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Article

Importance of the Voltage Dependence of Cardiac Na/K ATPase Isozymes

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ABSTRACT Cardiac cells express more than one isoform of the Na, K-ATPase (NKA), the heteromeric enzyme that creates the Na^+ and K^+ gradients across the plasmalemma. Cardiac isozymes contain one catalytic α-subunit isoform (α1, α2, or α3) associated with an auxiliary β-subunit isoform (β1 or β2). Past studies using biochemical approaches have revealed minor kinetic differences between isozymes formed by different α-β isoform combinations; these results make it difficult to understand the physiological requirement for multiple isoforms. In intact cells, however, NKA enzymes operate in a more complex environment, which includes a substantial transmembrane potential. We evaluated the voltage dependence of human cardiac NKA isozymes expressed in *Xenopus* oocytes, and of native NKA isozymes in rat ventricular myocytes, using normal mammalian physiological concentrations of Na^+_o and K^+_o. We demonstrate that although α1 and α3 pumps are functional at all physiologically relevant voltages, $\alpha2\beta1$ pumps and $\alpha2\beta2$ pumps are inhibited by ~75% and ~95%, respectively, at resting membrane potentials, and only activate appreciably upon depolarization. Furthermore, phospholemman (FXYD1) inhibits pump function without significantly altering the pump's voltage dependence. Our observations provide a simple explanation for the physiological relevance of the α2 subunit (~20% of total α subunits in rat ventricle): they act as a reserve and are recruited into action for extra pumping during the long-lasting cardiac action potential, where most of the Na^+ entry occurs. This strong voltage dependence of α2 pumps also helps explain how cardiotonic steroids, which block NKA pumps, can be a beneficial treatment for heart failure: by only inhibiting the α2 pumps, they selectively reduce NKA activity during the cardiac action potential, leading to an increase in systolic Ca^{2+}, due to reduced extrusion through the Na/Ca exchanger, without affecting resting Na^+ and Ca^{2+} concentrations.

INTRODUCTION

Cellular excitability and secondary active transport are among the many processes driven by the Na^+ and K^+ gradients built by the Na, K-ATPase (NKA or Na/K pump), a membrane protein that transports three Na^+ ions out of the cell in exchange for importing two K^+ ions at the cost of the hydrolysis of one ATP molecule (1). NKA is a P-type IIC ATPase formed by the obligatory association of a catalytic α subunit, which contains all the machinery for ion transport and ATP hydrolysis, with an accessory β-subunit, which is essential for membrane targeting and protein stability (2).

Vertebrates express multiple subunit isoforms with distinct tissue distributions. There are four α and three β subunits (reviewed in (3)): α1 is ubiquitous; α2 is present in all muscle types (4–6), glia (4,7), and neurons (8); α3 is expressed in neurons (9,10) and in the heart, especially in the conductive system (11); α4 is present in testis (12). This article focuses on those isoforms expressed in the heart. Cardiac cells express β1 and β2 (13) and coimmunoprecipitation experiments showed that β1 associates with α1 and α2, whereas β2 interacts with α2 and α3 (14). This association pattern can be even more intricate as some tissues express NKA trimers when a member of the single transmembrane domain FXYD protein family (isoforms 1 through 7) associates with the αxβx dimer. FXYD1 (aka phospholemman) participates in oxidative- (15) and kinase- (16) mediated regulation of the cardiac NKA formed by the α1 and α2 isoforms (17). Thus, mature NKA multimers, even within a single tissue, exhibit substantial molecular-level complexity.

A central question that remains unanswered is how the molecular variety translates into functional diversity? Several studies that have investigated NKA kinetics show that the isozymes exhibit relatively small differences in their affinity for ligands (see (3) for review). In addition, ion transport by all isoforms is electrogenic and hence affected by transmembrane voltage (18). Nearly all the voltage dependence arises from the transit of ions into and out of their binding sites through access channels (19). In the presence of both Na^+_o and K^+_o most of the voltage dependence arises from Na^+_o rebinding to externally facing sites. This induces voltage-dependent inhibition (VDI) at negative voltages and a positive slope in the current-voltage relationship (I_P-V) (20–23): The larger the $[Na^+]_o$ the stronger the

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VDI. Under external physiological conditions for mammals, where the $[K^+]_o$ does not saturate the K^+ binding sites, competition between Na^+ and K^+ is significant and VDI is even greater than at near-saturating $[K^+]_o$. Therefore, the chosen concentrations of Na^+ and K^+ can impact transport. The extent of VDI in cardiac cells, due to the individual sensitivities of the expressed isoforms, is presently unknown.

The comparison of physiological data from prior reports is confounded by the fact that, depending on the preparation, ionic conditions varied, as did the complexity of the underlying NKA isoforms. Using batrachian ionic conditions ($\leq$100 mM Na^+_o), α2-subunits showed greater VDI than α1 and α3 pumps (24,25), but these experiments failed to present concrete evidence for the role of voltage in determining the functional state of the α2 pumps. On the other hand, studies in cardiac myocytes have either measured the voltage dependence of all the pumps in the myocyte (20,26–28), or have measured the activity of α1 and non-α1 pumps only at 0 mV (29–32).

Here, we present a study of the voltage dependence of cardiac isozymes under mammalian physiological concentrations of Na^+_o (~150 mM) and K^+_o (~4.5 mM). Our results demonstrate that the voltage dependence of pumps including the α2 isoform is unusually steep over the physiological range. This characteristic suggests that they serve as a reservoir of pumping activity during the cardiac action potential but are inactive at ventricular resting potentials. In addition, we show that association with the β2 subunit exacerbates this voltage dependence. Furthermore, although FXYD1 does not significantly alter the voltage dependence of the isozymes, it affects the apparent affinity for K^+_o, which in turn depends on the external $[Na^+]_o$. Finally, using rat cardiac myocytes, where the contribution of different native isozymes can be distinguished, we demonstrate the relevance of isoform-specific voltage dependence to cardiac physiology.

MATERIALS AND METHODS

Cell preparation

Oocytes were isolated enzymatically, injected with a mixture of human $\alpha x\beta x$ cRNA, and maintained for 3–6 days postinjection as described previously (22).

Rat ventricular myocytes were isolated from 1 to 3 months old rats with slight modification of methods previously described for Guinea pigs (33). Briefly, the heart was removed under pentobarbital anesthesia and rapidly mounted on a Langendörf system and perfused with oxygenated Tyrode solution consisting of (in mM): 1.8 $CaCl_2$, 145 NaCl, 5 KCl, 1 $MgCl_2$, and 10 HEPES (pH 7.4) at 35°C. Once the coronaries were blood-free the heart was perfused with Ca^{2+}-free Tyrode for 5 min, followed by application of Ca^{2+}-free Tyrode with enzyme solution (1 mg/mL collagenase type 1A and 0.2 mg/mL protease type XIV (Sigma, St. Louis, MO)) until the digestion was obvious to sight and tact (~7 min). A solution containing (in mM): 70 K-glutamate, 25 KCl, 20 taurine, 10 KH_2PO_4, 1 $MgCl_2$, 0.5 EGTA, 20 glucose, and 10 HEPES (pH 7.3) was used to wash the enzyme, cut the heart, disperse the myocytes, and store them for up to 36 h at 4°C.

Molecular biology

Human NKA isoforms were amplified from the neuroblastoma cell line SHSY5Y cDNA using isoform-specific polymerase chain reaction primers. Sequences were validated by DNA sequencing and the open reading frames were subcloned into the Bgl II restriction enzyme site in the pBSTA vector for oocyte expression (34). Plasmids were linearized with Not I before in vitro transcription with T7 RNA polymerase. FXYD1, purchased from the mammalian gene collection (http://mgc.nci.nih.gov) was subcloned into the pSD5 vector and linearized with Bgl II before transcription with SP6.

