Analysis of Amino Acid Residues in the H5–H6 Transmembrane and Extracellular Domains of Na,K-ATPase α Subunit Identifies Threonine 797 as a Determinant of Ouabain Sensitivity†

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ABSTRACT: Several amino acid residues of the α subunit of the Na,K-ATPase have been identified which alter ouabain sensitivity. These residues are located in the N-terminal half of the α1 subunit suggesting that this portion of the molecule may represent the binding site for cardiac glycosides. However, not all extracellular and transmembrane regions have been investigated, including the H5–H6 membrane-spanning region. To determine if this region of the α subunit contributes to ouabain sensitivity, amino acids which have the potential to form hydrogen bonds were substituted with alanine, a non-hydrogen-bonding amino acid. cDNAs encoding enzyme containing these individual amino acid replacements were expressed in ouabain-sensitive HeLa cells, and the ability of the altered enzymes to confer ouabain resistance was examined. Nineteen amino acid substitutions were investigated. T797A (Thr 797 to Ala) was the only substitution which conferred ouabain resistance to sensitive HeLa cells. Three additional substitutions at this position (T797V, T797S, and T797D) were generated in order to examine the effects of the replacements of Thr 797 on ouabain inhibition of Na,K-ATPase activity. The T797V substitution conferred ouabain resistance, but T797S and T797D substitutions did not. The ouabain-resistant cell lines expressing the T797A and T797V substitutions exhibited Na,K-ATPase activity that was 60 and 70 times more resistant to ouabain than the endogenous HeLa or sheep enzymes. The absence of a hydroxyl group at amino acid 797 may be responsible for the reduced sensitivity of the enzyme with substitutions at this position. The T797 substitutions were further analyzed for inhibition of Na,K-ATPase activity by cardiac glycosides which vary only in a specific portion of the inhibitor. The I_{50} ratios for the paired drugs, ouabagenin–ouabain, dihydroouabain–ouabain, and digoxin–digitoxin, were similar for both the wild-type sheep and substituted enzymes suggesting that T797 does not interact directly with the sugar moiety, the unsaturated bond of the lactone ring, or the hydroxyl group at position C-12 of the steroid ring.

Na,K-ATPase[1] is a ubiquitous integral membrane protein which is responsible for establishing an electrochemical gradient of Na^+ and K^+ ions across the plasma membrane of mammalian cells, and these ion gradients are essential for maintaining cellular homeostasis (Ullrich, 1979; MacKnight & Leaf, 1977; Thomas, 1972). Na,K-ATPase is a heterodimer composed of a catalytic α subunit and a glycosylated β subunit. The α subunit is responsible for coupling the hydrolysis of ATP with Na^+ and K^+ transport across the membrane. The β subunit is required for proper assembly and targeting of the protein to the plasma membrane (McDonough, 1990). In addition, Na,K-ATPase is the receptor for cardiac glycosides, which are a class of drugs used in the treatment of congestive heart failure. At this time, the coupling mechanism of Na,K-ATPase is not well understood. However, understanding the interaction of cardiac glycoside with Na,K-ATPase and inhibitory action of these drugs on this transport protein may contribute to our knowledge of active cation transport.

The α subunit of the Na,K-ATPase was implicated in its binding of cardiac glycosides using photoactivable ouabain analogs (Forbush et al., 1978), and a series of experiments using chimeras between the ouabain-sensitive sheep and the ouabain-resistant rat Na,K-ATPase α1 subunits, along with site-directed mutagenesis studies, have identified the N-terminal half and, specifically, two charged amino acids at the border of the first extracellular loop (H1–H2) as important determinants in ouabain sensitivity (Price & Lingrel, 1988; Price et al., 1989). Subsequent studies identified a conserved Asp 121 in the first extracellular loop and two residues in the H1 transmembrane region, Tyr 108 and Cys 104, of the sheep α subunit as determinants of the affinity of the Na,K-ATPase for ouabain (Price et al., 1990; Schultheis & Lingrel, 1993). In the dog α1 subunit, Cys 113 in the H1 transmembrane domain (analogous to C104 in the sheep) and Tyr 317 in the H3–H4 extracellular loop have also been shown to influence the affinity of the enzyme for ouabain (Canessa et al., 1992, 1993). These studies indicate that the N-terminal half of the α subunit of Na,K-ATPase, and especially the H1–H2 transmembrane and extracellular domain, are important in determining cardiac glycoside sensitivity. However, when a monoclonal antibody recognizing the H1–H2 extracellular domain was bound to Na,K-ATPase, ouabain binding was not inhibited (Arystarkhova et al., 1992). Furthermore, the amino acids identified in the H1–H2 region do not specifically interact with the sugar moiety of cardiac glycoside (O'Brien et al., 1993). Therefore, the exact site of drug–enzyme interaction is not known and is probably complex involving the H1–H2 region in addition to other domains of the Na,K-ATPase.

