Original Article

Functional comparison of the reverse mode of $\text{Na}^+/\text{Ca}^{2+}$ exchangers NCX1.1 and NCX1.5 expressed in CHO cells

Yan LONG, Wei-ping WANG, Hui YUAN, Shi-ping MA, Nan FENG, Ling WANG, Xiao-liang WANG*

State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

Aim: To investigate the reverse mode function of $\text{Na}^+/\text{Ca}^{2+}$ exchangers NCX1.1 and NCX1.5 expressed in CHO cells as well as their modulations by PKC and PKA.

Methods: CHO-K1 cells were transfected with pcDNA3.1 (+) plasmid carrying cDNA of rat cardiac NCX1.1 and brain NCX1.5. The expression of NCX1.1 and NCX1.5 was examined using Western blot analysis. The intracellular Ca^{2+} level ($[\text{Ca}^{2+}]_i$) was measured using Ca^{2+} imaging. Whole-cell NCX currents were recorded using patch-clamp technique. Reverse mode NCX activity was elicited by perfusion with Na^+ -free medium. Ca^{2+} paradox was induced by Ca^{2+} -free EBSS medium, followed by Ca^{2+} -containing solution (1.8 or 3.8 mmol/L CaCl_2).

Results: The protein levels of NCX1.1 and NCX1.5 expressed in CHO cells had no significant difference. The reverse modes of NCX1.1 and NCX1.5 in CHO cells exhibited a transient increase of $[\text{Ca}^{2+}]_i$, which was followed by a Ca^{2+} level plateau at higher external Ca^{2+} concentrations. In contrast, the wild type CHO cells showed a steady increase of $[\text{Ca}^{2+}]_i$ at higher external Ca^{2+} concentrations. The PKC activator PMA (0.3–10 $\mu\text{mol/L}$) and PKA activator 8-Br-cAMP (10–100 $\mu\text{mol/L}$) significantly enhanced the reverse mode activity of NCX1.1 and NCX1.5 in CHO cells. NCX1.1 was 2.4-fold more sensitive to PKC activation than NCX1.5, whereas the sensitivity of the two NCX isoforms to PKA activation had no difference. Both PKC- and PKA-enhanced NCX reverse mode activities in CHO cells were suppressed by NCX inhibitor KB-R7943 (30 $\mu\text{mol/L}$).

Conclusion: Both NCX1.1 and NCX1.5 are functional in regulating and maintaining stable $[\text{Ca}^{2+}]_i$ in CHO cells and differentially regulated by PKA and PKC. The two NCX isoforms might be useful drug targets for heart and brain protection.

Keywords: $\text{Na}^+/\text{Ca}^{2+}$ exchanger; NCX1.1; NCX1.5; intracellular Ca^{2+} level; PKC; PKA; PMA; 8-Br-cAMP; KB-R7943

Acta Pharmacologica Sinica (2013) 34: 691–698; doi: 10.1038/aps.2013.4; published online 8 Apr 2013

Introduction

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) is an ion transporter that is expressed in the plasma membrane of almost all cell types. It plays a pivotal role in the regulation of homeostasis of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in cardiac, neuronal and kidney cells^[1–3]. So far, three mammalian isoforms of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger have been cloned^[4–6]. The isoforms NCX1, NCX2 and NCX3 are products of distinct genes. NCX1 is ubiquitously expressed in many tissues, including heart, brain and kidney, while NCX2 and NCX3 are mainly found in the brain and skeletal muscle^[7]. The transcripts coding for NCX1 show unique tissue-specific expression patterns. For instance,

splicing variant NCX1.1 (containing exons ACDEF) is dominant in the heart, while NCX1.5 (containing exons ADF) is the predominant splicing product expressed in the brain^[8].

To date, the interactions between NCX1 and various extracellular and intracellular factors such as Na^+ and Ca^{2+} , pH, adenosine triphosphate (ATP), phosphatidic acid (PA), phosphatidylinositol 4,5-bisphosphate (PIP_2), protein kinase A (PKA) and protein kinase C (PKC) have been extensively characterized^[9]. Although the activity of cardiac NCX1.1 is known to be regulated by PKC-mediated signaling^[10], the regulation of NCX1.1 activity by the PKA pathway is not fully defined^[11]. In addition, studies testing the regulation of NCX1.5 by PKA and PKC were reported rarely. Some authors reported that a phosphorylation step could accelerate the transporting function of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger^[12–14], but others failed to observe the same effect^[15, 16].

* To whom correspondence should be addressed.

