Mechanisms of Isoform-Specific Na/K Pump Regulation by Short- and Long-Term Adrenergic Activation in Rat Ventricular Myocytes

Jian Yin\textsuperscript{a,b} Hui-Cai Guo\textsuperscript{a,c} Ding Yu\textsuperscript{d} Hui-Ci Wang\textsuperscript{a,b} Jun-Xia Li\textsuperscript{a,b} Yong-Li Wang\textsuperscript{a,b}

\textsuperscript{a}The Key Laboratory of Pharmacology and Toxicology for New Drugs, \textsuperscript{b}Department of Pharmacology, \textsuperscript{c}Department of Toxicology, Hebei Medical University, \textsuperscript{d}Department of Cardiology, The Second Hospital of Hebei Medical University, Shijiazhuang, China

Key Words
Na\textsuperscript{+}•K\textsuperscript{+}-ATPase • Cardiomyocyte • Adrenoceptor • Long-term regulation • Rat

Abstract

Background: Many stressful conditions, including cardiovascular diseases, induce long-term elevations in circulating catecholamines, thereby leading to changes of the Na/K pump and thus affecting myocardial functions. However, only short-term adrenergic regulation of the Na/K pump has been reported. The present study is the first investigation of long-term adrenergic regulation of the Na/K pump and the potential mechanism. Methods: After acutely isolated Sprague-Dawley rat myocytes were incubated with noradrenaline or isoprenaline for 24 h, Na/K pump high- (\(I_{\text{PH}}\)) and low-affinity current (\(I_{\text{PL}}\)), \(\alpha\)-isoform mRNA, and \(\alpha\)-isoform protein were examined using patch-clamp, RT-PCR, and Western blotting techniques, respectively. Results: After the short-term incubation, isoprenaline reduced the \(I_{\text{PL}}\) through a PKA-dependent pathway that involves \(\alpha\)\(_{1}\)-isoform translocation from the membrane to early endosomes, and noradrenaline increased the \(I_{\text{PH}}\) through a PKC-dependent pathway that involves \(\alpha\)\(_{2}\)-isoform translocation from late endosomes to the membrane. After long-term incubation, isoprenaline increased the \(I_{\text{PL}}\), \(\alpha\)\(_{1}\)-isoform mRNA, and \(\alpha\)\(_{1}\)-isoform protein, and noradrenaline reduced the \(I_{\text{PH}}\), \(\alpha\)\(_{2}\)-isoform mRNA, and \(\alpha\)\(_{1}\)-isoform protein through a PKA- or PKC-dependent pathway, respectively. Conclusions: These results suggest that long-term adrenergic Na/K pump regulation is isoform-specific and negatively feeds back on the short-term response. Furthermore, long-term regulation involves transcription and translation of the respective \(\alpha\)-isoform, whereas short-term regulation involves the translocation of the available \(\alpha\)-isoform to the plasma membrane.
Introduction

The Na/K pump (Na+, K+-ATPase, NKP) is a ubiquitous plasma membrane enzyme that catalyses the ATP-dependent transport of three Na+ out of the cell and two K+ into the cell, thereby generating a net outward pump current (I_p) in nearly all eukaryotic cells [1]. The functional NKP is composed of a catalytic α-subunit and a glycosylated β-subunit. Four α-subunit isoforms (α_1-α_4) have been identified to date, and each has a unique tissue distribution and physiological role. The NKP α_1,isoform acts as a "housekeeper" to maintain low intracellular Na+ concentrations ([Na+]_i), whereas the NKP α_2,isoform regulates local [Na+] near the Na+, Ca2+-exchanger and thus governs cardiomyocyte Ca2+ levels and contractility [2, 3]. The NKP α-isofor-generated I_p is based on different cardiac glycoside affinities of the isoforms: the NKP α_1,isoform is cardiac glycoside-sensitive and generates the high-affinity pump current (I_pH), whereas the NKP α_2,isoform is cardiac glycoside-resistant and generates the low affinity pump current (I_pl) [4, 5]. The I_p is the sum of the I_pH and I_pl and is defined as the total NKP current. Furthermore, the I_p is a significant component of the total net current during the plateau phase of the cardiac action potential [5]. Hence, the regulation of NKP function may directly affect the [Na+]_i and the duration of the plateau phase and thus myocardial functions, such as rhythm, conduction, and contraction force.

The NKP can be regulated by various factors, including adrenergic neurotransmitters. Several studies from Mathias's laboratory demonstrated that the adrenoreceptor can regulate the NKP. After a short-term (10 min) treatment of guinea pig myocytes, α-adrenoreceptor (α-AR) agonists increased the I_pH without affecting the I_pl, and β-adrenoreceptor (β-AR) agonists inhibited the I_pH at low intracellular Ca2+ concentrations ([Ca2+]_i) but did not affect the I_pl [5-8]. However, although some evidence has indicated no effect [9, 10] or even inhibition during β-AR activation [5, 11, 12], most results demonstrate α_1- or α_2,-isoform stimulation after α-AR or β-AR activation [13-17]. These data are discrepant with the results from Mathias's laboratory, in which the short-term α-AR or β-AR agonist exposure had opposite effects on the I_p by affecting specific NKP α-isoforms. Therefore, it is necessary to further confirm whether α- and β-AR activation has opposite and isoform-specific effects on the NKP in rat ventricular myocytes similar to guinea pig ventricular myocytes.

Many stressful conditions, including various cardiovascular diseases, such as chronic heart failure, myocardial ischaemia, myocardial hypertrophy, Takotsubo stress cardiomyopathy, and hypertension, induce a long-term elevation in circulating catecholamines, thereby altering NKP activity, which affects myocardial function. For example, diminished myocardial NKP activity and ouabain binding sites have been observed in patients and experimental animals with congestive heart failure [18-21], and cardiac ischaemia-induced α,-adrenergic activation decreases automaticity, resulting from α,-receptor-mediated stimulation of an outward current that is carried by the NKP, as well as a decrease in intracellular sodium levels [22-25]. These data indicate that this alteration in NKP activity is caused, at least partially, by long-term heightened sympathetic nervous system activity. However, there are few reports regarding the regulatory effects of long-term adrenergic activation on the NKP. The present study sought to investigate the regulatory effect of long-term adrenergic activation on the NKP and the respective mechanisms of short- and long-term adrenergic regulation in isolated rat ventricular myocytes.

