0026-895X/04/6502-335–341$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:335–341, 2004

Vol. 65, No. 2
2923/1123086
Printed in U.S.A.

New Molecular Determinants Controlling the Accessibility of Ouabain to Its Binding Site in Human Na,K-ATPase α Isoforms

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Received August 26, 2004; accepted October 22, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Inhibition of Na,K-ATPase α2 isoforms in the human heart is supposed to be involved in the inotropic effect of cardiac glycosides, whereas inhibition of α1 isoforms may be responsible for their toxic effects. Human Na,K-ATPase α1 and α2 isoforms exhibit a high ouabain affinity but significantly differ in the ouabain association and dissociation rates. To identify the structural determinants that are involved in these differences, we have prepared chimeras between human α1 and α2 isoforms and α2 mutants in which nonconserved amino acids were exchanged with those of the α1 isoform, expressed these constructs in *Xenopus laevis* oocytes, and measured their ouabain binding kinetics. Our results show that replacement of Met^{119} and Ser^{124} in the M1–M2 extracellular loop of the α2 isoform by the corresponding Thr^{119} and Gln^{124} of the α1 isoform shifts both the fast ouabain association and dissociation rates of the α2 isoform to the slow ouabain binding kinetics of the α1 isoform. The amino acids at position 119 and 124 cooperate with the M7–M8 hairpin and are also responsible for the small differences in the ouabain affinity of the ouabain-sensitive α1 and α2 isoforms. Thus, we have identified new structural determinants in the Na,K-ATPase α-subunit that are involved in ouabain binding and probably control, in an α isoform-specific way, the access and release of ouabain to and from its binding site.

The ubiquitous Na,K-ATPase, which is responsible for the maintenance of the Na^+ and K^+ gradients in animal cells, functions as the pharmacological receptor for cardiac glycosides. Compounds such as digoxin, digitoxin, and ouabain are plant-derived steroids that bind to Na,K-ATPase with high selectivity and inhibit its transport activity. In myocardial cells, this inhibition results in a sequential increase in intracellular sodium and calcium concentrations and, consequently, an increase in the force of contraction. Especially digoxin is still widely used as an inotropic drug in the treatment of congestive heart failure despite its low therapeutic index. A better understanding of the structural features that determine cardiac glycoside interaction with Na,K-ATPase should help to develop inotropic drugs with better therapeutic effects and lower toxic effects.

The Na,K-ATPase is composed of a catalytic α-subunit with 10 transmembrane segments, which contains cation, ATP, phosphate, and cardiac glycoside binding sites as well as a β-subunit that is a type II membrane protein required for the structural and functional maturation of the α-subunit (Geering, 2001). Four α and three β isoforms exist that show a different tissue distribution (for review, see Blanco and Mercer, 1998) and that can produce Na,K-ATPase isozymes with different transport and pharmacological properties (Crambert et al., 2000).

This work was supported by Swiss National Science Foundation grant 31-64793.01 (to K.G.).

The contribution of different Na,K-ATPase isozymes to the pharmacological or toxic effects of cardiac glycosides is only partially understood. In rodents, the α1 isoform exhibits a nearly 1000-fold lower affinity for cardiac glycosides than α2 or α3 isoforms. Because rat cardiomyocytes express only α1 and α2 isoforms, it has been speculated that inhibition of the sensitive α2 isoform with low doses of cardiac glycoside produces the positive inotropic effect, whereas additional inhibition of α1 isoforms at higher doses leads to the toxic effect (Adams et al., 1982; Maixent et al., 1987). This hypothesis is supported by the recent observation that mouse hearts with genetically reduced levels of α2 isozymes are hypercontractile as a result of increased Ca^{2+} transients, which mimics the inotropic effect of cardiac glycosides. On the other hand, mouse hearts with reduced levels of α1 isoforms are hypocontractile, which resembles cardiac glycoside toxicity (James et al., 1999).

In humans, the situation is complicated by the fact that α1, α2, and α3 isoforms are present in the heart (Wang et al., 1996) and that all human isoforms have a similar high affinity for cardiac glycosides (Crambert et al., 2000; Wang et al., 2001). It has been speculated that an α2 isoform-specific function in the heart could be supported by a compartmentalization of the α2 isoform together with the Na^+/Ca^{2+} exchanger into microdomains, near the sarco-/endoplasmic reticulum (Juhaszova and Blaustein, 1997). Another argument for a role of human α2 isoforms in the positive inotropic effect of cardiac glycosides is that ouabain binding to α1

isoforms but not to α2 isoforms is efficiently antagonized by K^+ at physiological concentrations (Crambert et al., 2000).

Compared with α1 isoforms, human α2 isoforms display still another pharmacological difference that could be important for their implication in the inotropic effect of cardiac glycosides. Indeed, α2 isoforms have 5- to 10-fold faster ouabain association and dissociation kinetics than α1 isoforms (Crambert et al., 2000). Because association and dissociation rates vary in parallel, the K_d values are similar for α1 and α2 isoforms. So far, only α1 isoforms have been studied with respect to the localization of the binding site and the molecular determinants of the association and dissociation kinetics of cardiac glycosides. Experimental and modeling data suggest that cardiac glycosides bind to the extracellular surface of the α-subunit (Croyle et al., 1997; Middleton et al., 2000; Farr et al., 2002) and that the physical binding site for cardiac glycosides may be formed by the M3/M4 and M5/M6 hairpins (Koenderink et al., 2000). The association rate of ouabain seems to be dependent on the steroid moiety, whereas the dissociation rate depends both on the steroid and the sugar moieties (Yoda, 1974; Kawamura et al., 2001). For α1 isoforms, ouabain association rates are slow and independent of their sensitivity to cardiac glycosides, whereas dissociation rates are low in sensitive isoforms but high in resistant α1 isoforms (Yoda, 1974). In view of these data, it may be predicted that the differences in the ouabain association and dissociation rates in human α1 and α2 isoforms, which have a similar high sensitivity to cardiac glycosides, reflect differences in the accessibility of ouabain to its binding site rather than an alteration of the binding site itself.

In this study, we aimed to characterize the structural determinants that influence association and dissociation rates of cardiac glycosides to Na,K-ATPase and in particular to the 'inotropic' α2 isoform. We produced chimeras between human α1 and α2 isoforms or replaced amino acids in the α2 isoform by the corresponding amino acids of the α1 isoform, expressed the mutants in *Xenopus laevis* oocytes, and determined the association and dissociation rate and the equilibrium binding constants for ouabain. Our results indicate that the M1–M2 and the M7–M8 hairpins contain several specific amino acids that determine the differences in the ouabain binding kinetics in α1 and α2 isoforms and control the access of ouabain to its binding site.

