Original Paper

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

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Key WordsNa⁺ • K⁺-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_{PH}) and low-affinity current (I_{PL}), α -isoform mRNA, and α -isoform protein were examined using patch-clamp, RT-PCR, and Western blotting techniques, respectively. **Results:** After the short-term incubation, isoprenaline reduced the I_{PL} through a PKA-dependent pathway that involves α_1 -isoform translocation from the membrane to early endosomes, and noradrenaline increased the I_{PH} through a PKC-dependent pathway that involves α_2 -isoform translocation from late endosomes to the membrane. After long-term incubation, isoprenaline increased the I_{PL} , α_1 -isoform mRNA, and α_1 -isoform protein, and noradrenaline reduced the I_{PH} , α_2 -isoform mRNA, and α_2 -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 α -isoform, whereas short-term regulation involves the translocation of the available α -isoform to the plasma membrane.

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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 ($[\text{Na}^+]_i$), whereas the NKP α_2 -isoform regulates local $[\text{Na}^+]_i$ near the Na^+ , Ca^{2+} -exchanger and thus governs cardiomyocyte Ca^{2+} levels and contractility [2, 3]. The NKP α -isoform-generated I_p is based on different cardiac glycoside affinities of the isoforms: the NKP α_2 -isoform is cardiac glycoside-sensitive and generates the high-affinity pump current (I_{ph}), whereas the NKP α_1 -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_{pl} and I_{ph} 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 $[\text{Na}^+]_i$ 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_{pl} at low intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) but did not affect the I_{ph} [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_1 -adrenergic activation decreases automaticity, resulting from α_1 -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 α_1 -AR. Stock solutions of 10 mM noradrenaline, 1 mM isoprenaline, 10 mM propranolol, 1 mM prazosin, 1 M strophanthidin, 50 mM dihydroouabain (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 μ M isoprenaline, 10 μ M noradrenaline, 10 μ M propranolol, 1 μ M prazosin, 1 μ M staurosporine, 10 μ M H89, and 1 μ M 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 (I_p) 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 μ M noradrenaline in the presence of a β -AR blocker (10 μ M propranolol), and β -AR activation was achieved by applying 1 μ M isoprenaline. The I_p generated by the NKP α_2 - and α_1 -isoforms were distinguished as the I_{pH} and I_{pL}, respectively, based on their affinities for cardiac glycosides; 5 μ M DHO was used to detect the I_{pH}, and 500 μ M strophanthidin was used to detect the I_{pL} in the presence of 5 μ M DHO [7]. An Axopatch 700B amplifier (Axon Instruments, Inc.) was used to observe the cell membrane current. Patch pipette resistances were 2–4 M Ω 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 CdCl₂ 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 μ M DHO or 500 μ M strophanthidin and 1 μ M isoprenaline, 10 μ M noradrenaline, 10 μ M propranolol, 1 μ M prazosin, or 1 μ M 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.

Immunoblots

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 μ l/ml protease inhibitors. The cells were disrupted by sonication on ice using 4 $\times$ 2-s bursts. The supernatant (whole cell lysate) was collected by centrifugation at 120,000 $\times$ 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 $3,000 \times g$). 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 $110,000 \times g$ 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_1 -isoform (Santa Cruz, 1:500), NKP α_2 -isoform (Upstate, 1:200), α_1 -AR (Abcam, 1:500), and β_1 -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_1 - and α_2 -isoform-specific primers. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as a housekeeping gene. The PCR reactions contained $2 \times$ GoTaq Green Master Mix, 10 μ M forward primer, 10 μ M reverse primer, 2 μ l of template cDNA, and nuclease-free water in a final volume of 50 μ l. 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

Table 1. Primers for Na⁺-K⁺ pump α_1 -isoform and α_2 -isoform, GAPDH, and β -actin used in Real-time PCR and RT-PCR