Electrophysiology and solutions

Two-electrode voltage-clamp recordings were performed using either an OC-725C amplifier (Warner Instruments, Hamden, CT) or a CA-1B High Performance Oocyte Clamp amplifier (Dagan, Minneapolis, MN) in combination with a Digidata 1440 A/D board, a Minidigi 1A, and pClamp 10 software (Molecular Devices, Sunnyvale, CA). Signals were filtered at 2 kHz and digitized at 10 kHz. Resistance of both microelectrodes (filled with 3 M KCl) was 0.2–0.8 MΩ. Oocytes were Na-loaded by 20–30 min incubation in either a solution containing (in mM): 150 HEPES, 20 tetraethylammonium-Cl, and 0.2 EGTA (pH 7.2 with NaOH) or 90 Na-sulfamate, 20 Na-HEPES, 20 TEA-Cl, and 0.2 EGTA (pH 7.2). Both solutions produced indistinguishable results. The signals from α3 pumps were reduced with longer than 30-min incubations by a mechanism that was not studied further. After the 30-min incubation, oocytes were kept in 90 mM Na^+ solution until recording. All experiments were performed at room temperature (RT, 22–24°C) except those in Fig. 5 where a TC-10 temperature controller (Dagan) was used to set the temperature between 24 and 34°C.

The 90 mM Na^+ solution for two-electrode voltage-clamp (TEVC) was composed of (in mM): 130 methane sulfonic acid (MS), 90 NaOH, 35 *N*-methyl D-glucamine (NMG), 10 HEPES, 5 $Ba(OH)_2$, 0.5 $Ca(OH)_2$, 1 $Mg(OH)_2$ (pH 7.6, with NMG, 250–260 mOsm/kg). Temperature-controlled experiments were performed at 125 mM Na^+ (where 35 mM Na^+ was substituted for the NMG of the 90 mM Na^+ solution). The 150 mM Na^+ solution was used in TEVC and patch-clamp experiments and contained (in mM): 130 MS, 150 NaOH, 10 HEPES, 5 $BaCl_2$, 1 $CdCl_2$, 1 $MgCl_2$ (pH 7.4, 300–310 mOsm/kg).

A common strategy to completely eliminate contribution of endogenous pumps to the signals is to introduce ouabain resistance conferring mutations naturally occurring in the rat α1 subunit (35). However, such strategy produces significant changes in the affinity for both Na^+_o and K^+_o (36). Because the relevance of our results requires studying wild-type pumps under normal external mammalian solutions, we decided not to introduce any mutation of this sort. Therefore, to be sure that at least 90% of pump signals come from the exogenously expressed pumps we only used experiments that presented >200 nA of K^+-induced pump current. Small endogenous pump currents are shown in Fig. S1 in the Supporting Material along with averages from injected oocytes.

Turnover rates were estimated by dividing the pump current by the total ouabain-sensitive charge measured in the presence of Na^+ without K^+, essentially as described by Tavraz et al. (37). To obtain the total charge, the ouabain-sensitive transient currents when the pulses were turned off were integrated, the Q_{OFF}-V plots were fitted with a Boltzmann distribution $Q_{OFF} = Q_{min} + Q_{tot}/(1 + e(V-V_{1/2})/k)$, where Q_{tot} is the total charge, Q_{min} the charge moved by negative pulses, $V_{1/2}$ is the center of the distribution, and k is the slope factor.

Dose response curves from all experiments at each voltage were fitted globally with the Hill equation $I = I_{max} \times ([K^+]^n/([K_{0.5}]^n + [K^+]^n)$. The half maximal concentration ($K_{0.5}$) was shared and the Hill number was fixed ($n = 1.6$). In the few cases where unblocking of K^+-channel currents (38) at 4.5 or 10 mM K^+ was observed, evident as a second rising phase at $V \geq 0$ mV in the K^+-induced current versus voltage curve, only ouabain-sensitive currents were considered.

Whole-cell patch-clamp recording of rat ventricular myocytes was performed with an Axopatch 200A, a Digidata 1322 A/D board, and pClamp 10 software (all from Molecular Devices), with acquisition at 10 kHz, filtered at 2 kHz. Pipettes had resistances between 1 and 3 MΩ when filled with intracellular solution containing (in mM): 130 L-glutamic acid, 10 HEPES, 10 EGTA, 10 tetraethylammonium-Cl, 5 MgATP, 1 $MgCl_2$, and 85 NaOH, pH 7.4 with CsOH (~65 mM). The 150 mM external solution was heated to 35°C with an in-line heater connected to a temperature controller TC344B (Warner Instruments).

K^+ was added from a 450 mM K-MS stock. Bath electrodes were AgCl pellets connected by 3 M KCl agar bridges. NKA-mediated current was identified by inhibition with ouabain at concentrations of 200 μM in oocytes, and 5 μM for non-α1 pumps or 5 mM ouabain for α1 pumps in rat ventricular myocytes.

Western blotting

Membranes from ventricular tissue were prepared as described by Fuller, et al. (39). Protein electrophoresis was performed using Mini-Protean Tgx gels 7.5% (BioRad, Hercules CA) in Mini Protean Tetra System (BioRad). Protein was transferred onto 0.22 μm polyvinylidene fluoride membrane using Hoefer TE77. Transferred membranes were blocked with Tris-Buffered Saline (TBS) supplemented with 5% milk and Tween 20 (TBST) for 1 h, incubated overnight with TBS supplemented with 1% milk, 1% bovine serum albumin (BSA) and containing the TED primary antibody (40) diluted 1:100 (generous gift of Thomas A. Pressley, TTUHSC) followed by 1 h in TBST supplemented with 1% milk, 1% BSA and containing the horseradish peroxidase antirabbit (1:1000 dilution). Imaging was performed at the TTUHSC Molecular Biology Core Facility with Luminol (Santa Cruz Biotechnology, Dallas, TX) on an ImageQuant LAS 4000 (GE Healthcare, Pitsburgh, PA).

Immunofluorescence

After isolation cells were allowed to attach to poly-L-lysine-coated coverglass, fixed with paraformaldehyde for 15 min at RT, washed, permeabilized with triton X-100 for 10 min at RT, washed with phosphate buffered saline (PBS), and blocked in PBS supplemented with 3% BSA for 1 h. Samples were then incubated with primary antibody (PBS supplemented with 1% BSA) overnight, washed, and incubated with secondary antibody (PBS supplemented with 1% BSA) for 1 h. Permeabilized cells, blocked and treated only with secondary antibody were used as controls in each myocyte preparation (not shown). Images were acquired at the TTUHSC Image Analysis Core Facility on a Nikon Ti-E microscope.

RESULTS

Function of human NKA isozymes in *Xenopus* oocytes with mammalian osmolality

As mentioned above, all previous characterizations of NKA function in oocytes were made at 80–100 mM Na^+_o, with variable K^+_o concentrations (24,25). We first evaluated whether these batrachian ionic conditions might have led to an underestimation of the level of VDI observed at negative voltages with some isoforms, particularly α2 pumps. TEVC was used to address the effect of switching from 90 mM Na^+_o to 150 mM Na^+_o on the voltage dependence of the current induced by 4.5 mM K^+_o, the normal mammalian serum K^+_o concentration (Figs. 1 and 2).

