Substitution of Transmembrane Residues with Hydrogen-Bonding Potential in the α Subunit of Na,K-ATPase Reveals Alterations in Ouabain Sensitivity†

Patrick J. Schultheis and Jerry B. Lingrel*

Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0524

Received October 2, 1992; Revised Manuscript Received November 5, 1992

ABSTRACT: The role of H-bonding amino acids as determinants of ouabain affinity in the Na,K-ATPase was examined. Site-directed mutagenesis was used to substitute 21 conserved amino acid residues in the sheep α-subunit transmembrane regions. The amino acids were changed from residues which possess side chains capable of forming H-bonds with specific cardiac glycoside moieties such as the lactone ring or sugar(s) to residues unable to participate in H-bonding. The effect of each of these amino acid replacements on the affinity of the Na,K-ATPase for ouabain was initially assessed by screening the altered enzymes for the ability to confer ouabain resistance when expressed in otherwise sensitive HeLa cells. Three of the substitutions (Tyr-108 to Ala, Cys-104 to Ala, and Cys-104 to Phe) were able to confer resistance to the normally sensitive HeLa cells. Stable cell lines, each expressing one of the altered enzymes, were further characterized in terms of ouabain-inhibitable cell growth and Na,K-ATPase activity. Cell lines expressing the α1-isoform substitution Y108A, C104A, or C104F contained a Na,K-ATPase activity which gave an I_{50} value for enzyme inhibition 9-, 6-, and 150-fold greater, respectively, than the endogenous HeLa or sheep enzyme. These data show that Tyr-108 and Cys-104 of the α subunit are determinants of ouabain affinity. Cys-104 has also been shown to be a determinant of ouabain sensitivity in *Xenopus laevis* [Canessa, C. M., Horisberger, J.-D., Louvard, D., & Rossier, B. C. (1992) *EMBO J. 11*, 1681–1687]. The identification of an additional amino acid (Tyr-108) in the H1 transmembrane domain of the α subunit which influences the affinity of the Na,K-ATPase for ouabain provides additional evidence that this region is important in determining ouabain sensitivity.

Sodium- and potassium-activated adenosinetriphosphatase (Na,K-ATPase)[1] is an integral membrane protein present in all cells of higher organisms and is composed of both α and β subunits. The enzyme is responsible for establishing an electrochemical gradient of Na^+ and K^+ ions across the cell membrane which is essential for the maintenance of cellular homeostasis (Ullrich, 1979; MacKnight & Leaf, 1977; Thomas, 1972). Na,K-ATPase is also the target for a class of drugs known as cardiac glycosides (e.g., digoxin, digitoxin, ouabain) used in the treatment of congestive heart failure (Hansen, 1984; Schwartz et al., 1975). These drugs bind to and inhibit the enzyme, resulting in a transient increase in intracellular Na^+ levels which is thought to slow the outward transport of Ca^{2+} via a Ca^{2+}/Na^+ antiport protein. The resulting increase in the intracellular concentration of Ca^{2+} is responsible for the positive inotropic action of these drugs on cardiac muscle (Thomas et al., 1989). This is the basis for the extensive therapeutic use of cardiac glycosides in the treatment of congestive heart failure. Given the pharmacological significance of cardiac glycosides, it is of great interest to understand their mode of action and to elucidate the molecular nature of the interaction of these drugs with their receptor.

Initial biochemical studies using photoactivatable ouabain analogues suggested that the cardiac glycoside binding site is contained in the α subunit (Forbush, 1983; Deffo et al., 1983; Forbush et al., 1978; Goeldner et al., 1983; Jørgensen et al., 1982; Rogers & Lazdunski, 1979; Rossi et al., 1980; Ruoho & Kyte, 1974). In addition, species-specific variations in ouabain sensitivity have been noted (Periyasamy et al., 1983; Repke & Portius, 1965; Wallick et al., 1980). For example, the rat Na,K-ATPase is 1000-fold more resistant to ouabain than Na,K-ATPases from other sources such as sheep and human. The differential ouabain sensitivity is apparently due to large differences in the rate of dissociation of the drug from the binding site on these enzymes (Wallick et al., 1980; Tobin & Brady, 1972). In contrast, for a given set of binding conditions, association rates vary little among Na,K-ATPases from different species (Wallick et al., 1980).

Studies using chimeras between the ouabain-sensitive sheep and the ouabain-resistant rat Na,K-ATPase α1 subunits, coupled with site-specific mutagenesis, have identified three amino acids at the border of the first extracellular region (H1–H2) of the α subunit as important determinants of ouabain sensitivity (Price & Lingrel, 1988; Price et al., 1989, 1990). In these experiments, it was observed that the sheep α1 subunit contains the amino acids glutamine and asparagine at the border of the first extracellular domain while the rat α1 subunit contains the amino acids arginine and aspartate at these positions, respectively. Conversion of the border residues in the sheep to those found in the rat resulted in a 1000-fold increase in resistance to ouabain. Likewise, substitution of a conserved extracellular aspartic acid residue at position 121 of the sheep α subunit generated an enzyme with a 100-fold reduced affinity for ouabain. The *Bufo marinus* toad also expresses a ouabain-resistant Na,K-ATPase activity. The α1 subunit from this toad contains two positively charged amino acids at the N-terminal border of the H1–H2 extracellular region but no charged amino acid at the C-terminal

† Supported by Grant HL 28573 from the National Institutes of Health.
* To whom correspondence should be addressed. Telephone: (513)-558-5324. Fax: (513)-558-8474.
[1] Abbreviations: Na,K-ATPase, sodium- and potassium-activated adenosinetriphosphatase; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetate; bp, base pair(s).