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[1] Abbreviations: Na,K-ATPase, sodium- and potassium-activated adenosinetriphosphatase; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetate.

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Previous studies have indicated the involvement of the C-terminal half of α subunit for ouabain binding since both the N-terminal and the C-terminal portions of the α subunit are labeled by analogs of ouabain (Forbush et al., 1978; Forbush, 1983; Deffo et al., 1983; Goeldner et al., 1983; Jorgensen et al., 1982; Rogers & Lazdunski, 1979; Rossi et al., 1980; Ruoho & Kyte, 1974). It was recently shown that a chimeric protein in which the N-terminal region of the SR Ca-ATPase was replaced with the corresponding portion of the Na,K-ATPase α1 subunit (Met 1–Asp 200) exhibited ouabain-dependent inhibition (Ishii & Takeyasu, 1993). In addition, another chimeric enzyme composed of the N-terminal half of the rat gastric H,K-ATPase (which normally does not bind ouabain) and C-terminal portion of the rat Na,K-ATPase α1 isoform is also inhibited by ouabain (Blostein et al., 1993). These studies suggest that both N-terminal and C-terminal portions of the α subunit of Na,K-ATPase are able to support low-affinity ouabain binding. This was confirmed in a recent study which used random mutagenesis to identify an amino acid residue, arginine 880, in the C-terminal portion of sheep α subunit as a determinant of ouabain sensitivity (Schultheis et al., 1993). When this residue was mutated to a proline, the resulting enzyme exhibits a K_D for ouabain 5-fold higher than that of the wild-type sheep α1 isoform. Therefore, the determinants of ouabain binding are not restricted to the N-terminal half but also may involve the C-terminal portion of α subunit.

Understanding the interaction of cardiac glycoside with Na,K-ATPase is further complicated by the chemical structure of the drug. Cardiac glycosides, such as ouabain and digoxin, are composed of a steroid ring system, a 17β-lactone ring and a sugar residue(s) attached at C-3. It is reasonable to assume that multiple contacts exist between these drugs and the Na,K-ATPase. Extensive structure–activity relationship studies using a variety of cardiac glycoside analogs suggest that the Na,K-ATPase contains regions which interact with each of these chemical constituents. It is likely that Van der Waals and hydrogen-bonding forces are involved in the binding interaction (Thomas et al., 1989).

In this study, a systematic approach of site-directed mutagenesis was used to investigate the role of amino acids with hydrogen-bonding potential in the H5–H6 transmembrane and extracellular region for ouabain sensitivity. Among the 19 amino acids examined, Thr 797 was found to be a determinant of ouabain sensitivity. This amino acid has also been identified by Burns and Price (1993) in ouabain binding in the sheep α1 subunit. Here we report the substitution of this residue with other amino acids and characterize the binding to paired cardiac glycosides.

MATERIALS AND METHODS

Materials. Molecular biology reagents were purchased from Promega, New England Biolabs, Amersham Corp., and Pharmacia LKB Biotechnology, Inc., and tissue culture supplies were obtained from Fisher or GIBCO. Ouabain was purchased from Calbiochem. Digoxin, digitoxin, ouabagenin, dihydroouabain, pyruvate kinase, lactate dehydrogenase, ATP, NADH, and phosphoenolpyruvate were obtained from Sigma. Plasmid DNAs were purified using Qiagen columns, purchased from Qiagen, Inc., and radioisotopes were purchased from DuPont–New England Nuclear.

Mutagenesis and Cloning. Eukaryotic expression vectors, pKC4, containing either the wild-type sheep Na,K-ATPase α subunit cDNA (ouabain-sensitive form) or the sheep (RD) cDNA which was modified by substitutions Q111R and N122D at the border of the first extracellular domain to encode a ouabain-resistant form of the enzyme, were constructed as previously described (Price & Lingrel, 1988). Cassettes from the coding region of the sheep α1 cDNA were subcloned into M13. Site-directed mutagenesis was used to introduce the desired single mutation into the cassette (Kunkel, 1985). Mutant cassettes were sequenced in their entirety to verify that no other mutations were introduced during the mutagenesis procedure. The cassette carrying the nucleotide substitution was subcloned back into the context of both the sheep Na,K-ATPase α1 subunit cDNA and the sheep (RD) α1 cDNA in the pKC4 expression vector. Final constructs were analyzed by restriction analysis, as well as by sequencing across the mutation site. Nucleotide substitutions were made in the sheep α1 cDNA giving rise to the following amino acid replacements: H286A, T289A, E327A, T332A, K767A, S768A, Y771A, T772A, T774A, S775A, N776A, E779A, T781A, N790A, T797A, T797V, T797S, T797D, D804A, T807A, D808A, and S814A. All plasmids were purified on Qiagen column prior to their use for transfection.