Materials and Methods

Mutants and Chimeras. Chimeras of human α1 and α2 isoforms were produced by introducing restriction sites into the α1 cDNA that are present in the α2 cDNA. polymerase chain reaction fragments of α1 cDNA were digested and ligated into the α2 cDNA to replace the corresponding regions (Table 1). Point mutations were introduced into the human Na,K-ATPase α2 isoform, previously cloned into the pSD5 vector (Crambert et al., 2000), by the polymerase chain reaction-based method described by Nelson and Long (1989). The nucleotide sequences of all constructs were confirmed by dideoxy sequencing and cRNAs were prepared by in vitro translation (Melton et al., 1984).

Protein Expression in *X. laevis* Oocytes and Preparation of Microsomes. Stage V–VI oocytes were obtained from *X. laevis* as described previously (Geering et al., 1996). cRNAs coding for human Na,K-ATPase α1, α2, α1/α2 chimeras, or α2 mutants (10 ng/oocyte) were injected into oocytes in the presence of cRNA coding for the human Na,K-ATPase β1-subunits (1 ng/oocyte). Three days after cRNA injection, microsomes were prepared from oocytes as described previously (Geering et al., 1996). Protein concentrations were determined by the method of Lowry et al. (1951).

[^{3}H]Ouabain Binding Kinetics on Oocyte Microsomes. Ouabain binding kinetics were determined as described previously (Crambert et al., 2000). Briefly, oocyte microsomes (final concentration, 11 μg/ml), previously permeabilized by incubation with 0.15 μg of SDS/μg of protein for 25 min at 19°C, were added to a K^+-free medium containing various [^{3}H]ouabain (Amersham Biosciences, Piscataway, NJ) concentrations (from 3×10^{-9} to 5×10^{-8} M) and 4 mM ATP, 4 mM $MgCl_2$, 100 mM NaCl, and 30 mM imidazole/HCl, pH 7.4. After 2 h at 37°C, aliquots containing 5 μg of protein were removed, rapidly filtered under vacuum on glass-fiber filters (Whatman GF/C), and rinsed three times with 4 ml of an ice-cold buffer containing 100 mM NaCl and 30 mM imidazole/HCl, pH 7.4. Radioactivity bound to filters was counted after addition of 4 ml of scintillation solution (Emulsifier Scintillator Plus; PerkinElmer Life and Anylytical Sciences, Boston, MA). Ouabain binding experiments were performed under the same conditions on microsomes from noninjected oocytes of the same batch to determine ouabain binding to the oocyte, endogenous Na,K-ATPase, and the nonspecific binding. The mean values of these determinations were subtracted from ouabain binding data obtained with microsomes from cRNA-injected oocytes. Nonspecific binding, which was determined by addition of a 1000-fold excess of unlabeled ouabain, was not significantly different in different batches of oocytes and did not exceed 15% of the total binding. Wild-type and all mutant Na,K-ATPase α-β complexes were expressed in oocytes to a similar level, as reflected by the similar equilibrium [^{3}H]ouabain binding to oocyte microsomes (B_{eq}; see below and Table 2), and the expression of the exogenous Na,K-ATPase α-β complexes was 4 to 10 times above that of the endogenous oocyte Na,K-ATPase.

The association and dissociation kinetics of ouabain to wild-type and mutant Na,K-ATPase α-subunits were determined as specified in the figure legends. The dissociation rate constant (k_{-1}) was calculated from the slope of ln B/B_{eq} versus time plots; B_{eq} is specific [^{3}H]ouabain binding at equilibrium and B is specific [^{3}H]ouabain binding at several time points after addition of an excess of unlabeled ouabain. The observed first-order association rate constant (k_{obs}) of ouabain binding was determined as the slope of $\ln[(B_{eq} - B)/B_{eq}]$ versus time plots. Knowing k_{obs}, the ouabain concentration ([ouab]) used for association experiments, and the dissociation rate constant (k_{-1}), we determined the association rate constant (k_{+1}) with the equation $k_{obs} = k_{+1}[\text{ouab}] - k_{-1}$. All curve fittings were done with Kaleidagraph (Abelbeck/Synergy Software, Reading, PA) and unpaired Student's t test with Excel 98 (Microsoft, Redmond, WA) for the Apple Macintosh.

TABLE 1
Description of chimeras between Na,K-ATPase α 1 and α 2 isoforms.

Usual Name	Scientific Name
$\alpha1_{M4}/\alpha2$	$\alpha1Met^{1}$-$His^{390}/\alpha2Met^{389}$-$Tyr^{1020}$
$\alpha2_{M4}/\alpha1$	$\alpha2Met^{1}$-$His^{388}/\alpha1Met^{391}$-$Tyr^{1023}$
$\alpha1_{Nter}/\alpha2$	$\alpha1Met^{1}$-$Ala^{73}/\alpha2Arg^{72}$-Tyr^{1023}
$\alpha2/\alpha1_{M1-2}/\alpha2$	$\alpha2Met^{1}$-$Ala^{71}/\alpha1Arg^{74}$-$Val^{165}/\alpha2Pro^{164}$-$Tyr^{1020}$
$\alpha2/\alpha1_{M2-3}/\alpha2$	$\alpha2Met^{1}$-$Met^{162}/\alpha1Val^{165}$-$Leu^{302}/\alpha2Gly^{301}$-$Tyr^{1020}$
$\alpha2/\alpha1_{M7-8}/\alpha2$	$\alpha2Met^{1}$-$Asp^{827}/\alpha1Ile^{831}$-$Phe^{923}/\alpha2Ala^{921}$-$Tyr^{1020}$
$\alpha2/\alpha1_{M1-2}/\alpha2/\alpha1_{M7-8}/\alpha2$	$\alpha2Met^{1}$-$Ala^{71}/\alpha1Arg^{74}$-$Val^{165}/\alpha2Pro^{164}$-$Asp^{827}/\alpha1Ile^{831}$-$Phe^{923}/\alpha2Ala^{921}$-$Tyr^{1020}$

M, membrane domain; Nter , N-terminus.