Fig. 1 *A* shows a representative current recorded on a slow time base, from an oocyte expressing $\alpha2\beta1$ pumps held at −50 mV. The application of 4.5 mM K^+_o induced outward currents in both 90 mM and 150 mM external Na^+. Sharp vertical deflections in the continuous trace correspond to the application of brief (100 ms) voltage pulses. The current at the end of those pulses is plotted as a function of voltage in Fig. 1 *B*. Measurements in the absence of K^+ at the times marked *a*, *c*, and *e* in Fig. 1 *A* were then subtracted

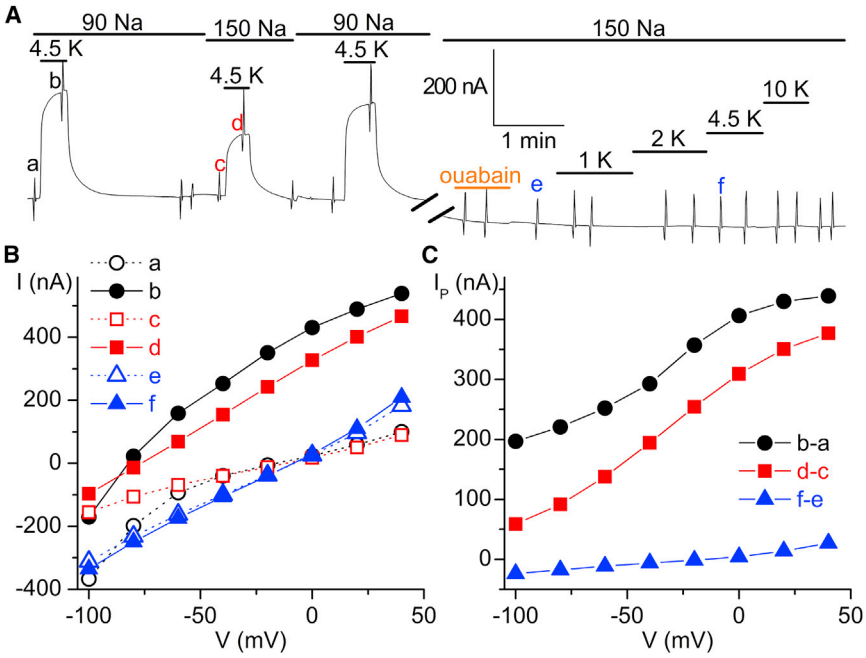

FIGURE 1 TEVC recording of a *Xenopus* oocyte 5 days after injection with human $\alpha2\beta1$ cRNAs. (*A*) Continuous recording at −50 mV. Application of a 4.5 mM K^+-induced outward pump current (I_P) in 90 as well as in 150 mM Na^+ (note significantly smaller current at 150 mM Na^+). 100 μM ouabain was applied after the 10-min break inhibiting K^+-induced I_P. Sharp vertical deflections correspond to 100 ms pulses used to obtain the current versus voltage (I-V) relationships. (*B*) I-V curves obtained at the times indicated by small cap letters in (*A*). (*C*) Voltage dependence of 4.5 mM K^+-induced currents at 90 mM Na^+, 150 mM Na^+, and 150 mM Na^+ with ouabain. Note the stronger VDI at 150 mM Na^+. To see this figure in color, go online.

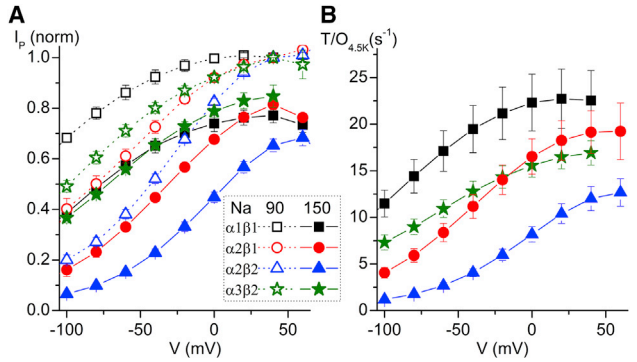

FIGURE 2 Voltage dependencies of all four cardiac isoforms at 4.5 mM K^+. (*A*) Voltage dependence of 4.5 mM K^+-induced I_P normalized to the value at +40 mV in 90 mM Na^+ to illustrate the effect of 150 mm Na^+, for $\alpha1\beta1$ (*squares*, $n = 7$), $\alpha2\beta1$ (*circles*, $n = 7$), $\alpha2\beta2$ (*triangles*, $n = 16$), and $\alpha3\beta2$ (*stars*, $n = 9$). (*B*) Turnover rate (T/O), for $\alpha1\beta1$ $n = 11$; $\alpha2\beta1$ $n = 8$; $\alpha2\beta2$ $n = 8$; and $\alpha3\beta2$ $n = 6$. To see this figure in color, go online.

from those marked *b*, *d*, and *f*, to obtain the K^+-induced currents plotted in Fig. 1 *C*. Thus, Fig. 1 *C* displays the K^+-induced currents with 90 mM or 150 mM Na^+_o in the absence of ouabain and in 150 mM Na^+_o after inhibition with 100 μM ouabain. Lack of current after ouabain demonstrates that K^+_o-induced current (I_P) is due to the Na/K pump. The activity of $\alpha2\beta1$ pumps expressed in this oocyte is highly dependent on voltage within the physiological voltage range; from −90 mV to +40 mV.

We performed similar experiments on oocytes expressing human $\alpha1\beta1$, $\alpha2\beta1$, $\alpha2\beta2$, and $\alpha3\beta2$ pumps because evidence suggests that these isoforms are present, and interact, in rat and guinea pig ventricular myocytes (14). Fig. 2 *A* plots the K^+-induced currents in 150 mM Na^+_o normalized to the same currents induced in 90 mM Na^+_o at +40 mV. To minimize the contribution of endogenous I_P to the signals (~25 nA, Fig. S1) we limit our analysis to oocytes showing currents ≥200 nA at our normalization point. It is evident that switching from 90 to 150 mM Na^+_o induces a strong current reduction in Na/K pumps created by all cardiac isoform combinations ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha2\beta2$, and $\alpha3\beta2$) and this effect is present over the entire range of physiological membrane potentials. Furthermore, 150 mM Na^+ induces a dramatic I_P reduction for all $\alpha2$-containing pumps at negative voltages. Endogenous pumps are responsible for most of the I_P remaining at −80 mV in $\alpha2\beta2$ expressing oocytes (cf. Fig. S1, *gray hexagons*).

The inhibitory effect of 150 mM Na^+_o can be better appreciated when the data is presented as turnover rate (Fig. 2 *B*). To obtain the turnover rate (T/O) under physiological conditions, one must estimate the number of pumps in each oocyte, and this was accomplished by measuring the total charge (Q_{tot}) moved by NKAs in the absence of K^+ (37). To be thorough, we also studied the function of $\alpha1\beta2$ and $\alpha3\beta1$ pumps. The association of any α-subunit with $\beta2$ shifts their voltage dependence toward positive voltages (Fig. S2).

To better understand how hypo- and hyperkalemia could affect cardiac pumps under physiological Na^+, we investigated the dose dependence for K^+_o activation in 150 mM Na^+_o (Fig. 3). A continuous current record from an oocyte expressing $\alpha1\beta1$ being held at −50 mV is shown in Fig. 3 *A* as an illustration of our experimental design. K^+ induces current in a concentration-dependent manner and saturates at ~10 mM. Fig. 3 *B* displays the K^+-induced currents as a function of voltage. Fig. 3 *C* shows the current as a function of the K^+ concentration at two

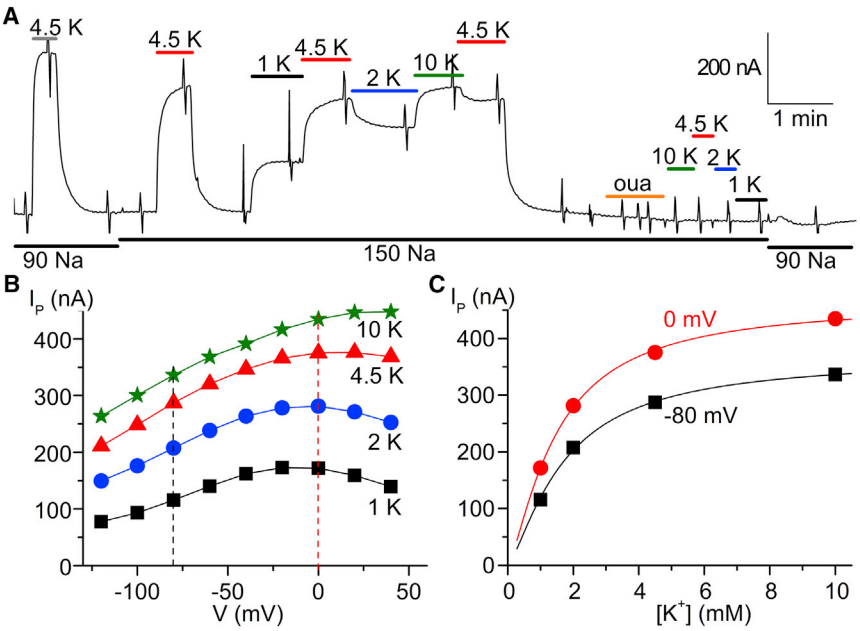

FIGURE 3 Dose response for K^+ in an $\alpha1\beta1$ expressing oocyte. (*A*) Continuous recording four days after injection. Step changes in [K^+] produced a concentration-dependent increase in outward current. (*B*) Voltage dependence of K^+-induced ouabain-sensitive currents at different [K^+]. (*C*) The I_P at different voltages were plotted against [K^+] and fitted with a Hill equation (*line plots*). Only two voltages are shown for clarity. The $K_{0.5}$ and the maximal turnover (I_{max}/Q_{tot}) are plotted as a function of voltage in Fig. 4. To see this figure in color, go online.

voltages, where the continuous lines are fits of the Hill equation to the data.

