Na\textsuperscript{+}/K\textsuperscript{+}-ATPase \(\alpha2\)-isoform preferentially modulates Ca\textsuperscript{2+} transients and sarcoplasmic reticulum Ca\textsuperscript{2+} release in cardiac myocytes

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Aims

Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (NKA) is essential in regulating [Na\textsuperscript{+}], and thus cardiac myocyte Ca\textsuperscript{2+} and contractility via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. Different NKA-\(\alpha\) subunit isoforms are present in the heart and may differ functionally, depending on specific membrane localization. In smooth muscle and astrocytes, NKA-\(\alpha2\) is located at the junctions with the endo(sarcoplasmatic) reticulum, where they could regulate local [Na\textsuperscript{+}], and indirectly junctional cleft [Ca\textsuperscript{2+}]. Whether this model holds for cardiac myocytes is unclear.

Methods and results

The ouabain-resistant NKA-\(\alpha1\) cannot be selectively blocked to assess its effect. To overcome this, we used mice in which NKA-\(\alpha1\) is ouabain sensitive and NKA-\(\alpha2\) is ouabain resistant (SWAP mice). We measured the effect of ouabain at low concentration on [Na\textsuperscript{+}], Ca\textsuperscript{2+} transients, and the fractional sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release in cardiac myocytes from wild-type (WT; NKA-\(\alpha2\) inhibition) and SWAP mice (selective NKA-\(\alpha1\) block). At baseline, Na\textsuperscript{+} and Ca\textsuperscript{2+} regulations are similar in WT and SWAP mice. For equal levels of total NKA inhibition (~25%), ouabain significantly increased Ca\textsuperscript{2+} transients (from \(F_0/F\approx 1.5 \pm 0.1\) to \(1.8 \pm 0.1\)), and fractional SR Ca\textsuperscript{2+} release (from 24 \pm 3 to 29 \pm 3%) in WT (NKA-\(\alpha2\) block) but not in SWAP myocytes (NKA-\(\alpha1\) block). This occurred despite a similar and modest increase in [Na\textsuperscript{+}], (~2 mM) in both groups. The effect in WT mice was mediated specifically by NKA-\(\alpha2\) inhibition because at a similar concentration ouabain had no effect in transgenic mice where both NKA-\(\alpha1\) and NKA-\(\alpha2\) are ouabain resistant.

Conclusion

NKA-\(\alpha2\) has a more prominent role (vs. NKA-\(\alpha1\)) in modulating cardiac myocyte SR Ca\textsuperscript{2+} release.

Keywords

Sarcolemma–SR junctions • Na\textsuperscript{+}/K\textsuperscript{+}-ATPase • Ouabain • Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger

1. Introduction

Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (NKA) is the main route for Na\textsuperscript{+} extrusion in cardiac myocytes and therefore is essential in regulating intracellular Na\textsuperscript{+} concentration ([Na\textsuperscript{+}]). Through the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX), [Na\textsuperscript{+}], and NKA activity are tightly linked to cardiac myocyte Ca\textsuperscript{2+} and contractility.\textsuperscript{1} There are multiple NKA isoforms in the heart. NKA-\(\alpha1\) is the dominant, ubiquitous isoform, whereas NKA-\(\alpha2\) and NKA-\(\alpha3\) are present in smaller amounts and their expression differs significantly between species. For instance adult rodent hearts express NKA-\(\alpha1\) and NKA-\(\alpha2\),\textsuperscript{2} whereas dogs and macaques express NKA-\(\alpha1\) and NKA-\(\alpha3\).\textsuperscript{3} All three NKA-\(\alpha\) isoforms are present in human hearts,\textsuperscript{4} with estimates of isoform distribution at the mRNA level ranging from relatively equal amounts of the three isoforms\textsuperscript{4} to NKA-\(\alpha1\) being dominant (62%) over NKA-\(\alpha2\) (15%), and NKA-\(\alpha3\) (23%).\textsuperscript{5}