0006-2960/93/0432-544$04.00/0

border (Jaisser et al., 1992). This enzyme exhibits an intermediate affinity for ouabain ($K_i \approx 50$ μM versus a K_i of ≈100 μM for rat α1), similar to that shown for sheep α1 Na,K-ATPase mutants in which a single charged residue was introduced at either the N- or the C-terminal border position (Price et al., 1990). Recently, mutation of a conserved cysteine residue in the H1 transmembrane domain of the normally ouabain-sensitive α subunit from *Xenopus laevis* has been shown to increase the inhibition constant (K_i) of ouabain more than 1000-fold when expressed in a *Xenopus* oocyte expression system (Canessa et al., 1992). Presumably, this amino acid, as well as the H1–H2 border residues, is somehow involved in the ouabain binding site. However, the identification of these amino acid residues as important determinants of ouabain affinity does not preclude the involvement of additional amino acids in ouabain binding.

Although it is well established that cardiac glycosides like ouabain act from the extracellular surface of the cell (Schwartz et al., 1975), this does not rule out a role for transmembrane amino acids in the interaction between ouabain and the Na,K-ATPase. It has been suggested, on the basis of the analysis of structure–activity relationships, that the cardiac glycoside binding site lies within a water-free cleft of the α subunit and that van der Waals and H-bonding forces are involved in the binding interaction between the receptor and ouabain (Thomas et al., 1989). The walls of such a cleft may be formed from amino acids contributed by the transmembrane domain(s) (e.g., H1 and H2) of the Na,K-ATPase α subunit.

In this report, we describe a systematic investigation of the role of H-bonding amino acids in ouabain binding. Site-specific mutagenesis was used to substitute 21 conserved amino acid residues in the α-subunit transmembrane regions. The amino acids were changed from residues capable of forming H-bonds with specific cardiac glycoside moieties such as the lactone ring or sugar side chain to residues unable to participate in H-bonding. Conserved residues were targeted since the rate at which ouabain binds to Na,K-ATPases from different species is similar (Wallick et al., 1980). This fact and the observation that ouabain binds to and can completely inhibit the relatively resistant rat α1 enzyme suggest that cardiac glycosides may interact with a site on the enzyme which is, at least, partially conserved among both sensitive and insensitive enzymes. The effect of the amino acid substitutions on the affinity of the Na,K-ATPase for ouabain was assessed by screening the altered enzymes for the ability to confer ouabain resistance when expressed in otherwise sensitive HeLa cells. We present evidence indicating that Tyr-108 is a determinant of the affinity of the Na,K-ATPase for ouabain. This amino acid is conserved in all Na,K-ATPases and is located in the H1 transmembrane domain of the α subunit, further implicating this region in ouabain binding. We also confirm that Cys-104 is a major determinant of ouabain sensitivity.

MATERIALS AND METHODS

Materials. Molecular biology reagents were purchased from New England Biolabs, Amersham Corp., Pharmacia LKB Biotechnology Inc., and Promega. Cell culture supplies were purchased from Gibco and Fisher. Ouabain was purchased from Calbiochem. Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, ATP, and NADH were from Sigma. Radioisotopes were from Du Pont—New England Nuclear. Plasmid DNA purification columns were purchased from Qiagen Inc. All othe reagents were of the highest quality available.

Site-Directed Mutagenesis. The sheep α1 cDNA expression vectors and methodology have previously been described in detail (Price & Lingrel, 1988). Briefly, cassettes from the coding region of the sheep Na,K-ATPase α-subunit cDNA were subcloned into the bacteriophage M13. The cDNA cassettes included a 430-bp *Sal*I–*Xba*I fragment, a 847-bp *Xba*I–*Pst*I fragment, and a 763-bp *Hin*dIII–*Bgl*II fragment containing the regions encoding amino acid residues 86–229, 230–512, and 691–945 of the α subunit, respectively. The Kunkel method of site-directed mutagenesis (Kunkel, 1985) was used to introduce the desired mutations in the cassettes. Cassettes containing the appropriate mutations were sequenced in their entirety to screen for unwanted mutations and then ligated back into the sheep Na,K-ATPase α-subunit cDNA, which is carried in the eucaryotic expression vector pKC4. All plasmids were purified on Qiagen columns prior to their use in transfection experiments.

Tissue Culture and Transfection of HeLa Cells. HeLa cells were maintained essentially as described elsewhere (Price & Lingrel, 1988). Transfections (20 μg of plasmid DNA) were performed by the modified calcium phosphate procedure of Chen and Okayama (1987). After 3-weeks selection in 0.1 μM ouabain, several colonies from each transfection were isolated and expanded into stable cell lines.