Tissue Culture and Transfection of HeLa Cells. HeLa cells were maintained essentially as described elsewhere (Price & Lingrel, 1988). Transfections (25 μg of each plasmid DNA) were carried out by the modified calcium phosphate procedure (Chen & Okayama, 1987). Two days after transfection, the transfected HeLa cells were selected in 0.2 μM ouabain for 3–4 weeks. Several ouabain-resistant 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 using a modification of the procedure of Chomczyski and Sacchi (1987). RNA (10 μg) from untransfected HeLa cells and HeLa cell transfectants was denatured in 1 M glyoxal, 54% DMSO, and 0.01 M $NaHPO_4$, pH 6.8, fractionated by electrophoresis in 1% agarose gel, 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 which contains sequences of the small t-intron and poly(A) addition signal. Hybridization and washes were carried out by the method of Church and Gilbert (1984). Following hybridization, the filter was washed twice in 0.5% bovine serum albumin, 1 mM Na_2 EDTA, 5% SDS, and 0.04 M $NaHPO_4$ (pH 6.8) at 65 °C for 15 min and twice in 1 mM Na_2 EDTA, 1% SDS, and 0.04 M $NaHPO_4$ (pH 6.8) at 65 °C for 15 min. The filter was blotted dry, and the signal was detected by autoradiography at −70 °C for 3 days to 1 week.

Isolation of Crude Plasma Membrane from HeLa Cells. Crude plasma membranes were prepared from transfected HeLa cells as described by Jewell and Lingrel (1991). Cells from six confluent 150-mm tissue culture dishes, grown in 0.2 μM ouabain, were washed twice with PBS and harvested via mild trypsinization. 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 by centrifugation at 3000g for 1 min, and the supernatant was treated with 6 M NaI as described (Lane et al., 1973). Membranes were pelleted at 48000g for 25 min and washed once with 1 mM Na_2EDTA/1 mM Tris (pH 7.4) buffer, and the pellet was resuspended in the same buffer at 1–2 mg/mL. The protein concentration of each membrane preparation was determined by a modification of the Bradford procedure (Bradford, 1976). NaOH was added to break up membrane fragments, and bovine serum albumin was used as a standard.

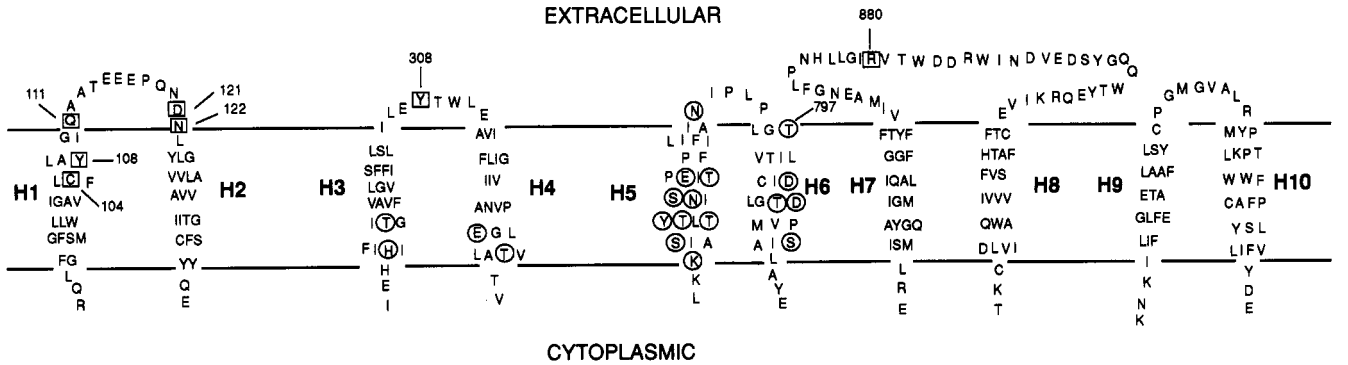

FIGURE 1: Transmembrane and extracellular domains of the 10-transmembrane model of the Na,K-ATPase sheep α1 subunit. Shown is the hypothetical membrane organization of the Na,K-ATPase α subunit. The circled residues were substituted by site-directed mutagenesis and their ouabain sensitivities examined. The substituted amino acids and replacements are listed in Table 1. The amino acids shown in squares are those which have been identified previously as determinants of ouabain sensitivity.

