Role of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in regulating cytosolic Ca\textsuperscript{2+} in cultured human pulmonary artery smooth muscle cells

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Zhang, Shen, Jason X.-J. Yuan, Kim E. Barrett, and Hui Dong. Role of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in regulating cytosolic Ca\textsuperscript{2+} in cultured human pulmonary artery smooth muscle cells. Am J Physiol Cell Physiol 288: C245–C252, 2005. First published September 29, 2004; doi:10.1152/ajpcell.00411.2004.—A rise in cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) in pulmonary artery smooth muscle cells (PASMC) is an important stimulus for cell contraction, migration, and proliferation. Depletion of intracellular Ca\textsuperscript{2+} stores opens store-operated Ca\textsuperscript{2+} channels (SOC) and causes Ca\textsuperscript{2+} entry. Transient receptor potential (TRP) cation channels that are permeable to Na\textsuperscript{+} and Ca\textsuperscript{2+} are believed to form functional SOC. Because sarcotubular Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger has also been implicated in regulating [Ca\textsuperscript{2+}]\textsubscript{cyt}, this study was designed to test the hypothesis that the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) in cultured human PASMC is functionally involved in regulating [Ca\textsuperscript{2+}]\textsubscript{cyt} by contributing to store-depletion-mediated Ca\textsuperscript{2+} entry. RT-PCR and Western blot analyses revealed mRNA and protein expression for NCX1 and NCKX3 in cultured human pulmonary artery smooth muscle cells. Removal of extracellular Na\textsuperscript{+}, which switches the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger from the forward (Ca\textsuperscript{2+} exit to reverse Ca\textsuperscript{2+} entry) mode, significantly increased [Ca\textsuperscript{2+}]\textsubscript{cyt}, whereas inhibition of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger with KB-R7943 (10 \mu M) markedly attenuated the increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} via the reverse mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. Store depletion also induced a rise in [Ca\textsuperscript{2+}]\textsubscript{cyt} via the reverse mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. Removal of extracellular Na\textsuperscript{+} or inhibition of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger with KB-R7943 attenuated the store depletion-mediated Ca\textsuperscript{2+} entry. Furthermore, treatment of human PASMC with KB-R7943 also inhibited cell proliferation in the presence of serum and growth factors. These results suggest that NCX is functionally expressed in cultured human PASMC, that Ca\textsuperscript{2+} entry via the reverse mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange contributes to store-depletion-mediated increase in [Ca\textsuperscript{2+}]\textsubscript{cyt}, and that blockade of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in its reverse mode may serve as a potential therapeutic approach for treatment of pulmonary hypertension.

sodium-calcium exchange; calcium homeostasis; vascular smooth muscle

intracellular Ca\textsuperscript{2+} is a critical second messenger that links external stimuli to cell contraction, proliferation, migration, and gene expression (3, 4). An increase in cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) in pulmonary artery smooth muscle cells (PASMC) is a major trigger for pulmonary vasoconstriction and an important stimulus for PASMC proliferation and migration (24, 31). [Ca\textsuperscript{2+}]\textsubscript{cyt} in PASMC can be increased by Ca\textsuperscript{2+} release from intracellular stores, such as the sarcoplasmic (or endoplasmic) reticulum (SR), and by Na\textsuperscript{+} influx through Ca\textsuperscript{2+}-permeable cation channels in the plasma membrane (24).

Binding of vasoconstrictive and mitogenic agonists with G protein-coupled receptors (GPCR) and receptor tyrosine kinases in the plasma membrane activates phospholipase C, which causes hydrolysis of phosphatidylinositol and generation of cytosolic inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and membrane-bound diacylglycerol. IP\textsubscript{3} induces a rapid Ca\textsuperscript{2+} release through IP\textsubscript{3} receptors at the SR membrane (14, 51), and the subsequent depletion of Ca\textsuperscript{2+} from the stores (i.e., the SR) opens a special family of Ca\textsuperscript{2+}-permeable channels, namely, store-operated Ca\textsuperscript{2+} channels (SOC), and elicits capacitative Ca\textsuperscript{2+} entry (CCE) (5, 34, 37, 38). The store depletion-activated SOC are believed to be formed heterogeneously by different isoforms of transient receptor potential (TRP) channels (5, 11, 26, 32, 59). The homo- or heterotetrameric TRP channels are generally nonselective cation channels that allow both Ca\textsuperscript{2+} and Na\textsuperscript{+} to go through (29, 50, 64). Therefore, opening of TRP channels would increase both [Ca\textsuperscript{2+}]\textsubscript{cyt} and cytosolic [Na\textsuperscript{+}] as a result of Ca\textsuperscript{2+} and Na\textsuperscript{+} influx via the channels.