Different NKA isoforms may function differently, depending on specific membrane localization. In smooth muscle and astrocytes, NKA-\(\alpha2\) and NKA-\(\alpha3\) are located at the junctions with the endo(sarcoplasmatic) reticulum, where they regulate local [Na\textsuperscript{+}], and, indirectly via NCX, junctional cleft [Ca\textsuperscript{2+}].\textsuperscript{6} In contrast, NKA-\(\alpha1\) is ubiquitously distributed and may regulate bulk [Na\textsuperscript{+}]. It is unclear whether this model holds for cardiac myocytes. The functional density of NKA-\(\alpha2\) is about four times higher in the T-tubules (vs. external sarcosome) in myocytes from both rats\textsuperscript{7,8} and mice,\textsuperscript{9} making this functionally plausible. However, its precise localization with respect to the junctions with the sarcoplasmic reticulum (SR), which occupy

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40–48% of T-tubular membrane, is unknown. Hearts with genetically reduced NKA- \( \alpha \)-2 levels are hypercontractile as a result of larger Ca\(^{2+}\) transients, whereas hearts with reduced levels of NKA-\( \alpha \)-1 are hypoorcontractile. Moreover, elevated NKA-\( \alpha \)-2 expression results in smaller NCX current and Ca\(^{2+}\) transients. Selective NKA-\( \alpha \)-2 inhibition was found to increase rat myocyte contractility, despite unchanged global [Na\(^+\)].

2. Methods

2.1 Generation of SWAP mice and cardiac myocyte isolation

All animal protocols were approved by the animal welfare committees at the University of California or University of California Davis and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Mice expressing ouabain-sensitive NKA-\( \alpha \)-1 and ouabain-resistant NKA-\( \alpha \)-2 (SWAP) were generated by mating mice with ouabain-sensitive NKA-\( \alpha \)-1 isoform (\( \alpha \)1RS) \(^{14}\) and mice with having an ouabain-resistant NKA-\( \alpha \)-2 isoform (\( \alpha \)2RS) \(^{14} \) as previously described. The ouabain-sensitive mouse NKA-\( \alpha \)-1 isoform was obtained by introducing the R111Q and D122N amino acid substitutions into the first extracellular domain of this isoform. Gln-111 and Asn-122 residues are naturally present in the low-affinity rodent NKA-\( \alpha \)-1 isoform, and the mutations did not alter the enzymatic activity of the two isoforms. \(^{14}\) The ouabain-sensitive mouse NKA-\( \alpha \)-1 isoform was obtained by introducing the R111Q and D122N amino acid substitutions into the first extracellular domain of this isoform. Gln-111 and Asn-122 residues are naturally present in the low-affinity rodent NKA-\( \alpha \)-1 isoform, and the mutations did not alter the enzymatic activity of the two isoforms. \(^{14}\) There are no differences in the haemodynamic parameters (both under basal conditions and during \( \beta \)-adrenergic stimulation) between WT and SWAP mice. \(^{14}\) Eleven SWAP, 10 WT, and 5 \( \alpha \)1RR \( \alpha \)2RR mice were used for this study.

Isolation of mouse ventricular myocytes was as previously described. \(^{19}\)

Briefly, SWAP mice and age-matched WT littermates (3–4 months age) were anaesthetized in a gas chamber with 3–5% isoflurane (100% O\(_2\)). Anaesthesia was confirmed by the complete lack of reflex to very firm toe pinch and/or touch of a suture thread to cornea of eye. Hearts were excised quickly, mounted on a gravity-driven Langendorff perfusion apparatus and digested by perfusion with 0.8 mg/mL collagenase (type B, Boehringer Mannheim, Indianapolis, IN, USA) in the presence of 20 \( \mu \)M Ca\(^{2+}\) and 1 mg/mL taurocholate. When the heart became flaccid (7–12 min), ventricular tissue was removed, dispersed, filtered, and myocyte suspensions were rinsed several times. Myocytes were used for experiments within 6 h after isolation.

