Functional analysis of Na\(^+\)/K\(^+\)-ATPase isoform distribution in rat ventricular myocytes

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Despa S, Bers DM. Functional analysis of Na\(^+\)/K\(^+\)-ATPase isoform distribution in rat ventricular myocytes. Am J Physiol Cell Physiol 293: C321–C327, 2007. First published March 28, 2007; doi:10.1152/ajpcell.00597.2006.—The Na\(^+\)/K\(^+\)-ATPase (NKA) is the main route for Na\(^+\) extrusion from cardiac myocytes. Different NKA α-subunit isoforms are present in the heart. NKA-α1 is predominant, although there is a variable amount of NKA-α2 in adult ventricular myocytes of most species. It has been proposed that NKA-α2 is localized mainly in T-tubules (TT), where it could regulate local Na\(^+\)/Ca\(^2+\) exchange and thus cardiac myocyte Ca\(^2+\). However, there is controversy as to where NKA-α1 vs. NKA-α2 are localized in ventricular myocytes. Here, we assess the TT vs. external sarcolemma (ESL) distribution functionally using formamide-induced detubulation of rat ventricular myocytes, NKA current (\(I_{\text{Pump}}\)) measurements and the different ouabain sensitivity of NKA-α1 (low) and NKA-α2 (high) in rat heart. Ouabain-dependent \(I_{\text{Pump}}\) inhibition in control myocytes indicates a high-affinity NKA isoform (NKA-α2, \(K_{1/2} = 0.38 \pm 0.16 \mu M\)) that accounts for 29.5 ± 1.3% of \(I_{\text{Pump}}\) and a low-affinity isoform (NKA-α1, \(K_{1/2} = 141 \pm 17 \mu M\)) that accounts for 70.5% of \(I_{\text{Pump}}\). Detubulation decreased cell capacitance from 164 ± 6 to 120 ± 8 pF and reduced \(I_{\text{Pump}}\) density from 1.24 ± 0.05 to 1.02 ± 0.05 pA/pF, indicating that the functional density of NKA is significantly higher in TT vs. ESL. In detubulated myocytes, NKA-α2 accounted for only 18.2 ± 1.1% of \(I_{\text{Pump}}\). Thus, ~63% of \(I_{\text{Pump}}\) generated by NKA-α2 is from the TT (although TT are only 27% of the total sarcolemma), and the NKA-α2/NKA-α1 ratio in TT is significantly higher than in the ESL. The functional density of NKA-α2 is ~4.5 times higher in the T-tubules vs. ESL, whereas NKA-α1 is almost uniformly distributed between the TT and ESL.

T-tubules; Na\(^+\)/K\(^+\) pump current; ouabain; external sarcolemma; detubulation

The Na\(^+\)/K\(^+\)-ATPase (NKA) is the main route for Na\(^+\) extrusion in cardiac myocytes and, therefore, is essential in the regulation of intracellular Na\(^+\) concentration ([Na\(^+\)]). Because [Na\(^+\)], is tightly linked to intracellular Ca\(^2+\) regulation, and thus contractility, via the Na\(^+\)/Ca\(^2+\) exchanger (NCX), alterations in NKA activity can have profound effects on cardiac myocytes contractility (2).

The NKA has two essential subunits: α and β. The α subunit (~110 kDa) contains the binding sites for Na\(^+\)/K\(^+\)-ATP and cardiac glycodies. The smaller β subunit (~50 kDa) has a role in the processing and in the proper membrane insertion of the pump. There are three α (α1–α3) and three β (β1–β3) NKA subunit isoforms in the heart, with any α-β combination resulting in a functional pump. NKA-α1 is present in the heart of all species studied, whereas the expression of NKA-α2 and NKA-α3 differs significantly between species (16). For instance, the adult rodent heart expresses NKA-α1 and NKA-α2, whereas dogs and monkeys do not have the NKA-α2 subunit (11). In humans, all three NKA-α isoforms can be detected (22).