Mammalian cells maintain a low cytoplasmic concentration of Na\textsuperscript{+} ([Na\textsuperscript{+}]\textsubscript{cyt}, \sim 10 mM) compared with the extracellular concentration of Na\textsuperscript{+} ([Na\textsuperscript{+}]\textsubscript{out}, \sim 140 mM) because of the activity of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (25, 58). The transmembrane Na\textsuperscript{+} gradient can be utilized to energize the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, which moves Na\textsuperscript{+} and Ca\textsuperscript{2+} across the membrane in the opposite direction. Two families of plasma membrane Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger proteins have been described in mammalian tissues (8, 30), one in which Ca\textsuperscript{2+} movement depends only on Na\textsuperscript{+} (NCX family) and the other in which Ca\textsuperscript{2+} movement depends on Na\textsuperscript{+} and K\textsuperscript{+} (NCKX family). The stoichiometry of NCX is 3 Na\textsuperscript{+} per 1 Ca\textsuperscript{2+}, whereas that for NCKX4 is 4 Na\textsuperscript{+} per 1 Ca\textsuperscript{2+} and 1 K\textsuperscript{+}. Both NCX and NCKX can operate in either a forward (Ca\textsuperscript{2+} exit) or reverse (Ca\textsuperscript{2+} entry) mode, depending on the Na\textsuperscript{+} and Ca\textsuperscript{2+} (and K\textsuperscript{+}) gradients and membrane potential (E\textsubscript{m}) (8, 14, 24). For a constant extracellular [Ca\textsuperscript{2+}] (1.8–2 mM) and [Na\textsuperscript{+}] (\sim 140 mM), [Ca\textsuperscript{2+}]\textsubscript{cyt} is a cubic function of [Na\textsuperscript{+}]\textsubscript{cyt} and an exponential function of E\textsubscript{m}. Therefore, a small change in [Na\textsuperscript{+}]\textsubscript{cyt} or E\textsubscript{m} can cause a large change in [Ca\textsuperscript{2+}]\textsubscript{cyt} (6).

Many functional studies have demonstrated that the plasma membrane Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is involved in regulating Ca\textsuperscript{2+} homeostasis of blood vessels (1, 8, 44, 45). The functional evidence for the sarcotubular Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in vessels is also supported by direct demonstration that the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is expressed in vascular smooth muscle and endothelial cells (23). However, the expression and function of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in human PASMC are unknown, al-

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though studies show that Na⁺/Ca²⁺ exchange activity is involved in the development of hypoxic pulmonary vasoconstriction in animals (8, 12, 27, 57). Recently, it was also suggested that there is a functional and physical interaction of TRP cation channels with NCX proteins (40). Therefore, the purpose of this study was to confirm the functional expression of NCX and NCKX proteins and to characterize the role of the Na⁺/Ca²⁺ exchanger in store depletion-mediated Ca²⁺ entry in human PASMC, as well as the potential role of the Na⁺/Ca²⁺ exchange-driven Ca²⁺ entry in PASMC proliferation.

