Overexpression of the Na^+/K^+ ATPase α2 But Not α1 Isoform Attenuates Pathological Cardiac Hypertrophy and Remodeling

Robert N. Correll,* Petra Eder,* Adam R. Burr, Sanda Despa, Jennifer Davis, Donald M. Bers, Jeffery D. Molkentin

Rationale: **The Na^+/K^+ ATPase (NKA) directly regulates intracellular Na^+ levels, which in turn indirectly regulates Ca^{2+} levels by proximally controlling flux through the Na^+/Ca^{2+} exchanger (NCX1). Elevated Na^+ levels have been reported during heart failure, which permits some degree of reverse-mode Ca^{2+} entry through NCX1, as well as less efficient Ca^{2+} clearance.**

Objective: **To determine whether maintaining lower intracellular Na^+ levels by NKA overexpression in the heart would enhance forward-mode Ca^{2+} clearance and prevent reverse-mode Ca^{2+} entry through NCX1 to protect the heart.**

Methods and Results: **Cardiac-specific transgenic mice overexpressing either NKA-α1 or NKA-α2 were generated and subjected to pressure overload hypertrophic stimulation. We found that although increased expression of NKA-α1 had no protective effect, overexpression of NKA-α2 significantly decreased cardiac hypertrophy after pressure overload in mice at 2, 10, and 16 weeks of stimulation. Remarkably, total NKA protein expression and activity were not altered in either of these 2 transgenic models because increased expression of one isoform led to a concomitant decrease in the other endogenous isoform. NKA-α2 overexpression but not NKA-α1 led to significantly faster removal of bulk Ca^{2+} from the cytosol in a manner requiring NCX1 activity. Mechanistically, overexpressed NKA-α2 showed greater affinity for Na^+ compared with NKA-α1, leading to more efficient clearance of this ion. Furthermore, overexpression of NKA-α2 but not NKA-α1 was coupled to a decrease in phospholemman expression and phosphorylation, which would favor greater NKA activity, NCX1 activity, and Ca^{2+} removal.**

Conclusions: **Our results suggest that the protective effect produced by increased expression of NKA-α2 on the heart after pressure overload is due to more efficient Ca^{2+} clearance because this isoform of NKA preferentially enhances NCX1 activity compared with NKA-α1. (*Circ Res*. 2014;114:249-256.)**

Key Words: calcium signaling ■ hypertrophy ■ myocardial contraction ■ myocytes, cardiac ■ sodium

Years of study have made it clear that Na^+ entry and exit pathways play an important role in the pathogenesis of heart disease because these systems are responsible not only for initiating the cardiac action potential (via voltage-gated Na^+ channels), but also for closely regulating the influx and efflux of Ca^{2+} through the Na^+/Ca^{2+} exchanger (NCX1).[1] NCX1 is an electrogenic exchanger that removes 1 Ca^{2+} ion in exchange for internalizing 3 Na^+ ions under normal conditions. However, the direction and rate of NCX1-mediated countertransport are determined by membrane potential and relative concentrations of Na^+ and Ca^{2+} inside and outside the myocyte. Multiple studies have indicated that intracellular Na^+ concentration ($[Na^+]_i$) is increased in numerous animal models of hypertrophy,[2] as well as in human heart failure.[3] This increased $[Na^+]_i$ during cardiac disease is likely an adaptive mechanism to reduce Ca^{2+} extrusion via NCX1 to augment contractility and cardiac function. However, this increase in cytosolic Ca^{2+} also increases the propensity for arrhythmias and may activate Ca^{2+}-dependent signaling pathways involved in the hypertrophic program and apoptosis.[4]

Numerous studies have characterized mechanisms by which $[Na^+]_i$ becomes elevated during cardiac disease, and they involve both entry and efflux pathways. Increased Na^+ influx via both tetrodotoxin-sensitive Na^+ channels and

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Nonstandard Abbreviations and Acronyms	
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
NCX	Na^+/Ca^{2+} exchanger
NFAT	nuclear factor of activated T cells
NKA	Na^+/K^+ ATPase
PLM	phospholemman
PLN	phospholamban
SERCA2	sarco(endo)plasmic reticulum Ca^{2+} ATPase2
TAC	transverse aortic constriction
Wt	wild type

Na^+/H^+ exchanger has been demonstrated in a rabbit model of heart failure[5,6] and in human heart failure.[7–9] Overexpression of Na^+/H^+ exchanger in the murine heart resulted in increased $[Na^+]_i$, increased $[Ca^{2+}]_i$ (likely because of decreased Ca^{2+} extrusion by NCX1), heart failure and premature death accompanied by arrhythmia, increased nuclear factor of activated T-cell (NFAT) translocation, and elevated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activity, resulting in exclusion of histone deacetylase 4 from the nucleus.[10] In addition, the late component of voltage-gated Na^+ channel activity (I_{NaL}) is enhanced in some models of heart failure,[11] whereas inhibition of I_{NaL} in CaMKII transgenic animals can improve diastolic function and eliminate premature arrhythmogenic contractions in papillary muscle preparations.[12]

