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Transport and Pharmacological Properties of Nine Different Human Na,K-ATPase Isozymes*

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Na,K-ATPase plays a crucial role in cellular ion homeostasis and is the pharmacological receptor for digitalis in man. Nine different human Na,K-ATPase isozymes, composed of 3 α and β isoforms, were expressed in *Xenopus* oocytes and were analyzed for their transport and pharmacological properties. According to ouabain binding and K^+-activated pump current measurements, all human isozymes are functional but differ in their turnover rates depending on the α isoform. On the other hand, variations in external K^+ activation are determined by a cooperative interaction mechanism between α and β isoforms with $\alpha2$-$\beta2$ complexes having the lowest apparent K^+ affinity. α Isoforms influence the apparent internal Na^+ affinity in the order $\alpha1 > \alpha2 > \alpha3$ and the voltage dependence in the order $\alpha2 > \alpha1 > \alpha3$. All human Na,K-ATPase isozymes have a similar, high affinity for ouabain. However, $\alpha2$-β isozymes exhibit more rapid ouabain association as well as dissociation rate constants than $\alpha1$-β and $\alpha3$-β isozymes. Finally, isoform-specific differences exist in the K^+/ouabain antagonism which may protect $\alpha1$ but not $\alpha2$ or $\alpha3$ from digitalis inhibition at physiological K^+ levels. In conclusion, our study reveals several new functional characteristics of human Na,K-ATPase isozymes which help to better understand their role in ion homeostasis in different tissues and in digitalis action and toxicity.

The Na,K-ATPase (Na,K-pump) belongs to the P-type ATPase family of cation transporters which are characterized by intermediate phosphorylation during the catalytic cycle. The Na,K-ATPase is an ubiquitous plasma membrane enzyme which transports 2 K^+ ions into and 3 Na^+ ions out of the cell by using the energy of the hydrolysis of 1 molecule of ATP. This enzyme plays a crucial role in cell homeostasis since it maintains Na^+ and K^+ gradients between the intra- and extracellular milieu which are necessary for the maintenance of the cell volume. Furthermore, the Na^+ gradient created by the Na,K-ATPase provides the energy for the transport activity of many secondary transporters which provide the cell with nutrients or regulate intracellular concentrations of ions which are implicated in specialized cellular functions such as muscle contraction, transmission of nerve impulses, or Na^+ reabsorption in the kidney. Moreover, the Na,K-ATPase is the pharmacological receptor for cardiac glycosides which are widely used in the treatment of heart failure because of their positive inotropic effect and is possibly also the physiological receptor for endogenous ouabain compounds. In view of its important "housekeeping" and specialized functions, it is expected that any dysfunction or dysregulation of the Na,K-ATPase may have important pathophysiological consequences (for review, see Ref. 1).

The minimal functional unit of Na,K-ATPase is composed of an α and β subunit. The α subunit has 10 membrane-spanning domains and exposes the N and C terminus to the cytoplasmic side while the β subunit is a type II glycoprotein with a single transmembrane segment, a short cytoplasmic tail, and a large ectodomain. The α subunit carries the functional properties of the Na,K-ATPase, namely it binds and transports the cations, hydrolyzes ATP, and is intermediately phosphorylated. Furthermore, the α subunit bears the binding site for cardiac glycosides (for review, see Ref. 2). The β subunit is necessary for the structural and functional maturation of the α subunit and also influences the K^+ and Na^+ activation kinetics of mature pumps (for references, see Ref. 3).

One of the remaining questions concerning the structure-function relationship in Na,K-ATPase is the functional role of existing α and β isoforms. Indeed, 4 α and 3 β isoforms have been identified which exhibit 85 and 45% of sequence identity, respectively, and which show a tissue-specific distribution and a developmentally regulated pattern of expression (for review, see Ref. 4). Biochemical evidence (5) and transfection studies (for review, see Ref. 4) suggest that α and β isoforms can assemble in different combinations and potentially form functional pumps. So far, analysis of functional differences among isozymes has mainly been performed with rat Na,K-ATPase. These studies have led to several hypotheses on the specific role of different isozymes in different tissues. $\alpha1$ Isoforms are ubiquitous and may assume a housekeeping function in all cells. In the adult rat, $\alpha2$ isoforms are expressed predominantly in brain, skeletal muscle, and heart, whereas $\alpha3$ isoforms are primarily expressed in brain. These α isoforms are thought to form auxiliary pumps working in particular physiological situations. For instance, compared with $\alpha1$ and $\alpha2$ isoforms, $\alpha3$ isoforms have a lower affinity for Na^+ and may only be active after an increase in intracellular Na^+ concentrations due to a series of action potentials (for review, see Ref. 4). On the basis

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of the large differences in ouabain sensitivities between α1 isoforms (ouabain-resistant) and α2 or α3 isoforms (ouabain-sensitive) of rat or dog, it was also speculated that "inotropic" and "toxic" isoforms determine digitalis action (6, 7).

Similar to the α1 isoform, the β1 isoform is expressed ubiquitously. In rat, the β2 isoform is mainly expressed in muscle and brain where it could have a complementary role as an adhesion molecule (8), whereas the β3 isoform is found in a variety of rat tissues. Some evidence exists that β isoforms may differentially influence the enzymatic and transport properties of Na,K-ATPase isozymes (for review, see Ref. 4) but the effects appear to be less pronounced than those of α isoforms.

Despite the physiological and pathophysiological importance of Na,K-ATPase and its role in digitalis action in the treatment of heart failure, little is known about the physiological and pharmacological properties of human Na,K-ATPase isozymes. It may indeed be expected that the extrapolations from data obtained with rat Na,K-ATPase isozymes on the pharmacological properties, *e.g.* therapeutic and toxic targets of digitalis, may not hold true for humans. In contrast to rat, the 3 α and the 3 β isoforms are present in the human heart, which raises the possibility that in this tissue, 9 different α-β complexes may exist with different transport and/or pharmacological properties. Furthermore, in the human heart, only one or two high affinity digitalis-binding sites were identified suggesting that, in contrast to rat Na,K-ATPase isozymes, human isozymes do not significantly differ in their digitalis sensitivity (for review, see Ref. 9).

To better understand the physiological and pharmacological relevance of the existence of different Na,K-ATPase isozymes in general, and in humans in particular, we expressed human α1, α2, or α3 cRNAs together with β1, β2, or β3 cRNAs in *Xenopus* oocytes and investigated several transport characteristics (turnover, Na^+ and K^+ affinities, voltage dependence) and pharmacological properties (K_d, k_{+1}, k_{-1} of ouabain binding and K^+/ouabain antagonism) of the 9 possible human Na,K-ATPase isozymes. The functional comparison in the same experimental system of the various α-β complexes revealed several new characteristics of Na,K-ATPase isozymes which are discussed with respect to their physiological and pharmacological relevance.

MATERIALS AND METHODS

Cloning of Human α2, α3, and β2 Isoforms of the Na,K-ATPase and cRNA Preparations—Based on the genomic or cDNA sequences available (see below), we cloned α2, α3, and β2 cDNAs from a human cardiac uncloned cDNA library (Marathon-ready cDNA, CLONTECH) using long distance PCR[1] (LD-PCR) technology (Advantage cDNA PCR kit, CLONTECH).

The α2 isoform (Ref. 10, GenBank™ accession number J05096) was cloned, using a sense oligonucleotide covering the sequence coding for Met^1 up to Ala^{12} and tailed with a sequence containing 10 nucleotides including an *Eco*RI restriction site. The antisense oligonucleotide covered the sequence coding for Pro^{1010} up to the stop codon and was tailed with a sequence coding for a *Hpa*I restriction site (blunt end). The LD-PCR was performed using a touchdown protocol with a first denaturing step (1 min at 94 °C), followed by two pre-amplification steps (5 cycles at 94 °C for 30 s and 72 °C for 5 min 30 s and 5 cycles at 94 °C for 30 s and 70 °C for 5 min 30 s) and the amplification step (25 cycles at 94 °C for 30 s and 68 °C for 5 min 30 s). Finally, a last elongation step was performed at 68 °C for 8 min. The α2 identity of the PCR product was confirmed by *Sma*I digestion. The α2 PCR product was then subcloned into a pSD5 vector (containing the *Xenopus* α1 cDNA), using *Eco*RI and *PmaCI* (blunt end) sites. The nucleotide sequences of clones were analyzed by dideoxy sequencing and, in the chosen clone, 2 mutations introduced by LD-PCR were identified and were corrected according to the published genomic sequence using PCR techniques (11). These clones were resequenced to verify their identity.