E-mail wangxl@imm.ac.cn

Received 2012-11-18 Accepted 2013-01-11

Our previous work demonstrated that NCX1 plays an important role in transient focal cerebral ischemia^[17]. We also reported that intracellular ATP depletion and acidification synergistically impaired Ca^{2+} extrusion via forward mode $\text{Na}^+/\text{Ca}^{2+}$ exchange in ventricular myocytes isolated from guinea pig^[18]. These results suggest that NCX1 plays a critical role in the pathophysiology of transient focal cerebral ischemia and cardiac ischemia, and it is due to the calcium overload induced by reverse mode $\text{Na}^+/\text{Ca}^{2+}$ exchange. Attempts to clarify the participation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in physiological and pathological processes have been made since this antiporter was found in 1969^[19], but these studies have been hampered by the lack of compounds that can selectively block this carrier. In 1996, Iwamoto *et al* reported that KB-R7943 could selectively inhibit the NCX reverse activity^[10], and subsequently, others found that KB-R7943 was also an effective inhibitor of nicotinic acetylcholine receptors (nAChRs), the *N*-methyl-*D*-aspartic acid (NMDA) receptor, ryanodine receptors (RyRs) and transient receptor potential (TRP) channels^[20–23]. Our laboratory found that KB-R7943 inhibited both directions of the exchange current with an almost equal potency^[24]. Currently, the exact role of NCX1 in physiological and pathophysiological conditions remains to be studied. The reverse mode activity of NCX may play an important role in enhancing intracellular Ca^{2+} levels especially under pathophysiological conditions. In this study, we expressed two isoforms of NCX1 (NCX1.1 and NCX1.5, mainly expressed in the heart and brain, respectively) in CHO cells and investigated the modulation of their reverse mode activity by PKC and PKA.

Materials and methods

Cell culture and transfection

CHO-K1 cells (ATCC, Manassas, VA, USA) were cultured in F12-K media (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. NCX1 plasmids were generously provided by Dr Jonathan LYTON (University of Calgary, Calgary, Alberta, Canada). Using Lipofectamine (Invitrogen, Carlsbad, CA, USA) transfection reagent, CHO-K1 cells were transfected with pcDNA3.1 (+) plasmid carrying rat cDNA of cardiac NCX1.1 and brain NCX1.5. To select the transfected cells, G418 (Sigma-Aldrich, St Louis, MO, USA) was applied to the transfected and non-transfected cells in graded concentrations. The effective concentration was determined to be the concentration that inhibited 100% of the non-transfected cells. Cells stably expressing high levels of NCX proteins were selected by a Ca^{2+} killing procedure^[25]. Single-cell clones that expressed similar levels of NCX1.1 and NCX1.5 proteins were chosen for the study.

Preparation of membrane proteins and Western blot analysis

Stably transfected CHO-K1 cells were collected and suspended in lysing buffer containing 10 mmol/L Tris-HCl, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L EDTA and 1 mmol/L dithiothreitol and then subjected to three freeze-thaw cycles. The particulate fraction was gathered by centrifugation

at 11000×g for 30 min at 4°C and resuspended in 5 mmol/L Tris-HCl at pH 8.0 and 10% sucrose. The protein concentration was determined by the Bradford method. The membrane protein samples (25 µg) were separated on 8% SDS-PAGE gels and transferred to PVDF membranes. The membrane was incubated overnight with a rabbit polyclonal antibody against NCX1 (Chemicon, Temecula, CA, USA) at a 1:400 dilution. After extensive washing, the membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the blot was rinsed and visualized using ECL (Fujifilm, Tokyo, Japan) and quantified using Kodak 1D software.

$[\text{Ca}^{2+}]_i$ imaging

For $[\text{Ca}^{2+}]_i$ measurement, CHO-K1 cells were grown on glass coverslips and incubated in bath buffer (140 mmol/L NaCl, 10 mmol/L glucose, 1 mmol/L MgCl_2 , 5 mmol/L KCl, 1 mmol/L CaCl_2 and 20 mmol/L HEPES, pH adjusted to 7.4 with Tris) containing 3 µmol/L fura 2-AM and 1% bovine serum albumin for 1 h at room temperature. After loading, the cells were washed three times with the bath buffer and used for measurements. A Lambda DG4 system (Sutter Instrument Co, Novato, CA, USA), under the control of Metafluor software (Molecular Devices, Sunnyvale, CA, USA), was used for Fura-2 excitation. The ratio within each cell was computed from images obtained at excitation wavelengths 340 and 380 nm and by subtracting the background fluorescence at each wavelength; emission was monitored at 510 nm. Time-lapse recording initially captured the images at 1-s intervals. To minimize cell photobleaching throughout the experiment, the intervals were increased to 3 s. Data presented were acquired at 3-s intervals. The experiment was repeated on multiple coverslips. Phorbol 12-myristate 13-acetate (PMA), 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) and other chemical reagents were purchased from Sigma unless otherwise indicated.