The Na^+/K^+ ATPase (NKA) is the primary Na^+ extrusion pathway in cardiac myocytes, consuming ATP to pump 3 Na^+ ions out in exchange for 2 K^+ ions, which determines the driving force for Na^+ entry into the myocyte. NKA is a heterodimer composed of an α subunit (α1 and α2 isoforms exist in the rodent heart) and β subunit (β1 is the only isoform in the heart) and is distributed both in the surface sarcolemma and in T-tubules. NKA is functionally coupled to NCX1 in cardiac myocytes[13] where even small changes in NKA activity may alter the local $[Na^+]_i$ environment to modulate Ca^{2+} extrusion through NCX1.[14–16] Accordingly, cardiac glycosides have been used for centuries to inhibit NKA activity, which during heart failure can blunt or reverse Ca^{2+} exit via NCX1 to enhance contractility.[17] NKA activity is either downregulated[18] or unchanged[5] during heart failure and can be further modulated by phosphorylation of the endogenous regulator protein phospholemman (PLM), which if hyperphosphorylated as shown in a rabbit model of heart failure enhances NKA activity.[19] Alternatively, reduced phosphorylation of PLM as shown in human heart failure[20] would lead to increased NKA inhibition.

The respective roles of the α1 and α2 subunits of NKA in the heart remain controversial. Early work suggested that α2 was directly coupled with NCX1 in T-tubules, whereas α1 regulated bulk Na^+ levels in the cytosol.[21] However, more recent work suggested that both isoforms are directly coupled to NCX1.[13] Nevertheless, it seems that although the α1 subunit shows localization to both the surface sarcolemma and T-tubules, the α2 isoform is enriched as much as 5× more in T-tubules, suggesting that α2 may play a preferential role in the generation of signaling domains within T-tubules that modulate NCX1 function.[22] Supporting this notion is a recent work demonstrating that partial inhibition of NKA-α2 but not NKA-α1 can increase Ca^{2+} transients, pointing to NKA-α2 as the isoform responsible for regulating NCX1 to control intracellular Ca^{2+} in the junctional space and subsequently affecting Ca^{2+} release from the sarcoplasmic reticulum (SR).[23]

We generated transgenic mice with cardiac-specific overexpression of the α1 or α2 subunit of NKA to determine whether altered expression of an Na^+ efflux protein could modulate the disease phenotype elicited by pressure overload in the heart. As suggested by previous reports,[21,24] we demonstrate that a true overexpression of either α isoform is impossible because increased expression of one isoform results in compensatory downregulation of the other endogenous α isoform. However, increased expression of the α2 isoform, which effectively results in replacement of α1 with α2, attenuated the hypertrophic response after transverse aortic constriction (TAC) and was associated with faster Ca^{2+} extrusion via NCX1. In addition, the protective effect of α2 overexpression is not mimicked by α1 overexpression, positioning NKA-α2 as the more important isoform affecting Ca^{2+} removal by NCX1 during disease.

Methods

cDNAs encoding rat NKA-α1 and ouabain-resistant rat NKA-α2 (gift from Dr Jerry Lingrel, University of Cincinnati) were cloned into the murine α-myosin heavy chain promoter expression vector and used to inject newly fertilized oocytes to generate transgenic mice (FVB/N background). NFAT-luciferase transgenic mice were previously described.[25] For cardiac pressure overload induction, mice aged 8 to 11 weeks were subjected to TAC or a sham surgical procedure, as previously described.[25] Mouse ventricular cardiomyocytes were isolated as previously described.[26] For Na^+ measurements, isolated myocytes were plated on laminin-coated coverslips and loaded with 10 μmol/L 1,3-benzenedicarboxylic acid, 4,4'-[1,4,10-trioxa-7,13-diazacyclopentadecane-7,13-diylbis(5-methoxy-6,12-benzofurandiyl)]bis-, tetrakis[(acetyloxy)methyl] ester (SBFI-AM) for 90 to 120 minutes (Invitrogen), as previously described.[27] NKA activity was determined using an enzyme-linked assay measuring the rate of ADP production as linked to the rate of NADH fluorescence decrease in the absence or presence of 10 mmol/L strophanthidin (Sigma Aldrich), as previously described.[28] Results are presented in all cases as mean±SEM. Values of $P<0.05$ were considered significant. See Online Data Supplement for expanded Materials and Methods.

Results

Overexpression of NKA α2 But Not α1 Reduces Hypertrophy After TAC

To determine the relative contribution of the α1 versus the α2 NKA isoform in the regulation of cytosolic Na^+ levels and cardiac hypertrophy during disease, we created transgenic mice overexpressing each isoform using the α-myosin heavy chain promoter (Figures 1A and 2A). We obtained 1 line of NKA-α2 transgenic mice that showed ≈3-fold overexpression of protein in the heart relative to wild-type (Wt) levels (Figure 1B). Echocardiographic, gravimetric, and histological analyses showed no baseline phenotype in NKA-α2 transgenic mice with aging nor was survival affected (data not shown; Online Figure I). We next performed TAC surgery to induce pressure overload hypertrophy. After 2 weeks of TAC stimulation, hearts from the NKA-α2 transgenic animals showed a significant reduction in ventricular weight normalized to body weight compared with Wt controls subjected to TAC (Figure 1C; Online Figure II), which became even more