The α3 isoform (Ref. 12, GenBank™ accession number X12910) was cloned by using a sense oligonucleotide covering the sequence coding for Met^1 up to Ser^{12} and containing a tail consisting of 10 nucleotides of the 5′-untranslated region of a N-terminal truncated *Xenopus* α1 cDNA (Xe 5′-UT (13)) and an antisense oligonucleotide covering the region coding for Gly^{986} up to the stop codon. This oligonucleotide was tailed with a sequence coding for *Eco*RI and *Eco*RV (blunt end) restriction sites. LD-PCR was performed as for α2 (see above). The α3 identity of the PCR product was confirmed by *Pvu*II digestion. We added the Xe 5′-UT sequence by performing an additional PCR between the human α3 PCR product (containing part of the Xe 5′-UT) and the entire Xe 5′-UT (containing a *Nhe*I site). This PCR leads to a cDNA of the full-length human α3 preceded by the entire Xe 5′-UT. This product was subcloned into a pSD5 vector using *Nhe*I and *PmaCI* (blunt end) sites. The addition of the Xe 5′-UT was previously shown to improve expression of foreign proteins in oocytes (14), but in our case, the expression of α3 was still low. We, therefore, subcloned the cDNA into the pNKS2 vector (kindly provided by G. Schmalzing). The human α3 cDNA in the pSD5 vector was removed by *Eco*RI digestion and blunted at both ends. It was then ligated with the pNKS2 vector previously digested with *Nco*I (fill-in) and *Sal*I (blunt). The nucleotide sequences of clones were analyzed by dideoxy sequencing. In the chosen clone, 2 mutations were identified and were corrected according to the published genomic sequence using PCR techniques (11). These clones were resequenced to verify their identity.

The β2 isoform (Ref. 15, GenBank™ accession number P14415) was cloned by using a sense oligonucleotide encompassing a sequence coding for Met^1 up to Glu^6 and containing a *Nco*I restriction site within the Met^1 codon. The antisense oligonucleotide covered a region coding for Leu^{295} up to the stop codon and was tailed with a sequence coding for a *Xba*I restriction site. The LD-PCR was similar to that used for α2 and α3 except that the amplification times were decreased to 2 min 30 s. PCR products obtained were checked by digestion with *Pvu*II and subcloned into the pSD5 vector between *Nco*I and *Xba*I restriction sites. The nucleotide sequences of β2 clones were analyzed by dideoxy sequencing and were found to differ from the sequence of Martin-Vassalo *et al.* (15) by a Leu replacement of Pro^{51}. This was not considered as a mutation since Ruiz *et al.* (16) have reported the same sequence for human β2 from fetal and adult retinal pigment epithelia (GenBank U45945).

The full-length human β3 cDNA was identified in the IMAGE Consortium clone number 133072 obtained from Research Genetics (Huntsville, AL) by restriction mapping and sequencing as described (17). To generate the pSD5 vector covering the entire β3 coding region preceded by Xe 5′-UT, the *Alwn*I-*Eco*RV fragment containing the coding region (without 36 nt at the 5′ end) and 15 nt of the 3′-UT, and a synthetic oligonucleotide linker which was designed to encode the first 12 amino acids and fit *Nco*I and *Alwn*I sites (sense strand of 37 nt and antisense strand of 30 nt), were ligated with the vector preliminary digested with *Nco*I and *Sma*I.

The full-length cDNA encoding human α1 isoform (18) was reconstructed from two overlapping clones, pSNa100 and pSN54, which were kindly provided by K. Kawakami. The *Nco*I-*Eco*RV fragment of α1 cDNA containing the entire coding part and 397 nt of 3′-UT was subcloned into the pSD5 vector containing Xe 5′-UT between *Nco*I and *Sma*I restriction sites. In order to improve the level of expression, the α1 cDNA was transferred into the pNKS2 vector.

The human β1 cDNA (19) (a gift from K. Kawakami) containing the entire coding part and 70 nt of 3′-UT was subcloned into the pSD5 vector containing Xe 5′-UT. cRNA coding for human α and β isoforms was obtained by *in vitro* translation (20).

Expression of the α-β Complexes of Human Na,K-ATPase in Xenopus laevis Oocytes and Detection by Nondenaturating Immunoprecipitation of α Subunits—Oocytes were obtained from *X. laevis* females, as described (21), and were injected with 10 ng of human α isoform cRNAs alone or together with 1 ng of β isoform cRNA. Oocytes were incubated for 24 or 72 h in modified Barth's medium containing 0.5–0.7 mCi/ml of [^{35}S]methionine (Hartman Analytic). In some experiments, oocytes were subjected to a 48-h chase period in the presence of 10 mM cold methionine. Digitonin extracts were prepared after the pulse and chase periods as described and α subunits were immunoprecipitated under nondenaturating conditions, as described (21), with a polyclonal anti-*Bufo* Na,K-ATPase α1 antibody. Reliable quantification of β subunits associated with α subunits is difficult due to the complex-type glycosylation of β subunits which can result in diffuse migration on gels of β subunits containing multiple glycosylation sites. Thus, to determine the

[1] The abbreviations used are: PCR, polymerase chain reaction; LD, long distance; UT, untranslated; nt, nucleotide(s); NMDG, *N*-methyl-D-glutamine.

ratio between α and β isoforms in Na,K-ATPase isozyme complexes, cRNA-injected oocytes were incubated in the presence of 5 μg/ml brefeldine A (Alexis Corp.) during a 24-h pulse period as described previously (21). Under these conditions, α-β complexes are retained in the endoplasmic reticulum and β subunits remain in their core-glycosylated form. This permitted removal of the sugar moiety from β subunits by treating the immunoprecipitated samples with endoglycosidase H as described previously (21) and allowed reliable quantification of the non-glycosylated species. The dissociated immune complexes were separated by SDS-polyacrylamide gel electrophoresis and labeled proteins were detected by fluorography and quantified by densitometry with a LKB 2202 Ultrascan.

Pump Current Measurements and Determination of Apparent K^+ and Na^+ Affinities and Voltage Dependence—3 days after cRNA injection, Na,K-pump currents were measured by using the two-electrode voltage-clamp method. The total currents measured in oocytes expressing exogenous Na,K-pumps are assumed to be the sum of the currents mediated by endogenous and exogenous Na,K-pumps. To identify the component which is due to the expressed Na,K-pumps, pump currents were measured in parallel under the same conditions in non-injected oocytes and the mean values were subtracted from those obtained in cRNA-injected oocytes of the same batch. To determine the apparent K^+ affinity of Na,K-pumps, oocytes were loaded with Na^+, as described previously (22), which increased the intracellular Na^+ concentration to about 70 mM (not shown). The electrophysiological measurements were done in the presence of external Na^+ (in mM, 80 sodium gluconate, 0.82 $MgCl_2$, 0.41 $CaCl_2$, 10 *N*-methyl-D-glutamine (NMDG)-Hepes, 5 $BaCl_2$, 10 tetraethylammonium chloride, pH 7.4). The current induced by increasing concentrations of K^+ (0.5, 1, 3, 5, 10 mM) was measured at -50 mV. The Hill equation (*I*) $I_K = I_{max}/[1 + (K_{½}\ K^+/[K])^{nH}]$ was fitted to the data of the current (I_K) induced by various K^+ concentrations ([K]) and yielded least-square estimates of the maximal current (I_{max}) and of the half-activation constant for K^+ ($K_{½}\ K^+$). A Hill coefficient (nH) of 1.6 was used, as described previously (22).

Measurements of the half-activation constant for internal Na^+ were performed as described previously (3). In brief, oocytes were injected with Na,K-ATPase α and β cRNAs along with cRNAs coding for α, β, and γ subunits (0.3 ng/subunit/oocyte) of the rat renal epithelial Na^+ channel (23). Injected oocytes were incubated for 3 days in a modified Barth's solution containing 10 mM Na^+. Before measurements, oocytes were incubated in a Na^+-free solution (50 mM NMDG-Cl, 40 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM NMDG-Hepes, pH 7.4), in order to maximally reduce the internal Na^+ concentration.

From five to eight pairs of measurements of $[Na]_i$ and of the K^+-activated Na,K-pump current were performed successively on each oocyte. Between each pair of measurements, oocytes were exposed to a 100 mM Na^+ solution (100 mM sodium gluconate, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM Na-Hepes, pH 7.4) in the absence of amiloride and at a holding potential of -50 to -100 mV to increase intracellular Na^+ concentrations.

Intracellular Na^+ concentrations were calculated from the reversal potential of the amiloride-sensitive current obtained from I-V curves recorded in the absence of amiloride in a solution containing 5 mM Na^+ (5 mM sodium gluconate, 0.5 mM $MgCl_2$, 2.5 mM $BaCl_2$, 95 mM NMDG-Cl, 10 mM NMDG-Hepes, pH 7.4). The K^+-activated Na,K-pump currents (I_K) were measured in the presence of 80 mM external Na^+ (see above), 10 mM K^+, and 20 μM amiloride.

To identify the component of the current mediated by exogenous Na,K-pumps in cRNA-injected oocytes, the endogenous pump currents (I_{Kendo}) were measured in parallel on non-injected oocytes and the $I_{max,endo}$ and the $K_{½}\ Na^+_{endo}$ were determined by fitting Equation 2, $I_{Kendo} = (I_{max,endo}/[1 + (K_{½}\ Na^+_{endo}/[Na]_i)^{nH}])$ to the K^+-induced currents (I_{Kendo}) and intracellular Na^+ concentrations $[Na]_i$ measured in non-injected oocytes. The values obtained were introduced into Equation 3, $I_K = I_{max,endo}/[1 + (K_{½}\ Na^+_{endo}/[Na]_i)^{nH}]) + I_{max,hum}/[1 + (K_{½}\ Na^+_{hum}/[Na]_i)^{nH}]$ and separate I_{max} ($I_{max,hum}$) and $K_{½}\ Na^+$ ($K_{½}\ Na^+_{hum}$) for the expressed, human pumps were obtained by fitting the entire Equation 2 to the I_K and $[Na]_i$ values measured in cRNA-injected oocytes. Parameter fitting was performed with a Hill coefficient (nH) of 3 as described previously (3).