2.2 Simultaneous measurements of [Na\(^+\)]\(_i\) and Ca\(^{2+}\) transients

Myocytes were plated on glass-coverslips and incubated with 1 \( \mu \)M Fluo-4 AM in the presence of Fluo-4 AM (0.05% w/v) for 90 min at room temperature. Fluo-4 AM (10 \( \mu \)M) was added during the last 35 min. The myocytes were allowed to recover for 20 min in normal Tyrode’s solution. Myocytes were placed on the stage of a confocal microscope and fluorescence was then alternatively excited at 340, 380 (for Fluo-4) and 480 nm (Fluo-4). For all excitation wavelengths, emission was recorded at 535 nm. For [Na\(^+\)]\(_i\) measurements, the \( F_{\text{340}}/F_{\text{480}} \) ratio was calculated after background subtraction and calibrated at the end of each experiment using divalent-free solutions with 0, 10, or 20 mM extracellular Na\(^+\) in the presence of 10 \( \mu \)M gramicidin and 100 \( \mu \)M 2,3-butanediol. \(^{19}\) Ca\(^{2+}\) transients were reported as the Fluo-4/Fluo-4 ratios. All measurements were at room temperature (21–25°C).

2.3 Ouabain-dose–response curves

Myocytes were pre-treated with 1 \( \mu \)M thapsigargin for 10 min and voltage clamped in the whole-cell configuration using patch electrodes made from borosilicate glass capillaries, as previously described. \(^{17} \) When filled with pipette solution, the electrode resistance was 1.5–2.5 M\( \Omega \). Current signals were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). Membrane capacitance and series resistance were calculated from a 5 \( \mu \)V voltage step from a holding potential of \( \approx 20 \) mV. The pipette solution contained (in millimolar): 30 NaCl, 70 NaOH, 70 aspartic acid, 20 K\(^{2+}\)-aspartate, 20 TEA-Cl, 10 HEPES, 5 Mg-ATP, 0.7 MgCl\(_2\) (1 mM free Mg), 3 BaCl\(_2\), 1.15 CaCl\(_2\) (100 mM free Ca\(^{2+}\)), pH 7.2. At this internal [Na\(^+\)], the pump operates near the maximum rate. \( I_{\text{Pump}} \) was activated in the whole-cell voltage clamp, by rapid switch from 0 to 4 mM external K at a holding potential of \( \approx 20 \) mV. We have shown previously that the K-activated current is ouabain sensitive. \(^{17} \) The external solution contained (in millimolar): 136 NaCl, 5 NiCl\(_2\), 2 BaCl\(_2\), 1 MgCl\(_2\), 5 HEPES, 5 glucose, \( \pm 4 \) KCl, pH 7.4.

After reaching the whole-cell configuration, myocytes were held at \( \approx 20 \) mV in K\(^{+}\)-free external solution for at least 8 min, to allow equilibration of intracellular and pipette Na\(^+\). Then, \( I_{\text{Pump}} \) was activated at \( \approx 20 \) mV by switching to 4 mM K\(^{+}\) external solution. Under these conditions, \( \approx 75\% \) of the pumps are activated (assuming a K\(_{1/2}\) for external K\(^{+}\) of 1.5 mM). We then recorded the current while adding increasing concentrations of ouabain. For each ouabain concentration, \( I_{\text{Pump}} \) was considered at steady state if the holding current was constant for at least 10 s. We also repeated the analysis using exponential extrapolation to idealized steady state at longer times, and obtained virtually identical results (not shown). Control experiments (not shown) indicated that \( I_{\text{Pump}} \) does not run down significantly during the duration of the experiment. We calculated the percentage of \( I_{\text{Pump}} \) inhibition for each ouabain concentration as 100 (\( I_{\text{Pump}} - I_{\text{PumpOuab}} \))/\( I_{\text{PumpOuab}} \) where \( I_{\text{Pump}} \) is \( I_{\text{Pump}} \) in the absence of ouabain (i.e. the outward current induced by adding 4 mM K\(^{+}\) to the external solution) and \( I_{\text{PumpOuab}} \) is \( I_{\text{Pump}} \) at the respective [ouabain]. The mean data were fit with a two-binding site equation: B_0([Ouabain]) / (K_1 + [Ouabain]) + (100-B_0)[Ouabain]/(K_2 + [Ouabain]), to derive the K\(_{1/2}\) for ouabain-binding to NKA-\( \alpha \)-1 and NKA-\( \alpha \)-2 and their relative contribution to \( I_{\text{Pump}} \). The fit was done using Origin software (Microcal Software, Inc., Northampton, MA, USA) and the best fit was identified by a minimum \( \chi^2 \) test.