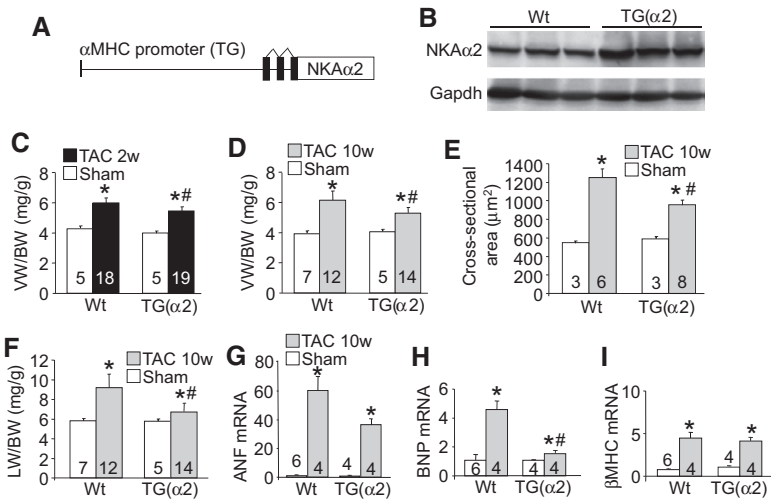

Figure 1. Na⁺/K⁺ ATPase (NKA)-α2 transgenic (TG) mice show less cardiac hypertrophy after pressure overload. A, Schematic representation of the transgene used to drive NKA-α2 expression in the mouse heart. **B**, Immunoblot for NKA-α2 and GAPDH protein from wild-type (Wt) and NKA-α2 transgenic heart homogenates. **C**, Ventricular weight (VW) to body weight (BW) ratios measured from Wt and NKA-α2 transgenic hearts after 2 weeks of transverse aortic constriction (TAC) or sham surgery. **D**, Ventricular weight to body weight ratios measured from Wt and NKA-α2 transgenic hearts after 10 weeks of TAC or sham surgery. **E**, Histological analysis of myocyte cross-sectional area using wheat germ agglutinin-Tetramethylrhodamine-5-(and-6)-Isothiocyanate (5(6) stain of heart histological sections from Wt and NKA-α2 transgenic mice after 10 weeks of TAC or sham surgery. **F**, Lung weight (LW) to body weight ratios measured from Wt and NKA-α2 transgenic mice after 10 weeks of TAC or sham surgery. **G–I**, mRNA levels of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC) measured via quantitative polymerase chain reaction from Wt and NKA-α2 transgenic hearts after 10 weeks of TAC or sham surgery. Wt controls were analyzed after 10 to 12 weeks of sham surgery. For each experiment, number of mice analyzed is given within the graph. **P*<0.05 vs sham; #*P*<0.05 vs Wt TAC.

apparent 10 weeks after TAC stimulation (Figure 1D). This reduction in cardiac hypertrophy in the transgenic mice after TAC correlated with reduced myocyte cross-sectional area (Figure 1E), reduced lung weight normalized to body weight (Figure 1F), a trend toward reduced mRNA expression of the hypertrophic marker atrial natriuretic factor, and a significant reduction in mRNA expression of brain natriuretic peptide (Figure 1G–1I).

We also obtained 2 lines of NKA-α1–overexpressing transgenic mice (Figure 2B). NKA-α1 transgenic mice failed to show any reduction in cardiac hypertrophy after 2 weeks of TAC stimulation compared with Wt mice (Figure 2C and 2D; Online Figure II). To extend these results, we performed 12 weeks of TAC stimulation on the slightly higher-expressing NKA-α1 line (line 21.4), which also failed to show any reduction in cardiac hypertrophic remodeling as measured by ventricular weight normalized to body weight (Figure 2E), myocyte cross-sectional area (Figure 2F), and expression of the hypertrophic markers atrial natriuretic factor, brain natriuretic peptide, and β myosin heavy chain (Figure 2G–2I). We also generated yet another separate cohort of Wt, NKA-α1, and NKA-α2 transgenic mice that were subjected to TAC surgery at the same time (exact same ages and sex) and followed for 2 weeks, which again showed attenuation of cardiac hypertrophy only in the NKA-α2 transgenic mice (Online Figure II). Direct comparison of NKA-α1 and NKA-α2 with Wt controls even after 16 weeks of TAC further confirmed these findings and demonstrated again that only overexpression of NKA-α2 could limit hypertrophy and remodeling as measured by echocardiography and gravimetry (Figure 3A and 3B). NKA-α2 transgenic mice were also protected from loss of cardiac ventricular performance compared with NKA-α1 and Wt hearts (Figure 3B). We also analyzed the degree of intracellular fibrosis during 16 weeks of TAC stimulation, but the α1 and α2 transgenic lines showed the same relative response as observed in Wt controls for this measure (Figure 3C). Thus, at multiple time points of TAC stimulation, NKA-α2 transgenic mice were significantly protected from the full extent of the hypertrophic response and loss of ventricular function compared with Wt or NKA-α1 transgenic mice.

Total NKA Protein Levels Are Conserved During Subtype Overexpression

Previous work has suggested that a change in the expression of a particular NKA isoform is balanced by altered expression of the other such that a constant level of NKA protein is maintained.[21,24] In agreement with these previous observations, our Western blot experiments from whole-cell extracts of NKA-α1, NKA-α2, and Wt hearts demonstrated that overexpression of one NKA isoform elicited downregulation of the other isoform in a manner that preserved total levels of NKA (Figure 4A). Consistent with these observations, measurement of total ATPase activity in cardiac lysates from NKA-α2 transgenic and Wt hearts did not differ (Figure 4B). Although other investigators have suggested a somewhat different subcellular localization of NKA-α2 versus NKA-α1,[22,29] it has been previously reported that both isoforms are expressed at the surface sarcolemma and T-tubules. Our findings agree with this because we were unable to see a difference in NKA localization when comparing myocytes from each transgenic line by confocal microscopy (Figure 4C). We also failed to observe any differences in localization of endogenous NKA-α1 versus NKA-α2 from isolated adult rat myocytes stained with the appropriate antibodies because both protein isoforms populated the sarcolemma and T-tubule at what seemed to be the same relative ratios (data not shown).