Gene name	Primer sequence (Forward/Reverse)	Length of amplified fragment (bp)	Oligonucleotide position
α_1 -isoform	5'-AAGGACGCCTTTCAGAATGCCT-3'	264	1617~1638
	5'-TGACCATGATGACCTTAATCC-3'		1863~1843
α_2 -isoform	5'-CACCTACTTTGTAATACTGGC-3'	247	2690~2710
	5'-ATCAGGATCTTGTCTTCATGCC-3'		2953~2931
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	452	550~569
	5'-TCCACCACCCTGTTGCTGTT-3'		1001~982
β -actin	5'-ACGGTCAGGTCATCACTATC-3'	90	812~901
	5'-TGCCACAGGATTCCATACC-3'		

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 α_1 - and α_2 -isoforms, β -actin, and GAPDH in 48-well optical reaction plates in triplicate. Each reaction contained 1× SYBR Premix E_x Taq (TaKaRa), 10 μ M forward and reverse primers, and 2 μ l of template cDNA in a final volume of 25 μ l. 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 (C_t) values were used to calculate the NKP α_1 - and α_2 -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 $\pm$ 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 α_1 - and α_2 - NKP isoforms. As the affinity for cardiac glucoside is approximately 100-fold lower for the α_1 - than for the α_2 -isoform, the two α -isoforms can be assessed separately using different concentrations of cardiac glucoside; 5 μ M DHO inhibits the α_2 -isoform only, and 500 μ M strophanthidin (or 1 mM DHO) inhibits both α -isoforms. Thus, when the NKP-generated I_p was assessed using the patch-clamp technique, the current that was sensitive to 5 μ M DHO was identified as the α_2 -isoform-generated $I_{pH'}$, and the current that was sensitive to 500 μ M strophanthidin in the presence of 5 μ M DHO was identified as the α_1 -isoform-generated I_{pL} . Figure 1A and 1B show the levels of I_{pL} and $I_{pH'}$, respectively. The averages of the control I_{pL} and $I_{pH'}$ 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 μ M noradrenaline in the presence of a β -blocker (10 μ M propranolol), and β -adrenergic activation was achieved by applying 1 μ M isoprenaline. The I_p value was normalised to the value obtained in the control condition. Our data demonstrated that short-term β -adrenergic activation reduced the I_{pL} but did not affect the $I_{pH'}$, whereas short-term α -adrenergic activation had no effect on the I_{pL} but increased the $I_{pH'}$ (Fig. 1). These findings suggest that short-term α - and β -adrenergic activation have opposite effects on NKP activity

Fig 1. The effects of short-term adrenergic activation on Na/K pump current (I_p). The cells were held at 0 mV, and the I_p was assessed using the patch-clamp technique. A) In the presence of 5 μ M dihydroouabain (DHO) to block high-affinity I_p , 0.5 mM strophanthidin (Str) treatment was used to demonstrate that 1 μ M isoprenaline (ISO) treatment inhibited low-affinity I_p , and 10 μ M noradrenaline (NA) treatment (with 10 μ M propranolol (Pro) to block the β -adrenoreceptor) had no effect on low-affinity I_p . B) 5 μ M DHO treatment was used to demonstrate that ISO treatment had no effect on high-affinity I_p and that NA treatment increased high-affinity I_p . C) The low affinity I_p was reduced by ISO treatment, and the high affinity I_p was increased by NA treatment. The normalised I_p represents the I_p value normalised to its control value (Con). Each column represents the mean value $\pm$ 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.

through α -isoform-specific regulation in rat ventricular myocytes, similar to guinea pig ventricular myocytes [7].

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_{max} . To investigate the mechanism of the short-term adrenergic stimulation effect on NKP activity, we first assessed α_1 - and α_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 α_1 - and α_2 -isoform mRNA levels were not affected by short-term α - and β -adrenergic activation. Furthermore, western blotting analysis demonstrated that short-term α - and β -adrenergic activation failed to alter α_1 - and α_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