2.4 Immunofluorescence

Myocytes from WT and SWAP mice were fixed with 4% paraformaldehyde, permeabilized with saponin (0.5 \( \mu \)g/mL for 2 min), blocked with 2% goat serum and labelled with primary antibodies that bind specifically to NKA-\( \alpha \)-1 (clone C464.6 from Millipore) and NKA-\( \alpha \)-2 (AB9094 from Boehringer Mannheim, Indianapolis, IN, USA) in the presence of 20 \( \mu \)M Ca\(^{2+}\) and 1 mg/mL taurocholate. When the heart became flaccid (7–12 min), ventricular tissue was removed, dispersed, filtered, and myocyte

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Millipore). Alexa-Fluor546-linked secondary antibodies were used for detection. Images were acquired with a laser scanning confocal microscope.

2.5 Statistical analysis
The statistical differences between the groups were determined using Student’s t-test. The data are presented as mean ± standard error. A statistical difference was achieved for a P-value < 0.05.

3. Results
3.1 Low ouabain concentration specifically inhibits NKA-α2 in WT and NKA-α1 in SWAP mice
In the mouse (and rat), NKA-α1 and NKA-α2 can be separated based on their different ouabain affinities. The dominant NKA-α1 isoform is ouabain resistant, whereas NKA-α2 is ouabain sensitive. SWAP mice carry two amino acid mutations in NKA-αisoforms so that NKA-α1 is ouabain sensitive and NKA-α2 is ouabain resistant. The dominant NKA-α is tissue distribution, and enzymatic activity of NKA-α1 and NKA-α2.

We measured the dose-dependent \( I_{\text{pump}} \) inhibition by ouabain in myocytes from WT and SWAP mice (Figure 1). For both WT and SWAP myocytes, data could be fit with a two-binding sites equation, which indicates the presence of two ouabain-binding sites (Figure 1). There was no significant difference in the ouabain \( K_{i2} \) for either the high \( (K_{i2} = 0.22 ± 0.02 \mu M \) vs. \( 0.25 ± 0.02 \mu M \) or the low \( (K_{i2} = 117 ± 5 \mu M \) vs. \( 105 ± 6 \mu M \) affinity sites between WT and SWAP mice. In WT mice, the low affinity sites (i.e. NKA-α1) are dominant (~72% of total NKA; Figure 1A). This is somewhat lower than we previously reported for mouse myocytes, may doubt to the different background mouse strain used (C57BL/6 previously vs. Black Swiss here). NKA-α1 is still the major isoform in SWAP mice (~80%), only here it is ouabain sensitive (Figure 1B). Thus, the relative proportion of NKA-α1 and NKA-α2 is comparable in WT and SWAP mice. Of note, we have shown previously\(^1\) that the apparent affinity for internal \( Na^+ \) is similar for the α1 and α2 isoforms, both under basal conditions and during activation of β-adrenergic receptors with isoprenaline.