**METHODS AND MATERIALS**

*Cell preparation and culture.* Human PASMC from normal subjects were purchased from Cambrex and used at the 4th-6th passages. The cells were plated onto coverslips or petri dishes and incubated in a humidified atmosphere of 5% CO₂ in air at 37°C in smooth muscle growth medium (SmGM; Cambrex, Walkersville, MD). The SmGM was composed of smooth muscle basal medium (SmBM) supplemented with 5% fetal bovine serum (FBS), 0.5% human epidermal growth factor, 2 ng/ml human fibroblast growth factor, and 5 μg/ml insulin. Cells were subcultured or plated onto 25-mm coverslips in trypsin-EDTA buffer when 70–90% confluence was achieved. The morphology of the cells was examined using an inverted phase-contrast microscope attached to a digital camera. Rat brain and pulmonary artery tissues were used in some experiments. Left and right branches of the main pulmonary artery along with intrapulmonary arterial branches were isolated from male Sprague-Dawley rats (125–250 g) (62). Brain tissues were usually removed from the same rats from which lungs were removed to prepare isolated extra- and intrapulmonary arteries.

*Western blot analysis.* Human PASMC were gently washed twice in cold PBS, scraped into 0.3 ml of radioimmunoprecipitation assay buffer [1% PBS, 1% Nonidet P-40 (Amresco, Solon, OH), 0.5% sodium deoxycholate, and 0.1% SDS], and incubated on ice for a 45-min period, during which the cell mixture was shaken for 30 s by vortex three times. Rat brain and pulmonary artery tissues were stored at −80°C and then homogenized in a glass tube with a Teflon Dounce pestle in 5 ml of ice-cold assay buffer as described above. The resulting cell and tissue lysates were sonicated and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants were collected, and protein concentrations were determined using Coomassie Plus protein standard. Protein (30 μg) was mixed and boiled in 2× sample buffer (0.25 M Tris-HCl, pH 6.8, 20% glycerol, 8% SDS, and 0.02% bromphenol blue). Protein suspensions were electrophoretically separated on an 8% acrylamide gel, and protein bands were transferred to nitrocellulose membranes by electroph blotting in a Mini Trans-Blot cell transfer apparatus (Bio-Rad, Hercules, CA) under conditions recommended by the manufacturer. After 1 h of incubation in a blocking buffer (0.1% Tween 20 and 5% nonfat dry milk powder), the membranes were incubated with R3F1 monoclonal antibody against NCX1 (Swant, Bellinzona, Switzerland) diluted in blocking buffer (1:5,000) overnight at 4°C. Finally, the membranes were washed and exposed to anti-mouse horseradish peroxidase-conjugated IgG for 60 min at room temperature. The bound antibody was detected with an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

*RT-PCR.* Total RNAs were extracted from human PASMC, rat brain, and rat pulmonary artery using the RNeasy mini kit (Qiagen, Valencia, CA). Genomic DNA was removed with RNase-free DNase according to the manufacturer’s instructions. SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA) was used to synthesize cDNA. RNA (2 μg) was first incubated with oligo(dT) (1 μl at 0.5 μg/μl) at 70°C for 10 min, and then 8 μl of a solution that contained 10× buffer, 10 mM dNTP, 20 mM MgCl₂, 0.1 M DTT, 40 U/μl RNaseOUT, and 50 U/μl SuperScript II reverse transcriptase was added to the samples and incubated for 10 min at 30°C, 60 min at 42°C, and 5 min at 95°C. RNase-H (1 μl at 2 U/μl; GibCO, Grand Island, NY) was added to each reaction. The sense and antisense primers were specifically designed from the coding regions of each gene (Table 1). The fidelity and specificity of the sense and antisense oligonucleotides were examined using the BLAST program.

*Immunofluorescence labeling.* Human PASMC on slides were fixed in 4% paraformaldehyde for 20 min. After blocking with 4% BSA for 20 min, a specific monoclonal antibody against NCX1 (R3F1; Swant) was applied to the cells, followed by a secondary antibody conjugated with green fluorescence (Alexa Fluor 488; Molecular Probes, Eugene, OR). The cells were then stained with the membrane-permeable nucleic acid stain 4',6'-diamidino-2-phenylindole (DAPI, 5 μM; Sigma, St. Louis, MO), and the blue fluorescence (emitted at 461 nm) was used to detect cell nuclei. The cell images were processed by three-dimensional deconvolution fluorescence microscopy with softWoRx (Applied Precision, Issaquah, WA) and analyzed using Matlab (Mathworks, Natick, MA).