The voltage dependence of the ouabain-sensitive currents of the human Na,K-pumps was investigated in non-injected oocytes and in oocytes expressing human Na,K-pump isozymes by measuring the currents activated by 10 mM K^+ during a series of ten 200-ms voltage steps ranging between -130 and $+50$ mV, before and after the addition of 100 μM ouabain in the presence of 90 mM external Na^+. Averaged currents of endogenous Na,K-pumps were subtracted from currents measured in oocytes expressing exogenous Na,K-pumps.

[^{3}H]Ouabain Binding on Intact Oocytes—Three days after cRNA injection, the total number of Na,K-pumps expressed at the cell surface was determined. For this purpose, oocytes were loaded with Na^+ for 2 h at 19 °C in a K^+-free solution (90 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4) before incubation with 1 μM ouabain (0.3 μM [^{3}H]ouabain (Amersham Pharmacia Biotech, 34.2–50 Ci/mmol) plus 0.7 μM unlabeled ouabain) for 30 min at room temperature as described previously (24). In preliminary experiments, we have determined that ouabain binding to all isozymes reaches a plateau after 20 min which persists up to 1 h indicating that ouabain does not gain access to an internal pool of binding sites during the assay period. After incubation with ouabain, oocytes were washed three times with 20 ml of the above solution and individually transferred to vials and solubilized with 100 μl of 5% (w/v) SDS before counting. Non-injected oocytes of the same batch were analyzed under the same conditions to determine the specific endogenous ouabain binding and the nonspecific component.

[^{3}H]Ouabain Binding Kinetics on Oocyte Microsomes—Three days after cRNA injection, microsomes were prepared from oocytes as described previously (21). Protein concentrations of microsomes were determined by the method of Lowry (25). Ouabain binding experiments were carried out as specified in the figure legends in the absence or presence of various concentrations of K^+ at 37 °C with various [^{3}H]ouabain concentrations (from 10^{-9} to 5×10^{-8} M) in a medium containing 4 mM ATP, 4 mM $MgCl_2$, 100 mM NaCl, 30 mM imidazole/HCl, pH 7.4 (Na-ATP conditions), or in a medium containing 4 mM H_3PO_4, 4 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.4 (Mg-P_i conditions). After temperature equilibration, binding reactions were initiated by addition of 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. After various time periods, 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 (Emulsifior scintillator plus, Packard). Equilibrium binding was reached within 90 min and 5 h in the absence and presence of K^+, respectively, with the lowest [^{3}H]ouabain concentrations used. Ouabain binding experiments were performed under the same conditions on microsomes from non-injected oocytes of the same batch to determine the endogenous, oocyte ouabain binding and the nonspecific binding and the mean values 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.

The association and dissociation kinetics of ouabain to each isozyme 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} = specific ouabain bound at equilibrium and B = specific ouabain bound at several time points after addition of an excess unlabeled ouabain. The observed first-order association rate constant (k_{obs}) of ouabain binding to each Na,K-ATPase isozyme was determined as the slope of $\ln((B_{eq} - B)/B_{eq})$ *versus* time plots; B_{eq} = specific [^{3}H]ouabain bound at equilibrium and B = specific ouabain bound at several time points. 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}) by using the equation $k_{obs} = k_{+1}[ouab] - k_{-1}$.

All curve fittings and unpaired Student's *t* test were done with Kaleidagraph software. When appropriate, two-way ANOVA variance analysis was performed by using Prism III software.

RESULTS

Cellular Expression and Processing of Human Na,K-ATPase Isozymes in Xenopus Oocytes

To test the cellular expression and processing of human Na,K-ATPase isozymes in *Xenopus* oocytes, we injected α1, α2, or α3 cRNAs alone or together with β1, β2, or β3 cRNAs, subjected the oocytes to a 24-h pulse with [^{35}S]methionine and a 48-h chase period and followed the stability of the α subunits. As previously shown for amphibian Na,K-ATPase (21), human Na,K-ATPase α isoforms expressed without β subunits were degraded during the chase period (Fig. 1*A, lanes 3–8*) and only the newly synthesized, endogenous, oocyte α subunits which are intrinsically stable (26), were immunoprecipitated (com-

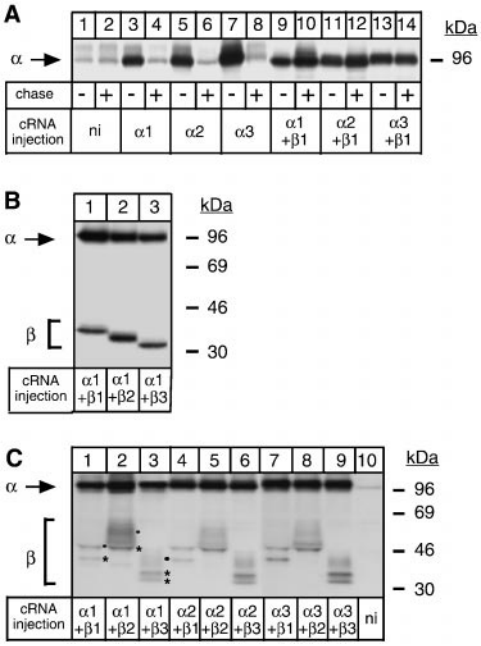

FIG. 1. **Cellular expression and processing of 9 possible, human Na,K-ATPase isozymes in *Xenopus* oocytes.** *A,* human Na,K-ATPase α isoforms need assembly with β subunits for stable cellular expression. *Xenopus* oocytes were injected with α1, α2, or α3 cRNAs alone (*lanes 1–8*) or together with β1 cRNAs (*lanes 9–14*) and labeled with [^{35}S]methionine. Digitonin extracts were prepared after a 24-h pulse and a 48-h chase period and α subunits were immunoprecipitated with a polyclonal α-antibody, resolved by SDS-polyacrylamide gel electrophoresis, and revealed by fluorography. The positions of α subunits and of a protein of known molecular mass are indicated. *B,* human Na,K-ATPase α-β isozyme complexes expressed in *Xenopus* oocytes exhibit a stoichiometry of close to 1. *Xenopus* oocytes were injected with α1 cRNAs together with β1, β2, or β3 cRNAs and subjected to a 24-h pulse. To enable reliable quantification of β subunits, oocytes were incubated during the pulse with brefeldine A which prevents endoplasmic reticulum exit of α-β complexes and full glycosylation of the β subunit (see "Experimental Procedures"). α-β Complexes were immunoprecipitated with an α-antibody under nondenaturating conditions and the immunoprecipitated samples were treated with endoglycosidase H to remove core sugars from the β subunit. Quantification of the α and the non-glycosylated β subunits revealed a β1/α1 ratio of 1.3 ± 0.14 (mean ± S.E., n = 7), a β2/α1 ratio of 1.1 ± 0.23 (n = 4), and a β3/α1 ratio of 1.4 + 0.17 (n = 3) by taking into account that human α1, β1, β2, and β3 isoforms contain 23, 4, 10, and 7 methionines, respectively. The positions of α1 isoforms and the non-glycosylated (ng) β isoforms are indicated. *C,* human α-β isozyme complexes expressed in *Xenopus* oocytes are targeted to the plasma membrane. *Xenopus* oocytes were injected with α1, α2, or α3 cRNAs together with β1, β2, or β3 cRNAs (*lanes 1–9*) and labeled with [^{35}S]methionine for 72 h. α-β Complexes were immunoprecipitated with an α-antibody under nondenaturating conditions. The positions of α isoforms and the core-glycosylated (*) and the fully glycosylated (●) β isoforms are indicated. The differences in the molecular masses of the core and fully glycosylated β isoforms correspond to the presence of 3, 8, and 2 putative glycosylation sites in β1, β2, and β3 isoforms, respectively. β3 Isoforms show two core-glycosylated species which are glycosylated on 1 and 2 glycosylation sites, respectively. *ni,* non-injected oocytes (*lane 10*).