Northern Analysis. Total RNA was isolated from confluent 100-mm tissue culture dishes by a modification of a previously described procedure (Chomczynski & Sacchi, 1987). RNA (10 μg) from control HeLa cells and HeLa cell transfectants was denatured in 1 M glyoxal, 54% DMSO, and 0.01 M $NaHPO_4$ (pH 6.8) (McMaster & Carmichael, 1977), fractionated by electrophoresis in 1% agarose, and transferred to a Nylon filter (Magna NT nylon transfer membrane, Micron Separations Inc.). The blot was probed with a 430-bp DNA fragment derived from the 3′-untranslated region of the pKC4 transcription unit. The probe contains sequences of the small t-intron and the poly(A) addition signal, both of which are of SV40 origin. Hybridization and washes were performed by the method of Church and Gilbert (1984). Briefly, hybridization was carried out overnight at 65 °C in 1 mM Na_2-EDTA, 7% SDS, 1% BSA, and 0.50 M $NaHPO_4$ (pH 6.8). The filter was washed twice in 0.5% BSA, 1 mM Na_2EDTA, 5% SDS, and 0.04 M $NaHPO_4$ (pH 6.8) at 65 °C, twice in 1 mM Na_2EDTA, 1% SDS, and 0.04 M $NaHPO_4$ (pH 6.8) at 65 °C, and twice in 0.1 × SSC [1 × SSC = 0.015 M sodium citrate (pH 7.0) and 0.15 M NaCl] and 0.5% SDS at 65 °C. The filter was blotted dry, and the signal was detected by autoradiography at −70 °C.

Isolation of Crude Plasma Membranes from HeLa Cells. Crude plasma membranes were isolated from wild-type and transfected HeLa cells as described by Jewell and Lingrel (1991). Five confluent 150-mm tissue culture dishes were washed twice with PBS and harvested by mild trypsinization (0.05% trypsin/0.02% Na_2EDTA). The cells were lysed in 1 mM $NaHCO_3$, 2 mM $CaCl_2$, and 5 mM $MgCl_2$ at 4 °C for 15 min and homogenized in a glass Dounce homogenizer. Intact cells, debris, and nuclei were pelleted with a 3000*g* centrifugation (1 min), and the supernatant was treated with NaI as described (Lane et al., 1973). Membranes were pelleted at 48000*g*, 25 min, and the pellet was washed once with 1 mM Na_2EDTA/1 mM Tris (pH 7.4) and resuspended in the same buffer at 1.5–3.0 mg/mL. The protein concentration of each membrane preparation was determined by a modification of the Bradford procedure (Bradford, 1976; Stoscheck, 1990) using bovine serum albumin as a standard.

Na,K-ATPase Assay. Total Na,K-ATPase activity in crude plasma membranes was quantitated using the spectrophotometric coupled enzyme assay (Schwartz et al., 1969).

Membranes prepared from the transfectants and wild-type HeLa cells were incubated for 15 and 120 min, respectively, in assay buffer (25 mM histidine, 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 1 mM phophoenolpyruvate, 5 mM ATP, 0.43 mM NADH, 5 units of pyruvate kinase, and 7 units of lactate dehydrogenase, pH 7.4) containing the desired concentration of ouabain prior to Na,K-ATPase activity measurements. Total Na,K-ATPase activity was determined by subtracting the ATPase activity obtained in the presence of 7.8×10^{-3} M ouabain from that observed in the absence of inhibitor. The data are presented as the percent activity remaining at a particular ouabain concentration relative to the total Na,K-ATPase activity in the absence of ouabain. The specific ouabain-inhibitable Na,K-ATPase activity of these preparations was 17–27 μmol of P_i (mg of protein)$^{-1}$ h^{-1}.

Ouabain-Inhibitable Cell Growth. The assay was performed as previously described (Price & Lingrel, 1988) with the following minor modifications. Trypsinized cells were resuspended at 1×10^5 cells/mL, and 0.5 mL (5×10^4 cells) was seeded into each well of a 24-well tissue culture plate containing 0.5 mL of media plus twice the desired concentration of ouabain. The cells were washed twice with PBS after 36-h incubation at 37 °C in 5% CO_2. Subsequent to solubilization of the cells with 1.0 N NaOH (100 μL) and neutralization with 1.0 N HCl (100 μL), a 50-μL aliquot was added to 500 μL of Bio-Rad protein assay dye reagent, and the absorbance at 595 nm was recorded. The data are presented as the percent of cell growth (measured as material absorbing at 595 nm) relative to growth in the absence of ouabain.

RESULTS

Naturally occurring cardiac glycosides such as ouabain and digoxin contain three different structural regions. These include a steroid ring system, a 17β-lactone, and a terminal sugar residue(s) at position C3. On the basis of analysis of structure–activity relationships, it is postulated that the Na,K-ATPase contains regions which interact with each of these chemical constituents. It is further suggested that H-bonding forces are involved in the interaction between cardiac glycosides and the binding site(s) on the enzyme (Thomas et al., 1989). To explore the role of H-bonding amino acids in ouabain binding, site-directed mutagenesis was used to convert transmembrane amino acids in the α1 subunit from residues with H-bonding potential to residues unable to form H-bonds, for example, tyrosine-108 to phenylalanine (Y108F). The effect of these amino acid substitutions on the affinity of the Na,K-ATPase for ouabain was initially assessed by screening the altered enzymes for the ability to confer ouabain resistance when expressed in otherwise sensitive HeLa cells. The wild-type isoform of the sheep Na,K-ATPase has an I_{50} for ouabain inhibition of 3×10^{-8} M and cannot confer ouabain resistance to the sensitive HeLa cells.