The voltage dependence of the Hill equation parameters fitted to the data from all cardiac isoforms combinations are presented in Fig. 4. The $K_{0.5}$ for K^+_o and the maximal turnover rates (T/O_{max}) as a function of transmembrane voltage are shown in Fig. 4, *A* and *B*, respectively. It must be noted that $\alpha2\beta2$ pumps have an extremely low apparent affinity for K^+_o, especially at negative voltages ($K_{0.5} \geq$ 10 mM at −60 mV); because of this strong voltage dependence neither the $K_{0.5}$ nor the I_{max} can be accurately determined below −60 mV. To extend our analysis beyond the cardiac isoforms combinations, we studied $\alpha1\beta2$ and $\alpha3\beta1$ (Fig. S3). The $\alpha1\beta2$ isozyme has a lower affinity for K^+ than $\alpha1\beta1$, but only at negative potentials.

The question arises of whether the lower than physiological temperature (T) used for the previous experiments (22–24°C) could have affected the NKA's voltage dependence. We measured the voltage dependence of pump function at 24°C and 34°C (Fig. 5), but with 125 mM Na^+ (oocytes could not withstand 150 mM Na^+ at high T). The top trace in Fig. 5 *A* shows a continuous recording at −50 mV (interrupted by 100 ms-long voltage pulses responsible for the sharp vertical deflections at this slow time base) from an oocyte injected with $\alpha2\beta2$. The I_P-V curve in the same oocyte is shown at the bottom. Fig. 5 *B* illustrates the mean turnover rate at each T measured in uninjected oocytes (uninj., *gray hexagons*) and in oocytes injected with $\alpha2\beta1$ (*circles*), or $\alpha2\beta2$ (*triangles*). Both, the lack of a strong effect on voltage dependence and the 2.5-fold change in NKA turnover rate, are consistent with previous electrophysiological studies addressing the effect of T on pump function (41–43).

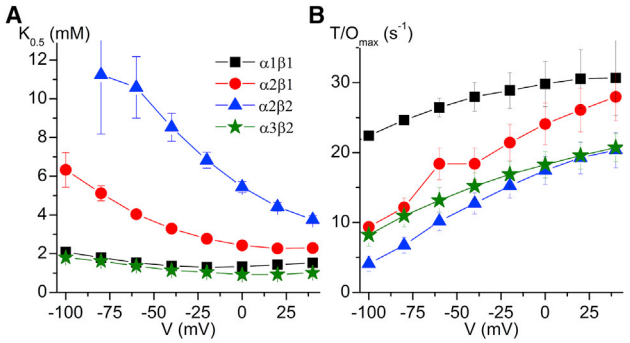

FIGURE 4 Voltage dependence of apparent affinity for K^+ and maximum turnover rate. (*A*) $K_{0.5}$ as a function of voltage at 150 mM Na^+. $\alpha1\beta1$ (*squares*, $n = 4$), $\alpha2\beta1$ (*circles*, $n = 3$), $\alpha2\beta2$ (*triangle*, $n = 6$), $\alpha3\beta2$ (*stars*, $n = 3$). (*B*) Maximum turnover rate (I_{max}/Q_{tot}) as a function of voltage. The I_{max} measured in the same experiments at +40 mV averaged 332 ±71 nA ($\alpha1\beta1$), 251 ±24 nA ($\alpha2\beta1$), 599 ±69 nA ($\alpha2\beta2$), and 338 ±81 nA ($\alpha3\beta2$). Note uncertainty of $K_{0.5}$ for $\alpha2\beta2$ at $V \leq -60$ mV due to unreliable measurements for $\alpha2\beta2$ at very negative potentials because of small endogenous pump signals (c.f. Fig. S1). To see this figure in color, go online.

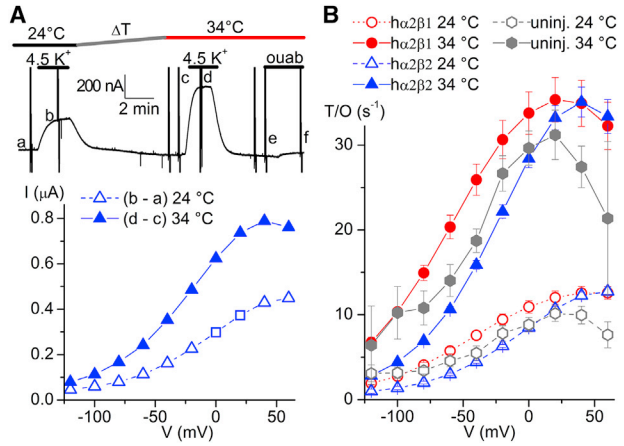

FIGURE 5 (*A*) Continuous trace at −50 mV (*top*) and I_P-V curves (*bottom*) from an oocyte expressing $\alpha2\beta2$ pumps bathed by 125 mM Na^+_o in which the T was increased from 24°C to 34°C. The small caps indicate when the current-voltage relationships shown subtracted in the I_P-V curves obtained. (*B*) Turnover rate as a function of V for $\alpha2\beta1$, $\alpha2\beta2$, and uninjected oocytes (average of four oocytes each with measurements at both T). To account for rundown, the I_P at each T was divided by the total charge at the same T. For example, in (*A*) the Q-V at 24°C was obtained by subtracting the traces in *a* minus the traces in *f*, and that at 34°C by subtracting traces in *e* minus traces in *f*. Rundown, was present in nearly all experiments. For injected oocytes, only those with currents ≥200 nA at 0 mV at both T, and without significant changes in linear capacitance (obvious in the transient currents and in Q-V curves) were included in (*B*). To see this figure in color, go online.

The striking difference in voltage dependence among the human isoforms carries profound physiological implications. We tested whether the differences in VDI that we measured in oocytes can also be observed in native rat ventricular myocytes (Fig. 6). In rodents the $\alpha1$ subunit has a relatively low affinity for ouabain (IC_{50} ~100 μM), whereas $\alpha2$ and $\alpha3$ pumps are far more sensitive ($IC_{50} < 1$ μM) (44). Thus, the function of the $\alpha1$ isoform can be readily separated from $\alpha2$ and $\alpha3$ using different ouabain concentrations (5 mM and 5 μM).