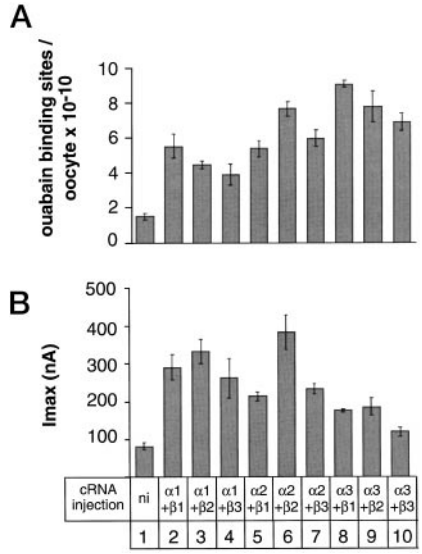

FIG. 2. **Cell surface expression and functionality of human Na,K-ATPase isozymes.** Oocytes were not injected (*ni*) or injected with different combinations of α and β isoform cRNAs as indicated and incubated for 3 days. *A,* maximal ouabain binding to intact oocytes. After electrophysiological measurements (shown in *B*), oocytes were transferred into a K^+-free solution and ouabain binding was determined as described under "Experimental Procedures." The results are expressed as the number of ouabain-binding sites per oocyte. *B,* maximal Na,K-pump current (I_{max}). Increasing concentrations of external K^+ were added to Na^+-loaded oocytes and the pump currents were measured by the two-electrode voltage-clamp technique as described under "Experimental Procedures." I_{max} values were determined by extrapolation of the K^+ activation curves. Data for I_{max} and ouabain binding are mean ± S.E. from 10–12 oocytes of one out of two to four similar experiments.

pare *lanes 4, 6*, and *8* to *lanes 1* and *2*). On the other hand, co-expression with β1 (*lanes 9–14*), β2, or β3 subunits (data not shown) permitted for the formation of stable α1, α2, and α3 isoforms indicating that all 3 human β isoforms can assemble with the 3 human α isoforms. Quantification of α and β subunits immunoprecipitated with an α-antibody under nondenaturing conditions which preserve α-β interactions revealed a ratio of β1, β2, and β3 over α1 isoforms close to 1 when corrected for the variable number of methionines present in the α1 and the various β isoforms (Fig. 1*B, lanes 1–3*). Finally, 3 days after cRNA injection, at the time point where functional assays were performed, the expression of all 9 α-β complexes was significantly higher (Fig. 1*C, lanes 1–9*) than that of Na,K-ATPase in non-injected oocytes (*lane 10*). Within a 72-h period, all β subunits were at least partially processed from a core glycosylated, endoplasmic reticulum form which was endoglycosidase H-sensitive (data not shown) to a fully glycosylated form which was endoglycosidase H-resistant indicating that the various α-β complexes were routed through a distal Golgi compartment to the plasma membrane. Altogether, these results show that human Na,K-ATPase isozymes are correctly processed and targeted in *Xenopus* oocytes and that, due to the rapid degradation of unassembled α subunits, the functional characteristics described in the following are exclusively determined by the presence of α-β complexes.

Cell Surface Expression and Transport Properties of Human Na,K-ATPase Isozymes

Cell Surface Expression of Functional Isozymes—To confirm the presence and the functionality of the 9 human α-β isozymes at the cell surface, maximal ouabain binding and pump current measurements were performed on intact oocytes. In oocytes expressing human α-β complexes, ouabain binding was 3–5-fold higher (Fig. 2*A, lanes 2–10*) than in non-injected oocytes (*lane 1*). The expression levels were similar for isozymes associated with different β isoforms confirming that all β isoforms can form functional pumps at the cell surface with each α isoform. The cell surface expression of human isozymes appears low compared with the large excess of human α isozymes synthesized over endogenous, oocyte α subunits (Fig. 1*C*). However, it has to be considered that the turnover of endogenous, oocyte α subunits is low (26) compared with that of exogenous α subunits and therefore the signal of biosynthetically labeled, immunoprecipitated α subunits does not necessarily reflect the endogenous Na,K-ATPase pool present in oocytes. Oocytes ex-

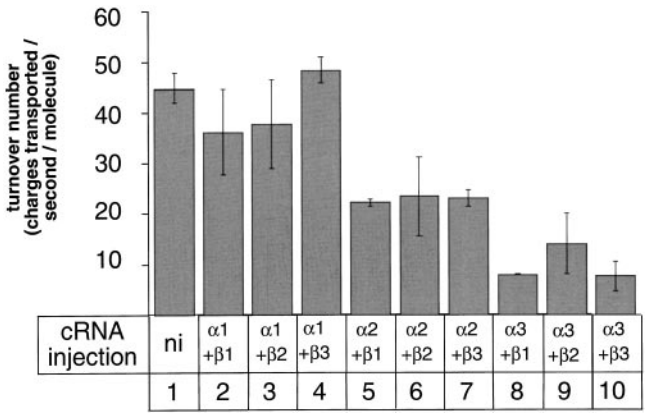

FIG. 3. **Turnover rates of human Na,K-ATPase isozymes.** The turnover number (charges transported/s/molecule) of the different Na,K-ATPase isozymes was calculated for individual oocytes as the ratio between the total pump current (as total charges transported/s) and the maximal ouabain binding sites. Data are mean ± S.E. of two to four experiments shown in Fig. 2 after subtraction of the endogenous Na,K-pump component. The turnover numbers of all α1-β complexes were significantly different from those of α3-β complexes ($p < 0.01$).

pressing exogenous Na,K-pump isozymes showed pump currents which were 2–4-fold higher (Fig. 2*B, lanes 2–10*) than those measured in non-injected oocytes (*lane 1*).

Na,K-Pump Turnover Rates—Assuming an identical stoichiometry, the ratio between the mean K^+-activated current and the mean number of ouabain-binding sites is a measure of the transport turnover rates of Na,K-pumps. Human Na,K-pump isozymes had different turnover rates which, according to variance analysis, depended on the α isoform ($p < 0.001$) and not the β isoform ($p = 0.56$) present in the α-β complexes (Fig. 3, Table I). α1-β complexes (*lanes 2–4*) had a similar turnover number than endogenous, oocyte Na,K-pumps (*lane 1*) which was higher than that of human α2-β (*lanes 5–7*) or of α3-β (*lanes 8–10*) complexes which had the lowest turnover rates.

Apparent K^+ Affinity—The apparent affinities for K^+ ($K_{½}$ K^+) of the 9 human Na,K-ATPase isozymes expressed in *Xenopus* oocytes were determined from K^+ activation curves of the Na,K-pump current measured (Fig. 4, *inset*). $K_{½}$ values for K^+ ranged between 0.9 and 2.7 mM (Fig. 4 and Table I). Variance analysis revealed that the K^+ affinity of human Na,K-pumps is determined by both the α isoform ($p < 0.0001$) and the β isoform ($p < 0.0001$) present in the isozyme complex as well as by the particular combination of α and β isoforms ($p < 0.0001$). The most pronounced effect of this cooperative mechanism was observed in α2-β2 complexes which exhibited a more than 2-fold increase in the $K_{½}$ value for K^+ compared with α2-β1 complexes.

Apparent Na^+ Affinity—The activation by internal Na^+ was investigated for human α1-β1, α2-β1, and α3-β1 complexes by using an electrophysiological technique involving the expression of the rat epithelial Na^+ channel along with the Na,K-pumps. The presence of rat epithelial Na^+ channel permitted to achieve a controlled, gradual increase in the intracellular Na^+ concentration from 2 to 70 mM, to measure intracellular Na^+ concentrations and to determine the Na^+ dependence of Na,K-pump currents as shown in Fig. 5*A*. The maximal pump currents of the various Na,K-ATPase isozyme complexes (Fig. 5*A, inset*), extrapolated from Na^+ activation curves (Fig. 5*A*), were similar to those extrapolated from K^+ activation curves (Fig. 2). This result indicates that the lower pump current measured for α3-β complexes compared with that measured for α1-β and α2-β complexes (Fig. 2*B*) is indeed due to a lower turnover rate and not to an inefficient activation by internal Na^+. As shown in Fig. 5*B* and Table I, α1-β1 complexes exhibited a high ($K_{½}$ Na^+ = 7.3 ± 0.9 mM), α2-β1 complexes an intermediate ($K_{½}$ Na^+ = 11.8 ± 2.9 mM), and α3-β1 complexes a low ($K_{½}$ Na^+ = 30 ± 5.2 mM) apparent affinity for internal Na^+.

Voltage Dependence—The voltage dependence of the ouabain-sensitive currents was investigated in oocytes expressing human α1-β1, α2-β1, and α3-β1 complexes (Fig. 6). The human α1-β1 and α2-β1 pump currents were voltage-sensitive over the whole potential range. The I-V curve profile of the human α1-β1 pump currents was similar to that obtained for the endogenous Na,K-pumps (data not shown). The α2-β1 pump complexes exhibited the most voltage-sensitive currents which at low membrane potentials of about −130 mV were nearly abolished. On the other hand, α3-β1 complexes produced pumps that were not significantly affected by voltage changes over the whole potential range.

Pharmacological Properties of Human Na,K-ATPase Isozymes

Ouabain Sensitivity—Because equilibrium binding studies on intact oocytes may be compromised by internalization and degradation of Na,K-pumps and recycling of ouabain, the equilibrium binding constants (K_d) for [^{3}H]ouabain was determined for each one of the 9 different Na,K-ATPase isozymes on oocyte microsomes. Scatchard plots of binding data showed that maximal ouabain binding on microsomes from cRNA-injected oocytes was 3–10-fold higher than that obtained on microsomes from non-injected oocytes (Fig. 7*A*). The maximal binding was not significantly influenced by the presence of different β isoforms (data not shown). After subtraction of binding data obtained on microsomes of non-injected oocytes, Scatchard plots obtained with microsomes from cRNA-injected oocytes were linear (Fig. 7*A*), reflecting a single population of [^{3}H]ouabain binding sites for each Na,K-ATPase isozyme. Ouabain affinity measured in Na-ATP conditions in the absence of K^+ was high and of similar magnitude, in the nanomolar range, for all Na,K-ATPase isozymes (Fig. 7*B* and Table I). However, α2-β isozymes had significantly higher K_d values (12–23 nM) than α1-β or α3-β isozymes (5–7 nM) (Fig. 7*B*, compare *lanes 4–6* to *lanes 1–3* and *7–9*). According to variance analysis, the α isoform ($p < 0.001$) and to a lesser extent also the β isoform ($p = 0.023$) present in the α-β complexes as well as the particular α-β combination ($p = 0.016$) affected the ouabain affinity of the Na,K-pumps.