It has been suggested that different NKA isoforms may function differently in the cell, depending on differential localization in the membrane. Juhaszova and Blaustein (14) showed that in smooth muscle NKA-α2 is localized at the junctions with the sarcoplasmic reticulum (SR) and regulates local Na\(^+\)/Ca\(^2+\) exchange and [Ca\(^2+\)](\(i\)). James et al. (13) showed that mouse hearts with genetically reduced levels of NKA-α were hypercontractile as a result of increased Ca\(^2+\) transients during the contractile cycle. In contrast, hearts with reduced levels of NKA-α2 were hypocontractile. Moreover, inhibition of NKA-α with ouabain increased the contractility of heterozygous NKA-α1 hearts. These data indirectly implicate a selective involvement of NKA-α2 in cardiac myocyte Ca\(^2+\) regulation. Further support for this idea came from the observation that increased expression of NKA-α2 decreased \(I_{\text{NCX}}\) and Ca\(^2+\) transients in mouse ventricular myocytes (24).

Immunofluorescence data indicated that NKA is located in both external sarcolemma (ESL) and T-tubules (TT) in ventricular myocytes (16, 21). The differential NKA-α1/NKA-α2 localization (TT vs. ESL) in cardiac cells is controversial. In the rat, McDonough et al. (16) found the NKA-α1 isoform to be preferentially distributed in the TT, while NKA-α2 was uniformly distributed in the TT and peripheral sarcolemma. Conversely, Silverman et al. (21) showed that in guinea pig ventricular myocytes NKA-α2 is mainly in TT and NKA-α1 is predominantly on the external sarcolemma. However, these are mostly qualitative data, limited by the selectivity of the antibodies and do not give information about the function of NKA isoforms in the TT vs. ESL.

Kawai et al. (15) developed a novel method to detubulate ventricular myocytes using formamide. Upon withdrawal of 1.5 M formamide, the TT seal off, and only currents carried by ESL channels and transporters are accessible. Notably, this procedure has no effect on ionic currents measured in atrial myocytes, which lack endogenous TT, indicating that formamide exposure and withdrawal do not by themselves alter ion channel function (3). We have previously used this method and...
Na+/K+ pump current (IPump) measurements in control and detubulated rat and mouse ventricular myocytes to show that the NKA functional density is significantly higher in the TT vs. ESL (1, 6).

The aim of this paper is to investigate the functional distribution of NKA-α1 and NKA-α2 in rat between the TT and ESL. In rat ventricular myocytes, we can distinguish the IPump generated by NKA-α1 and NKA-α2 based on their different ouabain affinities: NKA-α2 is ouabain sensitive, whereas NKA-α1 is ouabain resistant (12). We can also measure IPump due to whole cell NKA (measurements in control myocytes) and due to NKA in the ESL (measurements in detubulated myocytes). Obviously, the difference is the IPump in the TT. Here, we measured the dose-dependent IPump inhibition by ouabain in control and detubulated rat ventricular myocytes. Combining these approaches allowed us to directly assess what fraction of the external sarcolemmal IPump is due to NKA-α1 vs. NKA-α2 and likewise for the TT.

MATERIALS AND METHODS

Myocytes isolation and detubulation procedure. The procedure for isolation of rat ventricular myocytes has been described previously (7) and was approved by the Loyola University (Chicago, IL) animal welfare committee. Briefly, rats were anesthetized by intraperitoneal injection of nembutal (~1 mg/g). Hearts were excised quickly and placed on a Langendorff perfusion apparatus. Hearts were perfused for 5–7 min with nominally Ca2+-free Tyrode’s solution, then perfusion proceeded with added collagenase (1 mg/ml) and albumin (0.05%). When the heart became flaccid, the atrial and left ventricular tissues were excised and cut into small pieces for further incubation (5–10 min, with 0.4 mg/ml collagenase for the ventricular tissue and 12–20 min in the presence of 0.4 mg/ml collagenase and 0.2 mg/ml protease for the atria). The tissue was then filtered and Ca2+ concentration in the cell suspension was progressively increased to 1 mM. The standard Tyrode’s solution used in these experiments contained (in mM): 140 NaCl, 4 KCl, 1 MgCl2, 10 glucose, 5 HEPES and 1 CaCl2 (pH = 7.4). All experiments were done at room temperature (23–25°C).

Detubulation was induced by osmotic shock, as described previously (6, 15). Briefly, 1.5 M formamide was added to the cell suspension for 15–20 min; then the cells were returned to the standard solution. Upon formamide withdrawal, the T-tubules seal off and lose electrical contact with sarcolemma.