**Measurement of [Ca²⁺]ᵢ**. [Ca²⁺]ᵢ in single human PASMC was measured using the Ca²⁺-sensitive fluorescent indicator fura 2-AM. Cells on 25-mm coverslips were loaded with fura 2-AM (3 μM) for 30 min in the dark at room temperature (22–24°C) under an atmosphere of 5% CO₂-95% air. The fura 2-AM-loaded cells were then transferred to a perfusion chamber on the microscope stage and superfused with physiological salt solution (PSS) for 30 min to remove extracellular dye and allow intracellular esterases to cleave cytosolic fura 2-AM into active fura 2. The PSS contained (in mM) 141 NaCl, 4.7 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 10 glucose, pH 7.4. In Ca²⁺-free PSS, CaCl₂ was replaced by equimolar MgCl₂, and 0.1 mM EGTA was added to chelate residual Ca²⁺. Fura 2 fluorescence (510-nm light emission excited by 340- and 380-nm illuminations) from the cells, as well as background fluorescence, was collected at room temperature (22°C) with the use of a ×40 Nikon UV-Fluor objective and a charge-coupled device camera. The fluorescence signals emitted from the cells were monitored continuously using an Intracellular Imaging fluorescence microscopy system and were recorded in an IBM-compatible computer for later analysis. [Ca²⁺]ᵢ was calculated from fura 2 fluorescence emission excited at 340 and 380 nm.

### Table 1. Oligonucleotide sequences of primers used for RT-PCR

<table>
<thead>
<tr>
<th>Standard Name</th>
<th>Accession Number</th>
<th>Predicted Size, bp</th>
<th>Sense/Antisense</th>
<th>Location, nt</th>
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<tbody>
<tr>
<td>rNCX1</td>
<td>AF109166</td>
<td>231</td>
<td>5' - TGTGCATCTCAGCAATGTCA-3'</td>
<td>1556–1575</td>
</tr>
<tr>
<td>hNCX1</td>
<td>NM_021097</td>
<td>230</td>
<td>5' - CGAGATGCTGGCTCTTGGGTC-3'</td>
<td>1448–1467</td>
</tr>
<tr>
<td>rNCX3</td>
<td>AY009158</td>
<td>213</td>
<td>5' - CTCCTGATTCGAGGTACTC-3'</td>
<td>1770–1749</td>
</tr>
<tr>
<td>hNCX3</td>
<td>AF169257</td>
<td>188</td>
<td>5' - AAGAACGGGATGACATTGAC-3'</td>
<td>1754–1770</td>
</tr>
</tbody>
</table>

Accession numbers are GenBank accession numbers for the sequences used in designing the primers. r, Rat; h, human.
reagents were purchased from LC Laboratories (Woburn, MA), Seikagaku ON, Canada), or Sigma unless indicated otherwise. Pharmacological reagents were obtained from Fisher (Nepean, ON, Canada), BDH (Toronto, ON, Canada), and Sigma unless indicated otherwise. Pharmacological reagents were purchased from LC Laboratories (Woburn, MA), Seikagaku America (Falmouth, MA), or Calbiochem (San Diego, CA). Cell culture reagents were from Life Technologies (Rockville, MD).

Statistics and data analysis. Data are expressed as means ± SE; n represents the number of cells. Statistical analysis was performed using unpaired or paired Student’s t-test or ANOVA as indicated. Differences were considered to be significant at P < 0.05.

RESULTS

mRNA and protein expression of NCX and NCKX. With the use of rat brain tissues as positive controls, our RT-PCR experiments show that human PASMC expressed NCX1 and NCKX3 (Fig. 1A). The PCR products for NCX1 and NCKX3 obtained from human PASMC were sequenced by our core facility; the sequences matched with the GenBank sequences of NCX1 and NCKX3. Furthermore, our Western blot experiments revealed protein expression of NCX1 in human PASMC, although the protein expression level was much less than in rat brain tissues and rat pulmonary arteries (Fig. 1B). R3F1, an anti-NCX1 monoclonal antibody, recognized a pattern of protein expression typical of NCX1 in human PASMC comprising two bands with molecular masses of 120 and 70 kDa (17, 49). The weak band of 70 kDa corresponds to the short form of NCX1, which is either a proteolytically cleaved product or a functional, truncated form of NCX1 (53). In rat brain and pulmonary artery tissues, R3F1 recognized an additional band located between 120 and 70 kDa (that was not present in human PASMC), which is probably another proteolytically cleaved or truncated form of NCX1. In contrast, we could not detect NCKX3 protein expression in human PASMC, although mRNA expression was shown by RT-PCR analysis.