Immunofluorescence images of myocytes labelled with antibodies specific for NKA-α1 and NKA-α2 show that NKA-α1 is localized at the T-tubules and external sarcolemma, whereas NKA-α2 is present primarily at the T-tubules for both WT and SWAP mice (Figure 1C), consistent with prior analysis of \( I_{\text{pump}} \) distribution in WT mice.\(^7\)\(^-\)\(^9\)

Thus, swapping the ouabain sensitivity of NKA isoforms in the SWAP mice did not significantly affect NKA-α1 and NKA-α2 localization.

3.2 Selective NKA-α2 inhibition has greater consequences on cardiac myocyte \( Ca^{2+} \) than NKA-α1 blockade
In WT mice, 5 μM ouabain blocks NKA-α2 almost completely, whereas NKA-α1 is practically unaffected (Figure 1). In SWAP mice, 5 μM ouabain selectively blocks NKA-α1 isoform. Thus, we can use myocytes from WT and SWAP mice to determine the effect of selective NKA-α2 and NKA-α1 inhibition on cardiac myocyte \( Ca^{2+} \) cycling. However, because NKA-α1 represents ~80% of the total NKA, 5 μM ouabain would inhibit a much larger fraction of NKA in SWAP mice than in WT. This would likely lead to different elevations in \([Na^+]_i\), and SR \( Ca^{2+} \) content in WT and SWAP mice, making the SWAP mouse experiments a less appropriate control. Based on the dose–response curve in figure 1B, we used 0.1 μM ouabain in SWAP mice to attain a similar level of total NKA inhibition (~25%) as that produced by 5 μM ouabain in WT mice.

We measured simultaneously \([Na^+]_i\) and \( Ca^{2+} \) transients in myocytes contracting at 1 Hz under control conditions (Figure 2, point a) and then in the presence of low ouabain concentration (5 μM for WT, 0.1 μM for SWAP mice) (Figure 2, point b). Higher ouabain levels, 300 μM for WT and 5 μM for SWAP mice, were added in some experiments (Figure 2, point c). SR \( Ca^{2+} \) content (with and without ouabain) was determined in select experiments from the amplitude of the \( Ca^{2+} \) transient triggered by 10 mM caffeine.

Under control conditions, \( [Na^+]_i \) (10.2 ± 1.1 vs. 9.3 ± 0.9 mM), \( Ca^{2+} \) transient amplitude (\( \Delta F/F_0 = 1.5 ± 0.1 \) vs. 1.5 ± 0.1) and SR \( Ca^{2+} \) content (\( \Delta F/F_0 = 5.9 ± 0.4 \) vs. 6.3 ± 0.5) were similar in myocytes from WT and SWAP mice (Figures 3A–B and 4). Moreover, there was no difference in \( Ca^{2+} \) transient decay during both twitch (\( \tau = 437 ± 42 \) ms in WT and 446 ± 36 ms in SWAP mice) and caffeine application (\( \tau = 2.9 ± 0.4 \) s in WT and 2.7 ± 0.4 s in SWAP mice). Thus, at baseline the net function of \( Na^+ \) and \( Ca^{2+} \) cycling proteins is similar in WT and SWAP mice.

Upon selective NKA-α1/NKA-α2 blockade, \( [Na^+]_i \) increased modestly and to the same extent in myocytes from both mice (by 2.5 ± 0.4 mM in WT and by 2.1 ± 0.4 mM in SWAP mice; Figure 3A, B, and D). This indicates a similar level of total NKA inhibition in myocytes from WT and SWAP mice. However, NKA-α2 inhibition in WT mice resulted in significantly larger amplitude of \( Ca^{2+} \) transients (by 25 ± 4%, Figure 3D), whereas comparable NKA-α1 inhibition in SWAP mice had no effect (Figure 3B and D).