NCX reverse mode activity

Cells were incubated in bath buffer containing 0.1 mmol/L ouabain to increase the $[\text{Na}^+]_i$. To elicit reverse mode NCX activity, the bath buffer was switched to Na^+ -deficient NMDG⁺ medium (Na^+ -free) (5 mmol/L KCl, 140 mmol/L *N*-methyl glutamine, 1 mmol/L MgCl_2 , 1 mmol/L CaCl_2 , 10 mmol/L glucose, and 20 mmol/L HEPES, buffered to pH 7.4 with Tris). The rate of Ca^{2+} increase after Na^+ -free exposure was calculated as the slope of $[\text{Ca}^{2+}]_i$ plotted against time. All the results are presented as the cytosolic Ca^{2+} concentration. The fluorescent signal was calibrated *in vivo* at the end of each experiment using the method described by Grynkiewicz *et al*^[26]. The dissociation constant of Fura-2 for Ca^{2+} was considered to be 224 nmol/L. The maximum ratio (R_{max}) was obtained by permeabilizing the cells with a Ca^{2+} ionophore, ionomycin (10 µmol/L), in the presence of 1.5 mmol/L extracellular Ca^{2+} . The minimum ratio (R_{min}) was subsequently obtained by the exposure of cells to a Ca^{2+} -free solution (containing 10 mmol/L EGTA and 10 µmol/L ionomycin).

Calcium paradox injury

The calcium paradox model was constructed as follows: after loading Fura 2-AM in Earle's Balanced Salt Solution (EBSS, Invitrogen, Carlsbad, CA, USA) with 1.8 mmol/L CaCl_2 , Ca^{2+} paradox was induced by a 1-h incubation of cells in a Ca^{2+} -free EBSS medium, followed by a 1 h incubation in a Ca^{2+} -containing solution with 1.8 mmol/L or 3.8 mmol/L CaCl_2 at room temperature.

Electrophysiological recordings

The NCX currents were recorded from whole-cell patch configuration by the patch-clamp technique using EPC10 amplifiers (HEKA Electronics, Lambrecht, Germany) at room temperature (22–24°C). Currents were elicited by an ascending ramp pulse depolarized immediately from a holding potential of 0 mV to 100 mV and then hyperpolarized to -100 mV at 0.5 V/s. The external solution was 140 mmol/L NaCl, 2 mmol/L CaCl_2 , 1 mmol/L MgCl_2 , 0.33 mmol/L NaH_2PO_4 , 5 mmol/L HEPES, 5.5 mmol/L glucose, 0.02 mmol/L ouabain, and 0.01 mmol/L verapamil, pH 7.4. The pipette solution was 20 mmol/L NaCl, 3 mmol/L CaCl_2 , 0.5 mmol/L MgCl_2 , 50 mmol/L aspartic acid, 120 mmol/L CsCl, 5 mmol/L EGTA, 10 mmol/L HEPES and 5 mmol/L MgATP, pH 7.25. The NCX inhibitor KB-R7943 was kindly offered by Dr Chikaomi YAMADA of Nippon Organon KK in Japan, and other chemical reagents were purchased from Sigma.

Statistical analysis

The data are presented as mean $\pm$ SEM. Statistical significance was evaluated by Student's *t*-test or a one-way analysis of variance followed by Dunnett's test using SPSS software.

$P < 0.05$ was considered to be statistically significant.

Results

Expression and function of NCX1.1 and NCX1.5 in transfected CHO cells

The expression levels of NCX1 isoforms were analyzed to verify the transfection efficacy in CHO cells. As shown in Figure 1A and 1B, Western blot analysis of NCX-transfected CHO cells showed two proteins with molecular masses of 120 kDa and 160 kDa that were not present in the wide-type CHO cells. The 120 kDa and 160 kDa proteins correspond to the mature NCX1 protein and the intra-molecular disulfide protein^[27].

NCX1.1 and NCX1.5 activities were determined with a Na^+ gradient-dependent $[\text{Ca}^{2+}]_i$ increase in stably transfected CHO cells using the single cell Fura-2 microfluorimetry technique. The NCX reverse mode of operation was elicited by the perfusion of Na^+ -deficient NMDG⁺ medium (NaCl replaced with equimolar *N*-methyl-*D*-glutamine). The increased $[\text{Ca}^{2+}]_i$ rates elicited by the Na^+ -free solution were 7.00 ± 0.50 and 8.64 ± 0.48 $\text{nmol} \cdot \text{L}^{-1} \cdot \text{s}^{-1}$ in NCX1.1- and NCX1.5-expressing CHO cell lines, respectively. The two cell lines expressed comparable levels of NCX proteins. WT CHO cells did not respond to the Na^+ -free solution treatment (Figure 1C and 1D).

We examined the effects of KB-R7943, a potent NCX inhibitor, on the NCX current induced by a ramp pulse in the two transfectants. We observed that KB-R7943 inhibited the NCX current in a concentration-dependent manner at +80 mV. No significant differences were observed in the KB-R7943 inhibition of the NCX current between the two NCX1 isoforms (Figure 2A and 2B).