The 21 amino acids targeted for mutagenesis are listed in Table I. The location of these amino acids is shown in Figure 1 and is based on the eight-transmembrane model of the α1 subunit (Shull et al., 1985). Sheep α1 cDNAs encoding the altered enzymes were placed in the eucaryotic expression vector pKC4, and these constructs were independently transfected by $CaPO_4$ precipitation into ouabain-sensitive HeLa cells. The transfected cells were then cultured in the presence of 0.1 μM ouabain to select for cells expressing an ouabain-insensitive sheep Na,K-ATPase.

Ouabain-resistant colonies were obtained from cells transfected with the sheep α1 cDNA constructs encoding the substitutions Y108A, C104F, or C104A. When cells were transfected with the ouabain-sensitive sheep α1 cDNA or with the remaining 18 α1 cDNA mutant constructs, ouabain-resistant colonies were not observed (Table I). In order to demonstrate that the ouabain-resistant phenotype was due to the expression of the transfected sheep α1 cDNA mutant, Northern analysis was performed on RNA isolated from the ouabain-resistant transfectants and untransfected HeLa cells. The blots were probes with a 436-bp DNA fragment derived from the 3′-untranslated region of the pKC4 vector, which does not cross-react with the endogenous α1-subunit mRNA. As expected, RNA derived from untransfected HeLa cells does not hybridize to the DNA probe. Each ouabain-resistant cell line expresses a mRNA species identical in size to that seen when similar Northern blots are probed with an α-subunit cDNA (Figure 2). This probe hybridizes to endogenous as well as transfected α1 mRNA (Price & Lingrel, 1988).

Table I: Transfection of HeLa Cells with Mutant Sheep α1 cDNAs

sheep α1 mutant[a]	transmembrane location[b]	ouabain-resistant colonies
S94A	H1	–
W98A	H1	–
C104S	H1	–
C104A	H1	+
C104F	H1	+
Y108A	H1	+
Y108F	H1	–
G109F	H1	–
Y124A	H2	–
T136V	H2	–
C138F	H2	–
S298A	H3	–
S303A	H3	–
Y308A	H3–H4 extracellular loop	–
T309V	H3–H4 extracellular loop	–
N324V	H4	–
T799V	H5	–
C802F	H5	–
S844A	H6	–
Y847A	H6	–
W924F	H7	–

[a] Sheep α1 mutants were generated as described under Materials and Methods. A total of 20 μg of each construct was used to transfect HeLa cells. [b] The assignment of each mutant to the indicated transmembrane domains was based on the putative eight-transmembrane model of the Na,K-ATPase α subunit (Shull et al., 1985).

The ouabain-resistant transfectants and the untransfected HeLa cells were further characterized in terms of ouabain-inhibitable cell growth and Na,K-ATPase activity. The experiments were performed in order to demonstrate that the ouabain-resistant cell lines were generated as a direct consequence of the expression of an insensitive form of Na,K-ATPase in these cells rather than from the overexpression of the endogenous enzyme. As measured by ouabain-inhibitable cell growth, untransfected HeLa cells are sensitive to relatively low doses of ouabain ($I_{50} = 3.0 \times 10^{-8}$ M). The resistant transfectants survived higher levels of ouabain with an apparent I_{50} of 1.9×10^{-7}, 5.4×10^{-6}, and 3.1×10^{-7} M for mutants Y108A, C104F, and C104A, respectively (Table II and Figure 3). These data were experimentally verified by direct measurements of ouabain-inhibitable Na,K-ATPase activity in plasma membrane preparations isolated from the transfectants and from wild-type HeLa cells. Crude membranes prepared from wild-type HeLa cells and from the ouabain-resistant transfectants Y108A, C104F, and C104A exhibited ouabain-inhibitable Na,K-ATPase activity with half-maximal inhibition occurring at approximately 3.2×10^{-8}, 2.9×10^{-7}, 4.8×10^{-6}, and 2.0×10^{-7} M, respectively (Table II and