Materials and Methods

Acute myocyte isolation

All of the experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and were reviewed and approved by the Ethics Committee for the Use of Experimental Animals at Hebei Medical University (Permit Number: SCXK (HEBEI): 2008-1-003). Adult Sprague-Dawley rats (270 ± 20 g; provided by the Experimental Animal Center of Hebei Province, China) were anaesthetised with an injection of 1.2%...
sodium pentobarbital. The hearts were rapidly excised, mounted on a gravity-driven Langendorff perfusion apparatus, and perfused at 37 °C with Ca²⁺-free Tyrode's solution containing the following (in mM): NaCl 140.0 (Sigma), KCl 5.4 (Sigma), MgCl₂ 1.0 (Sigma), HEPES 10.0 (Merck, Germany), and D-glucose 10.0 (Sigma) that had been equilibrated with 100% O₂ (pH 7.4, adjusted with NaOH (Sigma)). This procedure was followed by 0.6% collagenase II perfusion (GIBCO, USA) in Ca²⁺-free Tyrode's solution for 15 min to digest the heart. Next, a piece of ventricle was cut out and teased into smaller pieces in Kraft-Brühe solution containing the following (in mM): KOH 80.0 (Sigma), KCl 40.0, KH₂PO₄ 25.0 (Sigma), MgSO₄ 3.0 (Sigma), L-glutamic acid 50.0 (Alfa Aesar, Germany), taurine 20.0 (Sigma), HEPES 10.0, EGTA 1.0 (Sigma), and D-glucose 10.0 (pH 7.2, adjusted with KOH). All of the solutions were bubbled with 100% O₂. The isolated cells were kept in Kraft-Brühe solution at room temperature for at least 1 h before the experiment. All of the experiments were performed within 36 h after ventricular myocyte isolation [26].

**Chemicals and treatments**

All of the chemicals were purchased from Sigma. The α-AR was activated with noradrenaline after blocking the β-AR with propranolol. The β-AR was activated with isoprenaline, and prazosin was used to block the α₁-AR. Stock solutions of 10 mM noradrenaline, 1 mM isoprenaline, 10 mM propranolol, 1 mM prazosin, 1 M strophanthidin, 50 mM dihydrouabain (DHO), 1 mM staurosporine, 10 mM H89, and 1 mM phalloidin were prepared with Milli-Q UF Plus water (Millipore) and stored at -20°C. The working concentrations were 1 μM isoprenaline, 10 μM noradrenaline, 10 μM propranolol, 1 μM prazosin, 1 μM staurosporine, 10 μM H89, and 1 μM phalloidin.

The cells were co-incubated with the α-AR agonist noradrenaline and propranolol or the β-AR agonist isoprenaline for 10 min (short-term) or 24 h (long-term) at room temperature. The other drugs were applied in the same manner as the noradrenaline and isoprenaline.

**NKP current (IP) determination**

We applied the whole-cell patch-clamp technique to examine the effects of α-AR and β-AR agonists on the I_p in rat ventricular myocytes. α-AR activation was achieved by applying 10 μM noradrenaline in the presence of a β-AR blocker (10 μM propranolol), and β-AR activation was achieved by applying 1 μM isoprenaline. The I_p generated by the NKP α₂- and α₁-isofoms were distinguished as the I_pα₂ and I_pα₁, respectively, based on their affinities for cardiac glycosides; 5 μM DHO was used to detect the I_pα₂ and 500 μM strophanthidin was used to detect the I_pα₁ in the presence of 5 μM DHO [7]. An Axopatch 700B amplifier (Axon Instruments, Inc.) was used to observe the cell membrane current. Patch pipette resistances were 2-4 MΩ before sealing. The pipette solutions contained the following (in mM): sodium aspartic acid 70.0 (Sigma), potassium aspartic acid 20.0 (Sigma), CsOH 30.0 (Sigma), TEACl 20.0 (Sigma), MgSO₄ 7.0, HEPES 5.0, EGTA 11.0, D-glucose 10.0, Na₂-ATP 5.0 (Sigma), and CaCl₂ 1.0 (Sigma) (pH 7.2, adjusted with CsOH). The external solutions contained the following (in mM): NaCl 137.7, NaOH 2.3, KCl 5.4, MgCl₂ 1.0, HEPES 5.0, D-glucose 10.0, BaCl₂ 2.0 (Sigma), and CaCl₂ 1.0 (pH 7.4; Sigma). The osmolality of each solution was between 290 and 310 mosmol (kg solvent)⁻¹. For the external solutions, Ca²⁺ was omitted, and Cd²⁺ and Ba²⁺ were added to suppress K⁺ and Ca²⁺ currents and Na⁺-Ca²⁺ exchange. For the pipette solutions, K⁺ was omitted, and ATP was added to suppress K⁺ currents and activate the NKP in the forward mode.

The cells were held at 0 mV after the formation of the whole-cell recording configuration. External solutions containing 5 μM DHO or 500 μM strophanthidin and 1 μM isoprenaline, 10 μM noradrenaline, 10 μM propranolol, 1 μM prazosin, or 1 μM phalloidin were perfused at 1.5–2 ml/min to observe changes in the I_p. All of the patch-clamp data were recorded using the data acquisition program AxoScope 9.2 (Axon Instruments, Inc.) for subsequent analysis. The sampling rate was 200 ms/point, and the data were low-pass filtered at 2 kHz.

**Immunoblot**

**Whole cell lysate preparation.** The acutely isolated cells were first treated as described in the Chemicals and Treatments section and then lysed with ice-cold lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 (NP-40), 0.5% Triton X-100, 0.1% SDS, 10% glycerol, and 10 μl/ml protease inhibitors. The cells were disrupted by sonication on ice using 4 × 2-s bursts. The supernatant (whole cell lysate) was collected by centrifugation at 120,000 × g for 30 min at 4°C, and the protein concentration was determined using the BCA Protein Assay Kit (Pierce).
Plasma membrane protein preparation. After treating the cells with isoprenaline or noradrenaline (propranolol was co-incubated to block β-ARs) for 10 min, we used the Cell Surface Protein Isolation Kit (Thermo) to extract plasma membrane proteins according to the manufacturer’s guidelines. This method involves four main steps: biotinylation, cell lysis, labelled protein isolation, and protein elution. Briefly, the cells were washed with pre-cooled phosphate-buffered saline (PBS, pH 7.2), which contained 100 mM sodium phosphate and 150 mM NaCl. The cells were then incubated with 1 mg/ml sulfo-NHS-SS biotin in PBS and gently agitated for 2 h at 4°C on a rocking platform. Unbound biotin was quenched with quenching solution containing 100 mM glycine for 15 min. The cells were washed with ice-cold PBS, lysed with lysis buffer, and disrupted by sonication on ice using five 1-s bursts. Cell suspensions were incubated for 30 min on ice, and the supernatants were collected by centrifugation. Protein concentrations of the supernatants were determined using the BCA Protein Assay Kit (Pierce). The supernatants were incubated overnight at 4°C with an equal amount of pre-washed immobilised streptavidin beads and centrifuged in micro-tubes for 2 min at top speed to collect the beads. The beads were washed four times with wash buffer, and SDS-PAGE sample buffer containing 50 mM DTT and bromophenol blue was added to the beads and incubated for 60 min at room temperature with end-over-end mixing on a rotator. The plasma membrane protein fractions were collected by centrifugation and analysed by western blot.