NKA Overexpression Does Not Alter Na⁺ Content But Modulates Ca^{2+} Efflux via an NCX1-Dependent Mechanism

To determine the mechanism whereby NKA-α2 overexpression reduced cardiac hypertrophy and negative remodeling after TAC, we examined whether increased expression of either NKA isoform could alter Ca^{2+} handling. NKA is known to colocalize with NCX1, and several studies (and our unpublished work) have demonstrated coimmunoprecipitation,[13] suggesting that NKA and NCX1 can coassociate as part of a complex to

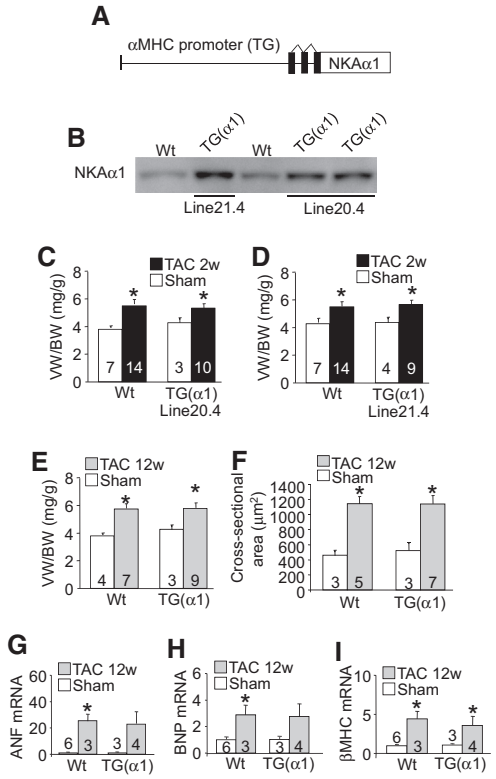

Figure 2. Na⁺/K⁺ ATPase (NKA)-α1 transgenic (TG) mice show the same cardiac hypertrophic response after pressure overload as wild-type (Wt) mice. A, Schematic representation of the transgene used to drive NKA-α1 expression in the mouse heart. **B**, Immunoblot for NKA-α1 protein from the hearts of Wt animals and 2 transgenic lines. Line 21.4 (high line) expresses more NKA-α1 protein than line 20.4 (low line). **C** and **D**, Ventricular weight (VW) to body weight (BW) ratios measured from Wt and line 20.4 or line 21.4 NKA-α1 transgenic mice after 2 weeks of transverse aortic constriction (TAC) or sham surgery. **E**, Ventricle weight to body weight ratios measured from Wt and high-line NKA-α1 transgenic mice after 12 weeks of TAC or sham surgery. **F**, Histological analysis of myocyte cross-sectional area using wheat germ agglutinin-tetramethylrhodamine-5-(and-6)-Isothiocyanate (5(6) (TRITC) stain of heart histological sections from Wt and high-line NKA-α1 transgenic mice after 12 weeks of TAC or sham surgery. **G–I**, mRNA levels of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and β-myosin heavy chain (β–MHC) measured via quantitative polymerase chain reaction from Wt and high-line NKA-α1 transgenic hearts after 12 weeks of TAC or sham surgery. Wt controls were analyzed after 10 to 12 weeks of sham surgery. For each experiment, number of mice analyzed is given within the graph. **P*<0.05 vs sham; #*P*<0.05 vs Wt TAC.

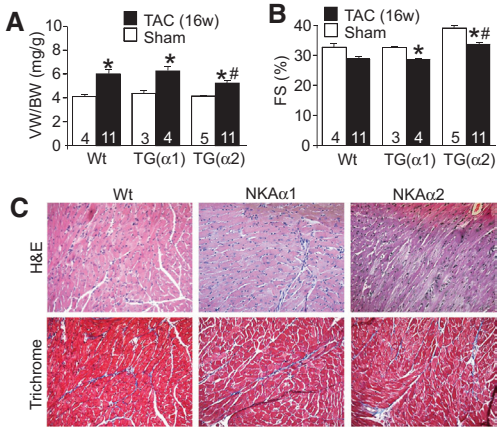

Figure 3. Na⁺/K⁺ ATPase (NKA)-α2 transgenic (TG) mice are protected from hypertrophy after 16 weeks of pressure overload. Ventricular weight (VW) to body weight (BW) ratio (**A**) and echocardiographic assessment of fractional shortening (FS%; **B**) in wild-type (Wt), high-line NKA-α1 transgenic, or NKA-α2 transgenic mice after 16 weeks of transverse aortic constriction (TAC) surgery or from 24-week-old baseline controls. **C**, Representative hematoxylin and eosin (H&E) and Masson trichrome–stained histological sections from hearts of Wt, high-line NKA-α1 transgenic, or NKA-α2 transgenic mice after 16 weeks of TAC surgery. Cardiac histology from corresponding baseline control animals is shown in Online Figure I.

regulate Na^+/Ca^{2+} countertransport. Interestingly, we found that increased expression of either NKA-α1 or NKA-α2 reduced the amplitude of the Ca^{2+} transient in myocytes isolated from these animals (Figure 5A), as well as total SR Ca^{2+} load (Figure 5B). Despite this, we found no change in baseline cytosolic Na^+ levels in myocytes isolated from either NKA-α1 or NKA-α2 transgenic mice either at rest or after a 2-Hz pacing protocol (Figure 5C and 5D), which is consistent with our observations that increased expression of one NKA subtype resulted in reduction of the other isoform to maintain a constant level of total NKA protein and activity. However, we are uncertain why overexpression of either isoform causes a decrease in the amplitude of the Ca^{2+} transient or reduced SR Ca^{2+} load, although total NKA activity is the same (see Discussion section).