Fig 2. The effects of short-term adrenergic activation on Na/K pump (NKP) protein and mRNA expression. A) Western blot analysis of NKP α_1 - and α_2 -isoform protein levels in whole cells was performed using specific anti- α_1 and anti- α_2 antibodies. Equal protein amounts were loaded for each sample. The protein levels were normalised to Con, and the data are summarised in the histogram. Neither 1 μ M ISO nor 10 μ M NA (with 10 μ M Pro) treatment affected NKP α_1 - and α_2 -isoform protein expression. B) Total RNA was amplified using NKP α_1 - and α_2 -isoform-specific primers. The products were subjected to electrophoresis on 2% agarose gels and visualised with GoldView. Amplification products at the predicted sizes (264 bp for the α_1 -isoform, 247 bp for the α_2 -isoform and 452 bp for GAPDH) were evident in RT-PCR reactions. The mean α_1 - and α_2 -isoform mRNA levels are demonstrated in the histogram. Neither ISO nor NA treatment affected NKP α_1 - and α_2 -isoform mRNA expression. C) For real-time PCR analysis, SYBR green, a non-specific fluorescent dye, was used to monitor gene amplification, and GAPDH and β -actin were used as two housekeeping genes to normalise α_1 - and α_2 -isoform mRNA expression levels. Neither ISO nor NA treatment affected NKP α_1 - and α_2 -isoform mRNA levels. Each column represents the mean value $\pm$ SE, and the number of myocytes studied is indicated within the column and comes from at least 3 different hearts.

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 α_2 - but not α_1 -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 α_2 - but not α_1 -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 α_2 -isoform, which may result from α_2 -isoform recycling from late endosomes to the plasma membrane. Short-term isoprenaline-mediated β -adrenergic activation reduced

Fig 3. The effects of short-term adrenergic activation on plasma membrane and endosomal NKP protein abundance. After treatment with 1 μ M ISO or 10 μ M NA (with 10 μ M Pro) for 10 min, plasma membrane proteins were extracted, and early and late endosomes (EE and LE, respectively) were fractionated by ultracentrifugation, with rab5 and lamp1 as markers for EE and LE, respectively. NKP α -isoform proteins in the plasma membrane and endosomes were analysed by western blot using specific anti- α_1 and anti- α_2 antibodies. Equal protein amounts were loaded for each sample. Protein levels were normalised to Con. A) NA treatment increased α_2 - but not α_1 -isoform amounts on the plasma membrane, which was reversed by 1 μ M prazosin (PZ) treatment, and ISO treatment decreased α_1 - but not the α_2 -isoforms on the plasma membrane, which was reversed by treatment with 10 μ M Pro. B) NA treatment decreased α_2 - but not α_1 -isoform levels in the LE but not the EE, and ISO treatment increased α_1 - but not the α_2 -isoform levels in the EE but not in the LE. Each column represents the mean value $\pm$ SE, and the number of myocytes studied is indicated within the column and comes from at least 3 different hearts. *P < 0.05, statistically significant relative to the Control.

α_1 - but not α_2 -isoform plasma membrane levels, which was reversed by incubation with the β -AR antagonist propranolol (Fig. 3A). This reduction was accompanied by increased NKP α_1 - but not α_2 -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 α_1 -isoform, which may be a consequence of NKP α_1 -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.

Phalloidin 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

Fig 4. The effects of phalloidin on the short-term adrenergic activation-mediated I_p and NKP translocation. A) The effect of 1 μ M phalloidin (Pha) on the I_p . The cells were held at 0 mV. In the presence of 5 μ M DHO to block high-affinity I_p and Pha to block actin-mediated protein trafficking, 0.5 mM Str treatment was used to demonstrate that 1 μ M ISO treatment did not affect the low affinity I_p . In the presence of Pha and 10 μ M Pro, 5 μ M DHO treatment was used to demonstrate that 10 μ M NA treatment had no effect on the high affinity I_p . The normalised I_p is the I_p value normalised to Con. B) The effects of Pha treatment on NKP translocation. Western blot analysis was used to analyse NKP α -isoforms on the plasma membrane using specific anti- α_1 and α_2 antibodies. Equal protein amounts were loaded for each sample. The protein levels were normalised to Con. Pha treatment counteracted the effect of ISO treatment on the NKP α_1 -isoform and the effect of NA treatment on the α_2 -isoform. Each column represents the mean value $\pm$ 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.