Figure 1. Expression and function of cardiac NCX1.1 and brain NCX1.5 in transfected CHO cells. (A) Immunoblotting and typical images of NCX and actin in wild-type (WT) cells and NCX-transfected CHO cells. (B) NCX1.1 and NCX1.5 protein levels in the transfected cells quantified by densitometry. (C) Representative traces showing $[\text{Ca}^{2+}]_i$ in WT CHO cells and cells transfected with NCX1.1, NCX1.4, and NCX1.5 under Na^+ -free conditions (NaCl replaced with NMDG). Experiments were repeated on 3 or 4 coverslips. For each coverslip, 20–40 individual cells were examined. (D) The initial increase rates of $[\text{Ca}^{2+}]_i$ in WT CHO and in cells transfected with NCX1.1 and NCX1.5 induced by Na^+ -free solution. $n=3-5$. Mean $\pm$ SEM.

Figure 2. Effect of NCX inhibitor KB-R7943 on I_{NCX} in cells stably transfected with NCX1.1 and NCX1.5. (A) Representative traces of the Na^+/Ca^{2+} exchange current in NCX cells before and after application of 10 μ mol/L KB-R7943. Currents were elicited by an ascending ramp pulse depolarized immediately from holding potential of 0 mV to 100 mV, then hyperpolarized to -100 mV at 0.5 V/s and subsequently returned to the holding potential at room temperature. (B) The inhibitory effects of KB-R7943 on I_{NCX} at +80 mV in stably transfected NCX cells. $n=3-5$. Mean $\pm$ SEM.

Functions of reverse mode NCX1.1 and NCX1.5 during the Ca^{2+} paradox

The calcium paradox model was used to investigate the functions of the transfected NCX isoforms in response to a change in the extracellular Ca^{2+} levels. As shown in Figure 3, in the absence of extracellular Ca^{2+} , the $[Ca^{2+}]_i$ levels were similar in both cell lines. After incubation with Ca^{2+} -containing solution, the levels of $[Ca^{2+}]_i$ in the transfected and non-transfected cells were markedly increased. When the incubation solution contained 1.8 mmol/L Ca^{2+} , the transient increase in WT cells was not significantly different, and the Ca^{2+} level plateau was not changed in transfected cells (Figure 3A–3C and 3G). However, when reperfused with 3.8 mmol/L Ca^{2+} , the transient increase in Ca^{2+} levels was higher in NCX1.1 and NCX1.5 cell lines than that in WT cells (Figure 3D–3F and 3H). Meanwhile, the Ca^{2+} level plateaus were lower in the transfected cell lines than that in WT cells (Figure 3G and 3H). Interestingly, after incubation with 3.8 mmol/L Ca^{2+} , the $[Ca^{2+}]_i$ increased continuously in WT cells, but it remained stable in the NCX1.1 and NCX1.5 transfectants, suggesting a potential protective role of NCX during calcium overload.

Effects of PMA on reverse mode activity of NCX1 transfectants

To provide insight into physiological modulation, we investigated the NCX reverse operation mode in response to PKC activation. When cells were incubated with Na^+ -free solution, the Ca^{2+} influx occurred immediately after reverse NCX activity was initiated. PMA (0.1–10 μ mol/L) increased $[Ca^{2+}]_i$ in NCX1.1 cells by 1.6 to 3.4-fold in a dose-dependent manner (Figure 4A and 4C), and a significant increase was observed from 0.3 μ mol/L PMA to a maximum level at 3 μ mol/L. The NCX1.5 transfectant was less sensitive to PKC activation compared to cells transfected with NCX1.1. A significant increase of $[Ca^{2+}]_i$ was observed at 10 μ mol/L PMA for NCX1.5. The concentration of PMA for the maximal $[Ca^{2+}]_i$ effect was 2.4-fold higher in the NCX1.5 cell line than that in NCX1.1 cells (Figure 4B and 4D). In contrast, PMA treatment did not induce Ca^{2+} uptake in wide-type CHO cells, indicating that NCX reverse activity provided the primary Ca^{2+} influx pathway under these experimental conditions. After pretreatment of NCX1.1- and NCX1.5-transfected cells with NCX1 antagonist KB-R7943 at 30 μ mol/L for 20 min, the Ca^{2+} uptake induced by 10 μ mol/L PMA was almost abolished in the two cell lines. This result suggests that PKC-activated Na^+/Ca^{2+} exchange might be inhibited by KB-R7943. In addition, the increased $[Ca^{2+}]_i$ was not due to endogenous Ca^{2+} activation because the $[Ca^{2+}]_i$ increase was completely abolished when 5 mmol/L EGTA was added to the extracellular solution (data not shown), suggesting that this $[Ca^{2+}]_i$ rise was dependent on external calcium.