The protein expression of NCX1 in human PASMC also was demonstrated using immunofluorescence analysis. As shown in Fig. 1C, human PASMC incubated with both primary (R3F1) and secondary antibodies showed strong fluorescent staining at the cell surface membrane (Fig. 1C, top); cells incubated with only secondary antibody showed no fluorescence (Fig. 1C, bottom). These results further suggest that NCX1 is expressed in human PASMC.

Functional role of Na+/Ca2+ exchanger in regulating [Ca2+]cyt. To test whether the Na+/Ca2+ exchanger is functionally involved in regulating [Ca2+]cyt, we first measured [Ca2+]cyt in human PASMC superfused with solutions with or without extracellular Na+. As shown in Fig. 2, removal of extracellular Na+ [by replacing Na+ in the bath solution with equimolar N-methyl-D-glucamine (NMDG+) or Li+] caused a rapid increase in [Ca2+]cyt as a result of switching the Na+/Ca2+ exchanger from the forward (Ca2+ exit) mode to the reverse (Ca2+ entry) mode (8, 28). Extracellular application of nifedipine (10 μM), a specific blocker of voltage-dependent Ca2+ channels, had no effect on the increase in [Ca2+]cyt driven by the reverse mode of the Na+/Ca2+ exchanger. However, extracellular application of KB-R7943, a potent and selective inhibitor of the Na+/Ca2+ exchanger (especially in its reverse mode), significantly attenuated the increase in [Ca2+]cyt induced by removal of extracellular Na+ (Fig. 2B). These results suggest that the Na+/Ca2+ exchanger can operate actively in the reverse mode in human PASMC.

The activity of the Na+/Ca2+ exchanges in human PASMC appeared to be independent of extracellular K+, because removal of extracellular K+ had little effect on the rise in [Ca2+]cyt due to the reverse mode of Na+/Ca2+ exchange (Fig. 2). These results suggest that the Ca2+ entry via the reversed mode of Na+/Ca2+ exchange is likely due to the function of NCX (rather than NCKX) in human PASMC.
**NCX in Pulmonary Arterial Myocytes**

Fig. 2. Activation of the reverse mode of Na⁺/Ca²⁺ exchange increases the cytosolic free Ca²⁺ concentration ([Ca²⁺]cyt) in human PASMC. **A**: a representative record (a) showing the time course of [Ca²⁺]cyt changes in cells before, during, and after extracellular Na⁺ was replaced with equimolar N-methyl-D-glucamine (NMDG⁺) in the presence (0Na⁺+5K) or absence (0Na⁺+0K) of 5 mM K⁺. Nifedipine (10 μM) was included in all solutions to block voltage-gated Ca²⁺ channels. Summarized data (means ± SE, b) show the amplitude of increases in [Ca²⁺]cyt in cells superfused with 0Na⁺+5K (+K, n = 33) or 0Na⁺+0K (–K, n = 33) solution. **B**: a representative record (a) showing the time course of [Ca²⁺]cyt changes induced by 0Na⁺+5K solution in the absence or presence of 10 μM KB-R7943 (KB-R). Summarized data (means ± SE, b) show the amplitude of increases in [Ca²⁺]cyt in cells superfused with 0Na⁺+5K solution in the absence (Cont, n = 30) or presence (KB-R, n = 50) of 10 μM KB-R7943. ***P < 0.001 vs. Cont.