Na,K-ATPase Assay. Total Na,K-ATPase activity in crude plasma membranes was measured using the spectrophotometric coupled enzyme assay (Schwartz et al., 1969). Membranes prepared from the transfected HeLa cells as well as purified sheep kidney Na,K-ATPase (provided by Dr. James Ball, University of Cincinnati) were incubated for 15 and 120 min in assay buffer (25 mM histidine, 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 1 mM phosphoenolpyruvate, 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 drugs before Na,K-ATPase activity measurements. NADH or phosphoenolpyruvate were added again if necessary. Total Na,K-ATPase activity was determined by subtracting the ATPase activity obtained in the presence of 0.01 M ouabain from that observed in the absence of inhibitor. The specific Na,K-ATPase activity of these preparations was 22–34 μmol of P_i/(mg·h). To generate dose–response curves for each drug, two membrane preparations from one clone were pooled together and assayed in duplicate at each drug concentration. For each mutant at least two independently cloned cell lines were assayed in this way. Ouabain and ouabagenin were dissolved in H_2O at 0.01 M and concentrations were verified by spectrophotometry at 220 nm; then they were diluted serially to the proper concentrations. Digoxin, digitoxin, and dihydroouabain were dissolved in DMSO in order to increase solubility and diluted serially in DMSO. The final concentration of DMSO in the assay of these drugs was 1%. The data are presented as the percent Na,K-ATPase activity remaining at a particular drug concentration relative to the total Na,K-ATPase activity in the absence of the drug. An I_{50} value is the concentration of drug that inhibits 50% of the maximum Na,K-ATPase activity, and all the data were fitted by a Kleidagraph program as one component. I_{50} ratios were the mean value of the I_{50} of the first drug divided by the mean value of the second drug, and standard deviations were calculated according to the formula described by Wilkinson (1961). Comparisons of I_{50} ratios were made between wild-type sheep enzyme and mutants using unpaired, two-tailed *t*-tests. A probability of less than 0.05 was the criterion used to determine statistical significance.

RESULTS

Fifteen conserved amino acids located in the H5–H6 membrane-spanning and extracellular domains and four in H3–H4 membrane-spanning regions, whose functional groups can participate in hydrogen-bonding, were substituted with alanine by site-directed mutagenesis of the sheep α1 cDNA. The methyl group of alanine is a poor hydrogen-bond former but is compatible with an α-helical structure (Chou & Fasman, 1974). The 10-transmembrane model of the α subunit is shown in Figure 1, and the substituted amino acids are indicated. The effect of each amino acid substitution on ouabain inhibition was assessed by its ability to confer ouabain resistance when expressed in sensitive HeLa cells.

Table 1: Summary of the Ability of Cells Expressing Sheep α1 with Various Amino Acid Substitutions To Survive in Ouabain

sheep α1 substitution[a]	transmembrane location[b]	growth in ouabain: substitution in sensitive isoform[c]	growth in ouabain: substitution in resistant isoform[d]
H286A	H3	–	+
T289A	H3	–	+
E327A	H4	–	–
T332A	H4	–	+
K767A	H5	–	+
S768A	H5	–	+
Y771A	H5	–	–
T772A	H5	–	+
T774A	H5	–	+
S775A	H5	–	–
N776A	H5	–	–
E779A	H5	–	+
T781A	H5	–	+
N790A	H5–H6 extracellular loop	–	+
T797A	H6	+	+
T797S	H6	–	+
T797V	H6	+	+
T797D	H6	–	–
D804A	H6	–	–
T807A	H6	–	+
D808A	H6	–	–
S814A	H6	–	+

[a] Sheep α1 substitutions were generated as described under Materials and Methods. [b] The location of each substitution is based on the 10-transmembrane model of the Na,K-ATPase α subunit. [c] Growth of HeLa cells transfected with the wild-type sheep α1 cDNA encoding the indicated substitutions and selected ouabain resistant colonies with 0.2 μM ouabain. [d] Growth of HeLa cells transfected with the modified sheep α1 (RD) cDNA encoding the indicated substitutions and assayed for survival in 0.5 μM ouabain. The (+) indicates that colonies appear in the presence of ouabain whereas the (–) indicates that no colonies are observed.

Ouabain-resistant colonies were obtained from cells transfected with the sheep α1 cDNA construct encoding the substitution T797A. No ouabain-resistant colonies were observed when cells were transfected with wild-type sheep α1 cDNA or with the 18 other amino acid substitutions (Table 1). In order to determine the functional and structural role played by the Thr 797 in ouabain binding, three additional substitutions were generated at this position including Thr to Ser (T797S), Thr to Val (T797V), and Thr to Asp (T797D).

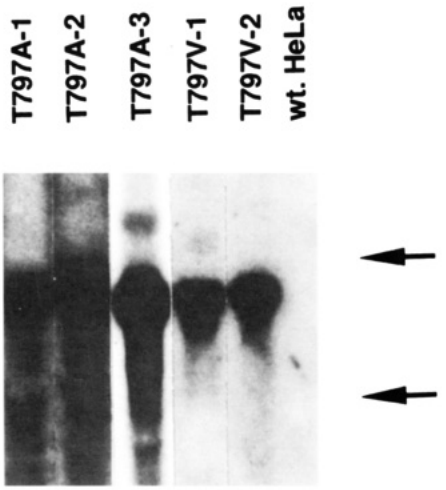

FIGURE 2: Northern analysis of RNA isolated from ouabain-resistant HeLa cell transfectants. Total RNA was isolated from ouabain resistant HeLa cell transfectants and from untransfected HeLa cells. Each lane contains 10 μg of RNA. Following electrophoresis and transfer to nylon membranes, hybridization was carried out with a ^{32}P-labeled DNA fragment derived from the 3′-untranslated region of pKC4. The blot was washed and autoradiography was carried out for 7 days. The arrows indicate the location of 28S and 18S ribosomal RNA. wt, wild type.