To examine the mechanism whereby NKA-α2 might be antihypertrophic, we more directly measured the effect of increased NKA-α2 on NCX1-mediated Ca^{2+} extrusion in isolated adult myocytes. We observed that the rate of $[Ca^{2+}]_i$ decline after caffeine-mediated depletion of SR stores, which is the result of both NCX1 and sarco(endo)plasmic reticulum Ca^{2+} ATPase2 (SERCA2) activity, was significantly faster in adult myocytes from NKA-α2 transgenic animals compared with NKA-α1 and Wt control myocytes (no Ni^{2+}), whereas inhibition of NCX1 by pretreatment with 10 mmol/L Ni^{2+} resulted in a slower $[Ca^{2+}]_i$ decay that was almost entirely reflective of SERCA2 activity and was not significantly different among the 3 groups (Figure 5E). These results suggest that NCX1

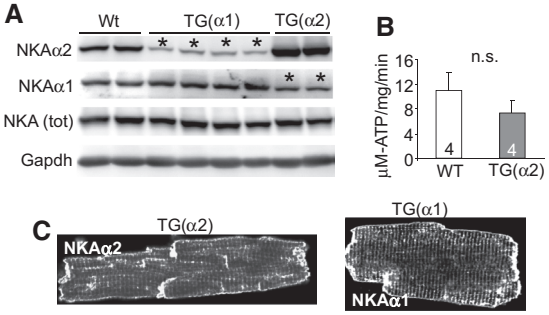

Figure 4. Compensation between Na⁺/K⁺ ATPase (NKA)-α1 and NKA-α2 in the hearts of transgenic (TG) mice. A, Immunoblots for NKA-α1, NKA-α2, total NKA, and GAPDH protein from wild-type (Wt) and transgenic heart homogenates. The asterisks show the lanes with reduced NKA protein expression because of compensation. **B**, NKA activity measured as strophanthidin-sensitive ATP turnover rate in crude homogenates from Wt and NKA-α2 transgenic hearts. **C**, Subcellular localization of NKA-α1 and NKA-α2 protein in NKA-α1– or NKA-α2–isolated transgenic myocytes, respectively, via immunofluorescence and confocal microscopy.

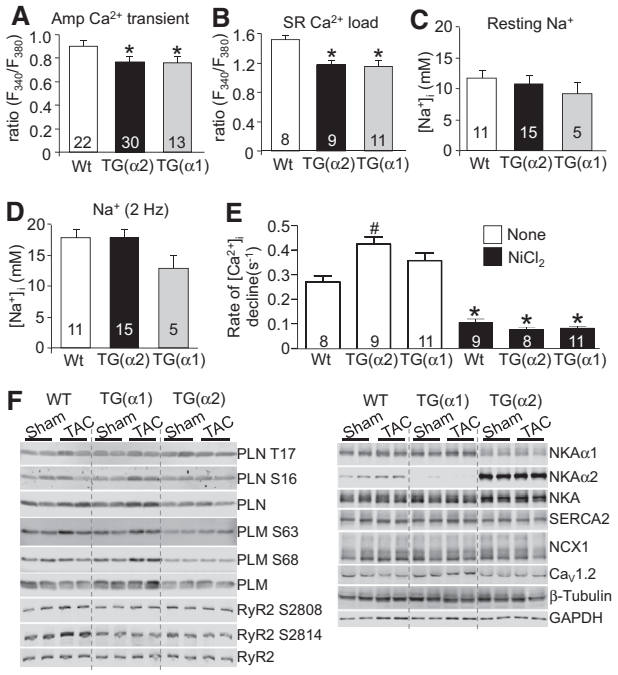

Figure 5. Analysis of intracellular Ca^{2+} and Na^+ handling in adult myocytes from Na^+/K^+ ATPase (NKA)-α1 and NKA-α2 transgenic (TG) mice. A, Mean amplitude of Ca^{2+} transients measured from wild-type (Wt), NKA-α2, and high-line NKA-α1–isolated transgenic cardiomyocytes paced at 0.5 Hz. **B**, Sarcoplasmic reticulum (SR) Ca^{2+} load measured from Wt, NKA-α2, and high-line NKA-α1–isolated transgenic cardiomyocytes via caffeine-induced Ca^{2+} release. **C** and **D**, $[Na^+]_i$ measured from Wt, NKA-α2, and high-line NKA-α1–isolated transgenic cardiomyocytes under resting conditions (**C**) and during stimulation at 2 Hz (**D**). Number of myocytes analyzed is given within the graph. *P<0.05 vs WT. **E**, Rate of $[Ca^{2+}]_i$ decline after caffeine-induced depletion of SR stores measured from Wt, NKA-α2, and NKA-α1 transgenic myocytes either in the presence of 10 mmol/L $NiCl_2$ (to block Na^+/Ca^{2+} exchanger) or in the absence of $NiCl_2$ (None). *P<0.05 vs control (no nickel); #P<0.05 vs Wt of same treatment. **F**, Immunoblots of phosphoprotein and total protein levels for the indicated Ca^{2+}- and Na^+-handling proteins from cardiac homogenates of Wt, high-line NKA-α1 transgenic, or NKA-α2 transgenic hearts after 16 weeks of transverse aortic constriction (TAC) or sham surgery. For each experiment, the number of mice analyzed is given in the graph. Quantification of the data is shown in Online Figure IV. *P<0.05 vs sham; #P<0.05 vs Wt TAC. PLM indicates phospholemman; PLN, phospholamban; RyR2, ryanodine receptor 2; and SERCA2, sarco(endo)plasmic reticulum Ca^{2+} ATPase 2.