Results

The N- and C-Terminal Parts of the Na,K-ATPase α-Subunit Contain Determinants for Ouabain Dissociation Kinetics. Fig. 1B shows the characteristic difference in the dissociation rate constant of ouabain between human Na,K-ATPase α1 and α2 isoforms. As described previously (Crambert et al., 2000), the ouabain dissociation rate constant of the α2 isoform was more than 10-fold greater than that of the α1 isoform. To localize the structural determinants that are responsible for these differences, we first prepared two chimeras, $\alpha1_{M4}/\alpha2$ and $\alpha2_{M4}/\alpha1$, in which the N-terminal part (up to M4) and the C-terminal part (including the large cytoplasmic loop up to M10) were exchanged between the two α isoforms (see Table 1 and Fig. 1A). The ouabain dissociation rate of the $\alpha1_{M4}/\alpha2$ chimera was seven times slower than that of the α2 isoform and two times faster than that of the α1 isoform (Fig. 1B). The reverse chimera $\alpha2_{M4}/\alpha1$ exhibited a 2-fold slower ouabain dissociation rate than the α2 isoform and a 5-fold faster rate than that of the α1 isoform. Thus, both the N-terminal part up to M4 and the subsequent C-terminal part comprising M5 to M10 of the α-subunit participate in the determination of ouabain dissociation rates with a predominant role of the N-terminal part.

Regions in the N- and C-Terminal Parts of the Na,K-ATPase α-Subunit Involved in the Ouabain Dissociation Kinetics. To define more precisely the region in the N-terminal part of the α-subunit that determines ouabain dissociation kinetics, we tested the effect of replacements of the cytoplasmic N terminus ($\alpha1_{Nter}/\alpha2$), of the M1–M2 region ($\alpha2/\alpha1_{M1-2}/\alpha2$) or of the M2-M3 region ($\alpha2/\alpha1_{M2-3}/\alpha2$) in the α2 isoform by the corresponding regions of the α1 isoform. As shown in Fig. 1B, the presence of the cytosolic N-terminal tail or the M2–M3 region of the α1 isoform did not significantly change the ouabain dissociation rate constant of the α2 isoform. On the other hand, the presence of the M1–M2 region of the α1 isoform decreased the ouabain dissociation rate constant of the α2 isoform near that of the $\alpha1_{M4}/\alpha2$ chimera, indicating that this region is an important although not unique determinant in the ouabain dissociation kinetics.

To define the contribution of the C-terminal part of the α-subunit in the ouabain dissociation rate, we produced a chimera in which the M7–M8 hairpin in the α2 isoform was replaced with that of the α1 isoform. This region was chosen because it has previously been identified to participate in ouabain binding (for references, see Croyle et al., 1997) and because it contains several amino acid differences among human α1 and α2 isoforms (see Fig. 2A). The $\alpha2/\alpha1_{M7-8}/\alpha2$ chimera showed a ouabain dissociation rate similar to that of the $\alpha2_{M4}/\alpha1$ chimera (Fig. 1B), indicating that the M7–M8 region, in addition to the M1–M2 region, influences ouabain dissociation kinetics. The essential role of these two regions was confirmed by testing a chimera in which both of these regions were replaced in the α2 isoform by the corresponding regions of the α1 isoform. This double chimera ($\alpha2/\alpha1_{M1-2}/\alpha2/\alpha1_{M7-8}/\alpha2$) exhibited an ouabain dissociation rate very near that of the α1 isoform (Fig. 1B; Table 2).

Amino Acids in the Na,K-ATPase α-subunit Involved in the Ouabain Dissociation Rate. Fig. 2A shows the alignment of the human α1 and α2 sequences in the M1–M2 and M7–M8 regions and the nonconservative amino acid differences in the two isoforms. To identify the amino acids in the M1–M2 and M7–M8 regions that are responsible for the α1 phenotype of the double chimera $\alpha2/\alpha1_{M1-2}/\alpha2/\alpha1_{M7-8}/\alpha2$ with respect to the ouabain dissociation kinetics, we tested the effect of the replacement in the α2 isoform of several nonconserved amino acids with the corresponding amino acids of the α1 isoform. Replacement of Met^{119} in the M1–M2 region of the α2 isoform with the corresponding Thr of the α1 isoform (α2M119T) produced a 3-fold reduction in the ouabain dissociation rate constant compared with that of the wild-type α2 isoform but it was still higher than that of the $\alpha2/\alpha1_{M1-2}/\alpha2$ chimera (Fig. 2B; Table 2). Simultaneous replacements in the α2 isoform of Met^{119} and Gly^{114} or Ala^{134} with the corresponding amino acid of the α1 isoform did not further decrease the ouabain dissociation rate compared with that of the α2M119T mutant. On the other hand, combination of the M119T mutation with a S124Q mutation (α2M119T/S124Q) reduced the ouabain dissociation rate of the α2 isoform 5-fold compared with that of the $\alpha2/\alpha1_{M1-2}/\alpha2$ chimera (Fig. 2B). This result shows that the only two non-

TABLE 2
Summary of results on the ouabain binding kinetics of chimeras between Na,K-ATPase α 1 and α 2 isoforms and of point-mutated α 2 isoforms

	k_{-1}	k_{obs}	k_{+1}	K_d	B_{eq}
	min^{-1}		$\mu M^{-1}\ min^{-1}$	nM	$pmol/mg$
α1	0.012 ± 0.001	0.034 ± 0.003	4.6 ± 0.4	5.1 ± 1.7	6.9 ± 0.7
α2	0.164 ± 0.01	0.21 ± 0.05	37.4 ± 5.8	17.9 ± 4.3	2.4 ± 0.1
$\alpha1_{M4}/\alpha2$	0.023 ± 0.001	N.D.	N.D.	N.D.	3.3 ± 0.1
$\alpha2_{M4}/\alpha1$	0.061 ± 0.011	N.D.	N.D.	N.D.	3.5 ± 0.9
$\alpha1_{Nter}/\alpha2$	0.14	N.D.	N.D.	N.D.	4 ± 0.3
$\alpha2/\alpha1_{M2-3}/\alpha2$	0.16	N.D.	N.D.	N.D.	3.1 ± 0.2
$\alpha2/\alpha1_{M1-2}/\alpha2$	0.034 ± 0.004	0.082 ± 0.004	11.6 ± 0.7	7.4 ± 1.4	5.2 ± 0.9
$\alpha2/\alpha1_{M7-8}/\alpha2$	0.088 ± 0.01	0.166 ± 0.04	25.4 ± 5.0	18.4 ± 5.5	3.9 ± 0.5
$\alpha2/\alpha1_{M1-2}/\alpha2/\alpha1_{M7-8}/\alpha2$	0.018 ± 0.003	0.043 ± 0.008	6.1 ± 1.1	5.1 ± 0.6	4.2 ± 0.4
α2M119T	0.055 ± 0.006	0.085 ± 0.009	14 ± 1.5	9.8 ± 0.5	8.1 ± 1.8
α2M119T/S124Q	0.033 ± 0.001	0.070 ± 0.001	10.2 ± 1.4	N.D.	6.2 ± 1.3
α2M119T/A134Q	0.056 ± 0.003	0.086 ± 0.022	14.2 ± 2.5	N.D.	3.8 ± 0.2
α2M119T/G114S	0.059 ± 0.001	0.079 ± 0.011	13.8 ± 1.15	N.D.	2.5 ± 0.2
α2T891W/M892I	0.168 ± 0.003	0.163 ± 0.05	33.1 ± 5.3	N.D.	5.5 ± 0.3
α2S878I	0.141 ± 0.007	0.233 ± 0.019	37.4 ± 2.6	N.D.	4.4 ± 0.2
α2E902Q	0.150 ± 0.032	0.225 ± 0.068	37.5 ± 10	N.D.	4.5 ± 0.2
α2S878I/E902Q	0.124 ± 0.015	0.223 ± 0.037	34.7 ± 5.2	N.D.	4 ± 0.6
α2S837P/Q838K	0.151 ± 0.020	0.198 ± 0.013	34.9 ± 3.3	N.D.	4.3 ± 0.3
α2M119T/S124Q/S878I/E902Q	0.026 ± 0.003	0.048 ± 0.005	7.4 ± 0.8	N.D.	3.2 ± 0.1