The T797S substitution represents a conservative replacement since serine has nearly the same hydrogen bonding characteristics and structure as threonine although the side chain is shorter. The T797V substitution eliminates hydrogen bonding by replacement of the hydroxyl group with a methyl group. T797D substitutes the hydroxyl containing side chain with a negatively charged side chain. When these three mutants were transfected in sensitive HeLa cells, only T797V generated ouabain-resistant colonies. Cells transfected with T797S or T797D cDNAs were not able to confer ouabain resistance. Ouabain-resistant colonies from the T797A and T797V substitutions were isolated and expanded into stable cell lines. Northern analysis was performed on RNA isolated from each cloned cell line to verify that the ouabain-resistant phenotype was due to the expression of the transfected sheep α1 cDNA carrying the amino acid replacement rather than from the overexpression of the endogenous enzyme. The DNA probe was derived from the 3′-untranslated sequence of the pKC4 vector, which does not cross-hybridize with the endogenous α1 subunit RNA. As expected, RNA isolated from untransfected HeLa cells does not hybridize to the DNA probe (Figure 2). All ouabain-resistant cell lines from the T797A and T797V transfections express a mRNA species hybridizing to the DNA probe (Figure 2). These cell lines were further characterized in terms of ouabain inhibitable Na,K-ATPase activity. The Na,K-ATPase in crude membranes prepared from the ouabain-resistant transfectants T797A and T797V exhibited an I_{50} for ouabain inhibition of 1.6×10^{-6} and 1.9×10^{-6} M, respectively. The wild-type sheep kidney enzyme gave an I_{50} of 2.4×10^{-8} M (Figure 3 and Table 2). The T797A and T797V substitutions lowered the ouabain sensitivity from the wild-type enzyme 60–70 fold. This indicates that Thr 797 in the H6 transmembrane domain of the α subunit is a determinant of ouabain sensitivity of the Na,K-ATPase.

Active enzyme is required for ouabain resistance to be conferred to sensitive HeLa cells; hence, it is possible that the remaining amino acid substitutions which did not produce ouabain-resistant colonies inactivated the enzyme. To test this possibility, T797S and T797D substitutions as well as the remaining amino acid replacements were introduced into a sheep α1 (RD) cDNA encoding a ouabain-resistant isoform.

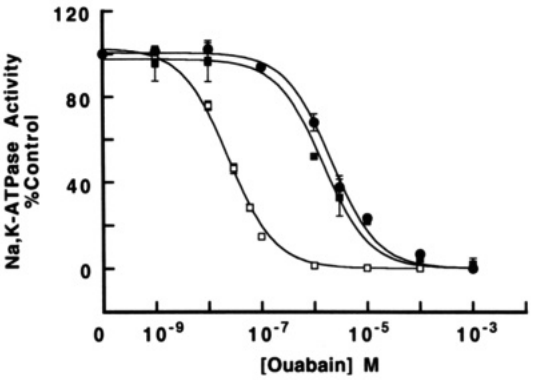

FIGURE 3: Ouabain-inhibitable Na,K-ATPase activity of wild-type sheep α1 and the functional T797 substitutions. The data are presented as the percent of Na,K-ATPase activity remaining at the indicated ouabain concentrations relative to the total Na,K-ATPase activity in the absence of ouabain. Membranes prepared from the ouabain-resistant transfectants and purified sheep kidney enzyme were preincubated with the appropriate concentrations of ouabain for 15min and 120 min, respectively, prior to Na,K-ATPase activity measurements. The plot represents the results of one experiment which was performed in duplicate using a pool of two membrane preparations. At least two independently cloned cell lines from each mutation were assayed. The specific activity (as micromoles of ATP hydrolyzed per milligram of protein per hour) of each membrane preparation in 0 mM ouabain was as follows: T797A = 24.7 ± 1.4; T797V = 25.4 ± 0.9; wild-type sheep enzyme = 725.3 ± 1.8. Open squares, wild-type sheep enzyme; filled squares, T797A; filled circles, T797V.

