding required the simultaneous presence of Na^+, Mg^{++}, and ATP. K^+ inhibited binding. No binding took place in the absence of Mg^{++}. Oxidized [^{3}H]ouabain was covalently attached to the membranes by reducing the ouabain-membrane complex with $NaBH_4$ at pH 9.0. The covalent nature of this binding was evident from its stability against dissociation by acid, 90% methanol, or heating. In the presence of Na^+, ATP, and Mg^{++} oxidized [^{3}H]ouabain was bound preferentially by an electrophoretically single membrane protein. This protein had an apparent molecular weight of 89,000 as estimated by polyacrylamide gel electrophoresis, and it is thought to be part of (Na^+ + K^+)-ATPase because (a) it bound ouabain in the presence of Na^+, Mg^{++}, and ATP but not in the presence of Tris-EDTA, and (b) it could be phosphorylated by [^{32}P]ATP in the presence of Na^+ and Mg^{++} but not in the presence of K^+ and Mg^{++}.

INTRODUCTION

Cardioactive digitalis glycosides, such as ouabain, specifically inhibit (Na^+ + K^+)-ATPase (EC 3.6.1.3), an enzymatic component of the Na^+ pump (1). This inhibition is related to the positive inotropic action of these drugs by an unknown mechanism (2, 3). The enzyme undergoes a cycle of phosphorylation, in which the γ-phosphate of ATP is transferred to and then released from the active center (4, 5). Na^+ and Mg^{++} stimulate this phosphorylation; on the other hand, K^+ enhances dephosphorylation. Ouabain binds preferentially to a transient phosphorylated form of the enzyme (3, 6). *In vitro*, Mg^{++} and P_i and to a lesser extent Mg^{++} alone can support ouabain binding, but the significance of these bindings *in vivo* is not clear (3, 7). Since all but one of the reported purified preparations of (Na^+ + K^+)-ATPase contain at least two subunits (8–10), it became an important issue to decide which component(s) of the enzyme binds digitalis. Ouabain-binding particles were isolated from different tissues (11–14), but the lability of the ouabain-enzyme complex in the presence of disrupting agents hampered characterization of the binding protein. Ruoho and Kyte (15) overcame this difficulty by photoaffinity labeling, and they have

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shown that in a highly purified (Na^+ + K^+)-ATPase the "large subunit" binds ouabain.

The goal of the present investigation was to find out whether (Na^+ + K^+)-ATPase is the only membrane protein that binds digitalis glycosides and, if so, which of its subunits is the binding protein. By affinity labeling one component of renal plasma membranes was identified as the main binding protein for ouabain. Functional tests indicated that this protein was the alleged large subunit of (Na^+ + K^+)-ATPase.

METHODS

[γ-^{32}P]ATP was synthesized enzymatically by the procedure of Post and Sen (16). Plasma membranes, rich in (Na^+ + K^+)-ATPase, were prepared from the red outer medulla of sheep kidney (17) according to Post and Sen (16) and further extracted with 2 M NaI, by a modification of the method of Nakao *et al.* (18, 19).

Ouabain was oxidized essentially by the procedure of Smith *et al.* (20). To 200 nmoles of [^{3}H]ouabain (1 μCi/nmole) solid $NaIO_4$ was added to a final concentration of 50 mM. After 30 min at room temperature, 3 μl of ethylene glycol were added to stop further oxidation by reacting with the unused iodate. Five minutes later the whole reaction mixture was filtered through an anion-exchange resin (Dowex 1-X1, chloride form) and the effluent was concentrated in a rotary evaporator. Oxidized [^{3}H]ouabain was then purified by thin-layer chromatography on silica gel G (21). By developing the plates with chloroform–ethanol (2:1) native ouabain stayed practically at the origin (R_F = 0.03). When ouabain was oxidized, another spot appeared ($R_F \cong 0.75$). This second fraction was eluted with 50% aqueous ethanol and used as oxidized ouabain in these experiments; it contained 60–70% of the original radioactivity.

Membranes were phosphorylated with [γ-^{32}P]ATP at 0° and pH 7.5 in the presence of either 100 mM NaCl and 1 mM $MgCl_2$ or 100 mM KCl and 1 mM $MgCl_2$ by the procedure of Post and Sen (16).