K^+ is known to antagonize ouabain binding. For α1-β complexes, 5 mM K^+ induced a larger increase in K_d values for ouabain (3–4-fold) than for α2-β and α3-β complexes (2–3 fold). As an exception, for α2-β2 complexes, the K_d value was not influenced by the presence of K^+ (Fig. 7*B, lane 5*) which may reflect the lower apparent K^+ affinity of these complexes (Fig. 4).

Association and Dissociation Rate Constants of Ouabain Binding—To investigate in more detail the ouabain binding kinetics, we determined the association rate constant (k_{+1}) and the dissociation rate constant (k_{-1}) for all 9 human Na,K-ATPase isozymes.

Fig. 8*A* shows representative dissociation kinetics of ouabain for α1-β1, α2-β1, and α3-β1 complexes which were similar to those obtained with isozymes containing β2 or β3 isoforms. The dissociation rate constants of ouabain for all 9 Na,K-ATPase isozymes were calculated from the slopes of the dissociation plots and are summarized in Fig. 8*B* and Table I. α-β complexes formed with α1 and α3 isoforms had slow dissociation rate constants corresponding to half-lives ($t_{½}$) between 30 and 80 min, whereas those formed with α2 isoforms had rapid dissociation kinetics with a $t_{½}$ of about 4–5 min.

Representative examples of [^{3}H]ouabain association kinetics for α1, α2, and α3 isoforms associated with β1 isoforms are

TABLE I
Transport and pharmacological properties of human Na,K-ATPase isozymes

	$\alpha1\beta1$	$\alpha1\beta2$	$\alpha1\beta3$	$\alpha2\beta1$	$\alpha2\beta2$	$\alpha2\beta3$	$\alpha3\beta1$	$\alpha3\beta2$	$\alpha3\beta3$
Turnover number									
charges/s/molecule	36.3 ± 8.5	37.8 ± 8.7	48.5 ± 2.6	23.3 ± 0.7	23.5 ± 7.8	23.2 ± 1.7	8.0 ± 0.1	14 ± 6	7.6 ± 3.0
$K_{1/2}$ K^+ (mM)	0.92 ± 0.11	1.16 ± 0.02	1.05 ± 0.12	1.30 ± 0.17	2.70 ± 0.30	1.80 ± 0.10	0.90 ± 0.27	1.60 ± 0.10	1.33 ± 0.17
$K_{1/2}$ Na^+ (mM)	8.3 ± 0.6	ND[a]	ND	12.8 ± 2.2	ND	ND	24.7 ± 2.4	ND	ND
K_d ouabain (nM)									
$-K^+$	4.9 ± 1.7	5.2 ± 1.5	7 ± 1.2	22.0 ± 4.5	25.8 ± 3.2	13.7 ± 1.7	4.6 ± 0.5	10.1 ± 2.1	6 ± 1.1
$+K^+$	19.5 ± 0.5	19.8 ± 2.1	19.1 ± 2.0	36.9 ± 3.1	26.5 ± 6.5	29.0 ± 5.0	14.3 ± 0.8	17.6 ± 2.0	12.5 ± 1.1
k_{-1} (min^{-1})	0.0085 ± 0.0015	0.0225 ± 0.0075	0.0097 ± 0.0003	0.156 ± 0.02	0.136 ± 0.004	0.114 ± 0.01	0.023 ± 0.001	0.032 ± 0.005	0.021 ± 0.003
$k_{+1} \times 10^{-7}$ ($M^{-1} \times min^{-1}$)	0.44 ± 0.02	0.69 ± 0.11	0.31 ± 0.05	3.05 ± 0.39	2.71 ± 0.13	2.78 ± 0.23	0.78 ± 0.01	0.97 ± 0.20	0.83 ± 0.26
$K_i K^+$, ouabain antagonism									
Na-ATP (mM)	2.9	ND	ND	5	ND	ND	7.2	ND	ND
Mg-Pi (mM)	0.77	ND	ND	3.75	ND	ND	1.9	ND	ND

[a] ND, not determined.

FIG. 4. **K^+ activation of human Na,K-ATPase isozymes.** Oocytes were injected with different α and β isoform cRNAs as indicated. The K^+ activation of the Na,K-pump currents was measured as described in the legend to Fig. 2 and under "Experimental Procedures" and the K^+ activation constants ($K_{½}$ K^+) were determined. The K^+ activation of the endogenous, oocyte Na,K-pumps was determined in parallel and the averaged, endogenous pump currents were subtracted from Na,K-pump currents measured in individual cRNA-injected oocytes at different external K^+ concentrations. Data are mean ± S.E. from 15 to 30 oocytes obtained from two to four different *Xenopus* females. *Inset,* representative examples of K^+ activation curves of exogenous Na,K-pump currents (I) determined in single oocytes injected with $\alpha1$ plus $\beta2$ (▲), $\alpha2$ plus $\beta2$ (■), $\alpha3$ plus $\beta2$ (○) cRNAs. The I_{max} values are represented in Fig. 2.

shown in Fig. 9*A*. The time required to reach equilibrium binding was in the range of 10 min ($\alpha2$-β) and 60 min ($\alpha1$-β, $\alpha3$-β). The observed association rate constants (k_{obs}) were calculated from the slopes of plots shown in Fig. 9*B* and the association rate constants (k_{+1}) (Fig. 9*C* and Table I) as described under "Experimental Procedures." Similar to dissociation kinetics, association kinetics of ouabain to Na,K-ATPase isozymes followed the order $\alpha2 \gg \alpha3 > \alpha1$. According to variance analysis, neither association ($p = 0.66$) nor dissociation ($p = 0.12$) rate constants of ouabain binding were influenced by the associated β isoform. The K_d values calculated from the ratio k_{-1}/k_{+1} were close to those measured by equilibrium binding for the different isozymes ($\alpha1$-β, 1–3 nM; $\alpha2$-β, 4–5 nM; $\alpha3$-β, 2.5–3 nM) which supports that human Na,K-ATPase isozymes have similar, low K_d values for ouabain. On the other hand, our results clearly indicate that despite similar K_d values, the association and dissociation kinetics of ouabain differ significantly among the different α isoforms.

K^+ Antagonism of Ouabain Binding—The K^+ antagonism of digitalis binding to the Na,K-pump is an important aspect in the pharmacology of these drugs. As shown in Fig. 7, K^+ affected the ouabain affinity of $\alpha1$-β complexes to a greater extent than that of $\alpha2$-β or $\alpha3$-β complexes. To better understand the K^+/ouabain antagonism on human Na,K-ATPases, we performed K^+ competition experiments on microsomes of oocytes expressing $\alpha1$-$\beta1$, $\alpha2$-$\beta1$, or $\alpha3$-$\beta1$ isozymes. When ouabain binding was performed under Na-ATP conditions, increasing concentrations of K^+ (up to 100 mM), present during the binding reaction, progressively decreased the level of bound ouabain for all isozymes (Fig. 10*A*). However, ouabain binding could not be completely abolished and reached a plateau at high K^+ concentrations. Residual ouabain binding amounted to 15% for $\alpha1$-$\beta1$, 30% for $\alpha3$-$\beta1$, and 50% for $\alpha2$-$\beta1$ complexes. The K_i values for the limited K^+ effect were 2.9, 5, and 7.2 mM for $\alpha1$-$\beta1$, $\alpha3$-$\beta1$, and $\alpha2$-$\beta1$ complexes, respectively (Table I). Under Na-ATP conditions, the addition of K^+ starts the catalytic cycle and rapidly results in a steady state where all

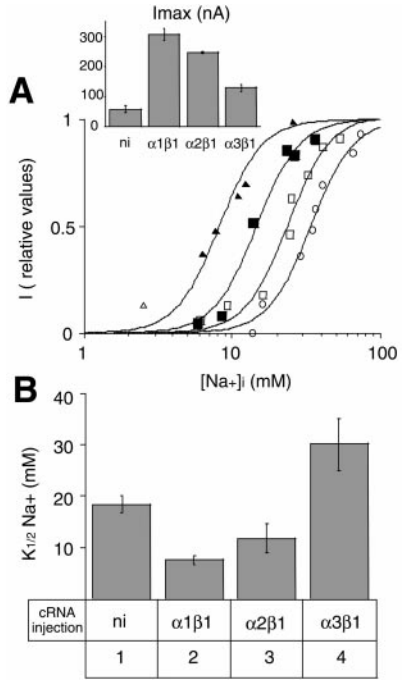

FIG. 5. **Na^+ activation of human Na,K-ATPase isozymes.** Oocytes were not injected (*ni*) or injected with α1 plus β1, α2 plus β1, or α3 plus β1 cRNAs of human Na,K-ATPase together with α and β and γ cRNAs of the rat epithelial Na^+ channel, and the Na^+ activation of the Na,K-pumps was determined as described under "Experimental Procedures." *A*, representative examples of Na^+ activation curves of Na,K-pump currents (*I*) determined in non-injected oocytes (□) or in oocytes injected with α1 plus β1 (▲), α2 plus β1 (■), α3 plus β1 (○) cRNAs. *Inset*, I_{max} extrapolated from Na^+ activation curves. *B*, Na^+ activation constants ($K_{½}$ Na^+) of the endogenous, oocyte (*lane 1*) and of the exogenous, human Na,K-pump isozymes (*lanes 2–4*). To determine the Na^+ activation of the exogenous, human Na,K-pumps, the currents which were mediated by the exogenous Na,K-pumps in cRNA-injected oocytes were calculated as described under "Experimental Procedures." To avoid artifactual results due to the low Na,K-pump current of the α3-β1 complexes, only oocytes were analyzed which exhibited at least 2 times higher currents than those measured in non-injected oocytes. Data are mean ± S.E. from 7 to 11 oocytes from 3 different *Xenopus* females. *Lane 1 versus lane 4*, $p > 0.05$; *lane 2 versus lane 3*, $p > 0.05$; *lanes 2* and *3 versus lane 4*, $p < 0.05$.