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 phalloidin-pretreated cardiomyocytes, which stabilises cytoskeletal actin and affects protein translocation. We used phalloidin 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 phalloidin, short-term treatment with α - and β -AR agonists did not induce any I_p 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.

Fig 5. The effects of long-term adrenergic activation on I_p , adrenoceptor (AR) density, and NKP α -isoform expression. After 1 μ M ISO or 10 μ M NA (with 10 μ M Pro) treatment for 24 h, I_p was assessed using the patch-clamp technique. The I_p density is 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 α_1 -AR and β_1 -AR antibodies; both ISO and NA treatment failed to alter α_1 - and β_1 -AR expression. C) Western blot analysis using specific anti- α_1 and anti- α_2 antibodies. ISO treatment up-regulated α_1 -isoform levels, which was reversed by 10 μ M Pro treatment, but α_2 -isoform expression remained unaltered, whereas NA treatment down-regulated α_2 - but not α_1 -isoform levels, an effect that was reversed by 1 μ M PZ treatment. Each column represents the mean value $\pm$ 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 affects NKP activity in an isoform-specific manner

To investigate the effects of long-term adrenergic activation on α_1 - and α_2 - 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. To assess the functional state of rat myocytes 24 h after isolation, their I_p densities (pA/pF) were measured and compared with the measurements that were taken 1 h after isolation. The results demonstrated that the control I_{pL} were 0.33 ± 0.04 and 0.35 ± 0.05 pA/pF, and the control I_{pH} were 0.14 ± 0.03 and 0.15 ± 0.04 pA/pF, respectively, suggesting that myocyte NKP activity is not changed after 24 h in Kraft-Brühe solution at room temperature. However, after incubating 24 h with adrenergic agonists, β -adrenergic activation selectively increased the I_{pL} but did not affect the I_{pH} , and α -adrenergic activation reduced only the I_{pH} and did not affect the I_{pL} (Fig. 5A). Our data demonstrate that long-term adrenergic NKP activity regulation is isoform-specific and that the effects of long-term α - and β -adrenergic activation are opposite of the short-term effects on NKP activity. These data suggest that long-term adrenergic NKP

Fig 6. The effects of long-term adrenergic activation on NKP α -isoform mRNA levels A) Total RNA was extracted following treatment with 1 μ M ISO or 10 μ M NA (with 10 μ M Pro) for 24 h and amplified using NKP α_1 - and α_2 -isoform-specific primers. The products were subjected to electrophoresis on 2% agarose gels and visualised with GoldView. Amplification products at the predicted sizes of 264 bp (α_1 -isoform), 247 bp (α_2 -isoform) and 452 bp (GAPDH) were evident in the RT-PCR reactions. The mean α_1 - and α_2 -isoform mRNA levels are summarised in the histogram. ISO treatment up-regulated α_1 -isoform mRNA, while α_2 -isoform mRNA was unaltered, and NA treatment down-regulated α_2 - but not the α_1 -isoform mRNA. B) For real-time PCR analysis, SYBR green, a non-specific fluorescent dye, was used to monitor amplification, and GAPDH and β -actin were used as two housekeeping genes to normalise α_1 - and α_2 -isoform mRNA expression levels. ISO treatment up-regulated α_1 - but not α_2 -isoform mRNA levels, which was reversed by treatment with 10 μ M Pro and NA, which down-regulated α_2 - but not α_1 -isoform mRNA levels, an effect that was reversed by 1 μ M PZ treatment. Each column represents the mean value $\pm$ SE, and the number of myocytes studied is indicated within the column and comes from at least 3 different hearts. * $P < 0.05$, statistically significant relative to the Control.

activity regulation may occur via different mechanisms than short-term regulation or that long-term adrenergic activation negatively feeds back on adrenoceptor expression.