8-Br-cAMP increased $[Ca^{2+}]_i$ in NCX1 transfectants at reverse mode

To determine the role of PKA phosphorylation in modulating NCX1 activity, we examined the effects of PKA activator 8-Br-cAMP on NCX reverse mode. When 8-Br-cAMP was added during perfusion, the Ca^{2+} influx was increased by about 3-fold in cell lines transfected with NCX1.1 or NCX1.5 (Figure 5). However, this result was different when compared to that of PKC activation; PKA activator 8-Br-cAMP caused an intracellular calcium oscillation in NCX-transfected cells even at the relatively lower concentration of 10 μ mol/L. The representative traces of $[Ca^{2+}]_i$ oscillations in response to 8-Br-cAMP treatment are shown in Figure 5A and 5B. To further determine whether the intracellular Ca^{2+} enhanced by 8-Br-cAMP was mediated by NCX, the NCX1 subtype cell lines were pre-incubated with 30 μ mol/L KB-R7943 for 20 min before PKA activation. KB-R7943 significantly reduced the Ca^{2+} uptake induced by 8-Br-cAMP ($P<0.01$, Figure 5C and 5D). However, the Ca^{2+} oscillation was not significantly inhibited. The Ca^{2+} oscillation could be abolished by 5 mmol/L EGTA (data not shown). This result indicates that the rise of $[Ca^{2+}]_i$ was mainly due to the extracellular solution.

Discussion

The reverse mode-mediated increase of $[Ca^{2+}]_i$ exhibited two phases: a transient increase and a steady-state level when

Figure 3. Effect of the Ca^{2+} paradox on $[\text{Ca}^{2+}]_i$ in WT-CHO cells and in stably transfected NCX1.1 and NCX1.5 cells. (A–C) Representative traces of mean $[\text{Ca}^{2+}]_i$ in WT-CHO and in stably transfected NCX1.1 and NCX1.5 cells exposed to Ca^{2+} -free solution for 60 min and then incubated in normal EBSS (1.8 mmol/L Ca^{2+}) solution for another 60 min. (D–F) Representative traces of mean $[\text{Ca}^{2+}]_i$ in CHO-WT and in stably transfected NCX1.1 and NCX1.5 cells exposed to Ca^{2+} -free solution for 60 min and then incubated in extracellular solution with 3.8 mmol/L $[\text{Ca}^{2+}]_o$ for another 60 min. For A–F, each treatment was repeated on 3 or 4 cover slips. For each cover slip, 20–40 individual cells were monitored. G and H: The quantification of the $[\text{Ca}^{2+}]_i$ increase elicited by 30 min of Ca^{2+} -free solution and 60 min of normal EBSS solution and extracellular solution with 3.8 mmol/L $[\text{Ca}^{2+}]_o$. ^b $P < 0.05$, ^c $P < 0.01$ vs WT CHO cells. ^e $P < 0.05$ vs WT CHO cells 1 h after incubation with 1.8 mmol/L $[\text{Ca}^{2+}]_o$. ^h $P < 0.05$ vs WT CHO cells 1 h after incubation with 3.8 mmol/L $[\text{Ca}^{2+}]_o$. $n = 3$ –5. Mean $\pm$ SEM.

$[\text{Ca}^{2+}]_o$ was increased from the baseline (Ca^{2+} -free). Under the physiological Ca^{2+} concentration (1.8 mmol/L Ca^{2+}), both the transient and steady-state $[\text{Ca}^{2+}]_i$ in NCX1.1- and NCX1.5-transfected cells were obvious. This result suggests that the transient NCX1-mediated increase of intracellular Ca^{2+} by reverse mode was switched off quickly, and therefore, the intracellular Ca^{2+} level might maintain homeostasis. Even under the Ca^{2+} paradox condition (3.8 mmol/L extracellular Ca^{2+}), the steady-state levels of intracellular Ca^{2+} were still close to the baseline in transfected cells. However, in control CHO cells, intracellular Ca^{2+} could not be stably maintained. Although a small Ca^{2+} influx was also observed in control CHO cells, this Ca^{2+} influx might be mediated partially by a Ca^{2+} -activated cationic channel that is constitutively expressed

in CHO cells^[28]. A similar phenomenon was observed by Chernysh^[29]. However, the $[\text{Ca}^{2+}]_i$ increase was more striking in the NCX transfected cells than in the wide-type cells because of the reverse mode activity^[30].

To date, the cardiac exchanger NCX1.1 has been extensively studied with respect to structure, function and regulation^[31, 32]. However, the isoform NCX1.5, expressed mainly in neuronal cells, is rarely reported. Our present results indicate that NCX1.1 and NCX1.5 exhibit similar responses to PKA activation with 8-Br-cAMP and a rapid rise and decline of intracellular Ca^{2+} , which is a specific property of NCX reverse mode activity. Furthermore, we found that 8-Br-cAMP repeatedly induced an intracellular Ca^{2+} increase after immediate exposure to the cells, *ie*, the oscillation of Ca^{2+} . This 8-Br-cAMP-