Binding of native or oxidized [^{3}H]ouabain was measured according to Matsui and Schwartz (22). The experimental sample contained [^{3}H]ouabain or oxidized [^{3}H]ouabain (concentrations are given in the legends to tables and figures), 20 μmoles of NaCl, 2 μmoles of Na_2ATP, 2 μmoles of $MgCl_2$, 50 μmoles of Tris-Cl (pH 7.5 or 9.0), and 0.1–0.5 mg of membrane protein; the final volume was 1.0 ml. Nonspecific binding was measured in an 1-ml control sample containing 10 μmoles of Tris-EDTA, 1 μmole of unlabeled ouabain, 50 μmoles of Tris-Cl (pH 7.5 or 9.0), 0.3–0.5 mg of membrane protein, and the radioactive substrate. Both samples were incubated at 37° for 60 min, then centrifuged, dissolved, and counted. "Specific binding" refers to the difference in the binding of either substrate between the experimental and control samples. About 30% of Na_2ATP was split in 60 min.

For affinity labeling 1 ml of the reaction mixture contained 0.5–1.0 mg of membrane protein, 20 μmoles of NaCl, 1 μmole of Na_2ATP, 1 μmole of $MgCl_2$, 0.5–1 nmole of oxidized [^{3}H]ouabain, and 50 μmoles of Tris-Cl (pH 9.0). In a control sample 10 μmoles of Tris-EDTA replaced NaCl, Na_2ATP, and $MgCl_2$. These mixtures were incubated at 37° for 2 hr, then cooled to 0°, and the unbound oxidized [^{3}H]ouabain was washed off with ice-cold 10 mM Tris-Cl (pH 9.0) by repeated centrifugation and resuspension of the membranes. After washing, the membranes were resuspended at 0° in the original media, but *without* oxidized [^{3}H]ouabain, and solid $NaBH_4$ was added to a final concentration of 0.05 M. After 6–8 hr at 0°, the membranes were washed three times with 20 mM imidazole HCl (pH 7.2) and finally were stored in this buffer at 0°.

For measuring total ATPase activity, the incubation medium (final volume, 1 ml) contained 100 μmoles of NaCl, 20 μmoles of KCl, 3 μmoles of $MgCl_2$, 2 μmoles of Tris-ATP, 20 μmoles of imidazole HCl (pH 7.4), and 20–30 μg of membrane protein. For measuring (Na^+ + K^+)-insensitive ATPase, this medium contained 1 μmole of ouabain in place of NaCl and KCl. The difference in the amount of inorganic phoshate liberated from ATP between these two media was taken as (Na^+ + K^+)-ATPase activity. Inorganic phos-

phate was measured by the method of Goldenberg and Fernandez (23). One unit of enzyme split 1 μmole of ATP per minute at 37°.

Protein was measured by Miller's modification (24) of the method of Lowry *et al.* (25).

For separation by polyacrylamide gel electrophoresis, membrane proteins were dissolved at room temperature either with 2% sodium dodecyl sulfate and 2% 2-mercaptoethanol in 0.06 M Tris-Cl (pH 6.8) or with 2% sodium dodecyl sulfate, 2% 2-mercaptoethanol, and 6 M urea in 0.12 M Tris-citrate (pH 2.9). In the first case the membranes were separated on a discontinuous gel (9) (stacking gel, 3% polyacrylamide, pH 6.8; running gel, 8% polyacrylamide, pH 8.8); in the second case the membranes were separated on a 6% polyacrylamide gel at pH 2.9 (26). The temperature was 18°. At the end of the electrophoresis one of two duplicate gels was sliced, the 1-mm slices were dissolved in 0.2 ml of 30% H_2O_2 at 50° overnight, and the radioactivity was determined by liquid scintillation counting. (At least 6000 counts were accumulated, and external standardization was used to correct for quenching.) The other gel was stained with Coomassie blue to visualize protein bands. Transferrin, serum albumin, ovalbumin, and ribonuclease A served as molecular weight standards. The tracking dye was either Pyronine Y (at pH 2.9) or bromphenol blue (at pH 8.8).

RESULTS

The binding of oxidized ouabain to plasma membranes was similar to that of native ouabain (Table 1). Thus maximal binding of oxidized ouabain required Na^+, Mg^{++}, and ATP; K^+ decreased binding, and over 90% of oxidized [^{3}H]ouabain could be displaced from its binding site by unlabeled native ouabain. In addition, the binding of oxidized ouabain was also temperature- and time-dependent (not shown). The stoichiometric relationship of ouabain binding to phosphorylation was nearly 1:1 (68.5 and 66.0 pmoles/mg of protein, respectively). By assuming that 1 mole of ouabain binds to 1 mole of enzyme, the turnover number would be about 9800/min.