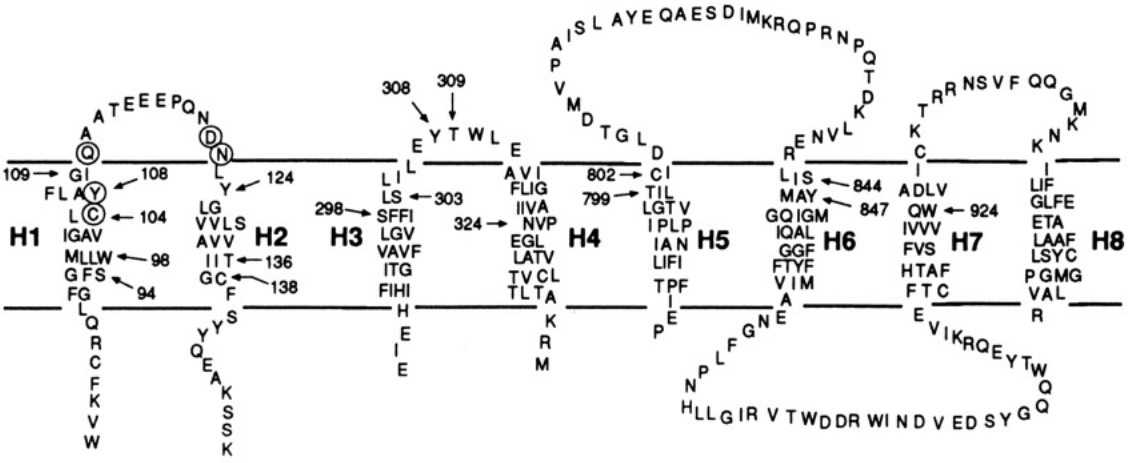

FIGURE 1: Eight-transmembrane model of the sheep Na,K-ATPase α subunit. Shown is the hypothetical membrane organization of the Na,K-ATPase α subunit which has been previously described (Shull et al., 1985). The intracellular region of the α subunit has been excluded for the sake of clarity and space. The circled residues in the H1–H2 region indicate amino acid residues which have been identified as determinants of ouabain affinity to date. The numbered residues were altered by site-directed mutagenesis in this study. The resultant mutants were screened for their ability to confer ouabain resistance in normally sensitive HeLa cells. The altered amino acids and their replacements are listed in Table I.

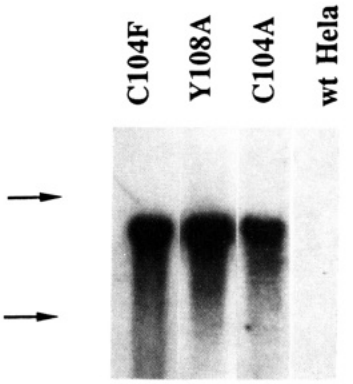

FIGURE 2: Northern analysis of RNA isolated from ouabain-resistant HeLa cell transfectants. Total RNA was isolated from ouabain-resistant HeLa cell transfectants and from untransfected HeLa cells. Each lane contains 10 μg of RNA which was hybridized with a DNA fragment from the 3′-untranslated region of pKC4. The autoradiograph was exposed for 5 days. The arrows indicate the location of the 28S and 18S ribosomal RNAs. wt, wild type.

Table II: Summary of I_{50} Values Determined by Ouabain-Inhibitable Cell Growth and by Direct Measurement of Ouabain-Inhibitable Na,K-ATPase Activity

	I_{50}, M	
mutant	ouabain-inhibitable cell growth	ouabain-inhibitable Na,K-ATPase activity
wild-type sheep	3.0×10^{-8}	3.2×10^{-8}
Y108A	1.9×10^{-7}	2.9×10^{-7}
C104A	3.1×10^{-7}	2.0×10^{-7}
C104F	5.4×10^{-6}	4.8×10^{-6}

Figure 4). The data from these experiments confirm that HeLa cells expressing the α1-isoform substitutions Y108A, C104F, and C104A were able to survive selection in 0.1 μM ouabain because the affinity of the transfected enzymes for ouabain was reduced.

Interestingly, the I_{50} values observed in the ouabain-inhibitable cell growth experiments correlate very well with the I_{50} values obtained by direct measurement of enzyme

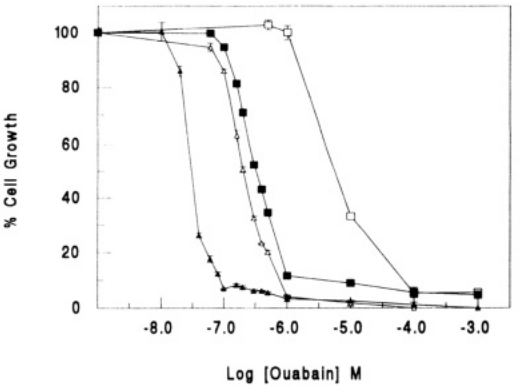

FIGURE 3: Ouabain-inhibitable cell growth. Untransfected HeLa cells and ouabain-resistant transfectants were grown in the presence of increasing concentrations of ouabain as described under Materials and Methods. The data are presented as percent of cell growth at the indicated ouabain concentrations relative to growth in the absence of inhibitor. At least two cloned cell lines from each transfection were assayed. This plot represents the result of one experiment which was performed in triplicate. Error bars represent the standard error of the mean of triplicate protein determinations. Symbols are as follows: (▲) wild type HeLa; (□) C104F; (■) C104A; (△) Y108A.

inhibition in the cell membrane preparations (Table II). The correlation was observed with the mutants as well as with the wild-type sheep enzyme. Therefore, determination of the I_{50} by ouabain-inhibitable cell growth would appear to be an accurate estimate of the I_{50} for inhibition of ATPase activity in cell membrane preparations. The correlation also indicates that the I_{50} for inhibition of enzyme activity in cell membranes is similar to the I_{50} for intact Na–K pump activity in whole cells.