Na+/K+ pump current measurements. Isolated ventricular and atrial myocytes were plated on laminin-coated glass coverslips and pretreated with 1 μM tachysagrin for 10 min. Control and detubulated myocytes were whole cell voltage-clamped using patch electrodes made from borosilicate glass capillaries, as previously described (6). When filled with the standard pipette solution, electrode resistance was 1.5–2.5 MΩ. Current signals were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Membrane capacitance (Cm) and series resistance were calculated from a 5-mV voltage step from a holding potential of −70 mV. The pipette solution contained (in mM): 30 NaCl, 70 NaOH, 70 aspartic acid, 20 K+-aspartate, 20 TEA-Cl, 1.15 CaCl2 (100 mM free Ca2+), pH = 7.2. IPump was activated in whole cell voltage-clamp by rapid switch from 0 to 4 mM external K+ at a holding potential of −20 mV. We have shown previously that the K+-activated current is ouabain sensitive (5). The external solution contained (in mM): 136 NaCl, 5 NiCl2, 2 BaCl2, 1 MgCl2, 5 HEPES, 5 glucose, ± 4 KCl, pH = 7.4.

Dose-dependent IPump inhibition by ouabain. After reaching the whole cell configuration, myocytes were held at −70 mV in K+-free external solution for at least 8 min, to allow equilibration of intracellular and pipette Na+. Then, IPump was activated at −20 mV by switching to 4 mM K+ external solution. Under these conditions, about 75% of the pumps is activated [assuming a Kp1 for external K+ of 1.5 mM (17)]. We then recorded the current, while adding increasing concentrations of ouabain. For each ouabain concentration, IPump
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\( \times \left( \frac{I_{\text{Pump}} - I_{\text{Pump OUa}}}{I_{\text{Pump}0}} \right) \), where \( I_{\text{Pump}0} \) 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 \( I_{\text{Pump OUa}} \) is \( I_{\text{Pump}} \) at the respective ouabain concentration. Mean data were fit with a two-binding site equation \( B_1 \times [\text{Ouabain}] / (K_1 + [\text{Ouabain}]) + (100 - B_1) \times [\text{Ouabain}] / (K_2 + [\text{Ouabain}]) \) 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, Northampton, MA) and the best fit was identified by a minimum \( \chi^2 \).

Statistical analysis. Data are expressed as means ± SE. Unpaired Student’s \( t \)-test was used for statistical discriminations, with \( P < 0.05 \) considered significant.

RESULTS

NKA functional density is significantly higher in the TT vs. ESL. The mean membrane capacitance of detubulated myocytes was 120 ± 8 pF (Fig. 1A). Upon detubulation, the TT seal off and lose electrical contact with sarcolemma; thus, \( C_m \) measured in detubulated cells is the capacitance of the membrane in the external sarcolemma. In control myocytes, both external sarcolemma and the TT contribute to the measured \( C_m \) (164 ± 6 pF). Thus, the difference between control and detubulated myocytes (44 pF) is the \( C_m \) of the membrane in the TT (Fig. 1A). These data show that the TT represent about 27% of the total sarcolemma, similar to our previous measurements (6) and electron microscopic morphological data in rat ventricular myocytes (18–20). Also, imaging of detubulated myocytes after staining with membrane-associated fluorescent dyes confirmed lack of TT (not shown). Thus, we are confident that our measurements in detubulated cells reflect almost exclusively external sarcolemma.