Fig. 6 *A* shows a continuous recording from a ventricular myocyte bathed by 145 mM Na^+_o held at −40 mV. Perfusion of 4.5 mM K^+_o activated outward I_P, which was inhibited by different ouabain concentrations. Application of 5 μM ouabain inhibited the current due to non-$\alpha1$ pumps and 5 mM ouabain inhibited all isoforms. Although a small rundown of the current cannot be ruled out, it is clear that only $\alpha1$ pumps recover significantly after washout, due to the faster ouabain unbinding from this isoform; the unbinding time constant at 37°C is ~4 min for $\alpha2$ pumps and ~100 min for $\alpha3$ pumps (45). The current from $\alpha1$ pumps is obtained by subtracting the current in 5 mM ouabain from that in 5 μM ouabain. The component from non-$\alpha1$ pumps is obtained by subtracting the current in 5 μM ouabain from that without ouabain. The I_P-V curves measured at the end of 100-ms step pulses (• = $\alpha1$; Δ = $\alpha2 + \alpha3$) are shown in Fig. 6, *B* and *C*. The non-$\alpha1$

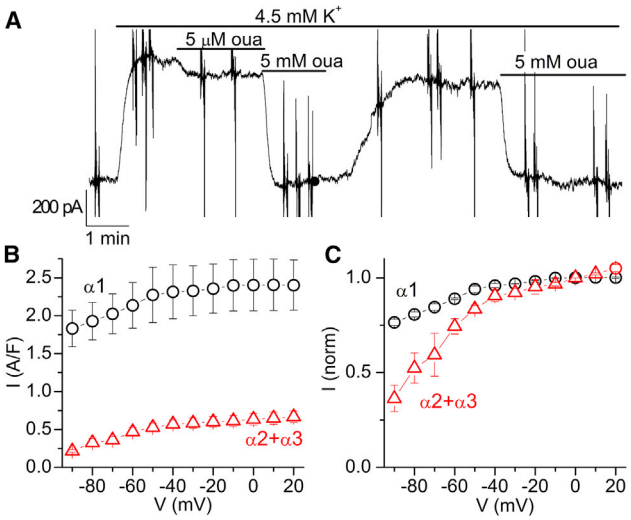

FIGURE 6 Voltage dependence of NKA isoforms in rat cardiac myocytes. (*A*) Current from a myocyte bathed by 145 mM Na^+ at −40 mV. K^+ application (4.5 mM) activates outward I_P, which was inhibited by a subsequent addition of ouabain; 5 μM inhibited non-α1 (α2 + α3) and 5 mM inhibited all isoforms. (*B*) I_P-V curve for the α1 and non-α1 pumps. I_P/cell capacitance accounts for myocyte size variation. (*C*) I_P-V curve for both components normalized to 0 mV. 85 mM Na^+_i, 5 mM MgATP, T = 35°C (n = 4, from three different animals aged 7, 8, and 16 weeks). To see this figure in color, go online.

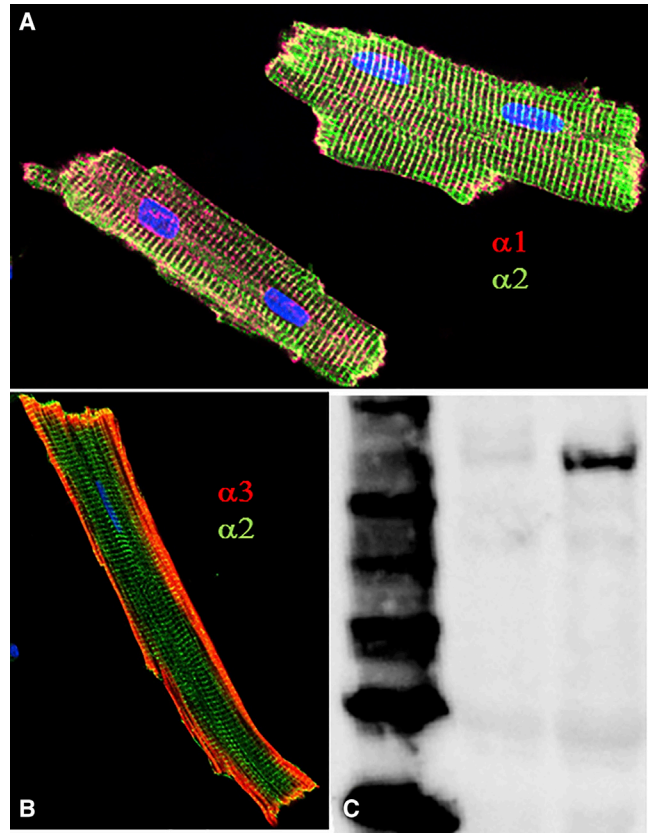

FIGURE 7 NKA catalytic subunit isoforms in ventricular myocytes. (*A*) Isolated myocytes stained against α1 (*red*) and α2 (*green*). Nuclei were stained with DAPI (*blue*). (*B*) Another representative myocyte stained against α3 (*red*) and α2 (*green*). (*C*) Western blot showing the ladder (*left lane*), boiled left ventricle membrane preparation (*middle lane*), and 65°C treated membrane preparation (*right lane*) probed with rabbit polyclonal antibody against α3 (TED).

component (α2 + α3) is ~20% of the total, consistent with previous reports at RT with similar ionic conditions (30). Fig. 6 *C* displays α1 and non-α1 components normalized to the current at 0 mV. Note that the stronger voltage dependence of the non-α1 component is consistent with a greater abundance of α2 pumps than α3 pumps in adult rat myocytes (46).

We tested whether the incomplete inhibition by voltage of the non-α1 component could be explained by a small percentage of α3 pumps, whose presence in adult rat hearts is still under debate. Coimmunostaining of ventricular myocytes with a monoclonal α1 antibody (α1-C464.6, Santa Cruz Biotech) and a rabbit-raised polyclonal α2 antibody (HERED (40)) always produced the same staining pattern (Fig. 7 *A*): α2 pumps appear mainly confined to T-tubules, whereas α1 pumps are present everywhere, consistent with previous functional (30) and immunostaining reports (14,47). We observed even α3 staining of the nontubular membrane with a monoclonal commercial α3 antibody (XVIF9-G10, Santa Cruz Biotech, Fig. 7 *B*). Immunostaining with this antibody produced identical results in all myocytes isolated from 11 adult animals (aged 3 weeks to 1 year). To confirm the presence of α3, we also performed Western blots with a rabbit polyclonal antibody (TED (40)). Some (e.g., Fig. 7 *C*), but not all, heart preparations showed a clear α3 band, consistent with previously reported animal-to-animal variation (11).

In the heart, transient modulation of NKA function by kinase-dependent phosphorylation is mediated by FXYD1 (48,49). It has been shown that dephosphorylated FXYD1 interacts with α1 and α2 pumps (16) and reduces their activity by lowering the apparent affinity for intracellular Na^+ and for extracellular K^+ (16,29,50,51). To ensure that FXYD1 was not phosphorylated by the variable and relatively high basal protein kinase A (PKA) activity in oocytes (52), we injected a PKA inhibitor peptide (PKI) 2 h before recording. PKI injection had no effect on the functional parameters of the three isozymes in the absence of FXYD1 (for clarity, only the results of the control with PKI-injected α1β1 pumps are shown in Fig. 8).

We tested whether switching from 90 mM to 150 mM Na^+ had a distinct effect on α1β1, α2β1, and α2β2 pumps interacting with FXYD1 (Fig. 8). Fig. 8 *A* shows the current induced by 4.5 mM K^+ in 150 mM Na^+, normalized to the current in 90 mM Na^+ at +40 mV (as in Fig. 2 *A*). For all three isoform combinations, FXYD1 causes a more pronounced reduction in pump current by switching to 150 mM Na^+, but this effect appears to be voltage independent. Fig. 8 *B* shows that pumps associated with FXYD1 have a reduced apparent affinity for K^+_o. Thus, Na^+_o is a

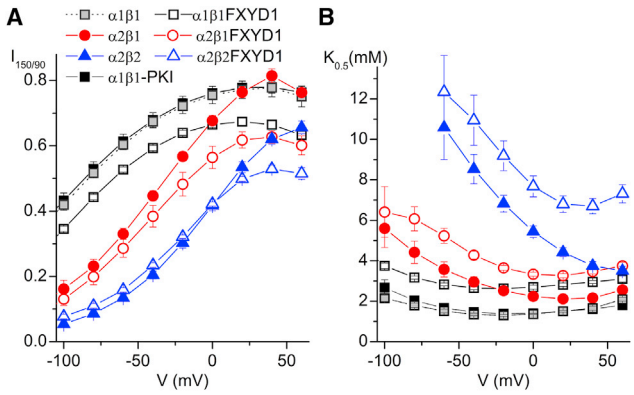

FIGURE 8 Effect of FXYD1 on $\alpha1$ and $\alpha2$ isozymes. (*A*) Voltage dependence of 4.5 mM K^+-induced I_P at 150 mM Na^+ normalized to the signal measured at +40 mV in 90 mM Na^+. (*B*) $K_{0.5}$ for K^+ activation as a function of voltage obtained in dose response experiments. Note that the $K_{0.5}$ for all pumps is increased by FXYD1. All FXYD1 experiments were performed after injection of PKI; no significant effect after PKI injection was observed for $\alpha1\beta1$ (*gray squares*), $\alpha2\beta1$ or $\alpha2\beta2$ (PKI injected $\alpha2$ pumps were excluded for clarity). To see this figure in color, go online.

more effective competitor for K^+_o and more potently reduces pump current. The fold change in K^+_o affinity induced by FXYD1 that we report here, however, is significantly larger than that in previous reports in oocytes (16,17).

