The EMBO Journal vol.11 no.5 pp.1681 - 1687, 1992

Mutation of a cysteine in the first transmembrane segment of Na,K-ATPase α subunit confers ouabain resistance

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Communicated by D.Louvard

The cardiac glycoside ouabain inhibits Na,K-ATPase by binding to the α subunit. In a highly ouabain resistant clone from the MDCK cell line, we have found two alleles of the α subunit in which the cysteine, present in the wild-type first transmembrane segment, is replaced by a tyrosine (Y) or a phenylalanine (F). We have studied the kinetics of ouabain inhibition by measuring the current generated by the Na,K-pump in *Xenopus* oocytes injected with wild-type and mutated α_1 and wild-type β_1 subunit cRNAs. When these mutations, α_1C113Y and α_1C113F [according to the published sequence [Verrey *et al.* (1989) *Am. J. Physiol.*, 256, F1034] were introduced in the α_1 subunit of the Na,K-ATPase from *Xenopus laevis*, the inhibition constant (K_i) of ouabain increased >1000-fold compared with wild-type. A more conservative mutation, serine α_1C113S did not change the K_i. We observed that the decreased affinity for ouabain was mainly due to a faster dissociation, but probably also to a slower association. Thus we propose that an amino acid residue of the first transmembrane segment located deep in the plasma membrane participates in the structure and the function of the ouabain binding site.

Key words: binding site mutation/Na,K-ATPase/ouabain/dog α subunit/*Xenopus laevis* oocyte

Introduction

Na,K-ATPase is the ouabain inhibitable sodium pump, a plasma membrane enzyme made of an $\alpha-\beta$ heterodimer which is present in almost all animal cells (Rossier *et al.*, 1987). Na,K-ATPase activity can be inhibited by an important class of drugs, the cardiac glycosides, such as ouabain, digoxine or digitoxine, which are used for their positive inotropic action on cardiac muscle and their effect of lowering heart rate (Smith, 1988). It is also postulated that Na,K-ATPase activity can be regulated by endogenous ligands, so called endo-ouabains, which have been characterized in plasma, adrenals and brain (Harber and Haupert, 1987; Hamlyn *et al.*, 1991). The ouabain binding site is therefore of primary importance, both for the clinician and the biologist.

Little is known about the molecular structure of this receptor. Site specific ligands (i.e. photoactivable ouabain analogues) have been used to localize the ouabain binding domain of the Na,K-ATPase α subunit (Forbush, 1983). Experiments using chimeric α_1 subunit molecules between species known for their resistance to ouabain (i.e. the rat) and α_1 subunit from species known for their sensitivity to the drug (i.e. sheep, human and chicken) clearly indicate that α subunits carry most if not all the ouabain sensitive or resistant phenotype (Price and Lingrel, 1988). Moreover, these experiments indicated that the first half of the molecule, i.e. the N-terminus, including the first four transmembrane domains, are involved. Site-directed mutagenesis experiments further delineated the ouabain resistant phenotype to the ectodomain loop between the first and second transmembrane hydrophobic domains (H_1 and H_2). The presence of charged amino acids at the border of the H_1-H_2 facing the external medium confers ouabain resistance, strongly suggesting their involvement in the ouabain binding site (Price *et al.*, 1990). The data obtained so far, however, do not exclude that other residues are involved in the ouabain binding site. To address this question, we have characterized at the molecular level, ouabain resistant α subunits from mutant cell lines which had been previously characterized at the pharmacological level (Soderberg *et al.*, 1983). MDCK (Madin-Darby Canine Kidney) cell lines were chemically mutagenized and ouabain resistant mutants which grow in up to 2000 times the ouabain dose necessary to inhibit the wild-type enzyme, were selected. The most resistant cell line was used in the present study. Inhibition of Na,K-ATPase activity by ouabain indicated that 50% of the cell enzyme displayed wild-type affinity and 50% a high resistance to ouabain with an estimated K_i of ~3 mM. The rate of synthesis as well as a total of enzyme molecules per unit of cell protein was unaltered in the mutant, suggesting that single mutations of one allele gene could explain the observed phenotype. We have therefore cloned and sequenced a 666 bp fragment of the MDCK α subunit from both ouabain sensitive and ouabain resistant cell lines that encompasses the first (H_1) and the third (H_3) hydrophobic domains, thus including the H_1-H_2 ectodomain so far characterized as a major site for ouabain binding. We found that the H_1-H_2 ectodomain is identical in the mutant cell line with respect to the wild-type sequence. By contrast, a cysteine found in the last third of the H_1 transmembrane domain was mutated to either tyrosine or phenylalanine. The introduction of this mutation in an ouabain sensitive α_1 subunit of *Xenopus laevis* confers high resistance to ouabain when expressed in the *Xenopus* oocyte expression system. We have therefore identified a novel residue that is an important determinant of ouabain binding kinetics.