Figure 4. Dose-dependent effects of PKC activator PMA on NCX reverse activity in NCX1.1 and NCX1.5 stably transfected cells. (A and B) Representative traces for the effect of Na⁺-free conditions on [Ca²⁺]_i in the absence and presence of different concentrations (1–10 μmol/L) of PMA and subsequently in the presence of KB-R7943 (30 μmol/L) in cells transfected with NCX1.1 and NCX1.5. Each treatment was repeated on 3 or 4 coverslips and 20–40 individual cells were examined per coverslip. (C and D) The initial rate of [Ca²⁺]_i increase induced by Na⁺-free conditions in the absence or presence of PMA and subsequently in the presence of KB-R7943 (30 μmol/L) in cells transfected with NCX1.1 and NCX1.5. ^a*P*<0.05, ^c*P*<0.01 vs 0 μmol/L PMA (Na⁺-free solution). ^f*P*<0.01 vs 10 μmol/L PMA. *n*=3–5. Mean±SEM.

induced calcium oscillation in NCX1 isoforms is likely attributed to the release of Ca²⁺ from the ER triggered by NCX^[33, 34]. Interestingly, the behaviors of intracellular Ca²⁺ activated by PKA were different from those by PKC activation. This result indicates that the targets of phosphorylation by PKC and PKA for modulation of Ca²⁺ release might be different.

Figure 5. Effects of PKA activator 8-Br-cAMP on NCX reverse activity in NCX1.1 and NCX1.5 stably transfected cells. (A and B) Representative traces for the effect of Na⁺-free conditions on [Ca²⁺]_i in the presence of different concentrations (0, 10, and 100 μmol/L) of 8-Br-cAMP and subsequently in the presence of KB-R7943 (30 μmol/L) in NCX1.1 and NCX1.5 transfected cells. Each treatment was repeated on 3 or 4 coverslips and 20–40 individual cells were examined per coverslip. (C and D) The initial rate of [Ca²⁺]_i increase induced by Na⁺-free conditions in the absence and presence of 8-Br-cAMP and subsequently in the presence of KB-R7943 (30 μmol/L). ^a*P*<0.01 vs 0 μmol/L 8-Br-cAMP (Na⁺-free solution). ^f*P*<0.01 vs 100 μmol/L 8-Br-cAMP. *n*=3–5. Mean±SEM.

In the present study, we demonstrated that NCX1.1 was more sensitive to PKC activation with PMA. At 0.3 μmol/L of PMA, the Ca²⁺ peak level was enhanced by two-fold and at the 3 μmol/L of PMA, the activity of NCX1.1 reverse mode reached its maximum. NCX1.5 was not sensitive to PMA activation. The half maximal activation and the maximal activation were about 3 and 10 μmol/L, respectively; they were

about 5 times higher than those in NCX 1.1. KB-R7943 abolished the effects of PMA on the Ca^{2+} increase in the two transfected cell lines. The $[\text{Na}^+]_i$ -dependent Ca^{2+} increase observed in our study is in accordance with previous reports^[35], and evidence for NCX1.1 phosphorylation by PKC has also been reported^[35]. Further studies performed in cardiac myocytes^[6, 7, 25] revealed that cardiac NCX (mainly 1.1 isoform) might be regulated by not only PKC but also PKA-mediated phosphorylation at the intracellular loop of the NCX^[10, 36–39]. The regulation of PKA and PKC on NCX1.5 was not reported previously. Our results demonstrate that PKA and PKC activation could enhance the activities of reverse mode NCX1.5. In addition, we also demonstrated that NCX1.5 showed a lower sensitivity to PKC and almost same sensitivity to PKA as NCX1.1.

Because of the broad tissue distribution of NCX1 isoforms and the absence of specific blockers for NCX1.1 and NCX1.5, it is difficult to investigate the regulation of NCX isoforms in native tissues. Our study demonstrates that NCX1.1 and NCX1.5 cell lines could be used to study some functions of NCX isoforms in heart tissue and in neurons, especially to investigate the regulations of the intracellular Ca^{2+} level by PKA and PKC through NCX1.1 and NCX1.5 (in heart and in neurons, respectively) under pathophysiological conditions. The data in our study showed that in NCX1.5 cell lines, the transient increase of intracellular Ca^{2+} induced by PKC was lower than that in NCX1.1, which might indicate the functional requirement for neuronal cells. In addition, because PKA and PKC are activated by various G protein-coupled receptors (GPCRs), both cell lines can be used as tools for studying the effects of certain GPCRs on intracellular Ca^{2+} through NCX in heart and brain tissues in the future.

These results suggest that NCX plays an important role in mediating a transient increase in Ca^{2+} levels and that NCX can prevent calcium overload under physiological or pathophysiological conditions. Cell lines stably transfected with NCX1 isoforms are therefore useful for further exploring the phosphorylation mechanisms involved in the regulation of NCX activity by protein kinases. NCX1.1 and NCX1.5 could be also validated as potential drug targets for protection of heart or brain injury from Ca^{2+} overload.