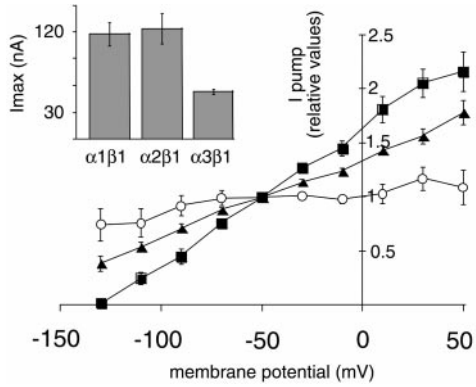

FIG. 6. **Voltage dependence of the human Na,K-ATPase isozymes.** Oocytes were not injected (ni) or injected with α1 plus β1 (▲), α2 plus β1 (■), or α3 plus β1 (○) cRNAs of the human Na,K-ATPase. K-activated and ouabain-sensitive Na,K-pump currents were determined at different membrane potentials as described under "Experimental Procedures." Averaged endogenous pump currents were subtracted from individual pump current measurements of cRNA-injected oocytes. Since α3-β1 complexes have low pump currents, we only analyzed oocytes which had at least 2-fold higher Na,K-pump currents than non-injected oocytes. Current values at −50 mV were used as reference to normalize data. Data are mean ± S.E. of 6 to 13 oocytes from two to four different batches. *Inset*, I_{max} values at −50 mV after subtraction of endogenous current.

enzyme forms are in equilibrium. This could partially explain the incomplete inhibition of ouabain binding by K^+ if one assumes that ouabain only binds to the phosphorylated E_2 form. The significant differences among the isozymes in the K^+ sensitivity of ouabain binding may indicate that equilibrium among the different enzymatic forms differs in the various isozymes. Alternatively, ouabain may bind to some forms of the enzyme other than E_2-P. To check this latter hypothesis, we performed K^+ competition experiments in Mg-P_i conditions in which the E_2 form of the enzyme is subject to "backdoor" phosphorylation and the catalytic cycle is blocked. After addition of K^+, the enzyme is dephosphorylated and mainly blocked in the E_2(K) form. Under these conditions, K^+ completely abolished ouabain binding to α1-β1 isozymes with a K_i value for K^+ of 0.77 mM (Fig. 10*B* and Table I). On the other hand, residual ouabain binding was still observed at high K^+ concentrations for α2-β1 (40%) and α3-β1 (20%) complexes and the K_i values for the limited K^+ effect amounted to 3.75 and 1.94 mM, respectively. These results suggest that ouabain may bind to non-phosphorylated, K^+-insensitive enzyme forms such as E_2(K) of human α2 and α3 isozymes.

DISCUSSION

To better understand the physiological and pharmacological role of Na,K-ATPase isozymes, we have expressed human Na,K-ATPase isozymes in *Xenopus* oocytes and investigated their functional differences under physiologically relevant, experimental conditions. For the first time, all combinations of α1, α2, α3 and β1, β2, and β3 isoforms of Na,K-ATPase were expressed in the same expression system and under the same experimental conditions which permits direct comparison of the functional properties of the isozymes formed, independent of environmental factors. All 9 different human Na,K-ATPase isozymes were efficiently expressed in *Xenopus* oocytes and sufficiently exceeded the endogenous, oocyte Na,K-pumps to permit their specific physiological and pharmacological characterization.

Transport Properties of Human Na,K-ATPase Isozymes

Turnover Rates of Human Na,K-ATPase Isozymes—All human Na,K-ATPase isozymes exhibited transport function but their turnover rates significantly differed depending on the α but not on the β isoform present in the enzyme complex (Table I). In particular, α1-β complexes showed high turnover rates compared with α3-β complexes. As to absolute values, all human Na,K-ATPase isozymes expressed in *Xenopus* oocytes exhibit relatively low turnover rates (8–50 s^{-1}) compared with those published for Na,K-ATPase isozymes expressed in mammalian or insect cells (100–200 s^{-1}), most likely due to the lower temperature used in the functional assay. In view of the variable environmental and experimental conditions used in different studies, our data on human isozymes cannot easily be compared with available data on Na,K-ATPase from other species and it is difficult to predict whether the high and low turnover rates observed for human α1-β and α3-β complexes, respectively, are common to Na,K-ATPase isozymes of other species. However, our observation that human α1-isozymes expressed in *Xenopus* oocytes have similar turnover rates than endogenous, oocyte α1-like isozymes (this study) or than expressed *Bufo* α1-isozymes (27) supports that, in general, α1-isozymes may have the highest turnover rates among the Na,K-ATPase isozymes. Of course, it cannot be excluded that there exist isoform-distinct regulatory factors which may differ among different tissues and species. Further analysis is needed to identify the intrinsic and environment dependent differences which determine the different turnover rates among different

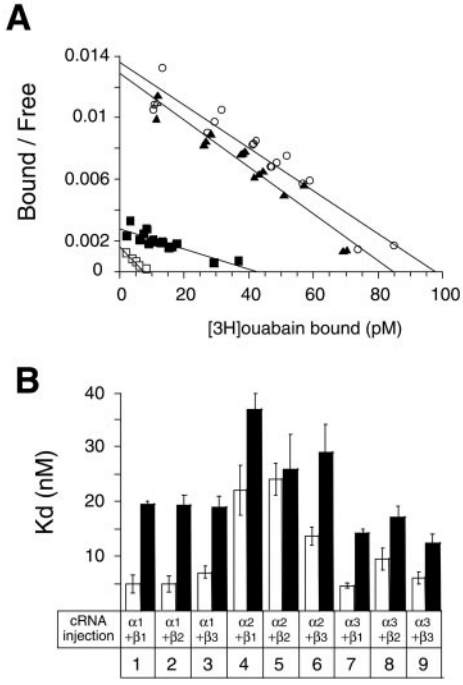

FIG. 7. **Ouabain affinity of human Na,K-ATPase isozymes.** Oocytes were not injected (*ni*) or injected with different α and β cRNAs as indicated. Three days later, microsomes were prepared as described under "Experimental Procedures." Ouabain binding measurements were carried out in Na-ATP conditions (see "Experimental Procedures") in the presence of 10^{-9} to 5×10^{-8} M [^{3}H]ouabain for 5 h either in the absence or presence of 5 mM K^+. *A*, representative Scatchard plots of ouabain binding data obtained with microsomes from non-injected oocytes (□) or from oocytes injected with α1 plus β1 (▲), α2 plus β1 (■), and α3 plus β1 (○) cRNAs in the absence of K^+. Data from equilibrium ouabain binding measurements obtained with microsomes from non-injected oocytes were subtracted from those obtained with microsomes from injected oocytes. One out of four similar experiments is shown. *B*, equilibrium dissociation constants (K_d) of the human Na,K-ATPases isozymes in the absence (*white bars*) or presence (*black bars*) of 5 mM K^+ as calculated from Scatchard plots. Data are mean ± S.E. of two to three experiments done in triplicate on two to three different microsomal preparations. *Lanes 4* and *5* ($-K^+$) *versus lanes 1–3* and *7–9* ($-K^+$) $p < 0.01$.

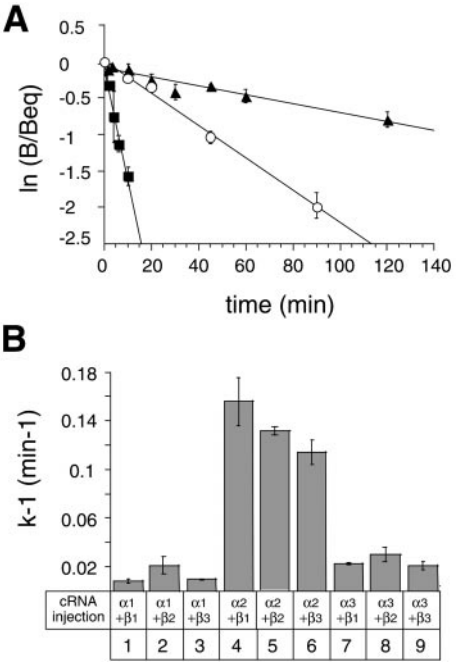

FIG. 8. **Dissociation rate constants (k_{-1}) of ouabain binding to human Na,K-ATPase isozymes.** Microsomes were prepared from non-injected oocytes or from oocytes injected with different α and β cRNAs. After incubation of microsomes for 1 h with 5×10^{-8} M [^{3}H]ouabain under Na-ATP conditions (see "Experimental Procedures"), unlabeled ouabain (5×10^{-5} M, final concentration) was added to initiate ouabain dissociation (time 0) and ouabain binding was determined after various time periods. Ouabain binding due to endogenous, oocyte Na,K-ATPase was subtracted from data obtained on microsomes from cRNA-injected oocytes. *A*, representative experiment of dissociation kinetics obtained with microsomes from oocytes injected with α1 plus β1 (▲), α2 plus β1 (■), and α3 plus β1 (○) cRNAs. The data are represented in a ln B/B_{eq} *versus* time plot; B_{eq} = specific ouabain bound at equilibrium and B = specific ouabain bound at several time points. *B*, dissociation rate constants (k_{-1}). k_{-1} values were calculated as the slopes of plots shown in *A*. Data are mean ± S.E. of three experiments done in triplicate. *Lanes 4, 5, 6 versus lanes 1–3* and *7* and *8*, $p < 0.01$.