**Involvement of Na⁺/Ca²⁺ exchanger in store depletion-induced Ca²⁺ entry.** Upon activation of GPCR or receptor tyrosine kinases, IP₃-mediated Ca²⁺ release (which induces an early transient increase in [Ca²⁺]cyt) depletes Ca²⁺ from intracellular stores (e.g., the SR). The store depletion-mediated opening of TRP channels would promote not only Ca²⁺ influx but also Na⁺ influx, because TRP channels are permeable to both Ca²⁺ and Na⁺ (35, 34). Because Ca²⁺ entry via the Na⁺/Ca²⁺ exchanger depends greatly on [Na⁺]cyt, the store depletion-mediated Na⁺ influx through TRP channels would increase [Na⁺]cyt, activate the reverse mode of Na⁺/Ca²⁺ exchange, and enhance Ca²⁺ entry. The next set of experiments was designed to test the hypothesis that in addition to triggering capacitative Ca²⁺ entry, the passive store depletion mediated by cyclopiazonic acid (CPA; 10 μM) induces Ca²⁺ entry as well via the Na⁺/Ca²⁺ exchanger.

In the absence of extracellular Ca²⁺ and presence of nifedipine (10 μM), extracellular application of CPA, a blocker of the Ca²⁺-Mg²⁺-ATPase in the SR (SERCA), induced a transient increase in [Ca²⁺]cyt due to Ca²⁺ leakage from the SR to the cytosol. Approximately 5–10 min later when the store was depleted (i.e., when the [Ca²⁺]cyt transients declined back to the basal level), restoration of extracellular Ca²⁺ ([Ca²⁺]o) (1.8 mM) induced a large increase in [Ca²⁺]cyt due to Ca²⁺ entry (Fig. 3A). Extracellular application of KB-R7943 (10 μM; an inhibitor of the reverse mode of the Na⁺/Ca²⁺ exchanger) significantly attenuated the sustained phase of the store depletion-mediated Ca²⁺ entry (Fig. 3). Furthermore, removal of extracellular Na⁺ (by replacing external Na⁺ with equimolar NMDG⁺ or Li⁺) also markedly inhibited the store depletion-mediated Ca²⁺ entry (Fig. 4, A and C). In the absence of extracellular Na⁺ (i.e., when Na⁺/Ca²⁺ exchange was in the reverse mode or Ca²⁺ entry mode), the CPA-induced transient [Ca²⁺]cyt rise and baseline [Ca²⁺]cyt were both enhanced as a result of inhibited Ca²⁺ extrusion via the forward mode (or Ca²⁺ exit mode) of Na⁺/Ca²⁺ exchange (Fig. 4, A and B). These results indicate that the Ca²⁺ entry via the reverse mode of Na⁺/Ca²⁺ exchange is involved in store depletion-mediated Ca²⁺ entry in human PASMC.

**Involvement of Na⁺/Ca²⁺ exchangers in human PASMC proliferation.** Store depletion-mediated elevation of [Ca²⁺]cyt is associated with human PASMC proliferation in response to serum and growth factors (19, 48, 60). As mentioned earlier, the store depletion-mediated increase in [Ca²⁺]cyt is partially due to the KB-R7943-sensitive Ca²⁺ entry via the reverse mode of Na⁺/Ca²⁺ exchange. To examine the possible involvement of the Na⁺/Ca²⁺ exchanger in human PASMC proliferation, we tested the effect of KB-R7943 on cell growth rate using flow cytometry. As shown in Fig. 5A, addition of serum and growth factors to culture medium (i.e., switching culture medium from SmBM to SmGM) significantly increased the number of cells that are in S/G2/M phases, whereas treatment of human PASMC with KB-R7943 (10–30 μM for 24 h) significantly reduced the counts of cells in S/G2/M phases. Furthermore, inhibition of the Na⁺/Ca²⁺ exchanger with KB-R7943 (3–30 μM for 48 h) also markedly inhibited the increase in cell numbers when human PASMC were cultured in growth medium (SmGM) (Fig. 5B). Compared with cells treated with vehicle, KB-R7943 reduced cell proliferation in a dose-dependent manner. At low doses (<1 μM), KB-R7943 inhibits only the forward mode of Na⁺/Ca²⁺ exchange, whereas at high