Acknowledgements

We thank Dr Jonathan LYTTON for generously providing NCX1.1 and NCX1.5 plasmids and Dr Nai-hong CHEN for technical assistance on calcium imaging. This work was supported by the National 973 Fundamental Project of China (2011CB504103) and the National Major Special Project on New Drug Innovation of China (No 2012ZX09301002-004).

Author contribution

Xiao-liang WANG designed the research and revised the paper; Yan LONG performed the research; Yan LONG, Weiping WANG, and Hui YUAN wrote the paper; Shi-ping MA, Nan FENG, and Ling WANG helped analyze data.

References

- Condrescu M, Opuni K, Hantash BM, Reeves JP. Cellular regulation of sodium-calcium exchange. *Ann N Y Acad Sci* 2002; 976: 214–23.
- Blaustein MP, Lederer WJ. Sodium/calcium exchange: its physiological implications. *Physiol Rev* 1999; 79: 763–854.
- Philipson KD, Nicoll DA, Ottolia M, Quednau BD, Reuter H, John S, et al. The $\text{Na}^+/\text{Ca}^{2+}$ exchange molecule: an overview. *Ann N Y Acad Sci* 2002; 976: 1–10.
- Nicoll DA, Quednau BD, Qui Z, Xia YR, Lusis AJ, Philipson KD. Cloning of a third mammalian $\text{Na}^+-\text{Ca}^{2+}$ exchanger, NCX3. *J Biol Chem* 1996; 271: 24914–21.
- Nicoll DA, Longoni S, Philipson KD. Molecular cloning and functional expression of the cardiac sarcolemmal $\text{Na}^+-\text{Ca}^{2+}$ exchanger. *Science* 1990; 250: 562–5.
- Li Z, Matsuoka S, Hryshko LV, Nicoll DA, Bersohn MM, Burke EP, et al. Cloning of the NCX2 isoform of the plasma membrane $\text{Na}^+-\text{Ca}^{2+}$ exchanger. *J Biol Chem* 1994; 269: 17434–9.
- Quednau BD, Nicoll DA, Philipson KD. Tissue specificity and alternative splicing of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger isoforms NCX1, NCX2, and NCX3 in rat. *Am J Physiol* 1997; 272: C1250–61.
- Kofuji P, Lederer WJ, Schulze DH. Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca exchanger. *J Biol Chem* 1994; 269: 5145–9.
- Annunziato L, Pignataro G, Di Renzo GF. Pharmacology of brain $\text{Na}^+/\text{Ca}^{2+}$ exchanger: from molecular biology to therapeutic perspectives. *Pharmacol Rev* 2004; 56: 633–54.
- Iwamoto T, Pan Y, Wakabayashi S, Imagawa T, Yamanaka HI, Shigekawa M. Phosphorylation-dependent regulation of cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger via protein kinase C. *J Biol Chem* 1996; 271: 13609–15.
- Zhang YH, Hancox JC. Regulation of cardiac $\text{Na}^+-\text{Ca}^{2+}$ exchanger activity by protein kinase phosphorylation — still a paradox? *Cell Calcium* 2009; 45: 1–10.
- DiPolo R, Berberian G, Delgado D, Rojas H, Beauge L. A novel 13 kDa cytoplasmic soluble protein is required for the nucleotide (MgATP) modulation of the Na/Ca exchange in squid nerve fibers. *FEBS Lett* 1997; 401: 6–10.
- Newell EW, Stanley EF, Schlichter LC. Reversed $\text{Na}^+/\text{Ca}^{2+}$ exchange contributes to Ca^{2+} influx and respiratory burst in microglia. *Channels (Austin)* 2007; 1: 366–76.
- He S, Ruknudin A, Bambrick LL, Lederer WJ, Schulze DH. Isoform-specific regulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in rat astrocytes and neurons by PKA. *J Neurosci* 1998; 18: 4833–41.
- Collins A, Somlyo AV, Hilgemann DW. The giant cardiac membrane patch method: stimulation of outward $\text{Na}^+-\text{Ca}^{2+}$ exchange current by MgATP. *J Physiol* 1992; 454: 27–57.
- Lin X, Jo H, Sakakibara Y, Tambara K, Kim B, Komeda M, et al. Beta-adrenergic stimulation does not activate $\text{Na}^+/\text{Ca}^{2+}$ exchange current in guinea pig, mouse, and rat ventricular myocytes. *Am J Physiol Cell Physiol* 2006; 290: C601–8.
- Li LL, Sun LN, Zhou HY, Li ZB, Wang XL. Selective alteration of expression of $\text{Na}^+/\text{Ca}^{2+}$ exchanger isoforms after transient focal cerebral ischemia in rats. *Neurosci Lett* 2006; 404: 249–53.
- Zhou HY, Han CY, Wang XL. Regulative effects of temperature, intracellular sodium, ATP and pH on $I_{(\text{Na/Ca})}$ of cardiac myocytes. *Sheng Li Xue Bao* 2006; 58: 136–40.
- Baker PF, Blaustein MP, Hodgkin AL, Steinhardt RA. The influence of calcium on sodium efflux in squid axons. *J Physiol* 1969; 200: 431–58.
- Sobolevsky AI, Khodorov BI. Blockade of NMDA channels in acutely