TABLE 1

Effects of ligands on binding of native or oxidized ouabain to plasma membranes

Binding of [^{3}H]ouabain or oxidized [^{3}H]ouabain was measured at pH 7.5 as described in METHODS. The final concentration of either substrate was 1 μM, and each sample contained 0.130 mg of membrane protein. Ligands were present at the concentrations indicated in the table. Note that oxidized [^{3}H]ouabain was not bound covalently in these experiments. These membranes had a (Na^+ + K^+)-ATPase activity of 0.67 unit/mg of protein, and specific phosphorylation was 66 pmoles of [^{32}P]phospho-protein per milligram of membrane protein with 0.05 mM [^{32}P]ATP as substrate.

Ligands (mM)	Substrate bound	
	[^{3}H]Ouabain	Oxidized [^{3}H]ouabain
	pmoles/mg protein	
NaCl (100), $MgCl_2$ (5), Na_2ATP (5)	68.5	35.7
KCl (100), $MgCl_2$ (5), Na_2ATP (5)	13.8	7.3
NaCl (100), $MgCl_2$ (5) Na_2ATP (5), ouabain (1.0)	2.2	1.7
Tris-EDTA (10), ouabain (1.0)	1.8	1.6

This value is in agreement with similar data reported previously (1). Phosphorylation and ouabain binding were nearly maximal in these experiments, since the K_m for ATP is less than 1 μM (3) and the K_m for ouabain was 0.12 μM (not shown). The membranes bound about half as much oxidized ouabain as native ouabain. Oxidized ouabain probably did not saturate the binding sites, because they have lower affinity for this substrate (27).

After sodium borohydride reduction at pH 9.0, the membrane–oxidized ouabain complex became largely resistant to heating or to extraction with acid or 90% methanol, although these treatments dissociated native, noncovalently bound ouabain (Table 2 and ref. 28). Without borohydride reduction, the membrane–oxidized ouabain complex was less resistant to these treatments (not shown), although oxidized ouabain was probably covalently bound via Schiff bases (see DISCUSSION). These

findings indirectly indicate that after borohydride reduction oxidized ouabain was bound to the membranes through a relatively stable covalent bond. (Borohydride reduction of the membranes *before* the addition of ouabain abolished binding activity.)

The specificity of the reaction between oxidized ouabain and the membranes is shown by the observation that 9–10 times more oxidized ouabain was bound to the membranes in the presence of Na^+, Mg^{++}, and ATP than in the presence of Tris-EDTA (in a typical experiment, 23.6 and 2.5 pmoles/mg of protein, respectively). This extra ouabain was bound to an electrophoretically single protein with an apparent molecular weight of 89,000 as estimated from its mobility (Fig. 1). When EDTA was the only ligand at labeling, a small amount of radioactivity appeared in some bands, but no single band was labeled preferentially. Some radioactivity, which did not enter the gels, was probably bound to incompletely solubilized proteins or represented unbound oxidized [^{3}H]ouabain that did not penetrate the gel either. Part of the radioactivity migrated with the tracking dye, probably bound to lipids (or proteolipids) since it could be extracted with chloroform–methanol (2:1). If the *native* [^{3}H]ouabain–membrane complex, formed in the presence of NaCl, $MgCl_2$, and ATP, was dissolved with sodium dodecyl sulfate and subsequently applied to polyacrylamide gel for electrophoresis, none of the radioactivity entered the gel (not shown).

To investigate the relationship of the ouabain-binding membrane protein to

TABLE 2

Stability of adsorptive and covalent binding

[^{3}H]Ouabain (final concentration, 1 μM) was bound noncovalently, and oxidized [^{3}H]ouabain (final concentration, 0.5 μM) was bound covalently, to plasma membranes. These binding procedures and the definition of "specific binding" are described in METHODS. Binding of either substrate was complete before washing with acid, extraction with methanol, or heating. In experiment A the membranes were washed three times with 5% trichloracetic acid (TCA) by centrifugation and resuspension at 0°; in experiment B the membranes stood in 90% methanol at room temperature for 30 min; and in experiment C the membranes were incubated at 50° for 30 min. After each treatment the membranes were sedimented, washed once with 10 mM imidazole HCl (pH 7.4), and then dissolved to measure bound radioactivity.