activity is responsible for the increased rate of Ca^{2+} extrusion in NKA-α2–overexpressing adult myocytes, whereas NKA-α1 does not seem to have this effect. This enhanced profile of NCX1 activity coupled to NKA-α2 could be protective and possibly antihypertrophic if it more effectively reduced intracellular Ca^{2+} or some aspect of its global signaling because of rate of removal during relaxation (see Discussion section).

NKA-α2 Overexpression Does Not Alter Hypertrophic Signaling Pathways But Does Alter Rate of Na^+ Removal

Given that overexpression of Na^+/H^+ exchanger, an Na^+ entry pathway, results in heart failure accompanied by increased NFAT nuclear translocation and CaMKII activity,[10] we performed experiments to determine whether activation of these prohypertrophic signaling pathways was altered after TAC in hearts of NKA-α2 transgenic mice. However, we failed to identify any reduction in cardiac NFAT-luciferase activity after TAC in NKA-α2 transgenic mice (Online Figure IIIA) nor was there a reduction in calcineurin activation as measured by calmodulin (CaM) coimmunoprecipitation with calcineurin B after TAC (Online Figure IIIB) or protein kinase Cα phosphorylation (data not shown). Similarly, assessment of CaMKII activity showed no difference between the NKA-α2 transgenic animals and Wt controls after 2 weeks of TAC stimulation (Online Figure IIIC). These results indicate that NKA-α2 overexpression is not acting in an antihypertrophic manner by affecting known Ca^{2+}-regulated signaling pathways in the heart.

To probe more carefully into the mechanisms that might be responsible or at least associated with the observed profile of altered Ca^{2+} and Na^+ handling in the hearts of NKA-α2 transgenic mice, we performed a series of Western blots for nodal ion-handling proteins, both at baseline and after TAC stimulation. We analyzed total phospholamban (PLN) protein levels and its phosphorylation status, ryanodine receptor 2 levels and phosphorylation, SERCA2, NCX1, the α1c subunit of the L-type Ca^{2+} channel, and PLM and its phosphorylation status (Figure 5F). We consistently observed that phosphorylation of PLM at both serine 63 and 68, as well as total PLM levels, was reduced in hearts of NKA-α2 transgenic mice compared with Wt or NKA-α1 mice, both at baseline in sham animals and after TAC stimulation (Figure 5F). PLM is known to inhibit NKA activity and alter NCX1 activity, and its reduction in NKA-α2 transgenic hearts could be protective and antihypertrophic by increasing NKA activity/effectiveness, further adding to the ability of the myocyte to deal with Na^+ and Ca^{2+} dysregulation that is often associated with hypertrophy and heart failure (see Discussion section). In addition, although total PLN levels seemed to be increased in both Wt and NKA-α1 hearts after TAC, no such increase was observed in NKA-α2 hearts likely because of the need to preserve SR load in the face of enhanced NCX1-mediated Ca^{2+} extrusion. We also observed that the increased ryanodine receptor 2 phosphorylation at S2814 after TAC in the Wt animals did not occur in either the NKA-α1 or the NKA-α2 transgenics after pressure overload. This agrees with our photometry data showing similar reductions in transient amplitude and reduction in SR load (Figure 5A and 5B) that could result in less CaMKII-mediated phosphorylation in the T-tubule/SR junctional space; however, there seems to be no loss of total CaMKII activity in any of the models after TAC (Online Figure IIIC).

NKA-α2 Isoform Has Greater Na^+ Affinity and Pump Activity

As demonstrated in Figure 5F, expression and phosphorylation of proteins such as PLN and PLM, which are important for controlling Na^+ and Ca^{2+} handling, are altered in NKA-α2 transgenic mice. To determine whether NKA-α2 and NKA-α1 functioned similarly in an uncompensated system, we carefully analyzed NKA activity in adult myocytes in which we overexpressed either the α1 or α2 isoform using adenoviral gene transfer (or a control β-galactosidase–expressing virus), followed by analysis of Na^+ pump activity as a function of $[Na^+]_i$.

Using an antibody that recognizes both NKA-α1 and NKA-α2, overexpression between the 2 isoforms was equivalent in total protein levels achieved after adenoviral infection (data not shown). The data show that NKA-α2–overexpressing myocytes had significantly greater affinity for Na^+ and a higher rate of activity compared with endogenous activity in control-infected myocytes (Figure 6A–6C). These results suggest that the NKA-α2 isoform, which may be less regulated by PLM (see Discussion section), is more effective than NKA-α1 in pumping Na^+ when expressed at high levels and should be more likely to maintain efficient forward-mode NCX1 activity during cardiac disease states, which we hypothesize would be cardioprotective (see Discussion section).