Direct effects on ryanodine receptors\(^2\) and \( Ca^{2+} \) influx through \( Na^+ \) channel,\(^3\) have also been proposed for explaining the inotropic effect of cardiac glycosides. To elucidate whether such mechanisms contribute to the ouabain-induced \( Ca^{2+} \) transient augmentation in WT mice, we repeated the measurements in mice in which both NKA-α1 and NKA-α2 are ouabain resistant (\( \alpha^{1\text{th}} \) \( \alpha^{2\text{RR}} \) mice; Figure 3C). As expected, ouabain had no effect on \( [Na^+]_i \) in these mice. Furthermore, ouabain did not alter \( Ca^{2+} \) transients either, which indicates that the enhancement observed in WT mice is caused by NKA-α2 inhibition.

\( SR \ Ca^{2+} \) content was not significantly altered by ouabain in either WT (\( \Delta F/F_0 = 6.3 ± 0.5 \) with ouabain vs. 5.9 ± 0.4 without) or SWAP mice (6.4 ± 0.6 vs. 6.3 ± 0.5; Figure 4). However, low concentrations of ouabain significantly increased the fractional SR \( Ca^{2+} \) release (calculated as the ratio between the amplitude of twitch- and caffeine-induced \( Ca^{2+} \) transients) in WT and had no effect (Figure 4).

4. Discussion
Whether NKA-α2 preferentially (vs. NKA-α1) regulates cardiac myocyte \( Ca^{2+} \), has not been determined unequivocally because the ouabain-resistant NKA-α1 cannot be selectively blocked to assess its effect. We overcame this by using mice in which NKA-α1 is ouabain sensitive and NKA-α2 is ouabain resistant (SWAP mice). At baseline, \( Na^+ \) and \( Ca^{2+} \) regulation are similar in WT and SWAP mice. For equal levels of total NKA inhibition (~25%), ouabain significantly increased \( Ca^{2+} \) transients and fractional SR \( Ca^{2+} \) release in WT (NKA-α2 block) but not in SWAP myocytes (NKA-α1 block), despite a similar \( [Na^+]_i \) rise. These findings support the hypothesis that,
similar to smooth muscle and astrocytes, NKA-α2 has a more prominent role in modulating cardiac myocyte $\text{Ca}^{2+}$ release.

### 4.1 Local $[\text{Ca}^{2+}]_i$ control by NCX and NKA-α2

In cardiac myocytes contracting at steady state, NCX must remove $\sim 10 \text{ μmol/L}\text{Ca}^{2+}$ at each beat, the amount that enters via $\text{Ca}^{2+}$-channels.\(^3\) Thus, at each beat NCX brings into $\sim 32 \text{ μmol/L}\text{Na}^+$.\(^{24}\)

To maintain low $[\text{Na}^+]$, and therefore facilitate NCX-mediated $\text{Ca}^{2+}$ extrusion, $\text{Na}^+$ must be pumped out of the myocytes by NKA. Thus, the interplay between NCX and NKA is essential for regulating $[\text{Na}^+]$, and $[\text{Ca}^{2+}]_i$, and profoundly influences cardiac contractility and arrhythmogenesis. Functional, co-immunoprecipitation and immunofluorescence studies support a mechanism where NCX and NKA interact functionally via local, microdomain $[\text{Na}^+]$, rather than bulk $[\text{Na}^+]$. Rapid NKA blockade can slow the decline of caffeine-induced $\text{Ca}^{2+}$ transients and NCX current\(^{15}\) and increase outward NCX current\(^{16}\) under conditions such that bulk $[\text{Na}^+]$, and SR $\text{Ca}^{2+}$ load are not expected to change. NCX and NKA have been shown to co-immunoprecipitate\(^{14,15}\) and co-localize\(^{15}\), which indicate a close physical association. The question is to what extent the microdomain controlled by NCX and NKA overlaps with the cleft at the sarcolemma–SR membrane junctions and if NKA isoform matters. During excitation, $[\text{Ca}^{2+}]_i$ increases faster and higher in the junctional cleft vs. bulk cytosol. NCX also senses an early rise in local $[\text{Ca}^{2+}]$ during SR $\text{Ca}^{2+}$ release,\(^{27-29}\) suggesting that it has some access to $\text{Ca}^{2+}$ in or near the junctional cleft. Indeed, $\text{Ca}^{2+}$ entry via reverse-mode NCX can trigger the SR $\text{Ca}^{2+}$ release, although with reduced efficiency compared with L-type $\text{Ca}^{2+}$ channels.\(^{30-32}\) Data from NCX-KO mice indicate that by priming the cleft with $\text{Ca}^{2+}$, NCX is essential for the normal triggering of $\text{Ca}^{2+}$ release.\(^{33}\) Moreover, a recent immunofluorescence study found that NCX partially co-localizes with ryanodine receptors (27%).\(^{34}\)