DISCUSSION

Our data provide conclusive evidence that the difference in the voltage dependence between the two main cardiac muscle NKA catalytic subunit isoforms is dramatic and relevant for ion homeostasis in myocytes. Furthermore, we demonstrate that the $\beta1$ and $\beta2$ subunits are important regulators of kinetics and this allows us to hypothesize a mechanism underlying kinetic differences observed between different preparations in previous reports, including significant differences on the magnitude of the inhibitory effects of FXYD1. These findings and their implications for the beneficial action of cardiotonic steroids in heart failure will be discussed next.

Which pump isozymes and isoforms are present in the heart?

The presence of $\alpha1$, $\alpha2$, $\beta1$, and $\beta2$ is well accepted in ventricular myocytes of several mammalian species (11). The $\alpha3$ isoform has also been reported in human ventricle, and reports have also shown that it is abundant in neonatal animals, but the amount diminishes as the animals approach adulthood (46). In this report, we present data that shows that there is a very small fraction of $\alpha3$ pumps in adult rat myocytes: immunostaining with $\alpha3$ antibodies (Fig. 7 *B*), Western blots showing detection in membrane preparations from some animals, but not all (Fig. 7 *C*), and pump current versus voltage plots where a small amount of non-$\alpha1$ component resists VDI at −100 mV (Fig. 6, *B* and *C*). These observations are fully consistent with a previous report using Western blots that showed variable $\alpha3$ presence in adult rats (11).

There are many possible combinations for functional pumps because all the $\alpha x\beta x$ form functional pumps in vitro (24). Nevertheless, studies in brain (53) and heart (14) indicate that $\alpha1$ does not associate with $\beta2$. Furthermore, Madin-Darby canine kidney cells expressing the $\alpha1$, $\beta1$, and $\beta2$ show a clear preference to form the $\alpha1\beta1$ isozyme (53) indicating that $\alpha1\beta2$ is not physiologically relevant. How much of the ventricular $\alpha2$ component may be formed by $\beta2$ isoforms? Because the $K_{0.5}$ for K^+_o for $\alpha2\beta2$ pumps in oocytes at mammalian physiological concentrations (Fig. 4 *B*, ~10 mM at −20 mV) is much higher than the $K_{0.5}$ values previously reported in myocytes for non-$\alpha1$ pumps (29) we think that most $\alpha2$ pumps in ventricular myocytes must be associated with $\beta1$ with a low expression level of $\beta2$, as previously suggested (13).

Functional characteristics of different isozymes in mammalian solutions

Several studies have investigated the kinetic differences between NKA isozymes, both biochemically and electrophysiologically. When coexpressed with $\beta1$, the affinity for Na^+_i is highest in $\alpha2$, intermediate in $\alpha1$, and lowest in $\alpha3$ subunits (54,55). All α subunits presented similar apparent affinities for K^+_o when measurements were taken without Na^+_o. However, the affinities for K^+_o differed in the presence of 100 mM Na^+_o, where the lower affinity for K^+_o corresponds to the pump with the highest Na^+ affinity ($\alpha2$) and the highest apparent affinity for K^+_o, to the catalytic subunit with lowest Na^+ affinity ($\alpha3$) (25). In other words, because external Na^+ competes with K^+ for the ion-binding sites, the pump's apparent affinity for K^+_o is highly dependent on the concentration of Na^+.

Our experiments highlight the importance of the actual Na^+_o concentration when trying to understand the physiological role of specific pumps. Indeed, we measured significantly (~2- to 3-fold) lower apparent affinities for K^+ at mammalian Na^+ concentrations than those previously reported for mammalian pump isoforms expressed in *Xenopus* oocytes (24,25,37,56,57). For instance, Larsen et al. (57) reported that the $K_{0.5}$ for K^+ of $\alpha2\beta2$, at −100 mV with 70 mM Na^+, was ~7 mM, although we estimate that it is on the order of 15 mM with 150 mM Na^+ (based on extrapolation). We think that due to the lower Na^+ concentration used in previous reports the physiologically significant lower affinity for $\alpha2\beta1$ pumps at negative voltages could not be observed. It is also possible that the use of ouabain-resistant pumps with altered function masked this observation.

The turnover rates we calculated are comparable to those previously measured in oocytes at RT. For example, the turnover rate of human $\alpha2\beta1$ pumps at −30 mV, 90 mM Na^+, and 10 mM K^+ reported by Tavraz et al. (37) was 13 s^{-1} nearly identical to the values we observe: 14 s^{-1} at −20 mV and 11 s^{-1} at −40 mV. Crambert et al. (24) reported the maximum turnover rate for all nine wild-type human isoforms at −50 mV and 90 mM Na^+ to be ~30 s^{-1} for $\alpha1\beta1$ and $\alpha1\beta2$; ~22 s^{-1} for $\alpha2\beta1$ and $\alpha2\beta2$; and ~10 s^{-1} for $\alpha3\beta1$ and $\alpha3\beta2$. At −40 mV and 150 mM Na^+ we report 19.5 ± 2.5 s^{-1} for $\alpha1\beta1$, 7.2 ± 0.4 s^{-1} for $\alpha1\beta2$, 11.2 ± 1.2 s^{-1} for $\alpha2\beta1$, 4.1 ± 0.4 s^{-1} for $\alpha2\beta2$, 18.5 ± 1.3 s^{-1} for $\alpha3\beta1$, and 12.8 ± 1.1 s^{-1} for $\alpha3\beta2$. The slightly lower turnover rates we report here for $\alpha1$ and $\alpha2$ pumps could be accounted for by the difference in ionic composition as Crambert et al. used 80 mM Na^+ with nearly saturating 10 mM K^+.

Voltage dependence of cardiac NKA isozymes at physiological Na^+ concentrations: the role of $\alpha2$ pumps and the $\beta2$ subunit

All pumps are subject to inhibition at negative potentials. This VDI depends on the concentration of external Na^+. The pump-current versus voltage curves shift to the right as the Na^+ concentration (20), or the Na^+ affinity of the sites in the pump is increased, but it shifts to the left as the concentration of K^+ (as a competitor for Na^+) is increased. Thus, the physiologic relevance of this process must be assessed at normal Na^+_o and K^+_o concentrations. Our results (Fig. 2) show that at physiological Na^+_o and K^+_o, $\alpha2$ pumps, when associated with $\beta1$, are ~75% inhibited at membrane potentials corresponding to the resting potentials in ventricular myocytes (~−75 mV (58)). When they associate with the $\beta2$ subunit, they are ~95% inhibited (percentages obtained after subtraction of endogenous currents, c.f. Fig. S1). We confirmed the stronger voltage dependence of $\alpha2$-pumps (~70% of non-$\alpha1$ pumps) in rat myocytes (Fig. 5). Our results strongly suggest heart $\alpha2$ pumps are not meant to pump continuously. This is in contrast to the $\alpha1$ pumps that perform a housekeeping role. Based on our data, we propose that $\alpha2$ pumps are inhibited at the resting membrane potential and do not significantly contribute to Na^+ pumping (nor to Ca^{2+} handling). During the cardiac action potential they are highly active when there is a need for increased Na^+ extrusion due to the large Na^+ influx through Na^+ channels and to the inversion of the Na/Ca exchanger (NCX) function due to the depolarized membrane potential.