Results

Cloning and sequencing of the H_1-H_3 segment of the α_1 subunit of Na,K-ATPase of MDCK cells

Using PCR and degenerated oligonucleotides corresponding to conserved hydrophobic regions (H_1 and H_3) of the α_1 subunit of Na,K-ATPase, we amplified a single fragment

of 666 bp in both wild-type and ouabain resistant MDCK cells. PCR products were subcloned in pBS vector. Ten colonies containing inserts from the wild-type cell line and 16 colonies with inserts from the ouabain resistant cell line were isolated and sequenced. The sequence of the wild-type fragment is shown in Figure 1 and was found to be identical in each of the 10 colonies selected and sequenced, indicating that no allelic variation and/or PCR errors were observed in the wild-type cell line. The sequence of the wild-type insert is consistent with that of previously published sequences of α_1 ouabain sensitive isoforms from various species (Kawakami *et al.*, 1985, 1986; Lebovitz *et al.*, 1989; Ovchinnikov *et al.*, 1986; Shull *et al.*, 1985, 1986; Takeyasu *et al.*, 1988). By contrast, sequence comparison of the wild-type segment with those from ouabain resistant cells revealed a 16% difference at the nucleotide level. Most of the changes were at the third base pair of codons and were scattered regularly over the whole length of the segment (Figure 1). The reason for this variability is unclear since such diversity is not expected after the chemical mutagenesis protocol using ethyl methane sulfonate. At the level of the amino acids (see Figure 2), there was a much higher conservation, since the 16% difference at the nucleotide level corresponded to only four amino acid substitutions at the protein level. Three of them were either conservative or found in ouabain sensitive α_1 isoforms, when comparison of the MDCK sequence was made with the published α subunit sequences from other species. The single most striking difference was the substitution of a highly conserved C113 for either Y or F in the first transmembrane domain.

Ouabain inhibition kinetics for wild-type and mutated α_1 subunit expressed in oocytes

Expression of wild-type and mutated α_1 cRNAs. In order to decide whether this mutation alone can confer ouabain resistance, we introduced these two mutations, or a more