Endosome preparation. Early and late endosomes were fractionated on a flotation gradient according to the technique described by Gorvel et al. [27]. The cells in suspension (1.5 mg protein/ml) were incubated with AR agonists for 10 min. The incubation was terminated by transferring the samples to ice and adding cold homogenisation buffer containing 250 mM sucrose and 3 mM imidazole (pH 7.4). To minimise endosomal damage, the cells were gently homogenised, and the homogenate was subjected to a brief (5 min) centrifugation (4°C at 3,000 × g). The supernatant was collected and adjusted to 40.6% sucrose. The supernatant (1.5 ml) was then pipetted onto the bottom of a 5.0-ml centrifuge tube. Then, 1.5 ml of 16% sucrose, 1 ml of 10% sucrose, and 0.5 ml of homogenisation buffer were sequentially overlaid to form a density gradient. Density gradient centrifugation was performed at 110,000 × g for 60 min at 4°C in a Beckman ultracentrifuge. Early endosomes (EE) were collected at the 16%-10% sucrose interface, while late endosomes (LE) were collected at the homogenisation buffer-10% sucrose interface. The isolation of EE and LE was confirmed by immunoblotting with antibodies against rab5 and lamp1 proteins, markers for EE and LE, respectively.

Western blot analysis

Equal amounts of protein were separated by electrophoresis on 10% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA), and blocked for 1 h at 37°C in 5% non-fat dry milk in TBST. The following primary antibodies were used: NKP α-isoform (Santa Cruz, 1:500), NKP α-isoform (Upstate, 1:200), α-AR (Abcam, 1:500), and β-AR (Abcam, 1:500). Anti-mouse and anti-rabbit horseradish peroxidase-conjugated IgGs (KPL, 1:1000) were used as secondary antibodies. The blots were developed using the enhanced DAB chromogenic reagent kit. The signal intensity of each lane was quantified by densitometry; the drug-treated sample intensity was compared with the control sample intensity.

mRNA level determination

Total RNA was extracted from adult rat myocytes using the SV Total RNA Isolation System (Promega) according to the manufacturer’s guidelines. RNA was eluted and immediately used as a template for cDNA synthesis. One microgram of total RNA was reverse transcribed using oligo (dT) primers with the Reverse Transcription System (Promega) according to the manufacturer’s guidelines. The PCR primers were synthesised by Invitrogen. The specific primers are listed in Table 1.

Reverse transcription polymerase chain reaction (RT-PCR)

The reverse transcription product (cDNA) was amplified using PCR with NKP α- and α-isoform-specific primers. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as a housekeeping gene. The PCR reactions contained 2× GoTaq Green Master Mix, 10 μM forward primer, 10 μM reverse primer, 2 μl of template cDNA, and nuclease-free water in a final volume of 50 μl. The PCR protocol was as follows: denaturation (94°C for 5 min), denaturation, amplification, and extension for 30 cycles (94°C for 40 s, 45°C for 1 min, and 72°C for 1 min). The PCR product was separated on a 1% agarose gel in TAE buffer. The gels were stained with ethidium bromide and visualised under UV light. The DNA bands were quantified by densitometry; the drug-treated sample intensity was compared with the control sample intensity.
for 45 s, and 72°C for 45 s, respectively), followed by a final extension (72°C for 10 min). The PCR products were analysed by electrophoresis on 2% agarose gels followed by GoldView II nucleic acid staining.

**Relative quantification of specific cDNAs by real-time PCR**

To ensure reliability of our results, we chose two housekeeping genes (β-actin and GAPDH). These primers are listed in Table 1. Real-time PCR was performed simultaneously for the NKP α₁- and α₂-isoforms, β-actin, and GAPDH in 48-well optical reaction plates in triplicate. Each reaction contained 1× SYBR Premix E, Taq (TaKaRa), 10 μM forward and reverse primers, and 2 µl of template cDNA in a final volume of 25 µl. The cycle conditions were as follows: denaturation (94°C for 5 min), denaturation, amplification, extension, and quantitation for 50 cycles (94°C for 40 s, 45°C for 45 s, and 72°C for 45 s, respectively, with a single fluorescence measurement), and a melt curve was performed at 60–95°C with a heating rate of 0.5°C/s and continuous fluorescence measurement. Cycle threshold (Cₜ) values were used to calculate the NKP α₁- and α₂-isoform mRNA expression relative to the housekeeping genes. All of the real-time PCR data were saved for subsequent analysis using the relative quantification method.

**Statistical analysis**

The data are expressed as the mean ± SE. The statistical discriminations were performed using Student’s t-tests (paired and unpaired) or one-way ANOVA as appropriate, and P < 0.05 was considered to be significant.

**Results**

**Short-term adrenergic activation regulates NKP activity in an isoform-specific manner**

Rat ventricular myocytes express both the α₁- and α₂- NKP isoforms. As the affinity for cardiac glucoside is approximately 100-fold lower for the α₁- than for the α₂-isoform, the two α-isoforms can be assessed separately using different concentrations of cardiac glucoside; 5 μM DHO inhibits the α₂-isoform only, and 500 μM strophanthidin (or 1 mM DHO) inhibits both α-isoforms. Thus, when the NKP-generated Iᵦ was assessed using the patch-clamp technique, the current that was sensitive to 5 μM DHO was identified as the α₂-isoform-generated Iᵦ and the current that was sensitive to 500 μM strophanthidin in the presence of 5 μM DHO was identified as the α₁-isoform-generated Iᵦ. Figure 1A and 1B show the levels of Iᵦ and Iᵦ, respectively. The averages of the control Iᵦ and Iᵦ were 29.3 ± 8.1 pA (n = 7) and 12.2 ± 4.3 pA (n = 10), respectively. α-Adrenergic activation was achieved by applying 10 μM noradrenaline in the presence of a β-blocker (10 μM propranolol), and β-adrenergic activation was achieved by applying 1 μM isoprenaline. The Iᵦ value was normalised to the value obtained in the control condition. Our data demonstrated that short-term β-adrenergic activation reduced the Iᵦ but did not affect the Iᵦ whereas short-term α-adrenergic activation had no effect on the Iᵦ but increased the Iᵦ (Fig. 1). These findings suggest that short-term α- and β-adrenergic activation have opposite effects on NKP activity.
Short-term adrenergic activation does not alter NKP α-isoform mRNA or protein levels