N.D., not determined.

conserved amino acids in the first extracellular loop of α1 and α2 isoforms contribute significantly to the differential ouabain dissociation rate of the two isoforms.

The M7–M8 region of the human α1 isoform contained in the $\alpha2/\alpha1_{M7-8}/\alpha2$ chimera shows six nonconservative amino acid differences compared with that of the α2 isoform (Fig. 2A). Several of these nonconserved amino acids in the α2 isoform were replaced alone or in combination with the corresponding amino acids of the α1 isoform, but none of these mutations reduced the ouabain dissociation rate of the α2 isoform to that of the $\alpha2/\alpha1_{M7-8}/\alpha2$ chimera (Fig. 2B; Table 2). Only the double mutant α2S878I/E902Q exhibited a significantly lower ouabain dissociation rate constant than the α2 isoform (Fig. 2B). Moreover, combination of the α2S878I/E902Q mutations in the M7–M8 extracellular loop with the M119T/S124Q mutations in the M1–M2 extracellular loop

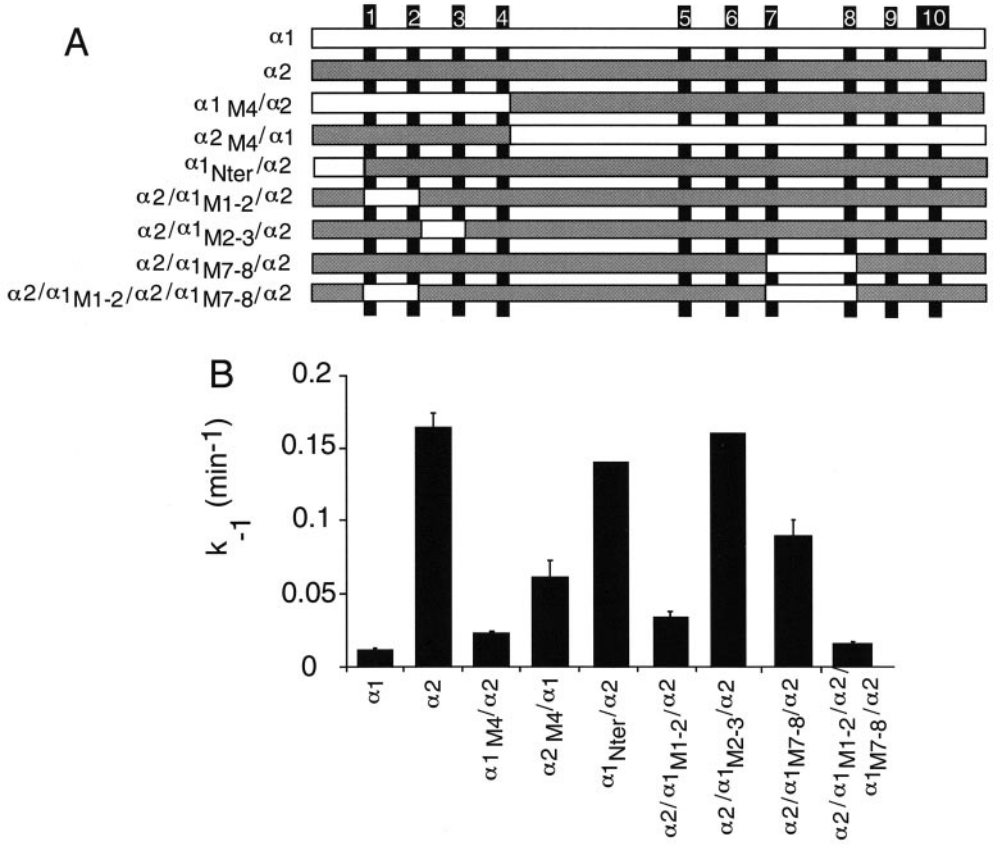

Fig. 1. Localization of regions involved in the dissociation rate constants (k_{-1}) of ouabain in human α1 and α2 isoforms and chimeras. A, schematic representation of chimeras between human α1 (□) and α2 (▨) isoforms. B, ouabain dissociation kinetics of wild-type α1 and α2 isoforms and of $\alpha1/\alpha2$ chimeras. Microsomes were prepared from noninjected oocytes or from oocytes injected with wild-type α1, α2, or $\alpha1/\alpha2$ chimera cRNAs together with β1 cRNA. After incubation of microsomes for 1 h with 5×10^{-8} M [^{3}H]ouabain, unlabeled ouabain (final concentration, 5×10^{-5} M) was added to initiate ouabain dissociation. Ouabain binding was determined after various periods. Ouabain binding caused by endogenous Na,K-ATPase was subtracted from data obtained on microsomes from the cRNA-injected oocytes. Dissociation rate constants (k_{-1}) were calculated as described under *Materials and Methods*. Data are means ± S.E. of three to nine experiments done in triplicate. $\alpha1_{M4}/\alpha2$ versus $\alpha1$, $p < 0.01$; $\alpha2/_{M1-2}/\alpha2$ versus $\alpha1_{M4}/\alpha2$, $p = 0.032$; $\alpha2/\alpha1_{M7-8}/\alpha2$ versus $\alpha1_{M4}/\alpha2$, $p = 0.12$; $\alpha2/\alpha1_{M1-2}/\alpha2/\alpha_{1M7-8}/\alpha2$ versus $\alpha1$, $p = 0.02$.