These constructs were transfected into ouabain-sensitive HeLa cells and selected for survival in the presence of 0.5 μM ouabain. After 3–4 weeks, ouabain-resistant colonies appeared with the sheep α1 (RD) cDNA (the positive control), as expected. Ouabain-resistant colonies appeared with the T797S substitution but not T797D substitution. This indicates that replacement of Thr 797 with serine does not inactivate the enzyme, while substitution of Thr 797 with aspartic acid does (Table 1). Since substitution at position 797 with a residue containing a hydroxyl group (T797S) results in a ouabain-sensitive enzyme and substitutions with residues lacking a hydroxyl group (T797A and T797V) produce a more resistant enzyme, it appears that the hydroxyl group of the wild-type Thr 797 is important in determining ouabain sensitivity and the hydroxyl group of this amino acid may be involved in hydrogen bonding with a specific moiety of the cardiac glycoside. Ouabain-resistant colonies were also observed with the following substitutions, H286A, T289A, T332A, K767A, S768A, T772A, T774A, E779A, T781A, N790A, T807A, and S814A (Table 1), when they encode ouabain-resistant isoform. These findings demonstrate that substitutions at these positions do not inactive the enzyme. Thus amino acids at positions 286, 289, 332, 767, 768, 772, 774, 779, 781, 790, 807, and 814 are ruled out as significant determinants of ouabain sensitivity. Substitutions at these positions do not inactive the enzyme, nor do they confer ouabain resistance. In contrast, ouabain-resistant colonies did not appear when cells were transfected with sheep α1 (RD) cDNA carrying the following substitutions: E327A, Y771A, S775A, N776A, D804A, and D808A (Table 1). As expected, ouabain-resistant colonies were not observed with mock transfected cells or cells transfected with wild-type sheep cDNA. These results indicate that the above six substitutions inactivated the enzyme and may be critical for overall activity. It is possible that these residues are involved in ouabain affinity, but this cannot be determined from our studies.

Ouabain, like other naturally occurring cardiac glycosides, is composed of three separate moieties: a lactone ring, a hydrophobic steroid ring system, and a sugar residue(s).

Table 2: Sensitivity to Cardiac Glycosides of Wild-Type Sheep Na,K-ATPase and Enzyme Carrying Substitutions in the α Subunit

substitution	I_{50}[a] (M)				
	ouabain	ouabagenin	dihydroouabain	digoxin	digitoxin
T797A (n = 3)	$(1.6 \pm 0.5) \times 10^{-6}$	$(1.1 \pm 0.3) \times 10^{-4}$	$(2.9 \pm 0.3) \times 10^{-5}$	$(6.5 \pm 1.5) \times 10^{-6}$	$(2.2 \pm 0.3) \times 10^{-6}$
T797V (n = 2)	$(1.9 \pm 0.3) \times 10^{-6}$	$(9.3 \pm 0.3) \times 10^{-5}$	$(3.7 \pm 1.1) \times 10^{-5}$	$(2.4 \pm 0.4) \times 10^{-6}$	$(1.4 \pm 0.7) \times 10^{-6}$
wt sheep enzyme (n = 3)	$(2.4 \pm 0.06) \times 10^{-8}$	$(9.3 \pm 0.2) \times 10^{-7}$	$(3.1 \pm 0.6) \times 10^{-7}$	$(2.2 \pm 0.3) \times 10^{-8}$	$(0.91 \pm 0.16) \times 10^{-8}$

[a] I_{50} values are the mean ± the standard deviation of (n) independent experiments. For mutant enzymes, n equals the number of independent clones. For wild-type (wt) sheep α1 enzyme, n equals the number of experiments for one preparation of sheep kidney enzyme.

Table 3: I_{50} Ratios[a]

sheep α1 substitution	ouabagenin/ouabain	dihydroouabain/ouabain	digoxin/digitoxin	digoxin/ouabain	digitoxin/ouabain
T797A (n = 3)	69 ± 29	18 ± 5.3	3.0 ± 0.8	4.1 ± 1.5[b]	1.4 ± 0.6[c]
T797V (n = 2)	49 ± 7.9	19 ± 6.4	1.7 ± 0.9	1.3 ± 0.3	0.7 ± 0.4
wt sheep enzyme (n = 3)	39 ± 1.3	13 ± 2.5	2.4 ± 0.5	0.92 ± 0.13	0.38 ± 0.07

[a] The I_{50} ratios are the mean value of I_{50} (shown in Table 2) of the first (top) drug divided by the mean value I_{50} of the second (bottom) drug, and standard deviations are calculated according to the formula described by Wilkinson (1961). [b] $p < 0.03$. [c] $p < 0.05$, when compared with the wild-type enzyme (group t-test). Other numbers are $p > 0.05$.