The I_{50} observed for untransfected HeLa cells was 3.0×10^{-8} M (Figure 4). This is approximately the same as the apparent I_{50} for ouabain inhibition of sheep kidney Na,K-ATPase (Wallick et al., 1980). However, to achieve half-maximal inhibition at 3.0×10^{-8} M ouabain, it was necessary to preincubate the untransfected plasma membrane preparation with the appropriate concentration of ouabain for 2 h.

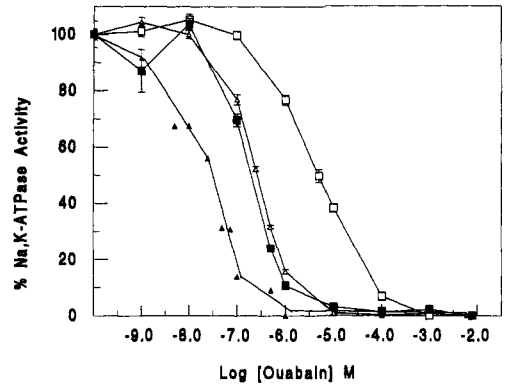

FIGURE 4: Ouabain-inhibitable Na,K-ATPase activity. The data are presented as the percent activity remaining at the indicated ouabain concentrations relative to the total Na,K-ATPase activity in the absence of inhibitor. Membranes were prepared and assayed from at least two independently cloned cell lines from each transfection. Membranes prepared from the ouabain-resistant transfectants and untransfected HeLa cells were preincubated with the appropriate concentrations of ouabain for 15 and 120 min, respectively, prior to Na,K-ATPase activity measurements. This plot represents the result of one experiment which was performed in triplicate using a single membrane preparation. Error bars represent the standard error of the mean of the triplicate velocity determinations. The specific Na,K-ATPase activity (as micromoles of ATP hydrolyzed per milligram of protein per hour) of each membrane preparation in 0 mM ouabain was as follows: wild-type HeLa = 26.7 ± 0.60; C104F = 22.1 ± 1.1; C104A = 17.4 ± 0.34; Y108A = 22.5 ± 0.34. Symbols are as follows: (▲) wild-type HeLa; (□) C104F; (■) C104A; (△) Y108A.

This was required to more closely approach equilibrium binding and maximal inhibition of enzyme activity at the lower concentrations of ouabain used in the assay (Wallick et al., 1980). In contrast, preincubating the ouabain-resistant transfectants with ouabain for 15 min was sufficient to obtain I_{50} values which reflect equilibrium binding conditions.

In none of the enzyme inhibition experiments were biphasic curves observed as would be expected if both sensitive and insensitive forms of the Na,K-ATPase are present in the ouabain-resistant transfectants (Figure 4). Presumably, this is the result of growing transfected cells in the presence of 0.1 μM ouabain, a concentration which would inhibit virtually all of the endogenous HeLa Na,K-ATPase. Membranes were prepared from these cells at 0 °C, and at this temperature, the rate of dissociation of ouabain from sensitive forms of the enzyme is estimated to be 2–3 days (E. Wallick, unpublished observations). Thus, it would not be unusual for the drug to remain bound to and inhibit the activity of the endogenous enzyme during the isolation of the membrane preparation. As a result, the activity contributed by the sensitive HeLa Na,K-ATPase was not detected during Na,K-ATPase activity measurements.

DISCUSSION

The goal of the present study was to investigate the role of H-bonding in the interaction between ouabain and the Na,K-ATPase. Site-directed mutagenesis was used to introduce amino acid substitutions which ablate the H-bonding potential of 21 conserved amino acids in the transmembrane regions of the α1 subunit. The effect of each of these amino acid replacements on the affinity of the Na,K-ATPase for ouabain was then assessed. Conserved residues were targeted with the intention of identifying residues which participate in the initial binding event between ouabain and the Na,K-ATPase. While a significant number of transmembrane amino acids were substituted, not all of the transmembrane amino acids capable of acting as H-donors or acceptors were altered. Except for the H1–H2 region, which has previously been implicated in ouabain binding, amino acid substitutions were limited to those portions of the transmembrane helices closest to the extracellular surface. We reasoned that if cardiac glycosides do partition in the membrane, the hydrophilic moieties of these drugs such as the lactone ring or sugar(s) might prevent them from penetrating too deeply in the membrane.

Twenty-one α1 cDNA mutant constructs, each encoding a unique amino acid substitution, were independently transfected into HeLa cells. Three of these (Y108A, C104A, and C104F) were able to confer ouabain resistance to the normally sensitive HeLa cells. It was further shown that the resistant phenotype was due to the expression of an ouabain-insensitive Na,K-ATPase in the otherwise sensitive cells, thus identifying tyrosine-108 and cysteine-104 of the α1 subunit as determinants of ouabain affinity. However, we cannot rule out a role for the remaining 18 amino acids in ouabain binding. An active enzyme is required for resistance to be conferred to sensitive HeLa cells, and it is possible that the some of the amino acid substitutions disrupted the function of the Na,K-ATPase.