Discussion

Removal of Na^+ from the cytosol of a cardiac myocyte is primarily accomplished through NKA-α1, which accounts for 88% of the NKA activity in the heart, whereas NKA-α2 accounts for the remaining 12%.[22] Although these proteins share >85% identity, published reports suggest that they are not completely redundant and likely have specialized functions. NKA-α2 heterozygous gene-deleted mice are hypercontractile for cardiac function, whereas NKA-α1 heterozygous gene-deleted mice are hypocontractile.[21] Although the reason for the profound phenotypic difference between these 2 heterozygous mouse models remains a mystery, especially because more recent studies suggest that both isoforms similarly interact with NCX1,[13] the ultimate mechanism may relate to the observations we have made here: that NKA has greater affinity and pump activity for Na^+ and that PLM levels are specifically reduced in NKA-α2–overexpressing hearts, effects that would tend to maintain proper Ca^{2+} handling in the face of disease insults. For example, transgenic mice expressing a mutant form of SERCA2 in the heart with greater Ca^{2+} affinity were protected from cardiac hypertrophy after TAC stimulation, presumably by better maintaining Ca^{2+} homeostasis during disease that would otherwise tend to secondarily lead to negative influences on the heart (ie, increased neurohumoral drive).[30]

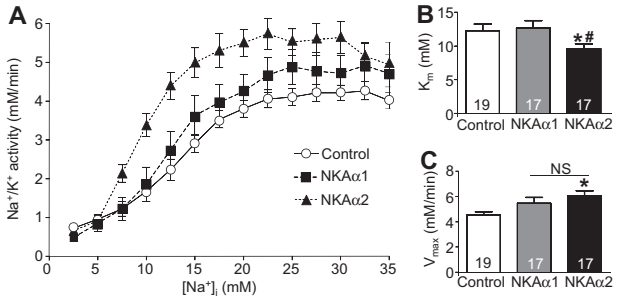

Figure 6. Affinity of the Na^+/K^+-ATPase pump for Na^+ is increased in myocytes with virus-mediated overexpression of Na^+/K^+ ATPase (NKA)-α2 but not NKA-α1. A, Na^+/K^+ ATPase pump rate as a function of intracellular Na^+ loading in adult rat myocytes 24 to 34 hours after infection with an adenovirus-expressing NKA-α1, NKA-α2, or β-galactosidase (control). K_m (**B**) and V_{max} (**C**) values for NKA-α1–infected, NKA-α2–infected, or β-galactosidase (control)–infected adult rat myocytes. For each experiment, number of myocytes analyzed is given within the graph. Data are from 2 separate myocyte isolations and viral infection experiments on 2 separate days. *P<0.05 vs control; #P<0.05 vs NKA-α1–infected myocytes.

Previous observations suggest that NKA-α1 and NKA-α2 differentially regulate cardiac contractility,[21] and here we provide further evidence demonstrating that these 2 isoforms play fundamentally different roles in the heart, this time in relation to regulation of the cardiac hypertrophic response. NKA-α1 transgenic mice showed a normal hypertrophic response after TAC surgery, whereas NKA-α2 transgenic mice were consistently less hypertrophic with less cardiac remodeling and signs of heart failure. The mechanism whereby increased NKA-α2 elicits such protection is intriguing, especially because overexpression of either NKA-α1 or NKA-α2 similarly reduces the Ca^{2+} transient amplitude and SR Ca^{2+} load. To determine whether NKA isoforms had differential effects on NCX1 activity, we used $NiCl_2$, a well-described inhibitor of the exchanger.[31] There was no difference in Ca^{2+} extrusion between control, NKA-α2, or NKA-α1 in the presence of Ni^{2+}, suggesting no change in the rate of Ca^{2+} decay when NCX1 was inhibited, and Ca^{2+} removal was primarily because of SERCA2 activity. However, when $NiCl_2$ was absent, the rate of Ca^{2+} decay was significantly increased in NKA-α2 but not significantly different in NKA-α1 transgenic myocytes after caffeine-mediated SR Ca^{2+} release (Figure 5E), suggesting that the rate of Ca^{2+} extrusion through NCX1 is more under the influence of NKA-α2.

We reasoned that NKA-α2 could generate a selective reduction in $[Na^+]_i$ that prevents the activation of prohypertrophic signaling pathways after TAC surgery by reducing resting $[Ca^{2+}]_i$ in T-tubule microdomains, which cannot be currently measured. However, this hypothesis lacks an ultimate molecular effector because there was no reduction in NFAT-luciferase activity, calcineurin/calmodulin interaction, or total CaMKII activity (Ca^{2+} dependent or Ca^{2+} independent, data not shown). These results suggest that normal hypertrophic signaling pathways are not differentially affected (reduced) in NKA-α2 transgenic hearts and that the protection we observed was possibly associated with a preservation in Na^+ and Ca^{2+} handling that would otherwise become dysregulated and lead to disease secondarily (ie, increased β-adrenergic drive that attempts to maintain contractile function). Despa et al[23] recently published more supportive evidence that NKA-α2 may have a selective effect on cardiac Na^+ and Ca^{2+} handling compared with NKA-α1. They showed that selective inhibition of NKA-α2 but not NKA-α1 could increase Ca^{2+} transients and fractional Ca^{2+} release from the SR, likely because of the sensitizing effect of increased cleft Ca^{2+} on ryanodine receptor 2. Our results may ultimately indicate that enhanced local Na^+ and Ca^{2+} removal elicited by NKA-α2 expression could subtly blunt alterations in abundance, post-translational modification, and activity of proteins in the T-tubule/SR junctional space to work against remodeling of the E–C coupling process and at least partially improve cardiac function, even in the face of activated hypertrophic signaling pathways such as calcineurin/NFAT and CaMKII, and that has the effect of reducing cardiac hypertrophy after pressure overload.