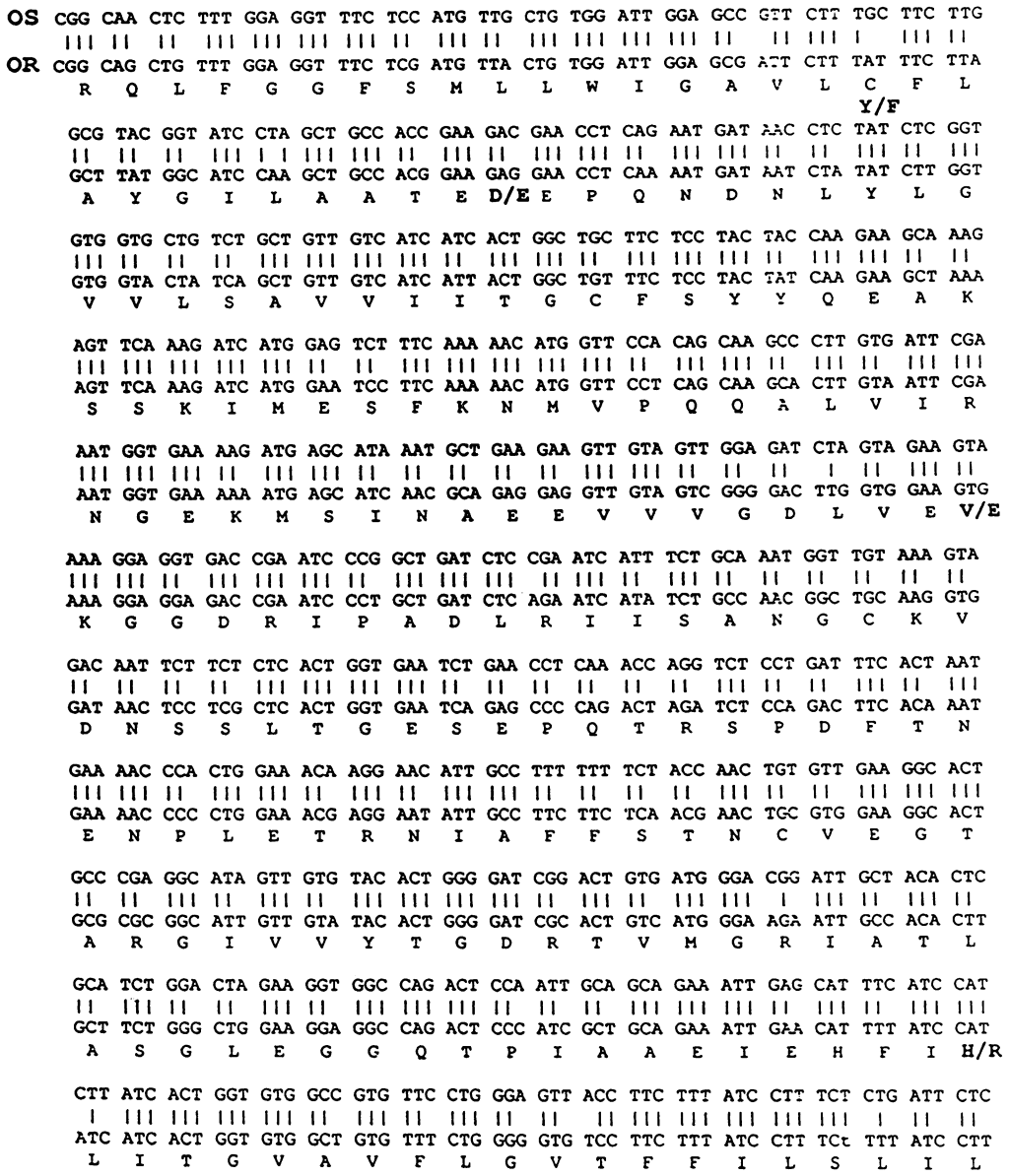

Fig. 1. Alignment of cDNA sequences and deduced amino acid sequences of ouabain sensitive (OS) and ouabain resistant (OR) MDCK cell lines. H_1-H_3 domain of α subunit of the Na,K-ATPase from OS or OR cells was amplified using degenerate oligonucleotides and PCR. The results represent the sequence of 10 OS and 16 OR clones. The absence of a vertical bar between both cDNAs indicates lack of homology. Amino acid differences are pointed out in bold. More than one amino acid in the sequence indicates the presence of different alleles in the OR cell line.

conservative S for C replacement at this single site in the ouabain sensitive α_1 subunit of *X.laevis* and expressed it in the oocyte system. We have previously shown that exogenous Na,K-pumps can be expressed in *X.laevis* oocyte by injection of cRNA encoding α_1 and β_1 subunits and the activity of the expressed pumps can be estimated by measuring the K^+-induced current (Horisberger *et al.*, 1991). As shown in Figure 3, coinjection of α_1 wild-type β_1 (lane 3) cRNA induced a highly significant increase ($P < 0.001$, $n = 10$) in Na,K-pump current compared with either the β_1 cRNA injected alone (lane 2) or the water injected oocyte control (lane 1). The injection of α_1 C113Y, C113F and C113S mutant cRNA led to identical levels of Na,K-pump current (see lanes 4–6) indicating that the mutation did not prevent the functional expression of new pumps at the cell surface.

Ouabain inhibition constant (K_i). As shown in Figure 4, the induction of Na,K-pump current by 10 mM K in the external medium is completely inhibited by 5 μM ouabain when α_1 and β_1 wild-type cRNAs have been coinjected. Wash out experiments indicated that the dissociation rate constant (K_{off}) for ouabain is very slow (see Figure 7), taking >60 min to recover ~10% of the original non-inhibited pump current. This observation was convenient in that it allowed us to eliminate the endogenous component of Na,K-ATPase which could disturb the measurement of ouabain sensitivity of the mutants. As shown in Figure 5, an experiment with an oocyte injected with α cRNA encoding for the C113Y mutant, we first eliminated the endogenous component by blocking the ouabain sensitive endogenous Na,K-ATPase by 5 μM ouabain, which should block >98% of the endogenous pump. Then, we washed out the ouabain and studied the sensitivity of the pump current by adding increasing concentrations of ouabain from 5 μM to 1 mM. From the data shown, one can deduce the K_i of ouabain for the mutant Na,K-pumps. As shown in Figure 6 and Table I, both mutants C113Y and C113F confer high resistance to ouabain in the *X.laevis* oocyte system such that 1 mM ouabain was not sufficient to block completely the inward K-induced current. By contrast, the C113S mutant (not shown) behaves like the α_1 wild-type.

Association rate constant. The association rate constant (K_{on}) can be estimated from the time course of the decrease of the current (see Materials and methods). In both the

	Frequency	113	125	195	295
Amino acid position					
Ouabain Sensitive	10/10	-- C --	D --	V --	H --
Ouabain Resistant	7/16	-- C --	E --	V --	H --
	5/16	-- Y --	E --	V --	H --
	3/16	-- F --	E --	V --	R --
	1/16	-- C --	E --	E --	H --

Fig. 2. Mutated amino acids in the four alleles cloned from the ouabain resistant cell line. The top line is the wild-type allele common to all 10 clones from the ouabain sensitive cell line. Only modified amino acids are represented, at the positions indicated by the numbers above the top line.