Na,K-ATPase isozymes.

Cation Activation of Human Na,K-ATPase Isozymes—The isoform composition of human Na,K-ATPase isozymes influences their cation activation (Table I). Our results indicate that variations in K^+ activation are determined by a cooperative mechanism among α and β isoforms which depends on the interaction of a particular β isoform with a particular α isoform. The additive K^+ effect is particularly pronounced in α2-β2 complexes which show a more than 2-fold lower apparent K^+ affinity than α2-β1 complexes.

Our results highlight the importance of the β subunit in the determination of the apparent K^+ affinity of Na,K-ATPase. K^+ effects of β subunits in general, have been reported by several groups (for references, see Ref. 3). As to the effects of β isoforms, our data are difficult to compare with other published data. Similar to our observations, after expression in *Xenopus* oocytes, Torpedo α1-mouse β2 complexes (28) or *Bufo* α1-*Xenopus* β3 complexes (22) expressed in *Xenopus* oocytes had only slightly lower K^+ affinities than α1-β1 complexes. Furthermore, human α1-β3 complexes expressed in Sf9 cells showed a similar K^+ activation than α1-β1 complexes (17). On the other hand, data on rat isozymes, which by themselves are controversial, are difficult to reconcile with our data. No differences in the K^+ activation could be detected between rat kidney α1-β1 and neuronal α2/α3 isozymes (29) or between rat kidney α1-β1 and rat pineal gland α3-β2 (30) isozymes studied in microsomes. Furthermore, expressed in HeLa cells, rat α1, α2 and α3 isozymes showed, in one study, similar K^+ affinities (31) and, in another study, a similar K^+ affinity for α1 and α2 and a higher K^+ affinity for α3 isozymes (32). Finally, the apparent K^+ affinity differed among rat isozymes in the order α1-β1 > α2-β1 = α2-β2 > α3-β1 = α3-β2 when expressed in Sf9 cells (for review, see Ref. 4). It is likely that most of the contradictory results in the literature are due to different experimental conditions or assays used to study Na,K-pump properties and/or to the use of different tissues rich in one isoform or the other. Therefore, as long as rat Na,K-ATPase isozymes have not been characterized in the same expression system and under similar experimental conditions than human isozymes, it cannot be decided whether they fundamentally differ from each other or from human isozymes in their K^+ activation properties.

Although we did not investigate the effect of β isoforms, variations in the apparent Na^+ affinity of human Na,K-ATPase isozymes are clearly dependent on the nature of the α isoform associated, in the order α1-β1 ≥ α2-β1 > α3-β1 (Table I). The $K_{1/2}$ values for internal Na^+ measured in this study for human α1 and α2 isozymes are in the same range than those previously reported for human kidney (33) and red blood cells (34). A low Na^+ affinity component corresponding to the α3 isoforms has not been observed in human heart (35) probably because α3 isoforms represent only a small fraction of the total α subunit

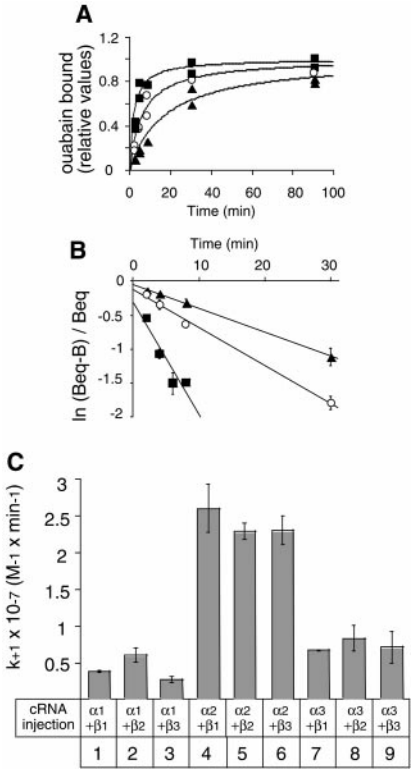

FIG. 9. **Association rate constants (k_{+1}) of ouabain binding to human Na,K-ATPase isozymes.** Microsomes were prepared from non-injected (*ni*) oocytes or from oocytes injected with different α and β cRNAs. *A,* representative experiment of association kinetics obtained with microsomes from oocytes injected with α1 plus β1 (▲), α2 plus β1 (■), and α3 plus β1 (○) cRNAs. Ouabain binding was carried out in Na-ATP conditions (see "Experimental Procedures") in the presence of 10^{-8} M [^{3}H]ouabain and determined after various time periods. Ouabain binding due to endogenous, oocyte Na,K-ATPase was subtracted from data obtained on microsomes from cRNA-injected oocytes. Shown is one out of three experiments done in triplicate on three different microsomal preparations. *B*, ln $(B_{eq} - B)/B_{eq}$ *versus* time plot of data shown in *A*. B_{eq} = specific [^{3}H]ouabain bound at equilibrium; B = specific ouabain bound at several time points. *C*, association rate constants k_{+1}. k_{+1} values were calculated as described under "Experimental Procedures." Data are mean ± S.E. of three experiments done in triplicate.

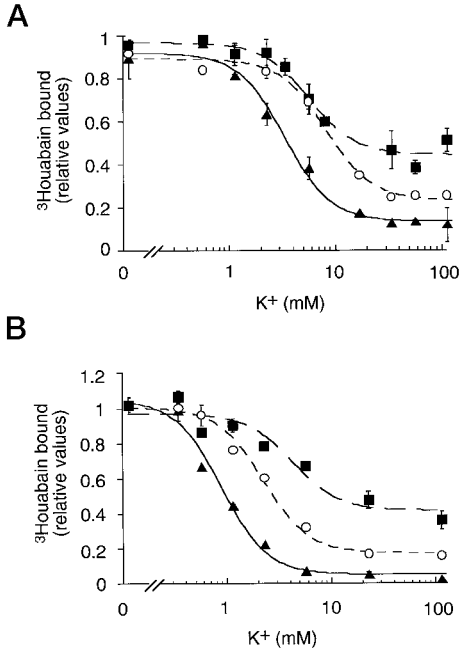

FIG. 10. **Competition of K^+ for ouabain binding to human Na,K-ATPase isozymes.** Microsomes were prepared from non-injected oocytes or from oocytes injected with α1 plus β1 (▲), α2 plus β1 (■), or α3 plus β1 (○) cRNAs. Ouabain binding on microsomes was performed for 1 h in the presence of 10^{-8} M or 5×10^{-8} M [^{3}H] ouabain for α1-β1 and α3-β1 complexes or for α2-β1 complexes, respectively, representing concentrations 2-fold superior to K_d values measured in the absence of K^+. *A*, experiments done in Na-ATP conditions (see "Experimental Procedures"). Data are mean ± S.E. from three experiments done in triplicate on three different microsomal preparations. *B*, experiments done in Mg-P_i conditions (see "Experimental Procedures"). Data are mean ± S.E. from two experiments done in triplicate on two different microsomal preparations.

population in this tissue (36, 37). In contrast to data on K^+ activation, our data on Na^+ activation of different human Na,K-ATPase isozymes compare well with data obtained for rat isozymes (for review, see Ref. 4). Our results indeed indicate that, irrespective of the species, α3 isozymes have the lowest Na^+ affinity among the different α isoforms.

Voltage Dependence of Human Na,K-ATPase Isozymes—Na,K-ATPase is electrogenic and voltage-dependent. The binding of extracellular Na^+ and K^+ are the voltage-dependent steps in the pump cycle (2). For the first time, we have measured the voltage dependence of Na,K-ATPase isozymes expressed in the same experimental system and we show that voltage-dependence is influenced by the α isoform present in the Na,K-pump. In the presence of external Na^+ and saturating concentrations of K^+, the profile of the I-V curve obtained for human α1-β1 complexes is similar to that previously reported for *Xenopus* α1-β1 (38). In comparison, human α2-β1 complexes show a steeper voltage dependence while α3-β1 complexes are nearly voltage-independent over the whole potential range. So far, we do not know the reasons for the differences in the voltage dependence among different isozymes. One interpretation may be that there is an isoform-specific difference in the backward rate constant for the Na^+-release step, *e.g.* a difference in binding of Na^+ at extracellular sites. Indeed, preliminary data (not shown) indicate that the voltage dependence of α1-β1 and α2-β1 complexes may mainly be determined by the presence of external Na^+ since, in the absence of external Na^+, the voltage dependence of these isozymes is virtually abolished. α3-β1 isozymes which are nearly voltage-independent may be due to a very low affinity for external Na^+.