NKP activity alterations are generally associated with changes in their plasma membrane protein levels, apparent Na affinity, and/or \( V_{\text{max}} \). To investigate the mechanism of the short-term adrenergic stimulation effect on NKP activity, we first assessed \( \alpha_1 \)- and \( \alpha_2 \)-isoform mRNA and protein expression levels by RT-PCR, quantitative real-time PCR, and western blotting to explore whether the short-term adrenergic activation NKP activity alterations are associated with changes in their mRNA or protein expression levels. The results from both PCR techniques revealed that \( \alpha_1 \)- and \( \alpha_2 \)-isoform mRNA levels were not affected by short-term \( \alpha \)- and \( \beta \)-adrenergic activation. Furthermore, western blotting analysis demonstrated that short-term \( \alpha \)- and \( \beta \)-adrenergic activation failed to alter \( \alpha_1 \)- and \( \alpha_2 \)-isoform protein expression levels (Fig. 2). These data suggest that the effects of short-term adrenergic activation on NKP activity do not result from changes in NKP α-isoform mRNA or protein expression levels.

Short-term adrenergic activation alters the plasma membrane to endosomal NKP ratio

Certain studies have indicated that membrane protein internalisation from the surface to form early and late endosomes can decrease current intensity without affecting total

\[ \text{Fig 1. The effects of short-term adrenergic activation on Na/K pump current (I}_p\text{) The cells were held at 0 mV, and the I}_p\text{ was assessed using the patch-clamp technique. A) In the presence of 5 μM dihydroouabain (DHO) to block high-affinity I}_p\text{, 0.5 mM strophanthidin (Str) treatment was used to demonstrate that 1 μM isoprenaline (ISO) treatment inhibited low-affinity I}_p\text{ and 10 μM noradrenaline (NA) treatment (with 10 μM propranolol (Pro) to block the β-adrenoreceptor) had no effect on low-affinity I}_p\text{. B) 5 μM DHO treatment was used to demonstrate that ISO treatment had no effect on high-affinity I}_p\text{ and that NA treatment increased high-affinity I}_p\text{. C) The low affinity I}_p\text{ was reduced by ISO treatment, and the high affinity I}_p\text{ was increased by NA treatment. The normalised I}_p\text{ represents the I}_p\text{ value normalised to its control value (Con). Each column represents the mean value ± SE, and the number of myocytes studied is indicated within the column and comes from at least 4 different hearts. *P < 0.05, statistically significant relative to the Control.} \]
Yin et al.: Adrenergic Regulation of Cardiac NKP

protein expression [27-29]. Our results demonstrated that altered NKP activities are not associated with changes in α-isoform mRNA and protein expression levels following short-term adrenergic activation. As the western blots were only able to quantify total whole-cell NKP protein expression levels, it remained undetermined whether plasma membrane (active) NKP levels were affected. Therefore, we assessed NKP plasma membrane protein levels using protein biotinylation. After short-term α- or β-adrenergic activation, isolated myocytes were subjected to protein biotinylation to label surface NKP, and the amount of biotinylated NKP was estimated by western blot analysis. Our results demonstrated that short-term noradrenaline-mediated α-adrenergic activation increased α₂- but not α₁-isoform abundance on the plasma membrane, which was negated by incubation with prazosin, an α-AR antagonist (Fig. 3A). This increase was accompanied by a corresponding decrease in α₂- but not α₁-isoform abundance in late endosomes but not in early endosomes (Fig. 3B). These results suggest that short-term α-AR activation increases incorporation of the plasma membrane α₂-isoform, which may result from α₂-isoform recycling from late endosomes to the plasma membrane. Short-term isoprenaline-mediated β-adrenergic activation reduced
α1- but not α2-isoform plasma membrane levels, which was reversed by incubation with the β-AR antagonist propranolol (Fig. 3A). This reduction was accompanied by increased NKP α1- but not α2-isoform expression in early endosomes but not in late endosomes (Fig. 3B). These results imply that short-term β-AR activation decreases plasma membrane incorporation of the α1-isoform, which may be a consequence of NKP α1-isoform translocation from the plasma membrane into early endosomes. Overall, these results suggest that short-term adrenergic activation alters the percentage of plasma membrane-localised NKP in cardiac myocytes by re-distributing the existing NKP from the plasma membrane and endosomal pools and does not affect NKP protein expression levels of either isoform in cardiomyocytes.

**Phallolidin blocks actin-mediated NKP trafficking between the plasma membrane and the endosomes**

Cells continuously internalise proteins from the plasma membrane to form early and late endosomes, which are then either recycled back to the membrane or transported to the lysosomes for degradation [30]. Actin polymerisation and depolymerisation provide a driving force for plasma membrane internalisation during endocytosis [31] as well as for endosomal...
and Golgi-derived vesicular movement [32]. If the equilibrium of NKP protein recycling between the plasma membrane and the endosomes is altered by short-term adrenergic activation, blocking actin-mediated protein trafficking may prevent this recycling. To test this hypothesis, the following series of experiments was performed in phallloidin-pretreated cardiomyocytes, which stabilises cytoskeletal actin and affects protein translocation. We used phallloidin to inhibit actin depolymerisation and protein trafficking and assessed NKP function with the patch-clamp technique and NKP plasma membrane and endosomal density with biotin labelling. Our data demonstrated that in the presence of phallloidin, short-term treatment with α- and β-AR agonists did not induce any IP changes (Fig. 4A), nor did the treatment alter plasma membrane or endosomal α-isoform levels (Fig. 4B). These results provide further evidence that the short-term adrenergic activation-mediated NKP α-isoform translocation results from actin-mediated NKP trafficking between the plasma membrane and the endosomes. Thus, the number of “working” pumps on the plasma membrane is changed without affecting total protein expression levels.
Long-term adrenergic activation affects NKP activity in an isoform-specific manner

To investigate the effects of long-term adrenergic activation on α₁- and α₂- NKP isoform activity, rat ventricular myocytes were isolated and incubated with isoprenaline or noradrenaline + propranolol at room temperature for 24 h, and NKP activity was assessed using the patch-clamp technique. The I_p density was defined as the ratio of I_p to capacitance. Whole cell lysates were prepared, equal protein amounts were loaded for each sample, and western blot analysis was used to analyse AR and NKP α-isoform expression. AR density and α-isoform values were normalised to their corresponding Con. A) ISO treatment increased the low-affinity I_p but had no effect on high-affinity I_p, and NA treatment reduced the high-affinity I_p but had no effect on the low-affinity I_p. B) Western blot analysis using specific α₁-AR and β₁-AR antibodies; both ISO and NA treatment failed to alter α₁- and β₁-AR expression. C) Western blot analysis using specific anti-α₁ and anti-α₂ antibodies. ISO treatment up-regulated α₁-isoform levels, which was reversed by 10 μM Pro treatment, but α₂-isoform expression remained unaltered, whereas NA treatment down-regulated α₂- but not α₁-isoform levels, an effect that was reversed by 1 μM PZ treatment. Each column represents the mean value ± SE, and the number of myocytes studied is indicated within the columns and comes from at least 3 different hearts. *P < 0.05, statistically significant relative to the Control.
Long-term adrenergic activation does not alter adrenoreceptor expression