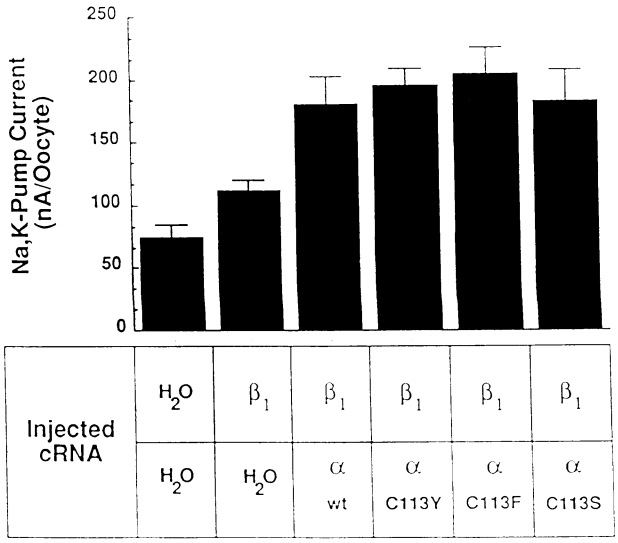

Fig. 3. Na,K-pump activity measured as the outward K^+ induced current in oocytes injected with β_1 and α_1 wild-type or β_1 and mutated α subunit cRNAs. Na,K-pump current induced by three different α subunit mutants was of the same magnitude as the current induced by wild-type. Each bar represents the mean of 10 oocytes per experimental condition. Na,K-pump current were measured as described in Horisberger *et al.* (1991).

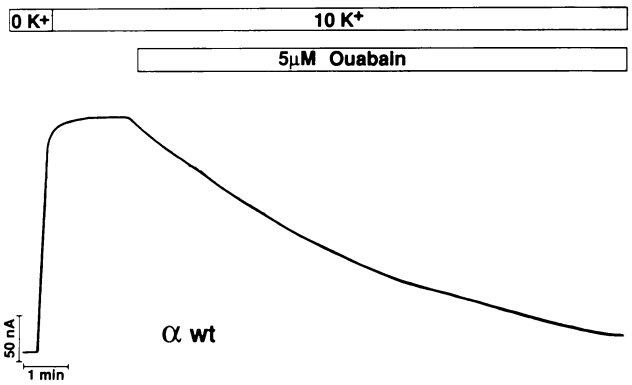

Fig. 4. Time course of ouabain inhibition of wild-type Na,K-pump. Tracing of an experiment showing Na,K-pump activity in an oocyte injected with wild-type β_1 and α_1 subunit cRNAs. Na,K-pump activity was measured as the current induced by 10 mM K^+ in the bathing solution at a holding potential of −50 mV. Addition of 5 μM ouabain produced complete inhibition of pump current.

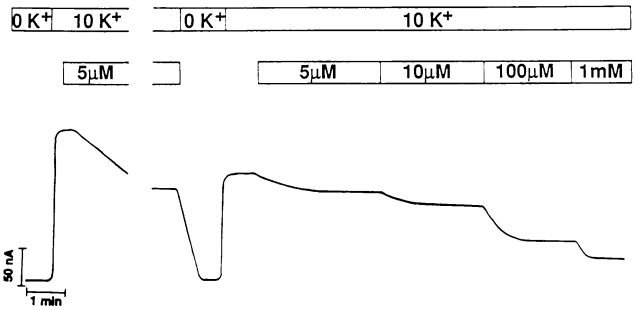

Fig. 5. Dose–response to ouabain of α_1C113Y mutant. Tracing of an experiment showing Na,K-pump activity measured as K^+ dependent current in an oocyte injected with β_1 wild-type and α_1C113Y subunit cRNAs. 5 μM ouabain produced inhibition of endogenous pump. A short wash out period with 0 mM K^+ was performed to verify the baseline. Increasing concentrations of ouabain from 5 μM to 1 mM were added to the bath and produced a stepwise decrease of current. At 1 mM it remained a fraction of pump current not inhibited by this concentration of ouabain.

C117Y and the C113F mutants there was a decrease of about one order of magnitude of the K_{on}, while with the C113S this variable was similar to the wild-type. Because of the high concentrations necessary to inhibit a sizeable part of the current due to resistant mutants, the time course of the onset of the effect is fast, not far from the bath exchange time, and therefore these K_{on} values (Table I) should be taken only as rough estimates. It should also be noticed that all K_{on} measurements were performed in the presence of a high extracellular K^+ concentration (10 mM), a condition known to slow down the association rate of ouabain (Forbush, 1983).

Dissociation rate constant. As expected from the known very slow dissociation rate (K_{off}) of ouabain [(half-life) ~150 min (Yoda, 1973)], when ouabain was washed out in the presence of 10 mM K^+, no recovery of the α wild-type injected oocytes could be observed. In contrast, the C113Y and the C113F mutants began to recover significantly their pump function within seconds. The dissociation for the wild-type and the α C113S mutant was so slow that it could not be measured accurately, because of the technical difficulty in maintaining stable impalement for a long time. To overcome this problem, we measured the K_{off} for strophanthidin, a cardiotonic steroid that lacks a sugar group at the third position of the steroid moiety and has been reported to have a much faster K_{off} for the wild-type Na,K-ATPase. As shown in Table I, dissociation rates for wild-type and the mutant C113S were similar (half-life ~150 and ~120 s, respectively), suggesting that the C to S substitution did not change the kinetics for the aglycone strophanthidin. By contrast, the mutants C113Y and C113F had a more rapid dissociation half time, respectively ~2 and ~7 s. These values have to be considered as upper limits as these rates are in the same range as the solution exchange time.