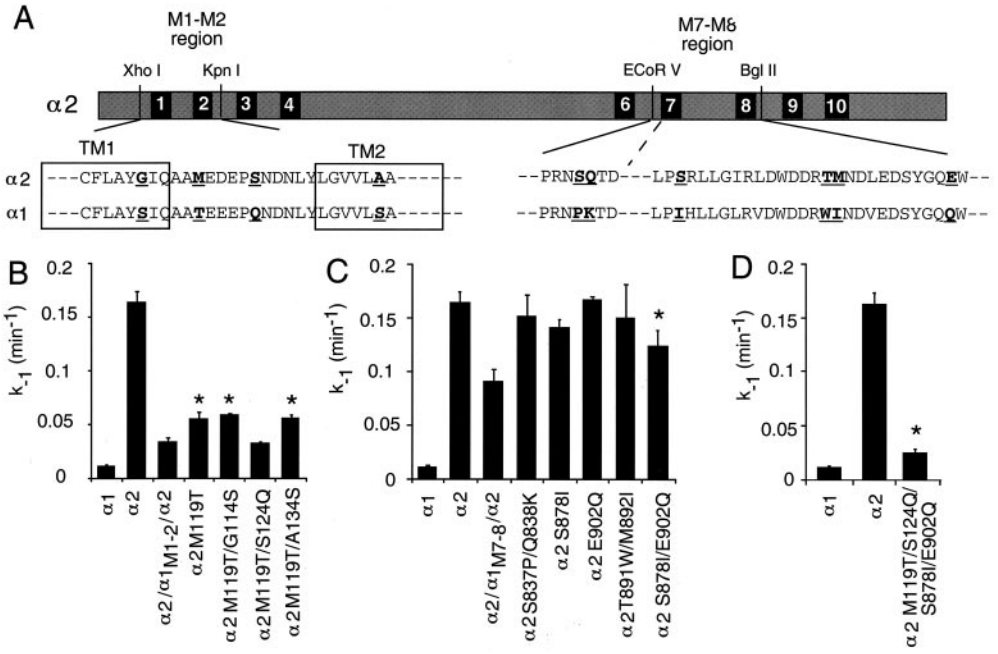

Fig. 2. Identification of amino acids involved in the dissociation rate constants (k_{-1}) of ouabain in human α isoforms. A, sequence alignment of human α1 and α2 isoforms. Shown are the regions in the M1–M2 and the M7–M8 hairpins that contain nonconservative amino acid differences in α1 and α2 isoforms (in bold). B to D, ouabain dissociation kinetics of mutant α2 isoforms. Measurement of ouabain dissociation was performed as described in the legend to Fig. 1. B, ouabain dissociation rate constants (k_{-1}) of α2 isoforms mutated in the M1–M2 region. *, $p < 0.05$, mutants versus the $\alpha2/\alpha1_{M1-2}/\alpha2$ chimera. C, ouabain dissociation rate constants (k_{-1}) of α2 isoforms mutated in the M7–8 region. *, $p < 0.05$ mutants versus the wild-type α2 isoform and the $\alpha2/\alpha1_{M7-8}/\alpha2$ chimera. D, ouabain dissociation rate constants (k_{-1}) of the α2 isoform mutated in the M1–M2 and M7–M8 regions. *, $p < 0.05$ mutant versus wild-type α1 isoform. Data are means ± S.E. of two to four experiments done in triplicate.

produced a α2M119T/S124Q/S878I/E902Q mutant with an ouabain dissociation rate that was slightly slower than that of the α2M119T/S124Q mutant, but still significantly different from that of the wild-type α1 isoform (Fig. 2B). Thus, despite the apparent, though minor contribution of the M7–M8 region in the determination of the ouabain dissociation rate of the α1 isoform, we were not able to fully mimic its effect by single or double mutations in the α2 isoform. This indicates that more complex intramolecular interactions are involved in the effect of the M7–M8 region of the α-subunit on the ouabain dissociation kinetics.

Regions and Amino Acids in the Human Na,K-ATPase α-Subunit Involved in the Ouabain Association Rate. As shown previously (Crambert et al., 2000), the association and dissociation rate constants of ouabain vary in parallel in human α1 and α2 isoforms. Therefore, we investigated whether the regions and amino acids in the α-subunit that are involved in the ouabain dissociation process were also important for the ouabain association kinetics. Fig.3 shows that, similar to previous findings (Crambert et al., 2000), the ouabain association rate constant of the human α2 isoform was about 8-fold greater than that of the α1 isoform. Replacement of the M1–M2 hairpin in the α2 isoform by that of the α1 isoform (α2/$\alpha1_{M1-2}$/α2) decreased the ouabain association rate by nearly 4-fold, whereas replacement of the M7–M8 hairpin (α2/$\alpha1_{M7-8}$/α2) had only a slight effect (Fig. 3; Table 2). However, when both membrane regions of the α2 isoform were replaced by those of the α1 isoform (α2/$\alpha1_{M1-2}$/α2/$\alpha1_{M7-8}$/α2), the ouabain association rate constant was similar to that of the wild-type α1 isoform (Fig. 3). Similar to the ouabain dissociation rate, the ouabain association rate of the α2 isoform was significantly decreased in the α2M119T mutant and even more in the α2M119T/S124Q mutant but was not influenced by mutations of other nonconserved amino acids in the M1–M2 or in the M7–M8 hairpins (see Table 2). However, the α2M119T/S124Q/S878I/E902Q mutant exhibited an ouabain association rate similar to that of the wild-type α1 isoform (Fig. 3). All together, these results indicate that the ouabain association and dissociation kinetics are determined by the same structural regions of the Na,K-ATPase α-subunit.

Regions In the Human α-Subunit That Are Involved in the Ouabain Association and Dissociation Rate Control the Ouabain Affinity of Ouabain-Sensitive Na,K-ATPases. All amino acids that so far have been identified to contribute to ouabain affinity of Na,K-ATPase (Croyle et al., 1997; Qiu et al., 2003) are identical in human α1 and α2 isoforms. It should thus be expected that these two isoforms have an identical ouabain affinity that is determined by the physical ouabain binding site and additional amino acids that influence the ouabain affinity through conformational changes. However, as shown previously (Crambert et al., 2000), although human α1 and α2 isoforms are both ouabain-sensitive, they differ about 4-fold in their affinity for ouabain (Fig. 4). Although the K_d values calculated from the k_{-1}/k_{+1} ratio (i.e., 2.6 and 4.4 nM for α1 and α2 isoforms, respectively) were lower than the K_d values determined by equilibrium binding (i.e., 5.1 and 17.9 nM for α1 and α2 isoforms, respectively), they varied in parallel between the two α isoforms. Here, we show that the same regions that influence the ouabain association and dissociation kinetics also influence the ouabain affinity of the human α isoforms. Indeed, the chimera α2/$\alpha1_{M1-2}$/α2 exhibited a K_d value for ouabain that was more than 2-fold lower than that of the wild-type α2 isoform. Replacement of the M7–M8 region did not change the affinity of the α2 isoform for ouabain, but the double chimera had the same ouabain affinity as the α1 isoform (Fig. 4; Table 2).