All these data are consistent with the idea that the junctional cleft and the microdomain controlled by NCX and NKA are functionally (and maybe also physically) overlapping.

Our data support this hypothesis and further point to α2 as the NKA isoform that is functionally important in controlling the cleft $[\text{Ca}^{2+}]_i$ and SR $\text{Ca}^{2+}$ release. The ouabain-induced local $[\text{Ca}^{2+}]_i$, increases faster and higher in the junctional cleft vs. bulk cytosol. NCX also senses an early rise in local $[\text{Ca}^{2+}]_i$ during SR $\text{Ca}^{2+}$ release,\(^{27-29}\) suggesting that it has some access to $\text{Ca}^{2+}$ in or near the junctional cleft. Indeed, $\text{Ca}^{2+}$ entry via reverse-mode NCX can trigger the SR $\text{Ca}^{2+}$ release, although with reduced efficiency compared with L-type $\text{Ca}^{2+}$ channels.\(^{30-32}\) Data from NCX-KO mice indicate that by priming the cleft with $\text{Ca}^{2+}$, NCX is essential for the normal triggering of $\text{Ca}^{2+}$ release.\(^{33}\) Moreover, a recent immunofluorescence study found that NCX partially co-localizes with ryanodine receptors (27%).\(^{34}\)

All these data are consistent with the idea that the junctional cleft and the microdomain controlled by NCX and NKA are functionally (and maybe also physically) overlapping.

For example, selective NKA-α2 inhibition was found to increase rat myocyte contractility by 40%\(^{30}\) and to slow NCX-mediated $\text{Ca}^{2+}$ extrusion\(^{12}\) although global $[\text{Na}^+]$ was not affected. However, this is the first study to demonstrate that selective NKA-α1 inhibition has a significantly smaller effect on the cardiac myocyte SR $\text{Ca}^{2+}$ release than the inhibition of a similar number of NKA-α2 pumps. We have previously shown that although NKA-α2 function is concentrated in the T-tubules, where $\text{Ca}^{2+}$ release sites are predominantly located, the amount of NKA-α1 and NKA-α2 in the T-tubules is comparable.\(^{7}\) Both NKA-α1 and NKA-α2 were found to co-immunoprecipitate with NCX\(^{14,15}\) thus both might create microdomains with NCX. It is possible, however, that the NCX–NKA-α2 microdomains are localized in the immediate vicinity of the sarcolemma–SR junctions, as in the smooth muscle and astrocytes. In contrast, NKA-α1 might predominate in the non-junctional area of the T-tubules. Alternatively, the NCX–NKA-α1 microdomains may also be localized at or near the junctions but the effect of NKA-α1 inhibition is diluted by the uniform distribution of NKA in the T-tubules and external sarcolemma. Both possibilities result in NKA-α1 being less important in regulating the local cleft $[\text{Na}^+]$, (and implicitly local $[\text{Ca}^{2+}]$). Instead, NKA-α1, as the dominant isoform, is central to determining global $[\text{Na}^+]$.