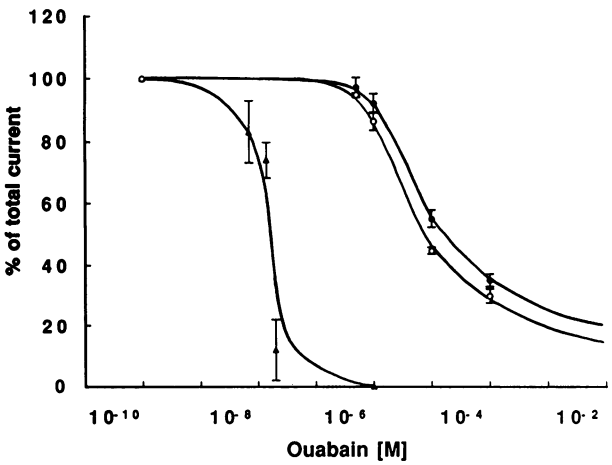

Fig. 6. Ouabain inhibition of Na,K-pump activity. Concentration dependence of ouabain inhibitable Na,K-pump activity measured as K^+ dependent current of whole oocytes injected with wild-type, C113Y or C113F α subunit cRNAs. For wild-type, duplicate measurements were taken at each ouabain concentration. For mutants, each point represents five individual oocyte measurements. The data are presented as percentage activity measured at a particular ouabain concentration relative to the total Na,K-pump current in the absence of ouabain.

The dissociation constants (K_ds) of ouabain that can be calculated by kinetic analysis ($K_d = K_{off} / K_{on}$) are within a factor of two to three of the measured equilibrium inhibition constant (K_i) (see Table I). The technical difficulties in obtaining precise measurements of the three variables under similar experimental conditions may explain the discrepancies: high rate constants may be underestimated because of the time needed to change the solution, while low K_i may be overestimated because of the long time needed to reach equilibrium at low concentration. Another possibility is that the binding of ouabain is a complex process with several bound states (Forbush, 1983).

Discussion

The tertiary structure of the Na,K-ATPase molecule is unknown. As for most membrane proteins, the limitation is the lack of three-dimensional crystals to allow electron or X-ray diffraction studies. Systematic site-directed mutagenesis of putative critical residue, has been undertaken to analyse structure–function relationship of membrane proteins (Roepe and Kaback, 1990). There are, however, difficulties interpreting results of such site-directed mutagenesis experiments because replacement of one amino acid can introduce unexpected changes in the proper folding of the protein, resulting in the absence of function. To avoid

Table I. Summary of inhibition constants (K_i), association (K_{on}) and dissociation (K_{off}) rate constants of ouabain on wild-type and mutated α subunit of Na,K-pump

	α wild-type		αC113Y		αC113F		αC113S	
	mean ± SEM	*n*	mean ± SEM	*n*	mean SEM	*n*	mean SEM	*n*
K_i (μM)	<0.1	3	250 ± 0.4	8	110 ± 10	8	<0.1	2
K_{on} (M^{-1}. s^{-1})	3140 ± 1370	5	495 ± 118	8	134 ± 11	7	2900 ± 590	4
K_{off} ouabain								
$K_{off}(s^{-1})\times10^{-3}$	<0.8	3	49 ± 5	12	4.8 ± 0.7	9	< 0.8	3
$t_{½}$(s)	>6000	3	15 ± 2	12	180 ± 30	9	>6000	3
K_{off} strophanthidin								
K_{off} $(s^{-1})\times10^{-3}$	4.7 ± 0.3	4	460 ± 50	4	114 ± 20	4	6 ± 1	4
$t_{½}$(s)	150 ± 9	4	1.5 ± 0.3[a]	4	7 ± 1.5[a]	4	120 ± 17	4

All measurements were made in the presence of 10 mM K^+.
K_{on} values were estimated from the time course of current inhibition by 5 or 10 μM ouabain in wild-type and C113S mutants, and by 100 μM ouabain in C113Y and C113F mutants.
K_{off} values were measured after maximal inhibition, from the time course of the current recovery when ouabain or strophanthidin was washed out.
[a]These values should be considered lower estimates as this half-life is similar to the solution exchange time.

some of the potential pitfalls of systematic site-directed mutagenesis, we have taken a ouabain resistant mutant clone of MDCK cells that was obtained by chemical mutagenesis followed by ouabain selection (Soderberg *et al.*, 1983). Although the ouabain resistance could be the result of amplification of the α subunit gene, it was shown (Soderberg *et al.*, 1983) that resistant clones produced equivalent amounts of Na,K-ATPase to wild-type. Other possibilities for acquired resistance include inactivation of the drug or regulation of ouabain resistance by another gene (English *et al.*, 1985). We hypothesized that if the observed resistance was due to a mutation in its binding site then the resulting α subunit should be correctly processed and targeted to the plasma membrane and that it should still be able to transport cations to maintain cell viability when grown with 4 mM ouabain in the culture medium. Therefore, we cloned and sequenced the segment of the α subunit containing a putative ouabain binding site.