It is therefore expected that this safety net function of the $\alpha2$ NKA also occurs in other tissues where it is highly expressed. For instance, $\alpha2$ pumps constitute >70% of the pumps in skeletal muscle, being principally targeted to the T-tubules (59) where it must associate with $\beta1$ and $\beta2$ subunits (60). In this muscle type the resting potentials can be as low as −90 mV, meaning $\alpha2$ pumps will be completely inactive, consistent with the minimal effect of NKA $\alpha2$ inhibition in the skeletal muscle resting potential (61). Although the skeletal muscle action potentials are very brief, a train of activity produces accumulation of K^+ in the T-tubule leading to a transient depolarization. This combination of elevated K^+_o and depolarization would activate $\alpha2$ pumps to clear K^+_o from the tubule. Consistent with this idea, a recent report by Di Franco et al. (62) showed a reduced apparent affinity for K^+ for $\alpha2$, compared to $\alpha1$ pumps, in mouse skeletal muscle fibers.

Previous reports have indicated that β subunits have relatively minor consequences on NKA kinetics (24,63,64). Our results contradict this idea. We show that compared to $\beta1$ isozymes, $\beta2$ isozymes induce drastic changes in pump function, significantly shifting the I_P-voltage curve toward depolarized potentials due to an increased affinity for Na^+_o. Within the physiologic voltage range, the effect is most pronounced when associated with $\alpha2$, as $\alpha2\beta2$ pumps are >80% inhibited below −60 mV due to the very low affinity for K^+ at this voltage (Fig. 4 *A*). This suggests that $\beta2$ association with $\alpha2$ is important to ensure that these pumps will activate only when there is a need to clear elevated external K^+. It is easy to envision how a mix of $\alpha2\beta1$ and $\alpha2\beta2$ can contribute when there is a need to clear different amounts of K^+_o accumulation. Larsen et al. (57) suggested a similar mechanism in glia, where the depolarization induced by the increase in K^+_o produces activation of the $\alpha2\beta2$ pumps when they are needed to clear K^+ from the extracellular space after K^+_o concentration increases following a train of action potentials or injury. Our results reinforce their proposal and show that differential interaction of $\alpha2$ with $\beta1$ and $\beta2$ may provide two safety net pumps with different K^+_o affinities.

NKA $\alpha2$ pumps, NCX, and the action of digitalis

Cardiotonic steroids (aka digitalis), such as ouabain and digoxin, are specific inhibitors of the NKA that have been used to treat heart failure since the 18th century (65). Cardiotonic steroids' therapeutic action occurs through NKA inhibition as the decrease in Na^+ electrochemical gradient reduces Ca^{2+} extrusion via NCX (66). This in turn leads to a higher Ca^{2+} load within the sarcoplasmic reticulum, thus allowing a larger Ca^{2+} release during the systole (67). Recent reports indicate that human $\alpha2$ pumps have a slightly higher affinity for the clinically preferred cardiotonic steroids than $\alpha1$ pumps (68). It is widely believed that the actions of cardiotonic steroids on cardiac contraction are mediated through the $\alpha2$ subunit, which occurs at a higher density in the T-tubules ((31) and Fig. 6 *A*). Although both $\alpha1$ and $\alpha2$ interact with NCX (69,70), $\alpha2$ and NCX interact within membrane micro-domains (71,72), and it was also shown that $\alpha2$ preferentially modulates Ca^{2+} transients (73,74).

The results described previously have led to the idea that by targeting the differentially expressed $\alpha2$ subunit, cardiotonic steroids could increase Ca^{2+}_i and contraction

force by binding preferentially to only ~25% of the total pumps, which account for up to 50% of the pumps in the T-tubules (31). Another reason why binding to $\alpha 2$ mediates the beneficial effects of cardiotonic steroids comes from their strong VDI; as $\alpha 2$ pumps are inactive at resting potentials, their inhibition would not affect the Ca^{2+} extrusion capacity at rest, but it would affect $[Ca^{2+}]$ handling during systole. The change in driving force due to inhibition of 50% of the tubular pumps may favor backward Ca^{2+} transport by NCX, also increasing systolic Ca^{2+}.

Hypokalemia, a pathophysiologic condition that increases the risk of ventricular arrhythmias, is another condition where the lower K^+-affinity and stronger VDI of $\alpha 2$ pumps appears to play an important role. It has been recently shown that hypokalemia hyperpolarizes rat ventricular myocytes and reduces the non-$\alpha 1$ pumping component, leading to larger Ca^{2+} transients with negligible changes in basal Ca^{2+} (cf. Figs. 1 and 2 in (74)) as we propose therapeutic concentrations of digitalis do.

The inhibitory effect of FXYD1

It has been shown that FXYD1 interacts with the $\alpha 1$ and $\alpha 2$ isoforms, but not with the $\alpha 3$ isoform (17), leading to partial inhibition of NKA. Förster resonance energy transfer experiments suggest that the relief of this inhibition through phosphorylation of FXYD1 by PKA and protein kinase C involves a separation of FXYD1 and α subunit at the intracellular ends of their transmembrane segments (51). The inhibition by FXYD1 at saturating Na^+_i reflects, at least in part, a reduction of the NKA's affinity for K^+_o. According to previous reports, FXYD1 induced a smaller increase in the $K_{0.5}$ for K^+_o in oocytes at 90 mM Na^+_o (~40%, 17) than in transgenic mice at 120 mM Na^+_o (~100%, 29). This difference brought into question the physiologic relevance of oocyte experiments.

Our results demonstrate that human FXYD1 induces physiologically relevant changes in apparent affinity for K^+_o of human $\alpha\beta$ isozymes, and again, quantitatively reconcile data obtained in different systems. In better agreement with the mice myocytes experiments, at -20 mV and 150 mM Na^+_o, the $K_{0.5}$ without and with FXYD1 varied, respectively, from 1.3 mM to 2.9 mM for $\alpha 1\beta 1$ and from 2.5 to 3.7 for $\alpha 2\beta 1$. Both the injection of PKI to inhibit all basal PKA activity and the use of mammalian $[Na^+]_o$ may have contributed to the observed differences. Finally, the finding that dephosphorylated FXYD1 does not affect the voltage dependence (neither the position of the I_P-V curve nor that of the $K_{0.5}$-V curve shift with respect to voltage) indicates that FXYD1 inhibition affects a completely different pathway. The larger inhibitory effect of 150 mM Na^+_o at 4.5 mM K^+_o may simply reflect the increased binding of Na^+_o secondary to the reduction in K^+_o affinity. A small increase in Na^+ binding affinity, however, cannot be ruled out by our experiments.

CONCLUSION

This work demonstrates a previously unnoted relevance for the NKA isozyme-specific voltage dependence. In particular, their voltage dependence allows $\alpha 2$ pumps to act as a safety net which, in the heart, will only increase Na^+ extrusion upon depolarization during an action potential. In this context, inhibition of $\alpha 2$ pumps with cardiotonic steroids should only increase intracellular Na^+ levels (and thus intracellular $[Ca^{2+}]$) during depolarization (controlled by $\alpha 2$ and $\alpha 1$ pumps), but not at rest (dependent on $\alpha 1$ pumps). On the other hand, although voltage dependence is apparently irrelevant for FXYD1-mediated regulation, future studies will test the possibility of voltage dependence being an important regulatory step for NKA function, as a small shift in the current voltage curve, particularly for $\alpha 2$ pumps, could have highly relevant consequences for pumping capacity.

SUPPORTING MATERIAL

Three figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(15)00952-2.

AUTHOR CONTRIBUTIONS

C.M.S., D.G.G., D.J.M., and P.A. performed and analyzed experiments. A.B. performed experiments, J.J.R. cloned human subunits and helped write the article, and P.A. and D.G.G. designed research and wrote the article.