### 4.2 NKA-α2 specific glycosides as inotropic agents in heart failure

Cardiac glycosides have been used for treating congestive heart failure (HF) for over 200 years. By raising $[\text{Na}^+]$, and thus impeding
Figure 2  Simultaneous $[\text{Na}^+]_i$ and $\text{Ca}^{2+}$ transient measurements; the effect of ouabain. (A) $[\text{Na}^+]_i$; (B) $\text{Ca}^{2+}$ transients. Representative example in a cell from WT mice. Myocytes dually loaded with SBFI and Fluo-4 were paced at 1 Hz until $[\text{Na}^+]_i$ and $\text{Ca}^{2+}$ transients reached steady state (point a). Selective NKA-α2 inhibition (5 μM ouabain) slightly raised $[\text{Na}^+]_i$ and significantly increased $\text{Ca}^{2+}$ transients (point b). A higher dose of ouabain (300 μM) was then applied. At this concentration, all NKA-α2 and ~70% of NKA-α1 are blocked. Both $[\text{Na}^+]_i$ and $\text{Ca}^{2+}$ transient amplitude were increased considerably (point c).

Figure 3  At low concentration, ouabain increases $\text{Ca}^{2+}$ transient amplitude in myocytes from WT but not SWAP mice. (A–C) The mean data showing the effect of the low concentration of ouabain on $\text{Ca}^{2+}$ transient amplitude and $[\text{Na}^+]_i$ in myocytes from (A) WT mice (NKA-α2 inhibition; n = 17 cells, 7 mice), (B) SWAP mice (NKA-α1 block; 15 cells, 7 mice), and (C) mice with both NKA-isoforms ouabain-resistant (no NKA inhibition; n = 10 cells, 5 mice). (D) Average change in $\text{Ca}^{2+}$ transient amplitude and $[\text{Na}^+]_i$ produced by selective NKA-α2 inhibition in WT mice and NKA-α1 inhibition in SWAP mice.
NCX-mediated Ca\(^{2+}\) extrusion, they increase the cellular and SR Ca\(^{2+}\) content and cause inotropy. However, their use is currently limited by the induction of lethal cardiac arrhythmias caused by spontaneous diastolic SR Ca\(^{2+}\) release. Our data indicate that selectively blocking NKA-\(\alpha_2\) may produce inotropy associated with a smaller rise in [Na\(^{+}\)], and SR Ca\(^{2+}\) load. Since diastolic SR Ca\(^{2+}\) release, the primary cause for triggered arrhythmias, depends steeply on the SR Ca\(^{2+}\) content, cardiac glycosides that specifically block NKA-\(\alpha_2\) may have a lower risk of triggering arrhythmias and thus a wider therapeutic window than regular cardiac glycosides. Cardiac glycosides may also have other adverse effects and using a glycoside targeting the NKA-\(\alpha_2\) isoform may result in an inotropic effect at a drug concentration that is low enough to minimize these adverse effects. In the human heart, NKA-\(\alpha_2\) represents 15–35% of the total NKA. All cardiac glycosides currently known have comparable affinity for the three human NKA isoforms. Thus, finding cardiac glycosides with selectivity for human NKA-\(\alpha_2\) may lead to new treatment options for patients with HF, especially those with low output syndrome, left ventricular systolic dysfunction, and low systolic blood pressure that need inotropic agents to improve symptoms and end-organ function.

In summary, we found that for a similar increase in [Na\(^{+}\)], selective NKA-\(\alpha_2\) inhibition generates a greater enhancement of cardiac myocyte Ca\(^{2+}\) transient and SR Ca\(^{2+}\) release than that produced by selective NKA-\(\alpha_1\) blockade. Thus, NKA-\(\alpha_2\) preferentially (vs. NKA-\(\alpha_1\)) regulates cardiac inotropy.

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