The molecular structure of digitalis compounds includes a steroid central body with a lactone ring at position C17; both constitute the genin moiety which is necessary for the inhibitory activity (Repke, 1985). One or more sugar residues (digitoxone, rhamnose or cymarose) are present at C3. The sugar moieties are not essential for the blocking activity but confer a more stable association with the α subunit (Yoda, 1973; Yoda and Yoda, 1976). Each of these three components of the glycoside has been postulated to bind to specific sites on the Na,K-ATPase. Yoda proposed that following ouabain binding there is a conformational change in the pump that renders the complex more stable, preventing ouabain dissociation, by exposing a binding site for the sugar moiety of cardiac glycosides (Yoda, 1973). Compounds that do not carry a sugar group, like strophanthidin, bind with a similar K_{on} but are released more rapidly as predicted by this hypothesis. Lingrel and his colleagues have shown that charged residues at the borders of the H_1-H_2 hydrophobic domain, present in the α_1 isoform of the rat, an ouabain resistant species or introduced by site-directed mutagenesis, render the sodium pump resistant to ouabain (from 1 μM up to 4 mM) probably by preventing the stabilization of the ligand−receptor complex (Price *et al.*, 1990).

Other domains in the protein like H_3-H_4 have also been implicated in ouabain binding. Ahmed *et al.* (1990) performed labelling studies with a photoactive probe ([^{3}H]24-azidodigitoxoside) located in the genin moiety of the digitalis molecule and found that most of the ^{3}H radioactivity was recovered in the second extracellular loop (80%) and the third transmembrane region (20%). Recently, however, Lingrel has introduced multiple non-conservative mutations in this region (Lingrel *et al.*, 1991) without changing ouabain sensitivity, suggesting that the region is probably not directly involved in ouabain binding.

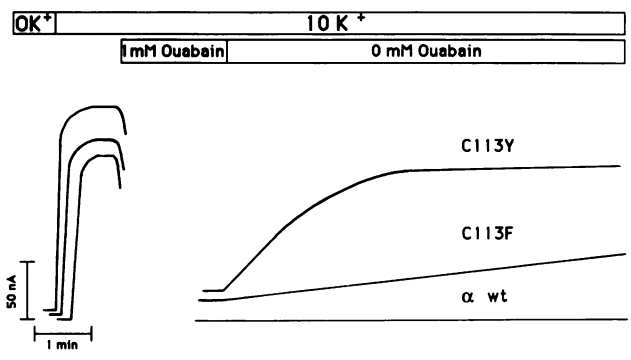

Fig. 7. Recovery of ouabain inhibitable Na,K-pump current. Whole oocyte K^+ dependent current was inhibited by 1 mM ouabain followed by a wash out period. In oocytes injected with wild-type α subunit cRNA, there was no recovery of activity during the wash out period. In oocytes injected with mutated α subunit cRNAs it was observed that recovery of pump current was faster for mutant C113Y than for C113F.

Characterization of ouabain inhibition kinetics of both wild-type and mutated *Xenopus* Na,K-ATPase in the oocyte system reveals that resistance in mutants C113Y and C113F was due to both a rapid K_{off} and a slower K_{on}. The slower association rate could be due to a decrease of the dwelling time of the Na,K-pump in the conformation able to bind ouabain (E2 conformation) (Forbush, 1987) or, alternatively, to an increase of an activation energy barrier in the binding and stabilization process. The substitution of Cys113 in the first transmembrane region by aromatic residues (tyrosine or phenylalanine) might prevent the accommodation of the ligand by virtue of the large side chains. Such a site could be like a cleft in the protein, consistent with the characteristic slow K_{on} of cardiac glycosides. Concerning the rapid dissociation rate in our mutants, we first postulated that Cys113 participates in the sugar binding. However, the faster K_{off} of the aglycone strophanthidin in the C113Y and C113F mutants rather supports the hypothesis that these mutations alter the binding of the genin moiety. Conceivably, the presence of bulky amino acids in the first transmembrane helix may hinder a conformational change that takes place subsequently to the initial binding of cardiac glycosides either in the form of a tilt or a rotation of H_1. Another interpretation is that at least part of the ouabain binding site is embedded in the membrane helices of the α subunit, somewhat similar to the interactions between rhodopsin and retinal (Nakayama and Khorana, 1991).