Treatment	Specific binding	
	[^{3}H]Ouabain	Oxidized [^{3}H]ouabain
	pmoles/mg protein	
Experiment A		
None	174.0	73.5
Washing with 5% TCA	1.8	51.5
Experiment B		
None	104.0	54.7
90% methanol for 30 min	2.8	49.4
Experiment C		
None	122.0	40.5
50° for 30 min	10.3	39.8

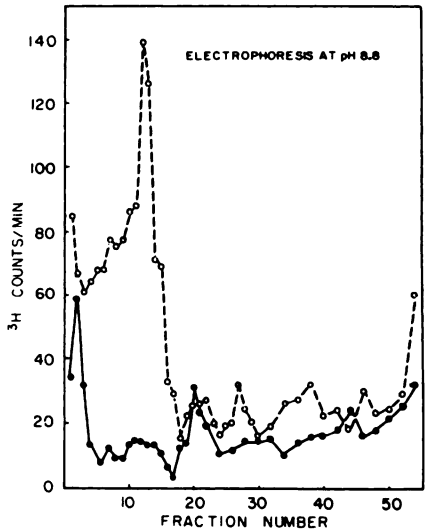

FIG. 1. *Separation of membrane proteins after affinity labeling with oxidized [^{3}H]ouabain*

[^{3}H]Ouabain (1 μM) was covalently bound to membranes in the presence of either Na^+, Mg^{++}, and ATP (O- - -O) or EDTA (●——●) as described in METHODS. Covalent labeling amounted to 23.6 pmoles/mg of protein in the first and to 2.5 pmoles/mg of protein in the second medium. The labeled membranes were dissolved at room temperature with 2% sodium dodecyl sulfate and 2% 2-mercaptoethanol in 0.06 M Tris-Cl (pH 6.8) and were separated on a discontinuous polyacrylamide gel (19). Onto each gel 84 μg of protein were charged, and 1-mm-thick gel slices were counted.

$(Na^+ + K^+)$-ATPase, the electrophoretic mobility of the ouabain-labeled protein was compared with that of the phosphorylated component ("large subunit") (8, 9) of $(Na^+ + K^+)$-ATPase (Fig. 2). The two mobilities were identical within the experimental error. In this experiment membrane proteins were separated at pH 2.9 to retard hydrolysis of the [^{32}P]phospho-enzyme. In the phospho-enzyme phosphate is bound to an aspartyl residue as acyl-phosphate, which is most stable at low pH (5). In addition, dissolution in sodium dodecyl sulfate also seemed to stabilize the phospho-enzyme. As a result, more than 90% of the initial radioactivity was still bound to proteins at the end of the electrophoresis.

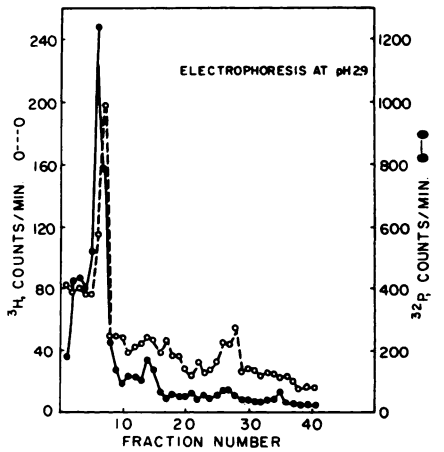

FIG. 2. *Electrophoresis of membrane proteins labeled with [γ-^{32}P]ATP or with oxidized [^{3}H]ouabain*

Membranes were phosphorylated with 40 μM [γ-^{32}P]ATP for 10 sec in the presence of either 100 mM NaCl and 1 mM $MgCl_2$ or 100 mM KCl and 1 mM $MgCl_2$ at pH 7.5. Phosphorylation was stopped with cold 5% $HClO_4$ containing 0.5 mM Na_2ATP and 10 mM H_3PO_4, and the membranes were washed three times with the same acidic mixture by centrifugation and resuspension. The washed membranes were dissolved with 2% sodium dodecyl sulfate, 2% 2-mercaptoethanol, and 6 M urea in 0.12 M Tris-citrate (pH 2.9) at 20°. Fifty micrograms of membrane protein were added to each gel column [6% polyacrylamide (26)]. Membranes labeled in the presence of Na^+ or K^+ were separated on two different columns. The *difference* in counts between corresponding 1-mm slices is plotted (●——●). Similarly, membranes were labeled with 0.5 μM oxidized [^{3}H]ouabain in the presence of either Na^+, Mg^{++}, and ATP or Tris-EDTA (see METHODS) and these two samples were placed on two different gels. Again the *difference* in counts between corresponding fractions is shown (O– – –O). The ouabain-labeled membranes were also washed with $HClO_4$ and dissolved in the same way as the phosphorylated membranes before electrophoresis. Onto each gel 60 μg of ^{3}H-labeled membranes were charged. Molecular weight standards (transferrin, serum albumin, ovalbumin, and ribonuclease A) were dissolved like the membranes, but were not precipitated with $HClO_4$ beforehand.