Extensive structure–activity relationship studies have indicated the importance of these three moieties in cardiac glycoside binding and suggest that the Na,K-ATPase contains specific regions or residues which interact with these moieties. The inhibition of the substituted Na,K-ATPase by paired cardiac glycoside analogs was examined to determine if Thr 797 interacted with a specific moiety of the inhibitor. These paired drug analogs were chosen such that they differ only in one specific portion of the molecule. The paired analogs were as follows: ouabain and ouabagenin, which vary only in the carbohydrate portion with ouabagenin lacking the sugar residue; ouabain and dihydroouabain, which have similar structures except dihydroouabain has a saturated lactone ring; and digoxin and digitoxin, which differ by a single hydroxyl group on the steroid ring with digoxin having an extra hydroxyl group at C-12. Data from drug pairs can be used as evidence to indicate which portion of the cardiac glycoside binds to the amino acid of interest, Thr 797. For example, if Thr 797 of the sheep α1 subunit binds to a specific structural moiety of the cardiac glycoside such as the sugar residue, substitution of T797 with an amino acid that cannot bind to the sugar moiety should result in an enzyme with a increase in the I_{50} for ouabain but not for ouabagenin. The ratio of the I_{50} for ouabagenin to the I_{50} for ouabain of the substituted enzyme would decrease compared with that of wild-type enzyme. If Thr 797 is not involved in binding to the sugar portion, substitution of T797 would result in an enzyme which exhibits an I_{50} ratio for this pair of analogs similar to that of the sheep α1 wild-type protein. Likewise, the I_{50} ratios for other drug pairs can be used to determine whether Thr 797 substitutions affect binding to the other two major moieties of cardiac glycosides.

The results of these studies are summarized in Tables 2 and 3. Wild-type sheep enzyme exhibits an I_{50} of 2.4×10^{-8} M for ouabain and 9.3×10^{-7} M for ouabagenin, and the I_{50} ratio for ouabagenin to ouabain is 39. I_{50} values of wild-type enzyme reported here correlate with the results obtained by Wallick et al. (1980). The T797A and T797V substitutions exhibit I_{50} values for ouabain of 1.6×10^{-6} and 1.9×10^{-6} M, respectively, and I_{50} values of 1.1×10^{-4} and 9.3×10^{-5} M for ouabagenin. The ratio of the I_{50} values for ouabagenin to ouabain for T797A and T797V is not significantly different from that of wild-type enzyme ($p > 0.05$). Therefore Thr 797 does not appear to be involved in the sugar binding. The wild-type enzyme with an I_{50} of 3.1×10^{-7} M for dihydroouabain has an I_{50} ratio of 13 for dihydroouabain to ouabain. The T797A and T797V substitutions yield I_{50} values of 2.9 $\times 10^{-5}$ and 3.7×10^{-5} M for dihydroouabain and I_{50} ratios of 18 and 19 (dihydroouabain to ouabain) which are not significantly different from the ratio of the wild-type enzyme ($p > 0.05$). These results suggest that T797 is also not primarily involved in binding to the unsaturated bonds of the lactone ring. In the case of digoxin and digitoxin the wild-type enzyme exhibits I_{50} values of 2.2×10^{-8} and 9.1×10^{-9} M, respectively. The T797A and T797V substitutions exhibit I_{50} values of 6.5×10^{-6} and 2.4×10^{-6} M for digoxin and I_{50} values of 2.2×10^{-6} and 1.4×10^{-6} M for digitoxin. The similar I_{50} ratios for digoxin and digitoxin among the wild-type enzyme and the T797 substitutions indicate that Thr 797 also does not interact with the hydroxyl group at position C-12 of the steroid ring. When the drug comparisons were carried out between digoxin and ouabain, as well as between digitoxin and ouabain, the wild-type enzyme showed an I_{50} ratio of 0.92 (digoxin to ouabain) and 0.38 (digitoxin to ouabain). The T797V substitution gave I_{50} ratios of 1.3 (digoxin to ouabain) and 0.7 (digitoxin to ouabain) with no significant difference from the wild type. However, the T797A substitution exhibited I_{50} ratios of 4.1 (digoxin to ouabain) ($p < 0.03$) and 1.4 (digitoxin to ouabain) ($p < 0.05$) with a significant difference from that of the wild-type enzyme.

DISCUSSION

Previously, a number of transmembrane amino acids with hydrogen-bonding potential have been examined for their roles in determining ouabain sensitivity (Schultheis & Lingrel, 1993). The objective of the present study was to further explore the role of hydrogen bonding in the interaction between ouabain and Na,K-ATPase. Fifteen potential hydrogen-bonding amino acids in the H5–H6 region (based on 10-transmembrane model) and four amino acids in the H3–H4 transmembrane region were substituted with alanine, an amino acid which does not form hydrogen bonds readily. Sheep α1 cDNAs carrying these substitutions were independently transfected into HeLa cells. One of these substitutions (T797A) was able to confer ouabain resistance to sensitive HeLa cells indicating that Thr 797 is a determinant of ouabain sensitivity. Following completion of this work, Burns and Price (1993) published that T797N was also able to confer ouabain resistance as identified by random mutagenesis.