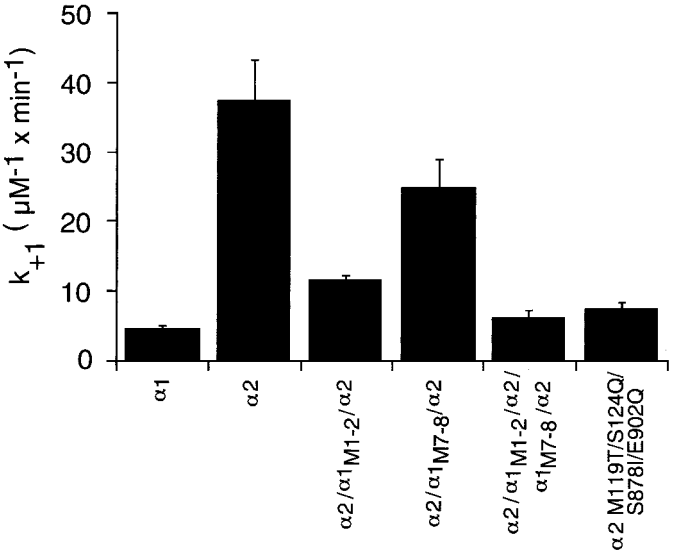

Fig. 3. Regions and amino acids involved in the association rate constants (k_{+1}) of ouabain in human α isoforms. Microsomes were prepared from noninjected oocytes or from oocytes injected with cRNAs coding for wild-type α1, α2, α1/α2 chimeras, or α2 mutants together with β1 cRNA. Ouabain binding was carried out in the presence of 1×10^{-8} M [^{3}H]ouabain and determined after various periods (between 0 and 90 min). Ouabain binding caused by endogenous Na,K-ATPase was subtracted from data obtained on microsomes from the cRNA-injected oocytes. Association rate constants (k_{+1}) were calculated from the observed first-order association rate constant (k_{obs}) and the dissociation rate constant as described under *Materials and Methods*. Data are means ± S.E. of two to four experiments done in triplicate. α2/$_{M1-2}$/α2 versus α1, $p = 0.002$; α2/$\alpha1_{M7-8}$/α2 versus α2, $p = 0.53$; α2/$\alpha1_{M1-2}$/α2/$\alpha1_{M7-8}$/α2 versus α1, $p = 0.35$; α2M119T/S124Q/S878I/E902Q versus α1, $p = 0.068$.

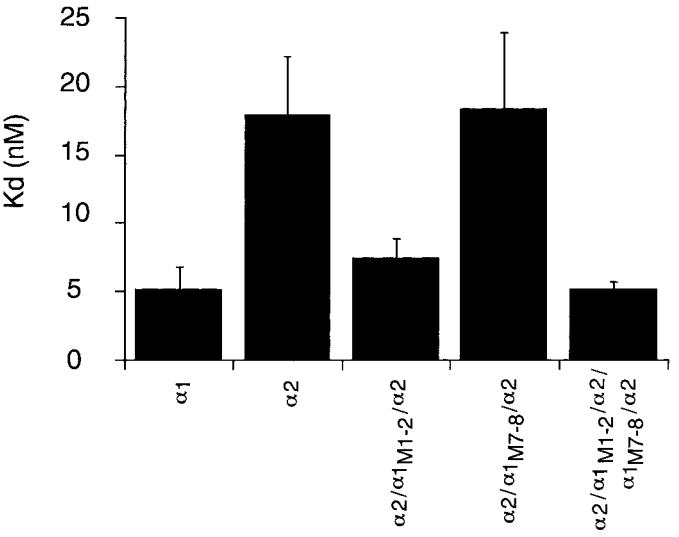

Fig. 4. Ouabain affinity of wild-type, human Na,K-ATPase α1 and α2 isoforms, α1/α2 chimeras, and α2 mutants. Ouabain binding experiments at equilibrium with various concentrations of [^{3}H]ouabain were performed as described under *Materials and Methods*. The equilibrium dissociation constants (K_d) were calculated from Scatchard plots. Data are means of two to three experiments done in triplicate.

Discussion

By using random mutagenesis (for references, see Croyle et al., 1997) or chimeras between Na,K- and H,K-ATPase (Qiu et al., 2003), nearly 20 amino acids have been identified that influence the sensitivity of Na,K-ATPase to ouabain. These amino acids are located in several transmembrane segments and extracellular loops of the Na,K-ATPase α-subunit and are hypothesized to comprise the physical binding site or to affect indirectly ouabain binding by conformational changes. In this study, we have investigated the structural determinants that are responsible for the α isoform-specific differences in the ouabain binding kinetics and could identify new amino acids that are involved in ouabain binding and play a role in the access and release of ouabain to and from its binding site.

Results from previous kinetic studies of ouabain binding, which were performed mainly on Na,K-ATPase α1 isozymes, have suggested that the rate of association of ouabain to enzymes from different sources, both sensitive or insensitive, is similar and that the ouabain sensitivity is mainly determined by differences in the dissociation rate (Yoda, 1974; Akera and Brody, 1977). Our observation that the human α2 isozyme exhibits 5- to 10-fold faster ouabain dissociation and association rates than the α1 isoform, despite their similar high ouabain sensitivity, indicates that this prediction is not valid for α isoforms other than α1 isoforms.

The amino acids that have previously been identified to be implicated in the ouabain sensitivity of Na,K-ATPase (Croyle et al., 1997; Qiu et al., 2003) are identical in human α1 and α2 isoforms. This points to a similar structure of the ouabain binding pocket in the two isoforms, which determines their similar high ouabain affinity. However, in the present study, we have identified 2 new amino acid positions in the M1–M2 hairpin that differ from those previously characterized and that are major determinants of the fast and slow ouabain binding kinetics of α2 and α1 isoforms, respectively. Replacement of the 2 amino acids at these positions in the α2 isoform by the corresponding amino acids of the α1 isoform decreases both the association and the dissociation rates of ouabain near those observed in the α1 isoform. Two other amino acids with a minor effect on ouabain binding kinetics were also identified in the M7–M8 extracellular loop. Although the precise mechanism is not known, our results suggest that the two extracellular loops cooperate in a synergistic way to control the accessibility of ouabain to its binding site.