As determined by direct measurement of ouabain-inhibitable Na,K-ATPase activity, HeLa cells expressing the α1-isoform substitutions Y108A and C104A are approximately 9- and 6-fold more resistant, respectively, to ouabain than untransfected HeLa cells (Table II). The α1-isoform substitution C104F confers an even greater level of resistance. Cells expressing this altered α1 subunit survive levels of ouabain 150-fold higher than wild-type HeLa cells. The equivalent C104F substitution in the *Xenopus laevis* α subunit gave an inhibition constant (K_i) for ouabain 1000-fold greater than the wild-type enzyme when assayed in *Xenopus* oocytes (Canessa et al., 1992). This result qualitatively but not quantitatively agrees with our data as this substitution in the sheep α1 subunit exhibited only a 150-fold reduced affinity for ouabain. The reason for this discrepancy is unclear. Significant differences in the methodologies used to measure ouabain-inhibitable Na,K-ATPase activity might be responsible. We directly measured Na,K-ATPase activity in plasma membrane preparations using the spectrophotometric-coupled enzyme assay (Schwartz et al., 1969) whereas Canessa et al. (1992) estimated the activity of expressed Na,K-ATPase pumps by measuring the outward K^+-induced current in *Xenopus laevis* oocytes. However, intrinsic differences between the sheep and *Xenopus* enzymes may best explain the observed differences in affinity. The sheep and *Xenopus laevis* α subunits, though closely related, contain differences in their primary amino acid sequence, and each is expressed in a unique lipid environment. These differences may influence ouabain binding kinetics at the macromolecular level either through the ouabain binding site or via allosteric effects.

The interaction between cardiac glycosides, such as ouabain, and the Na,K-ATPase enzyme is complex and, presumably, involves sites on the enzyme which interact with each of the structural regions of these drugs (Thomas et al., 1989). These regions include the steroid and lactone ring moieties and the sugar(s) at position C3. Kinetic and structure–activity relationship studies suggest that binding of cardiac glycosides to the Na,K-ATPase occurs in two steps (Yoda, 1974). In ouabain-sensitive Na,K-ATPases, the initial binding event is presumably followed by a structural change in the Na,K-ATPase resulting in the formation of a more stable enzyme–drug complex (Yoda, 1974; Adams et al., 1983; Hansen, 1984). It has been postulated that the uncharged amino acids occurring at the borders of the H1–H2 extracellular domain

of sensitive enzymes facilitate such a conformational change whereas in resistant species the presence of charged residues at these positions prevents the change in conformation necessary for stabilization of the drug–receptor complex (Price & Lingrel, 1988). This hypothesis is consistent with kinetic studies which have shown that species differences in sensitivity to the inhibitory effects of cardiac glycosides on Na,K-ATPase activity are due to differences in off-rates rather than on-rates (Wallick et al., 1980; Tobin & Brady, 1972; Thomas et al., 1989). In these studies, it was shown that sensitive and insensitive enzymes have similar association rate constants for binding cardiac glycosides but differ in their dissociation rate constants. The similar on-rates could be accounted for if cardiac glycosides initially bind to a site on the enzyme which is conserved among all species. The similar on-rates and the fact that ouabain binds to and can completely inhibit the relatively resistant rat α1 enzyme imply that the binding site is, at least, partially conserved among sensitive and insensitive enzymes. In contrast, the off-rate and the affinity of the Na,K-ATPase for ouabain may be determined by a different set of amino acids which are not conserved, for example, the H1–H2 border residues. The identity of the border residues may influence ouabain sensitivity via conformational effects subsequent to the initial interaction between drug and receptor. This is corroborated by studies using a monoclonal antibody specific for the H1–H2 extracellular loop (Arystarkhova et al., 1992). This antibody was shown to enhance ouabain binding rather than block it. An antibody to the ouabain binding site would have been expected to sterically hinder binding, not promote it. The antibody might alter the conformation of the Na,K-ATPase α subunit, effectively promoting or stabilizing ouabain binding. However, it is possible that the antibody interacts with only a portion of the H1–H2 extracellular loop while leaving the ouabain binding site accessible.

While substitution of an amino acid which H-bonds with ouabain could affect affinity by disrupting the H-bond, steric interference must also be considered. Evidence consistent with both of these effects was observed with our substitutions. For example, further increasing the hydrophobicity of the H1 transmembrane domain results in an increase in ouabain resistance: substitutions C104F, C104A, and Y108A conferred resistance in HeLa cells. Ouabain-resistant colonies were not observed when HeLa cells were transfected with substitution C104S. This represents an isosteric substitution effectively replacing a sulfhydryl with a hydroxyl group which retains the H-bonding potential. On the other hand, substitution Y108F, in which a hydroxyl group with H-bonding potential has been eliminated, does not fit this pattern since ouabain resistance was not demonstrated with this substitution. These observations could be explained if cysteine-104 H-bonds with a specific cardiac glycoside moiety such as the lactone ring, and tyrosine-108, while not participating in H-bonding, contributes to the specificity of the interaction between drug and receptor through van der Waals binding. The fact that substitution Y108A, but not Y108F, confers resistance could be explained by the sensitivity of van der Waals interactions to distance effects. The reduced affinity of these mutant enzymes for ouabain could also be due to steric effects. Substitution of C104 with phenylalanine increases resistance approximately 24-fold more than replacement of the same residue with alanine. Substitution with a residue containing a large side chain may sterically reduce the access of the inhibitor to the binding site. Furthermore, if Y108 and C104 actually are components of the putative cleft in which ouabain binds, then any substitution could have a subtle affect on ouabain affinity by disrupting van der Waals or H-bonding forces, the affect of which would be difficult to predict or explain without the three-dimensional structure of the enzyme. Finally, tyrosine-108 and cysteine-104 may not participate directly in ouabain binding. Substituting these residues may have altered the apparent affinity of the enzyme for ouabain by hindering a conformational change to a more stable enzyme–drug complex subsequent to the initial binding event, or by decreasing the time the Na,K-ATPase is in a conformation which can bind ouabain. Additional amino acid substitutions should help in defining the cardiac glycoside binding site in the Na,K-ATPase α subunit.