Thr 797 is predicted to reside in the putative H6 transmembrane region and is conserved among enzymes from various sources, including both sensitive (sheep) and insensitive (rat) enzymes (Lingrel et al., 1990). To further examine the

role of this Thr in ouabain sensitivity, additional amino acid substitutions were made. Substitution of Thr 797 with valine, which replaces the hydroxyl with a methyl group and disrupts possible hydrogen bonding, reduced ouabain affinity by 70-fold. Substitution of threonine with serine did not disrupt enzyme function and preserved the ouabain sensitivity exhibited by the wild-type enzyme. In contrast, the T797D substitution which introduces a negatively charged aspartic acid resulted in an inactive enzyme suggesting that the general structure and/or absence of a charged residue at this position must be maintained. These findings reveal the potential importance of the hydroxyl group and the structure of the threonine side chain in ouabain binding.

To characterize whether those substituted enzymes which were not able to confer ouabain resistance were functional, each individual substitution was introduced into a ouabain-resistant sheep (RD) cDNA expression vector. Substitutions of amino acids at positions H286, T289, T332, K767, S768, T772, T774, E779, T781, N790, T807, and S814 gave colonies when they were expressed in HeLa cells, indicating that they do not inactivate the enzyme. Because substitutions of these residues with alanine do not inactivate the enzyme and fail to give ouabain-resistant colonies when carried in a sensitive α1 subunit, it is unlikely that these amino acids play a major role in ouabain binding. Substitutions E327A, Y771A, S775A, N776A, D804A, and D808A were not able to generate colonies when carried in a ouabain-resistant α1 cDNA indicating that these amino acid substitutions prevented Na,K-ATPase function. These amino acids are either critical for the overall enzyme activity or preclude its synthesis, maturation, transport to the plasma membrane, or stability.

Recently, five carboxyl-containing amino acids located in the transmembrane regions of the rat α2 isoform were examined in terms of their importance in Na,K-ATPase function (Jewell-Motz & Lingrel, 1993). These residues were E327, E778, D803, D807, and D925. The present study adds three nonnegatively charged amino acids, Y771, S775, and N776, located in the putative H5 transmembrane region, to this list of residues important for enzyme function. The corresponding amino acids in the sarcoplasmic reticulum Ca-ATPase for E327, E778, D803, D807 (rat α2 Na,K-ATPase), S775, and N776 (sheep α1 Na,K-ATPase) are also important in Ca^{2+} ion transport (MacLennan, 1990). Y771 is also conserved in the sarcoplasmic reticulum Ca-ATPase. While colonies were not obtained for cells transfected with rat α2* containing the E778L substitution (Jewell-Motz & Lingrel, 1993), replacement of the corresponding amino acid, E779 in sheep α1, with alanine is functional enough to maintain cell growth. The different consequences of these two substitutions are probably due to the effect of the side chain of the substituted amino acid. Further experiments are under way to study the importance of E779 for the Na,K-ATPase as well as the basis for loss of enzyme activity by these substitutions.

The interaction between cardiac glycosides and the Na,K-ATPase is complex, including initial binding of the steroid moiety to the enzyme followed by a conformational change in the receptor which results in the exposure of a sugar binding site (Yoda,1974; Yoda & Yoda,1975). The additional binding forces stabilize the ouabain–enzyme complex and prevent the dissociation of the inhibitor from the binding site of the Na,K-ATPase (Yoda, 1974; Adams et al., 1983). Mutations perturbing either step would alter the apparent affinity for ouabain. Different species of Na,K-ATPase (sensitive and insensitive enzymes) have similar association rates for binding cardiac glycosides but differ in their dissociation rates (Wallick et al., 1980; Tobin & Brady, 1972). Therefore, the ouabain binding site is at least partially conserved among ouabain-sensitive and less sensitive enzymes. The conserved Thr 797 may therefore be part of the ouabain binding site.

Five pairs of cardiac glycoside analogs were used to investigate whether T797 is involved in binding to the sugar, lactone, or steroid moieties. Similar I_{50} ratios were observed with these pairs of analogs among the wild-type sheep enzyme and the T797 substituted sheep enzyme, suggesting that Thr 797 may not interact directly with the sugar residue or the unsaturated bond of the lactone ring or the hydroxyl group at position C-12 of the steroid ring. The T797A substitution exhibited slightly increased I_{50} ratios for digoxin to ouabain and digitoxin to ouabain. Since ouabain, digoxin, and digitoxin have several differences in their structure, it is not clear what part of the cardiac glycoside causes this effect. Additional studies with other cardiac glycoside analogs are needed. The fact that T797 substitutions reduce the affinity for all the cardiac glycoside analogs tested here suggests that Thr 797 may interact with a common site conserved among these analogs. It is also possible that the Thr 797 is not at the physical binding site and substitution of this threonine may alter ouabain binding through allosteric effects. This type of indirect effect has previously been observed with modification of two cysteine residues on the cytoplasmic side which alters ouabagenin binding on the extracellular side (Kirley & Peng, 1991). The studies described here provide further evidence that the C-terminal half of the α subunit of Na,K-ATPase plays a critical role in determining ouabain affinity.

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