Significantly, the amino acids identified in the α1 (Thr^{119}, Gln^{124}, Ile^{878}, and Gln^{902}) and α2 (Met^{119}, Ser^{124}, Ser^{878}, and Glu^{902}) isoforms that determine the slow and the fast ouabain binding kinetics, respectively, of the two isozymes, are conserved in all known mammalian α1 and α2 isoforms. The only exception is the highly ouabain-resistant rat α1, which bears a proline residue at the position of Gln^{124} in the human α1 isoform and a phenylalanine residue at the position of Ile^{878}. Interestingly, human α3 isoforms possess Thr^{119}, characteristic of α1 isoforms, and Ser^{124}, characteristic of α2 isoforms. This may reflect their ouabain association and dissociation rates, which are intermediate between those of α1 and α2 isoforms (Crambert et al., 2000) and are similar to those of the α2M119T mutant (this study). Besides our results on human Na,K-ATPase isozymes (Crambert et al., 2000), few reports exist on the ouabain binding kinetics of α isoforms other than α1. However, the ouabain dissociation rate constants reported for putative α2 isoforms in rat heart (Noel and Godfraind, 1984) or vas deferens (Noel et al., 1998) preparations or for rat α2 or α3 isoforms in transfected cells (O'Brien et al., 1994) are in good agreement with our values reported for human α2 and α3 isoforms (Crambert et al., 2000; this study), and are higher than the values reported for various α1 isoforms (Erdmann and Schoner, 1973; Schultheis et al., 1993). These data support the idea that the differences in the ouabain association and dissociation kinetics between α1 and α2 isoforms is a general phenomenon and not restricted to human Na,K-ATPase isozymes.

At present, how the particular amino acids at positions 119 and 124 in the α1 and α2 isoform mediate the slow and fast ouabain kinetics, respectively, is not known. It has been postulated that the interaction of cardiac glycosides with the Na,K-ATPase occurs in at least two steps: an initial rapidly reversible binding step, followed by a conformational change that permits the formation of a more stable ouabain-enzyme complex (Yoda, 1974). In this model, differences in both the association and dissociation rates in the absence of a significant difference in the apparent ouabain affinity, as observed for α1 and α2 isoforms, could be explained by a difference in the flexibility or the accessibility of the ouabain binding site in the two isoforms. Accordingly, in contrast to the α2 isoform, in the α1 isoform, the amino acids at positions 119 and 124 may induce a conformational state of the enzyme that decreases the flexibility or accessibility of the ouabain binding site. The α1 and α2 isoforms may have a different conformation in the ouabain binding state E2P or produce a different conformation after the initial binding step of ouabain. An even more appealing hypothesis is that in the α1 isoform, but not in the α2 isoform, a gate-like structure exists, formed by Thr^{119}, Gln^{124}, Ile^{878}, and Gln^{902}, that impedes ouabain association and dissociation. This concept could best explain our results that replacements of amino acids 119 and 124 in α2 isoforms with the corresponding amino acids of α1 isoforms changes the 'on' and 'off' rates of ouabain in parallel and that intramolecular interactions between the amino acids in the M1–M2 extracellular loop and the M7–M8 hairpin are involved in the control of the ouabain binding kinetics. It may be speculated that closure or opening of this putative gate-like structure could be mediated by conformational changes during pump cycling. We have indeed shown previously that binding of ouabain to the human α1 isoform is mainly restricted to the phosphorylated E2 conformation, whereas the α2 isoform can also bind ouabain in a nonphosphorylated K^+-occluded form of the enzyme (Crambert et al., 2000). We may speculate that the gate-like structure for ouabain in the α1 isoform is closed in all but the E2P conformation, whereas in the α2 isoform, this gate-like structure is less efficient and permits an access to the binding site also in the nonphosphorylated state of the Na,K-ATPase. In the α1 isoform, the presence of a threonine and a glutamine at positions 119 and 124, respectively, may promote strong interactions (hydrogen bonds for threonine and electrostatic interactions for glutamine) with other parts of the α-subunit that stabilize a closed state of the gate-like structure for ouabain. In addition to interactions with the M7–M8 extracellular loop, interactions of the M1–M2 loop with the M3–M4 extracellular loop could be imagined in view of the presence in the α1 isoform (but not in the α2 isoform) of

amino acids bearing hydroxyl groups (Tyr^{313} and Thr/Ser^{314}) or a negative charge (Glu^{310}). Considering the disposition of the transmembrane domains of the Na,K-ATPase α-subunit predicted from the Ca-ATPase structure (Toyoshima et al., 2000), an interaction between the extracellular loops could partially cover a putative ouabain binding site formed by the M3/M4 and M5/M6 hairpins (Koenderink et al., 2000), and thereby slow the kinetics of ouabain interaction. Unfortunately, because of large differences in the sequences of the extracellular loops between Na,K-ATPase and Ca-ATPase, the model of the structure of the Ca-ATPase does not permit structural and movement predictions of the extracellular domain of the Na,K-ATPase to verify this hypothesis. Nevertheless, it is interesting to note that results of a recent study (Reuter et al., 2003), based on a normal mode analysis of the Ca-ATPase, are compatible with the idea of gate-like structures in P-type ATPases. The results predict that movements of the membrane helices during the calcium transport cycle, 'twist open' the luminal side of the protein because of large rearrangements of the extracellular loops (Reuter et al., 2003).

Still another hypothesis could be considered to explain the differences in ouabain binding kinetics in α isoforms. It is most likely that the initial step in ouabain binding to the α-subunit is mediated by the D-ring of the steroid part and the lactone moiety (Forbush, 1983). This initial binding event opens, in a second, slower step, a binding site for the sugar moiety. This model, which implicates 'sugar-docking' to stabilize the interaction between cardiac glycosides and the α-subunit, could be fitted to the ouabain binding kinetics of α1 and α2 isoforms. Possibly, the slow sugar-docking step of ouabain binding occurs only in the α1 isoform and is responsible for the slow ouabain association and dissociation rates. On the other hand, in α2 isoforms, sugar docking may not be possible because of the specific amino acid composition of the M1–M2 extracellular loop, and this lack of sugar binding may lead to the rapid ouabain association and dissociation rates.

In conclusion, we have identified new amino acids in the Na,K-ATPase that differentially control discrete steps in the ouabain binding to α1 and α2 isoforms. These findings, which explain the isoform-specific differences in ouabain binding kinetics, may be of importance for the development of new drugs that are able to discriminate between the 'inotropic' α2 and the 'toxic' α1 isoform of Na,K-ATPase.