To measure \( I_{\text{Pump}} \), myocytes were held at −20 mV and NKA was activated by rapidly switching from 0 to 4 mM K\(^+\) in the external solution. Figure 1B shows that \( I_{\text{Pump}} \) was significantly lower in detubulated vs. control myocytes (122 ± 10 pA vs. 203 ± 9 pA). In control myocytes, \( I_{\text{Pump}} \) is generated by NKA present on both ESL and TT, whereas \( I_{\text{Pump}} \) in detubulated cells is due to NKA present on the ESL. The difference (81 ± 18 pA) represents \( I_{\text{Pump}} \) originating from the TT. Thus, 40 ± 7% of \( I_{\text{Pump}} \) originates from the TT, although they contain only 27% of the sarcolemma. This indicates that NKA is function-
ally concentrated in the TT. Indeed, I_{Pump} density was significantly higher in the TT vs. ESL (1.8 ± 0.3 vs. 1.02 ± 0.05 pA/pF; Fig. 1C). In atrial myocytes, which lack TT, the same formamide treatment affected neither the cell capacitance (47 ± 6 pF vs. 48 ± 4 pF in control cells) nor I_{Pump} density (0.94 ± 0.06 vs. 0.98 ± 0.05 pA/pF; five formamide-treated and seven control cells). This further indicates that changes observed in ventricular myocytes are entirely due to detubulation (vs. a direct effect of the formamide treatment). Interestingly, I_{Pump} density in atrial cells is similar to that in detubulated ventricular myocytes.

NKA-α₂ has a larger contribution to I_{Pump} in control vs. detubulated myocytes. Rat NKA-α₁ and NKA-α₂ have distinct ouabain affinities, with NKA-α₁ having a low affinity and NKA-α₂ having a high affinity. This allowed us to determine the fraction of I_{Pump} generated by NKA-α₁ and NKA-α₂ by measuring the dose-dependent I_{Pump} inhibition by ouabain in control and detubulated myocytes. Fig. 2A shows representative traces recorded in a control and a detubulated myocyte. At low concentrations (≤5 μM), ouabain had a significantly smaller effect in the detubulated cell, whereas at higher concentrations the effect was similar in control and detubulated myocytes.

Mean dose-response curves of I_{Pump} inhibition by ouabain in control and detubulated rat ventricular myocytes are shown in Fig. 2B. Data could be fit with a two-binding site equation, which indicates the presence of two ouabain binding sites: a high-affinity site, likely to be due to NKA-α₂, and a low-affinity site, which can be attributed to NKA-α₁ (Fig. 3A). There was no significant difference in the ouabain K_{1/2} for either NKA-α₁ (138 ± 17 vs. 145 ± 15 μM) or NKA-α₂ (0.36 ± 0.13 vs. 0.40 ± 0.17 μM) between control and detubulated myocytes (Fig. 3B). Thus, data were refitted with the same K_{1/2} for both NKA-α₁ and NKA-α₂, yielding K_{1/2} = 141 ± 17 and 0.38 ± 16 μM, respectively. These values are somewhat different from those found by Ishizuka et al. (12)—43 ± 14 μM and 1.0 ± 0.7 μM—maybe due to slight differences in the experimental protocol.

For ouabain concentrations in the 0.4–10 μM range, in which most of NKA-α₂ is inhibited, but there is little block of NKA-α₁, the dose-response curve in detubulated myocytes shows less I_{Pump} inhibition than in control cells (Fig. 2B). This suggests that there are fewer NKA-α₂ pumps in detubulated vs. control myocytes. The relative contribution of NKA-α₁ and NKA-α₂ to I_{Pump} was determined from the fit with a two-binding site equation. NKA-α₂ accounted for 29.5 ± 1.3% of

![Fig. 3](http://ajpcell.physiology.org/)
I_pump in control and only 18.2 ± 1.1% in detubulated myocytes (Fig. 3C). This suggests that the functional density of NKA-α2 is higher in TT vs. ESL.

On the basis of the contribution of NKA-α1 and NKA-α2 to I_pump (Fig. 3C) and the size of I_pump in control and detubulated myocytes (Fig. 1B), we estimated the relative number of functional NKA-α1 and NKA-α2 in the entire sarcolemma (ESL + TT), ESL, and TT (Fig. 4A). We found a larger NKA-α2 amount in TT vs. ESL (despite the smaller surface area of the TT vs. ESL). We then calculated the NKA-α1 and NKA-α2 density (number of pumps/μm²) in the entire sarcolemma (ESL + TT), ESL, and TT (Fig. 4, B and C), assuming that the total pump density in control myocytes is 1,000 pumps/μm² or a maximal turnover rate of 100/s (2). NKA-α1 is rather uniformly distributed between the TT (800 ± 370/μm²) and ESL (670 ± 70/μm²), whereas NKA-α2 is 4.5-fold concentrated in the TT (680 ± 120 vs. 150 ± 20/μm² in the external sarcolemma). This estimation of actual NKA-α1 vs. NKA-α2 site density assumes that the two isoforms have similar turnover rates, i.e., I_pump is proportional to the number of functional NKA molecules (although there are contradictory reports regarding this assumption) (4, 25). If turnover rates do differ between NKA-α1 and NKA-α2, the relative number of α1 vs. α2-pumps in the TT and ESL would change, but that does not affect our conclusion regarding the functional distribution of NKA-α1 and NKA-α2 in the TT vs. ESL.