DISCUSSION

Originally Erlanger and Beiser (29) proposed periodate oxidation of ribose in nucleotides or nucleosides for the covalent binding of these oxidized derivatives to proteins as haptens. Following this principle Smith *et al.* (20) coupled digoxin via oxidized digitoxose to serum albumin and produced antidigoxin antibodies of high affinity (ref. 30 summarizes this and similar procedures). Periodate oxidized vicinal hydroxyl groups in the sugar portion of the digitalis glycoside (rhamnose in ouabain) to dialdehydes. These aldehyde groups form Schiff bases with primary amino groups of proteins at high pH (pH 9.0 in these experiments). These Schiff bases are reduced with borohydride to stable secondary amines. This mechanism, although not proved directly, would indicate the presence of a primary amino group(s) at the site where the sugar portion of ouabain is bound. Even in a crude membrane preparation, bound ouabain formed a single peak on gel electrophoresis (Fig. 1). The protein in this peak binds about 90% of ouabain attached to the membranes. Several pieces of evidence indicate that this protein is part of $(Na^+ + K^+)$-ATPase: (a) it is phosphorylated by [γ-^{32}P]ATP in the presence of Na^+ and Mg^{++} but not in the presence of K^+ and Mg^{++} (Fig. 2); (b) this phosphorylation stimulates the binding of oxidized and native ouabain (Table 1); (c) oxidized ouabain can be displaced by native ouabain, which is a specific inhibitor of the enzyme (Table 1); (d) the apparent molecular weight of the ouabain-binding protein (89,000) was similar to the reported molecular weight of the "large subunit" of the enzyme (8, 9) when both were determined by electrophoresis. To enhance the selectivity of labeling only low concentra-

tions of oxidized ouabain (less than 1 μM) and only membranes rich in ($Na^+ + K^+$)-ATPase (more than 1 unit/mg of protein) were used. Under these conditions the molar ratio of free oxidized ouabain to enzyme was between 5:1 and 10:1.

These results confirm an earlier observation (15) that apparently only the large subunit of ($Na^+ + K^+$)-ATPase binds digitalis glycosides. No other membrane protein, including the alleged small subunit of the enzyme, was labeled significantly by oxidized ouabain. It has to be pointed out, however, that the oxidized ouabain was attached to the binding protein through its carbohydrate moiety and therefore its steroid portion might still bind to the small subunit. This idea would be consistent with the proven proximity of the two subunits (8) and with models of the digitalis binding sites based on kinetic evidence (31, 32). On the other hand, the small subunit may not bind digitalis at all, since a highly purified ($Na^+ + K^+$)-ATPase contained only one type of polypeptide (by its molecular weight the large subunit), yet it was inhibited by ouabain (10).

($Na^+ + K^+$)-ATPase is phosphorylated intracellularly (33) and binds digitalis glycosides extracellularly (34). These two reactions occur simultaneously and apparently on the same subunit. Ruoho and Kyte (15) discussed one implication of this fact, namely, that the large subunit has to "span" the membrane, which is possible in view of its size and shape (8). This fact also implies a stoichiometric relationship between binding of digitalis and phosphorylation. Indeed, most membrane preparations, including the one used in these experiments, bind 1 mole of digitalis glycoside per mole of phospho-enzyme (see Table 1). In some species (e.g., rat) (3, 35) or after treatment of the membranes with trypsin or with sulfhydryl reagents (36) this ratio is 2:1 or 3:1. This variation may be explained in several ways: (a) in some cases digitalis glycosides may be bound without prior phosphorylation (3, 7); (b) the large subunit may have more than one binding site for digitalis; or (c) phosphorylation-dependent binding of 1 molecule of a glycoside may induce an allosteric transition of the enzyme which allows binding to other sites within the oligomer without further phosphorylation (37–39).

An impressive body of indirect evidence points to a link between the inhibition of ($Na^+ + K^+$)-ATPase and the positive inotropic effect of digitalis glycosides (3). Several hypotheses were offered to explain this connection, but the exact mechanism is not known (for a summary, see ref. 40). It is not known either whether there are other "receptors" for digitalis besides ($Na^+ + K^+$)-ATPase, as some experiments suggested (41). The experiments presented here did not identify the pharmacological receptor site for the positive inotropic action of digitalis glycosides. These experiments did, however, show directly that about 90% of a digitalis glycoside (ouabain) bound to a single membrane protein and that this membrane protein was part of ($Na^+ + K^+$)-ATPase. These observations support the idea that, at least at the level of the cell membrane, ($Na^+ + K^+$)-ATPase is the main receptor for digitalis glycosides.

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