Taken together, our results show that human Na,K-ATPase isozymes exhibit major transport differences which could have important physiological consequences. The tissue distribution of α and β isoforms in humans is not yet well known and we therefore must rely on that reported for rat isozymes. The human isozymes formed with α1 isoforms are both very sensitive to K^+ and Na^+, have the greatest turnover, and exhibit an intermediate dependence on the membrane potential compared with other isozymes. These data confirm the housekeeping role of this ubiquitous isoform as previously defined (31, 32) since Na,K-ATPase with such characteristics should work at optimum rates under physiological conditions but cannot respond to increased physiological demands. On the contrary, the human Na,K-ATPase formed with α2 or α3 isoforms exhibit transport properties which confirm their auxiliary but indispensable role to restore resting conditions in muscles or nerves after a series of action potentials. Thus, α2-β2 isoforms expressed in skeletal muscle (39) may be crucial to restore external K^+ homeostasis after physical activity. During physical exercise, external K^+ increases to significant levels which, in turn, leads to depolarization of muscle cells. These two effects may specifically activate α2-β2 isoforms because of their low apparent K^+ affinity and their strong voltage dependence. A similar role for α2-β2 isozymes in external K^+ clearance after membrane depolarization may be predicted in the nervous system where

these isoforms are predominantly expressed in glial cells. On the other hand, $\alpha 3$ isozymes found in neuronal cells and which have a low Na^+ affinity, may be suited to restore intracellular Na^+ concentrations after a series of action potentials. The presence of the 3 α isoforms in human cardiomyocytes (36, 37) indicates that ion homeostasis is also finely regulated in these cells and that, as in skeletal muscles and neurons, the 3 Na,K-ATPase isozymes may act in concert to meet the physiological needs.

Pharmacological Properties of Human Na,K-ATPase Isozymes

Ouabain Sensitivity of Human Na,K-ATPase Isozymes—Inhibition of Na,K-ATPase in heart by digitalis reduces the driving force for Na^+ entry and, in consequence, Ca^{2+} extrusion via the Na/Ca exchanger which ultimately leads to increased intracellular Ca^{2+} levels and contractile force (40). This generally accepted mechanism of action is supported by the co-distribution of high ouabain affinity isoforms of Na,K-ATPase with the Na/Ca exchanger that overlie subplasmalemmal sarcoplasmic reticulum in rat cardiomyocytes (41). Although the molecular action of digitalis is well known, the question remained open whether the different Na,K-ATPase isozymes present in the human heart play a distinct role in the efficacy and/or the toxicity of cardiac glycosides.

Equilibrium binding on oocyte microsomes revealed that all human Na,K-ATPase isozymes exhibit a high affinity for ouabain in the low nanomolar range (Table I) in contrast to rat isozymes which exhibit a nearly 1000-fold difference in the ouabain sensitivity among $\alpha 1$ and $\alpha 2$ or $\alpha 3$ isozymes (for review, see Ref. 4). Although human Na,K-pump complexes containing $\alpha 2$ isoforms show a significant 4–5-fold higher K_d for ouabain compared with $\alpha 1$-β or $\alpha 3$-β complexes, this difference, which is decreased by about 2-fold in the presence of 5 mM K^+, is not likely to be of any pharmacological relevance in digitalis treatment. The concept of inotropic and toxic isoforms, based on different affinities for digitalis, is not justified in humans which may explain the narrow therapeutic range of digitalis.

The lack of important differences in ouabain affinity among different human Na,K-ATPase isozymes may explain the variable results reported on the number of ouabain binding sites in human tissues. Despite the possible presence of all isozymes, only one ouabain binding site was described in brain tissue (42) and either one (43) or two ouabain binding sites (35) in cardiac membranes. All these sites exhibited similar and high ouabain affinities as expected from our study. Similarly, in placenta (44), kidney (45), and HeLa cells (46), K_d values for ouabain ranged between 2 and 17 nM.

Despite the lack of differences in the intrinsic ouabain sensitivity, human Na,K-ATPase isozymes markedly differ with respect to the association and dissociation kinetics of ouabain binding with both these processes being much more rapid for $\alpha 2$-β complexes than for $\alpha 1$-β or $\alpha 3$-β complexes (Table I). Since association and dissociation rate constants vary in parallel in all isozymes, the calculated K_d values are similar to those measured under equilibrium conditions. It is generally accepted that ouabain association rate constants are slow and that variations in ouabain sensitivities among Na,K-ATPase isozymes are determined by the dissociation rate constants (for review, see Ref. 47). To our knowledge, differences in both association and dissociation rate constants among isozymes of similar ouabain sensitivity have so far not been reported. It may be speculated that $\alpha 2$-β isozymes which are characterized by rapid ouabain association and dissociation kinetics spend more time in the ouabain binding-competent state, E_2-P than $\alpha 1$-β or $\alpha 3$-β complexes. Alternatively, ouabain may have better access to and may be more rapidly released from the binding site in $\alpha 2$-β complexes than in $\alpha 1$-β or $\alpha 3$-β complexes (see also below).

The differences in the ouabain binding kinetics of human Na,K-ATPase isozymes reported in this study may be reflected in studies on human cardiac plasma membranes where the rapid dissociation process described ($t_{1/2}$ 10–13 min) (35, 48) could correspond to $\alpha 2$-β complexes ($t_{1/2}$ 4–5 min) while the slower process described ($t_{1/2}$ 39–75 min) (35, 48, 49) could correspond to $\alpha 1$-β and/or $\alpha 3$-β isozymes ($t_{1/2}$ 30–80 min). On the other hand, in human cardiac plasma membranes, only one association rate constant was described ranging from 0.12×10^7-0.7×10^7 M^{-1} min^{-1} (35, 49, 50).

The particular features of $\alpha 2$ isozymes concerning the ouabain binding kinetics allow speculations on their pharmacological role during digitalis treatment. Based on the important mass of muscle tissue in the body and the predominant expression of $\alpha 2$ isozymes in this tissue, several authors (for review, see Ref. 51) have speculated that this Na,K-ATPase isozyme may function as a store for digitalis during treatment of heart failure. We add to this idea the possibility that $\alpha 2$ isozymes may also be an important component of the regulation of the digitalis circulating concentration. Indeed, $\alpha 2$ isozymes may rapidly bind or release digitalis in response to changing digitalis plasma concentration, due to increased digitalis administration or metabolism and elimination, respectively.

K^+ Antagonism of Ouabain Binding to Human Na,K-ATPase Isozymes—External K^+ is known to antagonize ouabain binding by dephosphorylation of E_2-P (47). During hypokaliemia, often associated with congestive heart failure, the affinity of digitalis for Na,K-ATPase increases due to decreasing competition by K^+ which favors the susceptibility to digitalis toxicity (for review, see Ref. 9). We show in this study that equilibrium ouabain binding constants measured for $\alpha 1$-β complexes are more affected by the presence of 5 mM K^+ than those for $\alpha 2$-β or $\alpha 3$-β complexes (Table I). K^+ competition experiments under physiological Na-ATP or backdoor Mg-P_i conditions also revealed a lower K_i value for K^+ competition of ouabain binding to $\alpha 1$-β complexes than to $\alpha 2$-β or $\alpha 3$-β complexes. Ouabain binding to $\alpha 1$-β complexes is nearly completely abolished at 5 to 10 mM K^+ which is consistent with preferential binding of ouabain to the E_2-P conformation of this isozyme. Significantly, in $\alpha 2$ and $\alpha 3$ isozymes, an important fraction of ouabain remained bound at high K^+ concentrations (100 mM) in both Na-ATP and Mg-P_i conditions. These results may support earlier observations which led to the conclusion that ouabain may interact not only with E_2-P but with any enzyme conformation although at a finite and low rate (47). Our results suggest that in $\alpha 2$-β and $\alpha 3$-β isozymes, ouabain binding to other K^+-insensitive conformational species (*e.g.* E_2(K)) could be more pronounced than in $\alpha 1$-β isozymes. For $\alpha 2$ isozymes, our finding correlates with its rapid association and dissociation rate constants which may also reflect ouabain binding to multiple conformational species.

Thus, even though the intrinsic affinity for ouabain does not greatly differ among human Na,K-ATPase isozymes, the particular features of ouabain binding which distinguish the different isozymes, may be of pharmacological relevance. The effect of K^+ on ouabain binding is particularly interesting because our results suggest that at physiological K^+ concentrations, digitalis, at therapeutic doses, may predominantly bind to $\alpha 2$ and $\alpha 3$ isozymes present in human heart and to a lesser extent to $\alpha 1$ isozymes. Thus, in light of similar ouabain affinities of Na,K-ATPase isozymes, it is likely that, in humans, the distinct K^+/ouabain antagonism in different isozymes is at the origin of both beneficial and toxic effects of these com-

pounds because, at physiological K^+ concentrations, it would favor inhibition of the α2 and α3 isozymes present in neurons and heart and protect the ubiquitous α1 isoform. A small change in equilibrium among plasma K^+ (hypokaliemia) and digitalis (overdose) concentrations and Na,K-ATPase expression (hypo- or hyperthyroidism, diabetes etc.) may lead to toxic effects.

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