If C113 participated in a disulfide bond substitution by a serine could be responsible for the observed changes in the ouabain sensitivity. However, mutant C113S behaved like wild-type. In addition, mutagenesis of a H_2 cysteine (C146S) (data not shown) did not change ouabain sensitivity. The observed effects introduced by mutations C113Y and C113F seem to be dependent on the size of the amino acid substituted. Both tyrosine and phenylalanine are hydrophobic amino acids with bulky aromatic side chains. Similar observations were made on the murine acetylcholine receptor (Lo *et al.*, 1991) where substitution of C230 located in the putative first transmembrane region of the γ subunit does not change ion conductance or selectivity but has an effect upon gating which is proportional to the size of the substituted side chain.

Because ouabain only interacts from the extracellular side, it has always been assumed that the amino acids involved in binding are located in the H_1-H_2 and/or H_3-H_4 loops that face to the outside of the cell. Mutagenesis efforts so far have been concentrated in these two domains (Lingrel *et al.*, 1990; Price *et al.*, 1989). The data presented here bring attention to other residues located in transmembrane helices. The role of the other amino acid substitution observed in the H_2-H_3 cytoplasmic loop (e.g. V/E, D/E, H/R, see Figure 1) in determining the ouabain resistant phenotype has not yet been examined but could be tested easily with the present methodology. Other ouabain resistant MDCK clones are available in our laboratory and will be

studied, looking for other residues that participate in the ouabain binding site. Finally, the oocyte expression system used in this work offers the advantage of a better characterization of newly identified mutants because it is possible to determine independently each parameter of inhibition and allows a more complete understanding of the interactions between cardiac glycosides and the Na,K-ATPase.

Materials and methods

Cells and cell culture

MDCK cell lines, wild-type and resistant to 4 mM of ouabain, were previously characterized (Soderberg *et al.*, 1983). The ouabain resistant cell line was established by chemical mutagenesis using ethyl methane sulfonate followed by ouabain selection. Cells were grown in DMEM medium (Sigma) supplemented with 10% FCS (Gibco) and harvested when they reached confluency.

Cloning of the H1 – H3 fragment of the α subunit of the Na,K-ATPase from MDCK cells

Total RNA from wild-type and ouabain resistant MDCK cells was extracted by the citric acid method and the poly(A)$^+$ mRNA fraction was isolated by oligo(dT) cellulose column (Collaborative Research Incorporated). 5 μg of poly(A)$^+$ RNA from each clone were taken to synthesize single-stranded (ss) cDNA using oligo(dT) primers (Pharmacia).

Degenerated oligonucleotides corresponding to amino acids 89–95 and 317–325 of *X.laevis* α subunit were designed to amplify the H1–H3 fragment of the Na,K-ATPase α subunit by the polymerase chain reaction (PCR). To facilitate the subcloning of the PCR product, we added an *Eco*RI restriction site and a GC tail at the 5′ end of the sense oligo nucleotide and a *Bam*HI plus a GC tail at the 5′ end of the antisense oligonucleotide. The corresponding sequences were: sense: 5′-CG*GAATTC*CCIGA(A,G)T-GGATIAA(A,G)TT(C,T)TG-3′ and for antisense: 5′-CG*GGATCC*AAIA-CIGC(T,C)TCIA(A,G)CCAIGT (A,G)TA-3′. 0.01 vol of the final product of the ss cDNA reaction from each cell line were used as template for PCR amplification, plus 200 μM of dNTPs, 500 ng of each oligonucleotide and 1 unit of *Taq* polymerase (Cetus). Amplification was performed for 30 cycles in a Perkin Elmer Cetus DNA thermal cycler: 94°C for 1 min; 50°C for 1 min; 72°C for 2 min.

Sequencing of the H1 – H3 fragment

The PCR product and the cloning vector pBS were digested with *Eco*RI and *Bam*HI to create sticky ends. Digested PCR products and pBS were ligated with T4 ligase (Boehringer) at 4°C, overnight. 100 ng of each ligation product were used to transform competent bacteria. Inserts were sequenced in both directions by the chain termination method using sequenase (USBC).

Site directed mutagenesis of the α subunit of the Na,K-ATPase from X.laevis

Cysteine 113 in wild-type α subunit of *X.laevis* was replaced by Y, F and S as described by Nelson and Long (1989). Briefly, we used a three step PCR protocol. The first step consisted in a 20 cycle amplification using a 20 bp sense oligonucleotide containing the desired mutation and an antisense 31 bp oligo corresponding to bp 836–852 in *X.laevis* α subunit cDNA. At the 5′ end of this oligonucleotide were added 15 bp of a foreign viral sequence (VSVG). An aliquot of the first PCR product was used as primer for the following step. It consisted of a single 10 min synthesis PCR cycle at 72°C. The presence of the foreign sequence at the distal end of the first PCR amplification caused the extension reaction to proceed only in the direction towards the 5′ end of the cDNA. For the final step, we synthesized a sense oligonucleotide in the cloning vector pSD5 and an antisense 15 bp oligo corresponding to the VSVG sequence. The product of the third PCR was a 865 bp fragment containing single restriction sites for *Eco*RI at the 5′ end of the introduced mutation and *Hpa*I at the 3′ end. These enzymes were subsequently used to digest the mutated fragment and replace it in the wild-type cDNA. The wild-type and all the mutated α subunits were cloned in plasmid pSD5 which contains a PS6 promoter for synthesis of RNA and a poly(A)$^+$ tail.