Long-term adrenergic activation does not alter adrenoceptor expression

Because long-term α_1 - and β -adrenergic activation negatively feeds back on myocardial adrenoceptor expression [33-36], we further investigated whether long-term adrenergic activation negatively feeds back on adrenoceptor expression (*i.e.*, whether the long-term negative feedback on the I_p is a result of α_1 -AR and β_1 -AR up- or down-regulation). Western blotting indicated that α - and β -adrenergic activation for 24 h failed to alter α_1 -AR and β_1 -AR protein expression levels (Fig. 5B), suggesting that the negative feedback of long-term α - and β -AR activation on the I_p might be unrelated to modified myocardial α_1 - and β_1 -AR expression.

Fig 7. The roles of the PKA- and PKC-dependent pathways in the adrenergic NKP regulation Rat myocytes were treated with ISO, ISO + H89, ISO + staurosporine (Sta), NA, NA + H89 or NA + Sta for 10 min or 24 h; NA was co-incubated with Pro. The concentrations used were 1 μ M ISO, 10 μ M NA, 10 μ M Pro, 10 μ M H89, and 1 μ M Sta. Plasma membrane protein and whole cell protein samples were prepared, and NKP α -isoform expression levels were determined by western blot using specific anti- α_1 and anti- α_2 antibodies. Equal protein amounts were loaded for each sample, and protein levels were normalised to Con. A) H89 treatment completely reversed the effect of ISO treatment on α_1 -isoform translocation, and Sta treatment blocked the effect of NA treatment on α_2 -isoform plasma membrane translocation following short-term adrenergic activation. B) Sta treatment reversed the reduction in the NA and H89 treatment-induced α_2 -isoform expression levels and abolished the increased ISO-induced α_1 -isoform expression levels in whole cells following long-term adrenergic activation. Each column represents the mean value $\pm$ 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.

Long-term adrenergic-mediated NKP regulation involves mRNA and protein expression level alterations

We next assessed α_1 - and α_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 β -adrenergic activation, α_1 -isoform mRNA levels were significantly up-regulated, while α_2 -isoform mRNA levels were unaltered. In contrast, α_2 - but not α_1 -isoform mRNA levels were significantly down-regulated after 24 h of α -adrenergic activation. Western blots (Fig. 5C) from cardiac protein lysates further demonstrated that following 24 h of β -adrenergic activation, α_1 -isoform protein levels were significantly up-regulated, while the α_2 -isoform protein levels were unaltered. Conversely, α_2 - but not α_1 -isoform protein levels were significantly down-regulated after 24 h of α -adrenergic activation. These data indicate that steady-state mRNA and protein levels were increased for the NKP α_1 -isoform and decreased for the α_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 α_1 -isoform mRNA and protein levels were reversed by propranolol treatment, whereas the long-term α -adrenergic activation-mediated effects α_2 -isoform mRNA and protein levels were reversed by prazosin treatment, suggesting that the α_1 - and β_1 -ARs play a role in the events that decrease synthesis and/or increase degradation of the NKP α -isoforms.

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 I_p regulation is mediated by PKA and PKC, which are downstream β - and α -adrenergic activation signalling molecules, respectively, that play important roles in regulating α_1 - and α_2 -isoform 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 -isoform plasma membrane and endosomal localisation as well as the long-term adrenergic stimulation-mediated effects on the α -isoform 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 α_1 -isoform translocation, and staurosporine but not H89 treatment blocked the short-term effect of noradrenaline treatment on the α_2 -isoform translocation (Fig. 7A). Furthermore, PKC but not PKA inhibition reversed the long-term α -adrenergic activation-induced reduction in α_2 -isoform expression, and PKA but not PKC inhibition abolished the long-term β -adrenergic activation-mediated increase in α_1 -isoform expression (Fig. 7B). These results indicate that in rat ventricular myocytes, the effect of both short- and long-term β -AR activation on the α_1 -isoform involves the PKA-dependent pathway, and the effect of α -AR activation on the α_2 -isoform 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 -isoform protein expression are the same compared with the short-term effects of adrenergic agonists on NKP α_1 - and α_2 -isoform translocation. These PKC- and PKA-dependent changes in mRNA and protein expression after adrenergic activation also provide further evidence supporting similar I_p 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 α_2 -isoform-generated I_{pH} and decreased the α_1 -isoform-generated I_{pL} , respectively, in rat myocytes similar to guinea pig ventricular myocytes [6-8, 11] but also demonstrated that long-term α -AR activation reduced α_2 -isoform-generated I_{pH} and long-term β -AR activation increased the α_1 -isoform-generated I_{pL} . These results suggest that either short- or long-term α - and β -AR activation mediate opposite effects on the I_p in an α -isoform-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 I_p 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 $[Na]_i$ increase in stressful conditions.