Because long-term $\alpha_1$- and $\beta$-adrenergic activation negatively feeds back on myocardial adrenoreceptor expression [33-36], we further investigated whether long-term adrenergic activation negatively feeds back on adrenoreceptor expression (i.e., whether the long-term negative feedback on the $I_p$ is a result of $\alpha_1$-AR and $\beta_1$-AR up- or down-regulation). Western blotting indicated that $\alpha_1$- and $\beta$-adrenergic activation for 24 h failed to alter $\alpha_1$-AR and $\beta_1$-AR protein expression levels (Fig. 5B), suggesting that the negative feedback of long-term $\alpha_1$- and $\beta$-AR activation on the $I_p$ might be unrelated to modified myocardial $\alpha_1$- and $\beta_1$-AR expression.
We next assessed $\alpha_1$- and $\alpha_2$-isoform mRNA and protein expression levels to explore the mechanism by which long-term adrenergic stimulation effects NKP activity. RT-PCR and quantitative real-time PCR were performed to examine NKP mRNA levels. The results (Fig. 6) demonstrated that following 24 h of $\beta$-adrenergic activation, $\alpha_1$-isoform mRNA levels were significantly up-regulated, while $\alpha_2$-isoform mRNA levels were unaltered. In contrast, $\alpha_2$- but not $\alpha_1$-isoform mRNA levels were significantly down-regulated after 24 h of $\alpha$-adrenergic activation. Western blots (Fig. 5C) from cardiac protein lysates further demonstrated that following 24 h of $\beta$-adrenergic activation, $\alpha_1$-isoform protein levels were significantly up-regulated, while the $\alpha_2$-isoform protein levels were unaltered. Conversely, $\alpha_1$- but not $\alpha_2$-isoform protein levels were significantly down-regulated after 24 h of $\alpha$-adrenergic activation. These data indicate that steady-state mRNA and protein levels were increased for the NKP $\alpha_1$-isoform and decreased for the $\alpha_2$-isoform, a result that corroborates those from the functional studies. Furthermore, NKP mRNA level alterations might result in subsequent NKP protein level changes, suggesting that NKP mRNA and protein level alterations could be the...
mechanism that underlies the isoform-specific regulation of NKP activity during long-term α- and β-adrenergic activation. The long-term β-adrenergic activation-mediated alterations in α₁-isofrom mRNA and protein levels were reversed by propranolol treatment, whereas the long-term α-adrenergic activation-mediated effects α₂-isofrom mRNA and protein levels were reversed by prazosin treatment, suggesting that the α₁- and β₁-ARs play a role in the events that decrease synthesis and/or increase degradation of the NKP α-isofroms.

PKA- and PKC-dependent pathways mediate the isoform-specific regulation of short- and long-term adrenergic NKP activation

Previously published studies from Mathias’s laboratory demonstrated that short-term β- and α-adrenergic Iₚ regulation is mediated by PKA and PKC, which are downstream β- and α-adrenergic activation signalling molecules, respectively, that play important roles in regulating α₁- and α₂-isofrom activities in an isoform-specific manner [6-8, 11]. Here, we further assessed whether these downstream signalling pathways were also involved in the short-term adrenergic stimulation-mediated effects on the NKP α-isofrom plasma membrane and endosomal localization as well as the long-term adrenergic stimulation-mediated effects on the α-isofrom in whole cells. We applied H89 and staurosporine to inhibit PKA and PKC, respectively. As demonstrated in Figure 7, H89 but not staurosporine treatment completely reversed the short-term effects of isoprenaline treatment on the α₁-isofrom translocation, and staurosporine but not H89 treatment blocked the short-term effect of noradrenaline treatment on the α₁-isofrom translocation (Fig. 7A). Furthermore, PKC but not PKA inhibition reversed the long-term α-adrenergic activation-induced reduction in α₂-isofrom expression, and PKA but not PKC inhibition abolished the long-term β-adrenergic activation-mediated increase in α₁-isofrom expression (Fig. 7B). These results indicate that in rat ventricular myocytes, the effect of both short- and long-term β-AR activation on the α₁-isofrom involves the PKA-dependent pathway, and the effect of α-AR activation on the α₂-isofrom involves the PKC-dependent pathway. Although there are opposite consequences, the signalling pathways that are involved in the effects of long-term adrenergic activation on NKP α-isofrom protein expression are the same compared with the short-term effects of adrenergic agonists on NKP α₁- and α₂-isofrom translocation. These PKC- and PKA-dependent changes in mRNA and protein expression after adrenergic activation also provide further evidence supporting similar Iₚ characteristics that were observed in guinea pig ventricular myocytes [6-8, 11].

Discussion

The present study not only further confirmed that short-term α-AR and β-AR activation increased α₂-isofrom-generated Iₚ and decreased the α₁-isofrom-generated Iₚ, respectively, in rat myocytes similar to guinea pig ventricular myocytes [6-8, 11] but also demonstrated that long-term α-AR activation reduced α₂-isofrom-generated Iₚ and long-term β-AR activation increased the α₁-isofrom-generated Iₚ. These results suggest that either short- or long-term α- and β-AR activation mediate opposite effects on the Iₚ in an α-isofrom-specific manner, which may provide a balance between α- and β-AR activation on NKP function in cardiac myocytes [7]. Furthermore, the effect of either long-term α- or β-AR activation on the Iₚ negatively feeds back on the short-term response, which may reduce arrhythmia risk in patients with cardiovascular diseases by limiting the elevated circulating catecholamine-induced [Na] increase in stressful conditions.