Another profound change in NKA-α2 hearts was a dramatic reduction in PLM levels and phosphorylation. PLM is an inhibitor of NKA activity that functions in a manner analogous to PLN inhibition of SERCA2 such that it generally inhibits NKA activity.[32] Thus, its reduction in NKA-α2 transgenic

hearts would be predicted to lead to greater NKA activity, removing Na^+ more efficiently. PLM phosphorylated at S68 also functions as an inhibitor of NCX1,[33,34] and overexpression of a PLM phosphomimetic protein resulted in arrhythmia and heart disease associated with loss of NCX1 activity.[35] Therefore, the reduced phosphorylation of PLM we observed in our NKA-α2 transgenic mice, as well as the total reduction in PLM, may further increase NCX1 activity and enhance Ca^{2+} removal even during disease states that might otherwise lead to reverse-mode Ca^{2+} influx because of intracellular elevations in Na^+ (Online Figure V). Enhanced Ca^{2+} extrusion through NCX1 elicited by a steeper Na^+ gradient and reduced PLM phosphorylation would, in effect, prime the heart with bolstered forward-mode activity before induction of pressure overload, contributing to a positive profile of inotropy and lusitropy that should be cardioprotective. For example, mice lacking the gene for PLN are hypercontractile with optimized Ca^{2+} cycling, and crossing these mice with other mouse models of heart failure prevented or rescued disease likely by diminishing secondary neurohumoral signaling that is typically associated with reduced cardiac output and defects in Ca^{2+} cycling.[36] Similarly, as stated above, overexpression of a mutant form of SERCA2 that enhances Ca^{2+} cycling in the hearts of transgenic mice produced less cardiac hypertrophy with pressure overload stimulation.[30] Reciprocally, ventricle-specific deletion of the gene encoding NCX1 resulted in increased fibrosis and arrhythmias at baseline and increased cardiac hypertrophy after pressure overload stimulation that resulted in the death of all animals within 3 weeks after surgery,[37] a result that is consistent with less cardiac hypertrophy because of optimized NCX1 activity in our NKA-α2 transgenic mice. Thus, NKA-α2 overexpression could be cardioprotective, producing less pressure overload–induced hypertrophy simply by optimizing Ca^{2+} handling and cardiac contractile performance (less need for neuroendocrine drive).

In addition to optimizing cardiac contractile function, NKA-α2 enhancement could be cardioprotective by affecting one or more microdomains of Na^+. For example, Na^+ overload during ischemia results in damage to mitochondria and reduction in ATP production that can be ameliorated via inhibition of voltage-gated Na^+ channels, Na^+/H^+ exchanger,[38,39] and NCX1.[40] A recent article by Liu and O'Rourke[41] further demonstrated that in a guinea pig aortic constriction model of heart failure, elevated $[Na^+]_i$ results in decreased mitochondrial Ca^{2+} uptake, which the authors suggest can negatively affect mitochondrial energy production, diminish mitochondrial Ca^{2+} buffering, and reduce the ability of the cell to respond to reactive oxygen species. Thus, augmented NKA-α2 expression in transgenic hearts may provide protection after TAC solely through a reduction in local $[Na^+]_i$. Our current data certainly strengthen the case that the NKA subtypes are not functionally redundant and further suggest that NKA-α2 controls Na^+ with a more proximal influence on NCX1 activity, which positively affects contractile parameters of the heart and imparts protection from pressure overload stimulation.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- The Na^+/K^+ ATPase (NKA) controls Na^+ efflux from cardiac myocytes, a process that regulates Ca^{2+} homeostasis through the Na^+/Ca^{2+} exchanger.
- There are 2 isoforms of NKA (α1 and α2) in the heart, of which the α2 subunit shows preferential localization to T-tubules and may generate a unique signaling domain.
- Intracellular Na^+ concentration is increased in many models of heart disease and may play a role in disease progression.

What New Information Does This Article Contribute?

- The expression of NKA α subunit is regulated such that increased expression of one isoform leads to downregulation of the other isoform.
- Increased expression of the α2 subunit reduces phospholemman expression and phosphorylation and enhances Ca^{2+} efflux through forward-mode Na^+/Ca^{2+} exchanger activity.
- Increased expression of the α2 subunit increases the affinity of NKA for Na^+ compared with α1 overexpression.

This study was designed to examine whether increased expression of NKA, the primary Na^+ efflux pathway in cardiac myocytes, could prevent the increase in intracellular Na^+ associated with heart disease and mitigate hypertrophic remodeling. Our results show that upregulation of this pathway is protective, and we provide new insights into the respective roles of the NKA isoforms during disease. We found that increased NKA-α2 (but not α1) expression reduced disease after pressure overload and enhanced Ca^{2+} removal via upregulation of forward-mode Na^+/Ca^{2+} exchanger activity. In addition, increased NKA-α2 resulted in a profile that is predicted to more effectively remove Na^+ from the myocyte, thus collectively providing a cardioprotective profile that resists dysregulation of Na^+ and Ca^{2+} levels. These results reveal a unique role of the α2 subunit of NKA in mitigating cardiac hypertrophic remodeling during pressure overload.