ACKNOWLEDGMENT

We thank Dr. Earl Wallick for helpful advice on technical matters, for useful discussions, and for reviewing the manuscript.

REFERENCES

Adams, R. J., Wallick, E. T., Asana, G., DiSalvo, J., Fondacaro, J. D., & Jacobson, E. D. (1983) *J. Cardiovasc. Pharmacol. 5*, 468–482.

Arystarkhova, E., Gasparian, M., Modyanov, N. N., & Sweadner, K. J. (1992) *J. Biol. Chem. 267*, 13694–13701.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Canessa, C. M., Horisberger, J.-D., Louvard, D., & Rossier, B. C. (1992) *EMBO J. 11*, 1681–1687.

Chen, C., & Okayama, H. (1987) *Mol. Cell. Biol. 7*, 2745–2752.

Chomczynski, P., & Sacchi, N. (1987) *Anal. Biochem. 162*, 156–159.

Church, G. M., & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1991–1995.

Deffo, T., Fullerton, D. S., Kihara, M., McParland, R. H., Becker, R. R., Simat, B. M., From, A. H., Ahmed, K., & Schimerlik, M. I. (1983) *Biochemistry 22*, 6303–6309.

Forbush, B., III (1983) *Curr. Top. Membr. Transp. 19*, 167–201.

Forbush, B., III, Kaplan, J. H., & Hoffman, J. F. (1978) *Biochemistry 17*, 3667–3676.

Goeldner, M. P., Hirth, C. G., Rossi, B., Ponzio, G., & Lazdunski, M. (1983) *Biochemistry 22*, 4685–4690.

Hansen, O. (1984) *Pharmacol. Rev. 36*, 143–163.

Jaisser, F., Canessa, C. M., Horisberger, J., & Rossier, B. C. (1992) *J. Biol. Chem. 267*, 16895–16903.

Jørgensen, P. L., Karlish, S. J. D., & Gitler, C. (1982) *J. Biol. Chem. 257*, 7435–7442.

Jewell, E. A., & Lingrel, J. B. (1991) *J. Biol. Chem. 266*, 16925–16930.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.

Lane, L. K., Copenhaver, J. H., Lindenmayer, G. E., & Schwartz, A. (1973) *J. Biol. Chem. 248*, 7197–7200.

MacKnight, A. D. C., & Leaf, A. (1977) *Physiol. Rev. 57*, 510–573.

McMaster, G. K., & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4835–4838.

Periyasamy, S. M., Huang, W.-H., & Askari, A. (1983) *Comp. Biochem. Physiol. 76B*, 449–454.

Price, E. M., & Lingrel, J. B. (1988) *Biochemistry 27*, 8400–8408.

Price, E. M., Rice, D. A., & Lingrel, J. B. (1989) *J. Biol. Chem. 264*, 21902–21906.

Price, E. M., Rice, D. A., & Lingrel, J. B. (1990) *J. Biol. Chem. 265*, 6638–6641.

Repke, K. E. M., & Portius, H. J. (1965) *Biochem. Pharmacol. 14*, 1785–1802.

Rogers, T. B., & Lazdunski, M. (1979) *Biochemistry 18*, 135–140.

Rossi, B., Vuilleumier, P., Gache, C., Balerna, M., & Lazdunski, M. (1980) *J. Biol. Chem. 255*, 9936–9941.
Ruoho, A., & Kyte, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2352–2356.
Schwartz, A., Allen, J. C., & Harigaya, S. (1969) *J. Pharmacol. 168*, 31–41.
Schwartz, A., Lindenmeyer, G. E., & Allen, J. C. (1975) *Pharmacol. Rev. 27*, 3–134.
Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature 316*, 691–695.
Stoscheck, C. M. (1990) *Methods Enzymol. 182*, 62–63.
Thomas, R. C. (1972) *Physiol. Rev. 52*, 563–594.
Thomas, R., Gray, P., & Andrews, J. (1989) *Adv. Drug Res. 19*, 311–561.
Tobin, T., & Brady, T. M. (1972) *Biochem. Pharmacol. 21*, 1553–1560.
Ullrich, K. J. A. (1979) *Annu. Rev. Physiol. 41*, 181–195.
Wallick, E. T., Pitts, B. J. R., Lane, L. K., & Schwartz, A. (1980) *Arch. Biochem. Biophys. 202*, 442–449.
Yoda, A. (1974) *Ann. N.Y. Acad. Sci. 242*, 598–616.