In human hearts, all of the three α-isofroms are detected, and the estimated stoichiometric distribution is the α₁-isofrom being dominant (62%) over the α₂-isofrom (15%) and the α₃-isofrom (23%) [37]. Moreover, the inhibition of Iₚ by DHO are also present in the cells, and the dissociation constants for inhibition by DHO are similar for the three α-isofroms [38]. The Iₚ averaged from 12 atrial cells in the control were 0.29 ± 0.06 pA/pF [39], which was similar to the Iₚ (0.33 ± 0.04 pA/pF) in this study and was significantly increased by α- but not β-adrenergic activation [39]. These results suggest that the Iₚ in human atrial cells
has similar properties to the $I_{\text{pH}}$ in rat and guinea pig ventricle cells. Moreover, our results in Figure 1C indicated a 26% increase of $I_{\text{pH}}$ by $\alpha$-adrenergic activation, which is similar to the 24% increase in $I_{\text{pH}}$ observed in human atrial myocytes [39]; therefore, the $\alpha_2$-isoform is responsible for approximately 100% of the $I_{\text{pH}}$ in human atrial myocytes, which is not consistent with this study in rat ventricular myocytes (29.5%) and the $I_{\text{pH}}$ in most reports (10–20%) [40, 41]. Moreover, $\beta$-adrenergic activation had no effect on the $I_{\text{pH}}$ in human atrium, which is also not consistent with the presence of the $\alpha_1$-isoform, unless $\alpha_1$-isoform regulation in human atrium differs from that in rat and guinea pig ventricle.

Generally, the mechanism by which adrenergic regulation activates NKP is associated with a change in “working” NKP levels at the cell surface, which depends on the translocation and/or synthesis of $\alpha$-isoform proteins. However, the present result indicates that long- but not short-term adrenergic activation altered whole cell $\alpha_1$- and $\alpha_2$-isoform protein or mRNA expression. It is possible that different $\alpha$-isoform pools (plasma membrane and endosomal) are recruited for plasma membrane insertion or translocated to endosomes after plasma membrane invagination [28, 42]. Expectedly, short-term $\alpha$-AR activation increased plasma membrane $\alpha$-isoform incorporation accompanied with a decrease of $\alpha_2$-isoform in late endosomes, and short-term $\beta$-AR activation decreased the content of plasma membrane $\alpha_1$-isoform accompanied with an increase of $\alpha_1$-isoform in early endosomes. These effects were prevented by stabilising the actin cytoskeleton with phalloidin, suggesting that short-term adrenergic activation alters the percentage of plasma membrane-localised NKP in cardiomyocytes by re-distributing the existing NKP. These results indicate that the short-term $\alpha$-AR and $\beta$-AR activation-mediated increase in the $I_{\text{pH}}$ and reduction in the $I_{\text{pH}}$ cannot be attributed to $\alpha_1$-isoform synthesis or $\alpha_2$-isoform degradation in whole cells. Rather, this effect may be attributed to actin-mediated NKP protein trafficking between the plasma membrane and the endosomes to alter the number of functional plasma membrane-localised NKP without affecting total protein expression levels. Therefore, the short-term adrenergic activation-mediated NKP regulatory mechanism involves pre-existing NKP $\alpha$-isoform translocation between the plasma membrane and the endosomes in an isoform-specific manner, which explains the isoform-specific regulation of the $I_{\text{pH}}$ by short-term adrenergic activation.

Another important finding of the present study is that long-term adrenergic regulation of the $I_{\text{pH}}$ and NKP $\alpha$-isoform abundance by either $\alpha$- or $\beta$-AR activation negatively feeds back on the short-term response. Considering that negative feedback is generally associated with altered effector density and that prolonged exposure to adrenergic agonists may up- or down-regulate adrenoreceptor density [34, 43, 44], we investigated whether long-term $\alpha$- and $\beta$-adrenergic activation altered the respective receptor amounts. However, our data indicated that $\alpha$- and $\beta$-adrenergic activation for 24 h failed to alter $\alpha_1$- and $\beta$-AR expression levels, implying that the negative feedback on the $I_{\text{pH}}$ by long-term $\alpha$- and $\beta$-AR activation may be because of altered NKP $\alpha$-isoform mRNA and protein expression levels but not myocardial $\alpha_1$- and $\beta$-AR density. This result may also be because the 24 h incubation period was not long enough to initiate transcriptional and/or translational events because most adrenoreceptor down-regulation occurred after weeks or even months [34, 42, 45]. However, our data obtained from western blotting, RT-PCR, and real-time PCR analyses demonstrate that long-term $\alpha$-AR activation decreased $\alpha_2$-isoform mRNA and protein levels and that long-term $\beta$-AR activation increased $\alpha_1$-isoform mRNA and protein levels in rat myocytes, further confirming that long-term adrenergic regulation involves protein synthesis or degradation. These results agree with the changes in the $I_{\text{pH}}$ suggesting that the reduced rat myocyte $I_{\text{pH}}$ may be associated with decreased $\alpha_2$-isoform mRNA and protein levels and that the increased $I_{\text{pH}}$ may be related to increased $\alpha_1$-isoform mRNA and protein expression levels.

Certain evidence has also indicated that increased NKP membrane expression via PKA or PKC-mediated phospholemman (PLM, FXYD1) phosphorylation [28, 42] may be involved in short-term adrenergic NKP regulation. PLM associates with NKP and mediates its adrenergic regulation in a tissue-specific manner [46-50]. PLM is the only FXYD protein family member that is present in cardiomyocytes, where it is a major substrate for PKA and
PKC phosphorylation. PLM inhibits the NKP by reducing its affinity for Na⁺, whereas PLM that has been phosphorylated by PKA or PKC relieves this inhibition and, thus, mediates PKA/PKC-dependent NKP stimulation during adrenergic activation [13, 16, 46, 50, 51]. Certain evidence indicated that β-AR-mediated PKA activation can target α₁- and α₂-isoform-associated PLM to increase the Iₚ whereas α-AR-mediated PKC activation can target α₂-isoform-associated PLM to increase the Iₚ [13, 41, 51]. Although this hypothesis is supported at least in part by the results from the present study, in the present study, β-AR activation targeted only the α₁-isoform via the PKA-dependent pathway, and α-AR activation targeted only the α₂-isoform via the PKC-dependent pathway. It is likely that PLM is required for the PKA- and PKC-mediated activity of the NKP, whereas the PLM phosphorylation state is regulated by the kinase activity of PKA and PKC and phosphatase activity of PP-1 and PP2-A. PKA and PKC phosphorylate PLM and, thus, stimulate NKP, whereas PP-1 and PP2-A remove phosphates from PLM and, thus, inhibit NKP [37]. Bibert et al. found that while PKA activation increases the apparent Na affinity of the α₁- and α₂-isofoms, PKC had no effect on the apparent Na affinity of either α₁- or α₂-isoform but increased the Vₘₗ of the α₂-isoform only [52]. Furthermore, the activation of these pathways always had the opposite effects, suggesting that PLM phosphorylation is important, but is not the sole factor contributing to short-term adrenergic stimulation-mediated NKP regulation. Hence, NKP α-isofom translocation may be another short-term adrenergic regulatory mechanism, and the relationship of NKP α-isofom translocation and PLM phosphorylation during short- and long-term adrenergic activation deserves further investigation.