- isolated rat hippocampal neurons by the $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibitor KB-R7943. *Neuropharmacology* 1999; 38: 1235–42.
- 21 Kraft R. The $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibitor KB-R7943 potently blocks TRPC channels. *Biochem Biophys Res Commun* 2007; 361: 230–6.
- 22 Pintado AJ, Herrero CJ, Garcia AG, Montiel C. The novel $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. *Br J Pharmacol* 2000; 130: 1893–902.
- 23 Barrientos G, Bose DD, Feng W, Padilla I, Pessah IN. The $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibitor 2-(2-(4-(4-nitrobenzyloxy)phenyl)ethyl)isothiourea methanesulfonate (KB-R7943) also blocks ryanodine receptors type 1 (RyR1) and type 2 (RyR2) channels. *Mol Pharmacol* 2009; 76: 560–8.
- 24 Lu J, Liang Y, Wang X. Amiloride and KB-R7943 in outward $\text{Na}^+/\text{Ca}^{2+}$ exchange current in guinea pig ventricular myocytes. *J Cardiovasc Pharmacol* 2002; 40: 106–11.
- 25 Iwamoto T, Shigekawa M. Differential inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchanger isoforms by divalent cations and isothiourea derivative. *Am J Physiol* 1998; 275: C423–30.
- 26 Gryniewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; 260: 3440–50.
- 27 Santacruz-Toloza L, Ottolia M, Nicoll DA, Philipson KD. Functional analysis of a disulfide bond in the cardiac $\text{Na}^+-\text{Ca}^{2+}$ exchanger. *J Biol Chem* 2000; 275: 182–8.
- 28 Gamper N, Stockand JD, Shapiro MS. The use of Chinese hamster ovary (CHO) cells in the study of ion channels. *J Pharmacol Toxicol Methods* 2005; 51: 177–85.
- 29 Chernysh O, Condrescu M, Reeves JP. Calcium-dependent regulation of calcium efflux by the cardiac sodium/calcium exchanger. *Am J Physiol Cell Physiol* 2004; 287: C797–806.
- 30 Matsuda T, Takuma K, Nishiguchi E, Hashimoto H, Azuma J, Baba A. Involvement of $\text{Na}^+-\text{Ca}^{2+}$ exchanger in reperfusion-induced delayed cell death of cultured rat astrocytes. *Eur J Neurosci* 1996; 8: 951–8.
- 31 Matsuoka S, Nicoll DA, Reilly RF, Hilgemann DW, Philipson KD. Initial localization of regulatory regions of the cardiac sarcolemmal $\text{Na}^+-\text{Ca}^{2+}$ exchanger. *Proc Natl Acad Sci U S A* 1993; 90: 3870–4.
- 32 Nicoll DA, Philipson KD. Molecular studies of the cardiac sarcolemmal sodium-calcium exchanger. *Ann N Y Acad Sci* 1991; 639: 181–8.
- 33 Brini M, Manni S, Carafoli E. Recombinant expression of the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger affects local and global Ca^{2+} homeostasis in Chinese hamster ovary cells. *J Biol Chem* 2002; 277: 38693–9.
- 34 Fameli N, van Breemen C, Kuo KH. A quantitative model for linking $\text{Na}^+/\text{Ca}^{2+}$ exchanger to SERCA during refilling of the sarcoplasmic reticulum to sustain $[\text{Ca}^{2+}]$ oscillations in vascular smooth muscle. *Cell Calcium* 2007; 42: 565–75.
- 35 Iwamoto T, Wakabayashi S, Shigekawa M. Growth factor-induced phosphorylation and activation of aortic smooth muscle $\text{Na}^+/\text{Ca}^{2+}$ exchanger. *J Biol Chem* 1995; 270: 8996–9001.
- 36 Caroni P, Carafoli E. The regulation of the $\text{Na}^+-\text{Ca}^{2+}$ exchanger of heart sarcolemma. *Eur J Biochem* 1983; 132: 451–60.
- 37 Ruknudin A, He S, Lederer WJ, Schulze DH. Functional differences between cardiac and renal isoforms of the rat $\text{Na}^+-\text{Ca}^{2+}$ exchanger NCX1 expressed in *Xenopus* oocytes. *J Physiol* 2000; 529: 599–610.
- 38 Schulze DH, Polumuri SK, Gille T, Ruknudin A. Functional regulation of alternatively spliced $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX1) isoforms. *Ann N Y Acad Sci* 2002; 976: 187–96.
- 39 Ruknudin A, Schulze DH. Phosphorylation of the $\text{Na}^+/\text{Ca}^{2+}$ exchangers by PKA. *Ann N Y Acad Sci* 2002; 976: 209–13.