The final products of PCR mutation reactions were sequenced in both strands to verify the presence of the desired mutation and to rule out any misincorporations introduced during amplification.

Synthesis of cRNA and injection of α mutants Y, F and S in oocytes

cRNA from each mutant was synthesized using SP6 RNA polymerase (Pharmacia). β_1 subunit cRNA from the Na,K-ATPase of *X.laevis* was synthesized in the same way. Stage V–VI oocytes were injected using an automatic pressure injection system (Inject+matic, Geneva) with 50 nl of water or with 6 ng of β_1 cRNA only, or the combination of 6 ng of β_1 cRNA plus 18 ng of cRNA of α wild-type or 18 ng of each of the α mutants: Y, F and S. In all cases the total volume of injection was kept at 50 nl. After injection, oocytes were incubated at 19°C for 3–4 days in a modified Barth's solution containing (in mM): 85 NaCl, 2.4 $NaHCO_3$, 1 KCl, 0.8 $MgSO_4$, 0.3 $Ca(NO_3)$, 0.4 $CaCl_2$ and HEPES buffered to pH 7.2, plus penicillin (10 mg/ml) and streptomycin (5 mg/ml).

Measurements of the Na,K-pump current and ouabain inhibition kinetics

Electrophysiological experiments were performed 4–6 days after cRNA injection, according to a technique described earlier (Horisberger *et al.*, 1991). Briefly, the oocytes were first loaded with sodium by incubation for 2 h in a solution containing K^+-free and Ca^{2+}-free solution containing 0.5 mM EDTA. Later, oocytes were transferred to a solution of similar composition but containing 0.41 mM Ca^{2+} and no EDTA until measurements were performed. The sodium-loaded oocytes were then placed in a Lucite chamber with a volume of 0.3 ml connected to a flow system for continuous circulation of the bathing solutions at a flow rate of 10–20 ml/min. Using the two-electrode voltage clamp technique, Na,K-pump current was measured as the inward current induced by addition of 10 mM K^+, at −50 mV, to a previously K^+-free solution (in mM, Na^+ 97, K^+ 0.0, Ba^{2+} 5, Mg^{2+} 0.82, Ca^{2+} 0.41, gluconate 90, Cl^- 22.5, HCO_3^- 2.4, MOPS 10, pH 7.2). Measurements were restricted to oocytes with a membrane resistance >0.5 MOhm. Experiments were performed at room temperature. Then, in the presence of 10 mM K^+, 5 μM ouabain was added, a concentration sufficient to inhibit all the current due to sensitive Na,K-pumps (see Figure 3). The association rate constant (K_{on}) of ouabain could be obtained by fitting the decrease of the K^+-activated current to a single exponential equation. Because of the slow K_{on} of ouabain, in wild-type or ouabain sensitive mutants only an upper limit estimate of the inhibition constant (K_i) of ouabain could be obtained by measuring the inhibition produced by exposure to 0.1–10 μM ouabain, for up to 1 h (see Figure 6).

In ouabain resistant mutants inhibition kinetics were studied after inhibition of the ouabain sensitive endogenous Na,K-pump by a previous 5 min exposure to 5 μM ouabain. Then K_i was estimated in the presence of 10 mM K^+, by increasing the concentration of ouabain in a stepwise fashion from 0 to 5, 10, 100 and 1000 μM. Several minutes were allowed to reach a new current plateau before proceeding to a higher concentration (see example in Figure 5). The K_{on} was estimated from the time course of the decrease of the Na,K-pump current produced by addition of 100 μM ouabain, taking into account that the rate of the decrease of the current was equal to the sum of the on and off rate constants. After maximal inhibition of the Na,K-pump current, ouabain was rapidly washed and the evolution of the current recorded in the presence of 10 mM K^+ was used to calculate the dissociation rate constant (K_{off}). All rate constants were estimated by fitting the current data to a single exponential equation. The baseline current was checked several times during the course of each experiment by switching to the K^+-free solution.

Results are expressed as mean ± SEM (standard error of the mean) and the Student's *t* test was used to evaluate the statistical significance of differences between means.

Acknowledgements

We would like to thank Mrs Nicole Skarda-Coderey for her secretarial work, Jim Schafer and Edward Moczydlowski for their helpful comments and suggestions and Manual Peitsch. This study was supported by the Swiss National Fund for Scientific Research (grant #31–26497.89).

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Received on December 4, 1991; revised on February 17, 1992