In summary, the results of the present study in rat cardiomyocytes demonstrate that NKP is regulated by adrenergic activation in an isoform-specific manner. The NKP α₁-isoform is regulated by α-AR activation via a PKC-dependent pathway, and the α₂-isoform is regulated by β-AR activation via a PKA-dependent pathway. The long-term adrenergic stimulation-mediated NKP regulation negatively fed back on the short-term response. However, the NKP regulatory mechanism mainly involves the translocation of pre-existing NKP α-isofoms between the plasma membrane and the endosomes for short-term adrenergic activation, protein synthesis, and degradation for long-term adrenergic activation. These results suggest that the effect of changed NKP function (by long-term elevations in circulating catecholamine levels under stressful conditions of heart diseases) on heart function should be considered during cardiovascular disease treatment. However, considering the limitations of 24 h treatment with β- and α-adrenergic agonists as a model of chronic adrenergic stimulation, further in vivo studies are necessary to confirm the results of the present study and their functional significance.

**Acknowledgments**

This work was supported by the Natural Science Foundation of Hebei Province of China (No 301360).

**References**

1. Skou JC, Esmann M: The Na,K-ATPase. J Bioenerg Biomembr 1992;24:249-261.
2. James PF, Grupp IL, Grupp G, Woo AL, Askew GR, Croyle ML, Walsh RA, Lingrel JB: Identification of a specific role for the Na,K-ATPase alpha 2 isoform as a regulator of calcium in the heart. Mol Cell 1999;3:555-563.
3. Yamamoto T, Su Z, Moseley AE, Kadono T, Zhang J, Cognon M, Li F, Lingrel JB, Barry WH: Relative abundance of alpha2 Na⁺ pump isoform influences Na⁺-Ca²⁺ exchanger currents and Ca²⁺ transients in mouse ventricular myocytes. J Mol Cell Cardiol 2005;39:113-120.
4. Ishizuka N, Fiedling AJ, Berlin JR: Na pump current can be separated into ouabain-sensitive and -insensitive components in single rat ventricular myocytes. Jpn J Physiol 1996;46:215-223.
Yin et al.: Adrenergic Regulation of Cardiac NKP

5 Mathias RT, Cohen IS, Gao J, Wang Y: Isoform-Specific Regulation of the Na⁺-K⁺ Pump in Heart. News Physiol Sci 2000;15:176-180.

6 Gao J, Mathias RT, Cohen IS, Wang Y, Sun X, Baldo GJ: Activation of PKC increases Na⁺-K⁺ pump current in ventricular myocytes from guinea pig heart. Pflugers Arch 1999;437:643-651.

7 Gao J, Wymore R, Wymore RT, Wang Y, McKinnon D, Dixon JF, Mathias RT, Cohen IS, Baldo GJ: Isoform-specific regulation of the sodium pump by alpha- and beta-adrenergic agonists in the guinea-pig ventricle. J Physiol 1999;516:377-383.

8 Wang Y, Gao J, Mathias RT, Cohen IS, Sun X, Baldo GJ: alpha-Adrenergic effects on Na⁺-K⁺ pump current in guinea-pig ventricular myocytes. J Physiol 1998;509:117-128.

9 Ishizuka N, Berlin JR: Beta-adrenergic stimulation does not regulate Na pump function in voltage-damped ventricular myocytes of the rat heart. Pflugers Arch 1993;424:361-363.

10 Main MJ, Grantham CJ, Cannell MB: Changes in subsarcolemmal sodium concentration measured by Na-Ca exchanger activity during Na-pump inhibition and beta-adrenergic stimulation in guinea-pig ventricular myocytes. Pflugers Arch 1997;435:112-118.

11 Gao J, Cohen IS, Mathias RT, Baldo GJ: The inhibitory effect of beta-stimulation on the Na/K pump current in guinea pig ventricular myocytes is mediated by a cAMP-dependent PKA pathway. Pflugers Arch 1998;435:479-484.

12 Gao J, Mathias RT, Cohen IS, Baldo GJ: Isorenaline, Ca²⁺ and the Na⁺-K⁺ pump in guinea-pig ventricular myocytes. J Physiol 1992;449:689-704.

13 Bossuyt J, Despa S, Han F, Hou Z, Robia SL, Lingrel JB, Bers DM: Isoform specificity of the Na/K-ATPase association and regulation by phospholemman. J Biol Chem 2009;284:26749-26757.

14 Glitsch HG: Electrophysiology of the sodium-potassium-ATPase in cardiac cells. Physiol Rev 2001;81:1791-1826.

15 Kockskamper J, Erlenkamp S, Glitsch HG: Activation of the cAMP-protein kinase A pathway facilitates Na⁺ translocation by the Na⁺-K⁺ pump in guinea-pig ventricular myocytes. J Physiol 2000;523:561-574.

16 Silverman B, Fuller W, Eaton P, Deng J, Moorman JR, Cheung JY, James AF, Shattock MJ: Serine 68 phosphorylation of phospholemman: acute isoform-specific activation of cardiac Na/K ATPase. Cardiovasc Res 2005;65:93-103.

17 Wanless RB, Noble MI, Drake-Holland AJ: The mechanisms of action of noradrenaline on ouabain-sensitive rubidium uptake in guinea-pig myocardium. Clin Sci 1985;69:737-743.

18 Dzimir N: Regulation of beta-adrenergic receptor signaling in cardiac function and disease. Pharmacol Rev 1999;51:465-501.

19 Fan TH, Frantz RP, Elam H, Sakamoto S, Imai N, Liang CS: Reductions of myocardial Na-K-ATPase activity and ouabain binding sites in heart failure: prevention by nadolol. Am J Physiol 1993;265:H2086-2093.

20 Kim CH, Fan TH, Kelly PF, Himura Y, Delehanty JM, Hang CL, Liang CS: Isoform-specific regulation of myocardial Na,K-ATPase alpha-subunit in congestive heart failure. Role of norepinephrine. Circulation 1994;89:313-320.

21 Steinberg SF: The molecular basis for distinct beta-adrenergic receptor subtype actions in cardiomyocytes. Circ Res 1999;85:1101-1111.

22 Anyukhovsky EP, Rosen MR: Abnormal automatic rhythms in ischemic Purkinje fibers are modulated by a specific alpha 1-adrenergic receptor subtype. Circulation 1991;83:2076-2082.