In human hearts, all of the three α -isoforms are detected, and the estimated stoichiometric distribution is the α_1 -isoform being dominant (62%) over the α_2 -isoform (15%) and the α_3 -isoform (23%) [37]. Moreover, the inhibition of I_p by DHO are also present in the cells, and the dissociation constants for inhibition by DHO are similar for the three α -isoforms [38]. The I_p averaged from 12 atrial cells in the control were 0.29 ± 0.06 pA/pF [39], which was similar to the I_{pH} (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 I_p in human atrial cells

has similar properties to the I_{pH} in rat and guinea pig ventricle cells. Moreover, our results in Figure 1C indicated a 26% increase of I_{pH} by α -adrenergic activation, which is similar to the 24% increase in I_{pT} observed in human atrial myocytes [39]; therefore, the α_2 -isoform is responsible for approximately 100% of the I_{pT} in human atrial myocytes, which is not consistent with this study in rat ventricular myocytes (29.5%) and the I_{pT} in most reports (10–20%) [40, 41]. Moreover, β -adrenergic activation had no effect on the I_{pT} in human atrium, which is also not consistent with the presence of the α_1 -isoform, unless α_1 -isoform regulation in human atrium differs from that in rat and guinea pig ventricle.

Generally, the mechanism by which adrenergic activation regulates NKP is associated with a change in “working” NKP levels at the cell surface, which depends on the translocation and/or synthesis of α -isoform proteins. However, the present result indicates that long- but not short-term adrenergic activation altered whole cell α_1 - and α_2 -isoform protein or mRNA expression. It is possible that different α -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 α -AR activation increased plasma membrane α_2 -isoform incorporation accompanied with a decrease of α_2 -isoform in late endosomes, and short-term β -AR activation decreased the content of plasma membrane α_1 -isoform accompanied with an increase of α_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 α -AR and β -AR activation-mediated increase in the I_{pH} and reduction in the I_{pL} cannot be attributed to α_2 -isoform synthesis or α_1 -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 α -isoform translocation between the plasma membrane and the endosomes in an isoform-specific manner, which explains the isoform-specific regulation of the I_p by short-term adrenergic activation.

Another important finding of the present study is that long-term adrenergic regulation of the I_p and NKP α -isoform abundance by either α - or β -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 α - and β -adrenergic activation altered the respective receptor amounts. However, our data indicated that α - and β -adrenergic activation for 24 h failed to alter α_1 - and β_1 -AR expression levels, implying that the negative feedback on the I_p by long-term α - and β -AR activation may be because of altered NKP α -isoform mRNA and protein expression levels but not myocardial α_1 - and β_1 -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 α -AR activation decreased α_2 -isoform mRNA and protein levels and that long-term β -AR activation increased α_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_p , suggesting that the reduced rat myocyte I_{pH} may be associated with decreased α_2 -isoform mRNA and protein levels and that the increased I_{pL} may be related to increased α_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 α_1 - and α_2 -isoform-associated PLM to increase the I_p , whereas α -AR-mediated PKC activation can target α_2 -isoform-associated PLM to increase the I_p [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 α_1 -isoform via the PKA-dependent pathway, and α -AR activation targeted only the α_2 -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 α_1 - and α_2 -isoforms, PKC had no effect on the apparent Na affinity of either α_1 - or α_2 -isoform but increased the $V_{\max}$ of the α_2 -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 -isoform translocation may be another short-term adrenergic regulatory mechanism, and the relationship of NKP α -isoform 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 α_2 -isoform is regulated by α -AR activation via a PKC-dependent pathway, and the α_1 -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 -isoforms 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).

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