**DISCUSSION**

There is controversy as to where NKA-α1 and NKA-α2 are located in ventricular myocytes and whether they have different physiological roles. Immunofluorescence studies (16) suggested that in rat ventricular myocytes NKA-α1 is preferentially distributed in the TT and NKA-α2 is homogeneously

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**A**

Number of Pumps (%)

- ESL + TT
- ESL
- TT

**B**

Density

- ESL + TT
- ESL
- TT

**C**

- α1: 670/μm²
- α2: 150/μm²
- α1: 800/μm²
- α2: 880/μm²

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Fig. 4. Estimated number (A) and density (B and C) of functional NKA-α1 and NKA-α2 in the entire sarcolemma (ESL + TT), ESL, and TT. A: we used the ratio between I_pump measured in detubulated and control myocytes to calculate the relative number of pumps (including both NKA-α1 and NKA-α2) in the external sarcolemma. The remaining pumps are located in the T-tubules. Then, we used the relative contribution of NKA-α1 and NKA-α2 to I_pump in control and detubulated myocytes (see Fig. 3C) to determine the number of NKA-α1 and NKA-α2 in ESL + TT and ESL, respectively. The difference between total sarcolemma and ESL was the number of NKA-α1 and NKA-α2 in the TT. B: these numbers were divided by the membrane capacity of each component (see Fig. 1A) to derive the pumps density (as % divided by pF) in the ESL + TT, ESL, and TT. The result was converted in terms of pumps/μm² assuming that the total pump density in control myocytes is 1,000 pumps/μm² (2) and 1 μF/cm².

C: cartoon showing the estimated NKA-α1 and NKA-α2 density in the TT and ESL.
distributed in the TT and external sarcolemma. Conversely, in guinea pig myocytes, NKA-α1 was localized predominantly in the peripheral sarcolemma, while NKA-α2 was mainly in the TT (21). The differential sensitivity of NKA-α1 and NKA-α2 antibodies limits the interpretation of these data to qualitative description, without the possibility of comparing quantitatively the density of NKA-α1 and NKA-α2 in the TT and external sarcolemma. Furthermore, immunofluorescence data do not give information about the function of NKA isoforms in the TT vs. ESL. So far, there are no quantitative determinations of the relative NKA-α1/NKA-α2 distribution in the TT vs. external sarcolemma in cardiac myocytes, except our recent study in mouse ventricular myocytes using the same approach used here for rat (1).

Here, we investigated the functional distribution of NKA-α1 and NKA-α2 between the TT and external sarcolemma by taking advantage of 1) the different affinity for ouabain of NKA-α1 and NKA-α2 in rat and 2) our ability to detubulate ventricular myocytes and thus measure the I_pump generated by NKA located in the external sarcolemma only. We found that the functional density of NKA-α2 is ~4.5 times higher in the TT vs. external sarcolemma, whereas NKA-α1 is practically uniformly distributed between the TT and ESL (Fig. 4, B and C). However, the amount of NKA-α1 and NKA-α2 in the TT is comparable (Fig. 4). Our results here for rat are qualitatively similar to our recent results in mouse (in which 12% of total and 6% of surface NKA function was attributable to NKA-α2 and that NKA-α2 function was 5.3 times higher in T-tubular relative to ESL. So far, there are no quantitative determinations of the relative NKA-α1/NKA-α2 distribution in the TT vs. ESL. So far, there are no quantitative determinations of the relative NKA-α1/NKA-α2 distribution in the TT vs. external sarcolemma in cardiac myocytes, except our recent study in mouse ventricular myocytes using the same approach used here for rat (1).

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GRANTS

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