23 del Balzo U, Rosen MR, Malfatto G, Kaplan LM, Steinberg SF: Specific alpha 1-adrenergic receptor subtypes modulate catecholamine-induced increases and decreases in ventricular automaticity. Circ Res 1990;67:1535-1551.

24 Shah A, Cohen IS, Rosen MR: Stimulation of cardiac alpha receptors increases Na/K pump current and decreases gK via a pertussis toxin-sensitive pathway. Biophys J 1988;54:219-225.

25 Zaza A, Kline RP, Rosen MR: Effects of alpha-adrenergic stimulation on intracellular sodium activity and automaticity in canine Purkinje fibers. Circ Res 1990;66:416-426.

26 Xiong C, Li JX, Guo HC, Zhang LN, Guo W, Meng J, Wang YL: The H(1)-H(2) domain of the alpha(1) isofrom of Na⁺-K⁺ ATPase is involved in ouabain toxicity in rat ventricular myocytes. Toxicol App Pharmacol 2012;262:32-42.

27 Gorvel JP, Chavrier P, Zerial M, Gruenberg J: rab5 controls early endosome fusion in vitro. Cell 1991;64:915-925.

28 Bertorello AM, Katz AI: Short-term regulation of renal Na-K-ATPase activity: physiological relevance and cellular mechanisms. Am J Physiol 1993;265:F743-755.
Liu J, Shapiro Jr: Regulation of sodium pump endocytosis by cardiotic steroids: Molecular mechanisms and physiological implications. Pathophysiol 2007;14:171-181.

Putheenweedu MA, Laufer B, Temkin P, Vistein R, Carlton P, Thorn K, Taunton J, Weiner OD, Parton RG, von Zastrow M: Sequence-dependent sorting of recycling proteins by actin-stabilized endosomal microdomains. Cell 2010;143:761-773.

Fomina AF, Deerinck TJ, Ellisman MH, Cahalan MD: Regulation of membrane trafficking and subcellular organization of endocytic compartments revealed with FM1-43 in resting and activated human T cells. Exp. Cell Res 2003;291:150-166.

Co C, Wong DT, Gierke S, Chang V, Taunton J: Mechanism of actin network attachment to moving membranes: barbed end capture by N-WASP WH2 domains. Cell 2007;128:901-913.

Butterfield MC, Chess-Williams R: Potentiation of alpha-adrenoceptor-mediated responses following chronic beta-adrenoceptor stimulation in the rat heart. Br J Pharmacol 1993;108:658-662.

Dong E, Yatani A, Mohan A, Liang CS: Myocardial beta-adrenoceptor down-regulation by norepinephrine is linked to reduced norepinephrine uptake activity. Eur J Pharmacol 1999;384:17-24.

Karliner JS, Barnes P, Brown M, Dollery C: Chronic heart failure in the guinea pig increases cardiac alpha 1- and beta-adrenoceptors. Eur J Pharmacol 1980;67:115-118.

Sato S, Nomura R, Kawano E, Tanahata J, Tachiyaishi K, Imaizumi K: Effects of the beta2-agonist clenbuterol on beta1- and beta2-adrenoceptor mRNA expressions of rat skeletal and left ventricle muscles. J Pharmacol Sci 2008;107:393-400.

Pavlovic D, Fuller W, Shattock MJ: Novel regulation of cardiac Na pump via phospholemman. J Mol Cell Cardiol 2013;61:83-93.

Shamraj OI, Grupp IL, Grupp G, Melvin D, Gradoux N, Kremers W, Melvin D, Gradoux N: Functional analysis of Na/K-ATPase isoform distribution in rat ventricular myocytes. Cardiovasc Res 2003;291:150-166.

Gao J, Wymore RS, Wang Y, Gaudette GR, Krukenkamp IB, Cohen IS, Mathias RT: Isoform-specific Stimulation of Cardiac Na/K Pumps by Nanomolar Concentrations of Glycosides. J Gen Physiol 2002;119:297–312.

Berry RG, Despa S, Fuller W, Bers DM, Shattock MJ: Differential distribution and regulation of mouse cardiac Na/K-ATPase alpha1 and alpha2 subunits in T-tubule and surface sarcolemmal membranes. Cardiovasc Res 2007;73:92-100.

Despa S, Bers DM: Functional analysis of Na/K-ATPase isoform distribution in rat ventricular myocytes. Am J Physiol Cell Physiol 2007;293:C321-327.

Chibalin AV, Pedemonte CH, Katz AI, Feraill E, Berggren P-O, Bertorello AM: Phosphorylation of the catalytic alpha-subunit constitutes a triggering signal for Na+, K+-ATPase endocytosis. J Biol Chem 1998;273:8814-8819.

Cohn JN, Levine TB, Olwari MT, Garberg V, Lura D, Francis GS, Simon AB, Rector T: Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. N Engl J Med 1984;311:819-823.

Ufer C, Germack R: Cross-regulation between beta 1- and beta 3-adrenoceptors following chronic beta-adrenergic stimulation in neonatal rat cardiomyocytes. Br J Pharmacol 2009;158:300-313.

Osadchii OE, Norton GR, McKechnie R, Deftereos D, Woodiwiss AJ: Cardiac dilatation and pump dysfunction without intrinsic myocardial systolic failure following chronic beta-adrenoceptor activation. Am J Physiol Heart Circ Physiol 2007;292:H1898-1905.

Despa S, Bossuyt J, Han F, Ginsburg KS, Jia LG, Kutchai H, Tucker AL, Bers DM: Phospholemman-phosphorylation mediates the beta-adrenergic effects on Na/K pump function in cardiac myocytes. Circ Res 2005;97:252-259.

Garty H, Karlish SJ: FXYD proteins: tissue-specific regulators of the Na, K-ATPase. Semin Nephrol 2005;25:304-311.

Garty H, Karlish SJ: Role of FXYD proteins in ion transport. Annu Rev Physiol 2006;68:431-459.

Geering K: FXYD proteins: new regulators of Na-K-ATPase. Am J Physiol Renal Physiol 2006;290:F241-250.

Han E, Bossuyt J, Despa S, Tucker AL, Bers DM: Phospholemman phosphorylation mediates the protein kinase C-dependent effects on Na+/K+ pump function in cardiac myocytes. Circ Res 2006;99:1376-1383.

Han E, Bossuyt J, Martin JL, Despa S, Bers DM: Role of phospholemman phosphorylation sites in mediating kinase-dependent regulation of the Na+/K+-ATPase. Am J Physiol Cell Physiol 2010;299:C1363-1369.

Bibert S, Ray S, Schaer D, Horisberger JB, Geering K: Phosphorylation of phospholemman (FXYD1) by protein kinases A and C modulates distinct Na, K-ATPase isozymes. J Biol Chem 2008;283:476-486.