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Supplemental Information

Importance of the Voltage Dependence of Cardiac Na/K ATPase Isozymes

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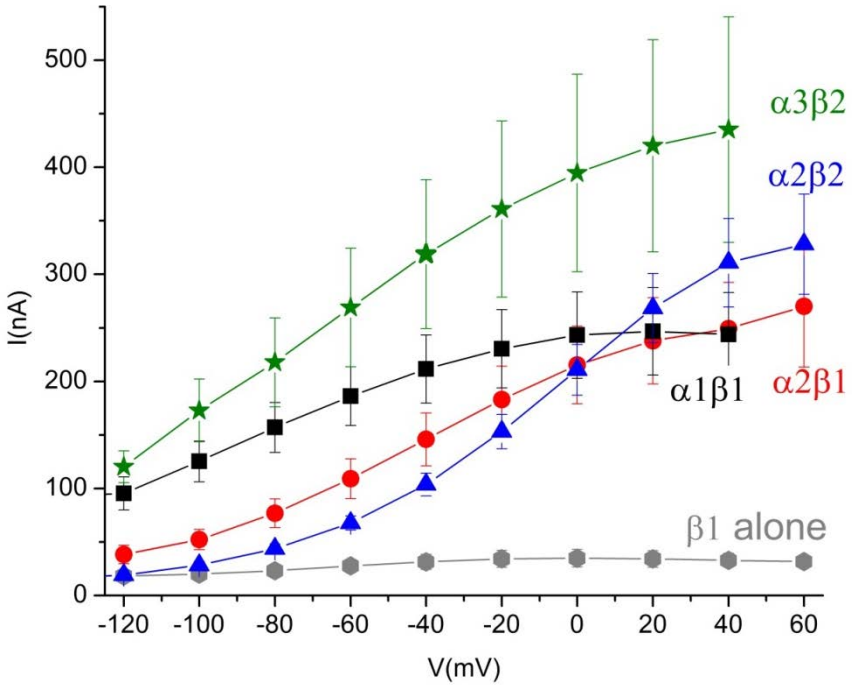
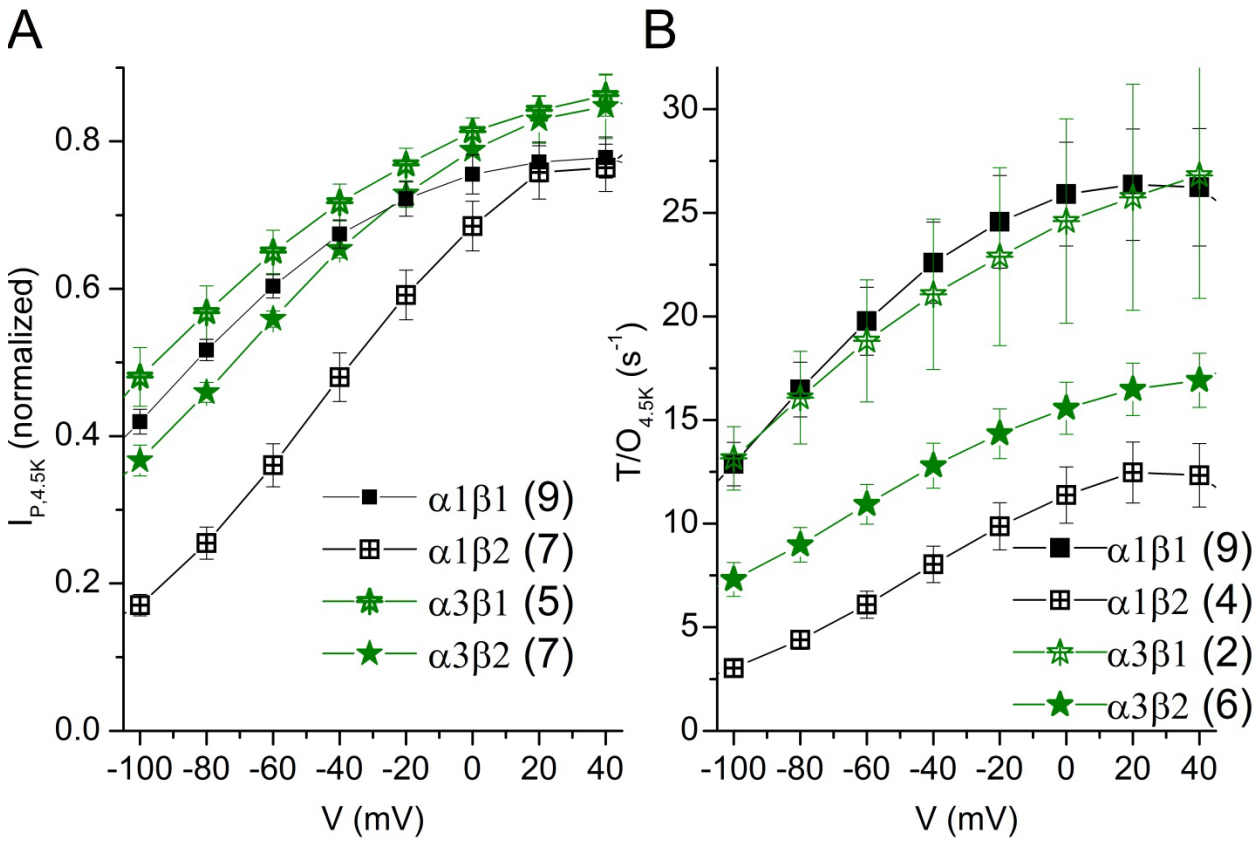
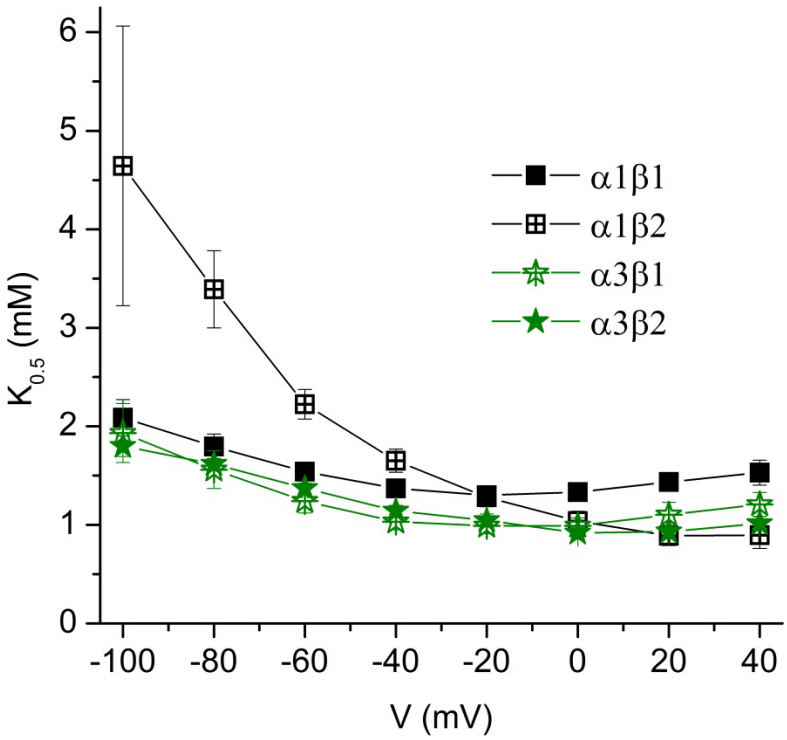
Supplementary Information



Supplementary Figure 1. Mean current induced by 4.5 K^+ in all oocytes shown in Fig. 1 injected with the human cardiac isozymes or with human β1 alone. Uninjected oocytes produced identical signals to β1 injected oocytes (not shown).



Supplementary Figure 2. Voltage dependencies of the two isozymes not shown in Fig. 2 at 4.5 mM K^+, together with α1β1 and α3β2 for comparison **A)** Voltage dependence of 4.5 mM K^+ induced I_P normalized to the value at +40 mV in 90 mM Na^+ to illustrate the effect of 150 mm Na^+, for α1β1 (black solid squares), α1β2 (black crossed squares) α3β1 (green crossed stars) and α3β2 (green solid stars). **B)** T/O rate for the same isozymes. The number of experiments are given in parenthesis.



Supplementary figure 3. Voltage dependence of apparent affinity for K^+ of the isozymes not shown in Fig. 4A. **A)** $K_{0.5}$ as a function of voltage at 150 mM Na^+. α1β1 (black solid squares); α1β2 (black crossed squares), α3β1 (crossed green stars), α3β2 (solid green stars).