Acknowledgments

We thank Jean-Daniel Horisberger for helpful discussions

References

Adams RJ, Schwartz A, Grupp G, Grupp I, Lee SW, Wallick ET, Powell T, Twist VW, and Gathiram P (1982) High-affinity ouabain binding site and low-dose positive inotropic effect in rat myocardium. *Nature (Lond)* **269:**167–169.

Akera T and Brody TM (1977) The role of Na^+,K^+-ATPase in the inotropic action of digitalis. *Pharmacol Rev* **29:**187–220.

Blanco G and Mercer RW (1998) Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol* **275:**F633–F650.

Crambert G, Hasler U, Beggah AT, Yu C, Modyanov NN, Horisberger JD, Lelievre L, and Geering K (2000) Transport and pharmacological properties of nine different human Na,K-ATPase isozymes. *J Biol Chem* **275:**1976–1986.

Croyle ML, Woo AL, and Lingrel JB (1997) Extensive random mutagenesis analysis of the Na^+/K^+-ATPase α subunit identifies known and previously unidentified amino acid residues that alter ouabain sensitivity—implications for ouabain binding. *Eur J Biochem* **248:**488–495.

Erdmann E and Schoner W (1973) Ouabain-receptor interactions in (Na^++K^+)-ATPase preparations from different tissues and species. Determination of kinetic constants and dissociation constants. *Biochim Biophys Acta* **307:**386–398.

Farr CD, Burd C, Tabet MR, Wang X, Welsh WJ, and Ball WJ Jr (2002) Three-dimensional quantitative structure-activity relationship study of the inhibition of Na^+,K^+-ATPase by cardiotonic steroids using comparative molecular field analysis. *Biochemistry* **41:**1137–1148.

Forbush IIIB (1983) Cardiotonic steroid binding to Na,K-ATPase, in *Current Topics in Membranes and Transport* (Bronner F and Kleinzeller A eds) vol. 19, pp 167–201, Academic Press, New York.

Geering K (2001) The functional role of β subunits in oligomeric P-type ATPases. *J Bioenerg Biomembr* **33:**425–438.

Geering K, Beggah A, Good P, Girardet S, Roy S, Schaer D, and Jaunin P (1996) Oligomerization and maturation of Na,K-ATPase: functional interaction of the cytoplasmic NH_2 terminus of the β subunit with the α subunit. *J Cell Biol* **133:**1193–1204.

James PF, Grupp IL, Grupp G, Woo AL, Askew GR, Croyle ML, Walsh RA, and Lingrel JB (1999) Identification of a specific role for the Na,K-ATPase α 2 isoform as a regulator of calcium in the heart. *Mol Cell* **3:**555–563.

Juhaszova M and Blaustein MP (1997) Na^+ pump low and high ouabain affinity α subunit isoforms are differently distributed in cells. *Proc Natl Acad Sci USA* **94:**1800–1805.

Kawamura A, Abrell LM, Maggiali F, Berova N, Nakanishi K, Labutti J, Magil S, Haupert GT Jr, and Hamlyn JM (2001) Biological implication of conformational flexibility in ouabain: observations with two ouabain phosphate isomers. *Biochemistry* **40:**5835–5844.

Koenderink JB, Hermsen HPH, Swarts HGP, Willems PHGM, and De Pont JJHHM (2000) High-affinity ouabain binding by a chimeric gastric H^+,K^+-ATPase containing transmembrane hairpins M3–M4 and M5–M6 of the α 1-subunit of rat Na^+,K^+-ATPase. *Proc Natl Acad Sci USA* **97:**11209–11214.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Maixent JM, Charlemagne D, de la Chapelle B and Lelievre LG (1987) Two Na,K-ATPase isoenzymes in canine cardiac myocytes. Molecular basis of inotropic and toxic effects of digitalis. *J Biol Chem* **262:**6842–6848.

Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, and Green MR (1984) Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res* **12:**7035–7056.

Middleton DA, Rankin S, Esmann M, and Watts A (2000) Structural insights into the binding of cardiac glycosides to the digitalis receptor revealed by solid-state NMR. *Proc Natl Acad Sci USA* **97:**13602–13607.

Nelson RM and Long GL (1989) A general method of site-specific mutagenesis using a modification of the *Thermus aquaticus* polymerase chain reaction. *Anal Biochem* **180:**147–151.

Noel F and Godfraind T (1984) Heterogeneity of ouabain specific binding sites and (Na^+ + K^+)-ATPase inhibition in microsomes from rat heart. *Biochem Pharmacol* **33:**47–53.

Noel F, Quintas LE, Freitas E, Caricati-Neto A, Lafayette SS, Wanderley AG, and Jurkiewicz A (1998) Quantitative analysis of the high-affinity binding sites for [^{3}H]ouabain in the rat vas deferens and their immunological identification as the α 2 isoform of Na^+/K^+-ATPase. *Biochem Pharmacol* **55:**1531–1535.

O'Brien WJ, Lingrel JB, and Wallick ET (1994) Ouabain binding kinetics of the rat α-2 and α-3 isoforms of the sodium potassium adenosine triphosphate. *Arch Biochem Biophys* **310:**32–39.

Qiu LQ, Koenderink JB, Swarts HGP, Willems PHGM, and De Pont JJHHM (2003) Phe^{783}, Thr^{797}, and Asp^{804} in transmembrane hairpin M5–M6 of Na^+,K^+-ATPase play a key role in ouabain binding. *J Biol Chem* **278:**47240–47244.

Reuter N, Hinsen K, and Lacapère JJ (2003) Transconformations of the SERC1 Ca-ATPase: a normal mode study. *Biophys J* **85:**2186–2197.

Schultheis PJ, Wallick ET, and Lingrel JB (1993) Kinetic analysis of ouabain binding to native and mutated forms of Na,K-ATPase and identification of a new region involved in cardiac glycoside interactions. *J Biol Chem* **268:**22686–22694.

Toyoshima C, Nakasako M, Nomura H, and Ogawa H (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. *Nature (Lond)* **405:**647–655.

Wang J, Schwinger RH, Frank K, Muller-Ehmsen J, Martin-Vasallo P, Pressley TA, Xiang A, Erdmann E, and McDonough AA (1996) Regional expression of sodium pump subunits isoforms and Na^+-Ca^{++} exchanger in the human heart. *J Clin Investig* **98:**1650–1658.

Wang J, Velotta JB, McDonough AA, and Farley RA (2001) All human Na^+-K^+-ATPase αsubunit isoforms have a similar affinity for cardiac glycosides. *Am J Physiol* **281:**C1336–C1343.

Yoda A (1974) Association and dissociation rate constants of the complexes between various cardiac monoglycosides and Na,K-ATPase. *Ann NY Acad Sci* **242:**598–616.

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