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Fig. 3. Inhibition of Na⁺/Ca²⁺ exchanger with KB-R7943 attenuates store depletion-mediated Ca²⁺ entry. **A**: representative records showing the time course of [Ca²⁺]cyt changes in human PASMC in response to cyclopiazonic acid (CPA; 10 μM) in the presence or absence (0Ca) of extracellular Ca²⁺. KB-R-7943 (10 μM) or vehicle (DMSO) was applied to the cells when the CPA-mediated [Ca²⁺]cyt increase reached the maximal level in the presence of extracellular Ca²⁺. Nifedipine (10 μM) was included in all solutions to eliminate the contribution of voltage-gated Ca²⁺ channels to the [Ca²⁺]cyt changes. **B**: summarized data (means ± SE) showing the amplitude of CPA-mediated increases in [Ca²⁺]cyt after treatment with vehicle (n = 36) or KB-R7943 (n = 48). ***P < 0.001 vs. vehicle control.
the Na-PASMC is likely due to its inhibition of the reverse mode of KB-R7943-mediated antiproliferative effect on human PASMC. Absence of extracellular Ca\textsuperscript{2+} replaces with equimolar Li\textsuperscript{+}. Ca\textsuperscript{2+} expression (4, 9). A rise in [Ca\textsuperscript{2+}]\textsubscript{cyt} changes in response to CPA (10 \mu M) in the presence or absence (0Ca) of extracellular Ca\textsuperscript{2+}. Cells were superfused with solutions containing 140 (140Na) or 0 mM Na\textsuperscript{+} (0Na). In these experiments, extracellular Na\textsuperscript{+} was replaced with equimolar Li\textsuperscript{+}, and nifedipine (10 \mu M) was included in all solutions to eliminate the contribution of voltage-gated Ca\textsuperscript{2+} channels to the [Ca\textsuperscript{2+}]\textsubscript{cyt} changes. B: summarized data (means ± SE) showing the amplitudes of CPA-induced increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to Ca\textsuperscript{2+} leakage or release in the absence of extracellular Ca\textsuperscript{2+} (shaded bars) and the basal levels of [Ca\textsuperscript{2+}]\textsubscript{cyt} after CPA-induced Ca\textsuperscript{2+} release (solid bars) in cells bathed in Na\textsuperscript{+}-containing (140Na, n = 38) or Na\textsuperscript{+}-free (0Na-) solution. C: summarized data (means ± SE) showing the amplitudes of CPA-induced increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to Ca\textsuperscript{2+} entry or influx in the presence of extracellular Ca\textsuperscript{2+} in cells bathed in Na\textsuperscript{+}-containing (140Na, n = 38) or Na\textsuperscript{+}-free (0Na-) solution. Extracellular Na\textsuperscript{+} was replaced with NMDG (n = 63) or LiCl (n = 53). ***P < 0.001 vs. 140Na.

Fig. 4. Switching from the forward mode to the reverse mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange by removing extracellular Na\textsuperscript{+} inhibits store depletion-mediated Ca\textsuperscript{2+} entry. A: representative records showing the time course of [Ca\textsuperscript{2+}]\textsubscript{cyt} changes in response to CPA (10 \mu M) in the presence or absence (0Ca) of extracellular Ca\textsuperscript{2+}. B: summarized data (means ± SE) showing cell numbers (n = 12 experiments) of human PASMC cultured in SmBM and SmGM with or without 3, 10, or 30 \mu M KB-R7943 (for 48 h). ***P < 0.001 vs. SmBM without KB-R7943 (solid bar).
in the reverse mode to promote Ca\(^{2+}\) entry and increase [Ca\(^{2+}\)\(_{\text{cyt}}\)], and 3) removal of extracellular K\(^+\) has no effect on the Ca\(^{2+}\) entry via the reverse mode of Na\(^+/Ca\(^{2+}\) exchange. These observations provide strong evidence that Ca\(^{2+}\) entry via the reverse mode of the Na\(^+/Ca\(^{2+}\) exchange is a critical mechanism that regulates intracellular Ca\(^{2+}\) homeostasis in human PASMC. The NCX1 is probably the major Na\(^+/Ca\(^{2+}\) exchanger isofrom in human PASMC that functions in the reverse mode when [Na\(^{+}\)\(_{\text{cyt}}\)] is increased but works in the forward mode when [Ca\(^{2+}\)\(_{\text{cyt}}\)] is increased and [Na\(^{+}\)\(_{\text{cyt}}\)] remains unchanged. The protein expression level of NCX1 in cultured human PASMC was noticeably much smaller than that in freshly isolated rat pulmonary arteries (Fig. 1B), although the immunocytochemistry data clearly showed the surface expression of NCX1 in cultured PASMC (Fig. 1C). There are several possibilities that explain the discrepancies: 1) expression level of NCX1 may be significantly different between human and rat PASMC, and 2) expression level of NCX1 in human pulmonary arteries may be changed (e.g., downregulated) in cultured PASMC because of phenotypic changes (e.g., from contractile phenotype to synthetic or proliferative phenotype). As mentioned earlier, the stoichiometry of the NCX-encoded Na\(^+/Ca\(^{2+}\) exchanger proteins is 3 Na\(^+\) per 1 Ca\(^{2+}\). Thus [Ca\(^{2+}\)\(_{\text{cyt}}\)] determined by NCX is mainly related to [Na\(^{+}\)\(_{\text{cyt}}\)] by the following equation:

\[
\left[\text{Ca}^{2+}\right]_{\text{cyt}} = \left[\text{Ca}^{2+}\right]_{\text{out}} \times \frac{([\text{Na}^{+}]_{\text{cyt}}/[\text{Na}^{+}]_{\text{out}})^3 \times e^{(F/T)}}{e^{(F/R)} - 1}
\]

where \(F\) is the Faraday constant, \(R\) is the gas constant, and \(T\) is absolute temperature. The equation indicates that for a constant [Ca\(^{2+}\)\(_{\text{cyt}}\)] and [Na\(^{+}\)\(_{\text{cyt}}\)], [Ca\(^{2+}\)\(_{\text{cyt}}\)] is positively proportional to the third power of [Na\(^{+}\)\(_{\text{cyt}}\)]; that is, a small increase in [Na\(^{+}\)\(_{\text{cyt}}\)] can cause a large increase in [Ca\(^{2+}\)\(_{\text{cyt}}\)] due to Na\(^+/Ca\(^{2+}\) exchange.

In PASMC, activation of GPCR (e.g., endothelin receptor) or receptor tyrosine kinases (e.g., platelet-derived growth factor receptor) often leads to an increased synthesis of IP\(_3\) that mediates Ca\(^{2+}\) mobilization from the SR to the cytosol by activating IP\(_3\) receptors (9, 15, 18, 36). The subsequent depletion of Ca\(^{2+}\) from the SR opens SOCs in the plasma membrane and causes further Ca\(^{2+}\) influx to maintain a high level of [Ca\(^{2+}\)\(_{\text{cyt}}\)] during contraction or proliferation. The store depletion-mediated Ca\(^{2+}\) entry (SDCaE) has been demonstrated to result mainly from capacitative Ca\(^{2+}\) entry through Ca\(^{2+}\)-permeable SOC (5, 56, 63). However, the canonical TRP (TRPC) channels that form functional SOC (5, 10, 33, 52, 55) are also permeable to other cations, including Na\(^+\); some of the TRPC channels are actually more permeable to Na\(^+\) than to Ca\(^{2+}\) (5, 13, 19). In other words, store depletion-mediated opening of TRPC channels would allow both Ca\(^{2+}\) and Na\(^+\) to enter the cell. The store depletion-mediated Na\(^+\) entry (SDNaE) would thus induce a local rise in [Na\(^{+}\)\(_{\text{cyt}}\)], which activates the operation of Na\(^+/Ca\(^{2+}\) exchanges in the reverse mode and subsequently increases [Ca\(^{2+}\)\(_{\text{cyt}}\)].

The results from this study demonstrate that 1) the CPA-induced passive depletion of Ca\(^{2+}\) from the intracellular stores (i.e., the SR) increases [Ca\(^{2+}\)\(_{\text{cyt}}\)] as a result of store depletion-mediated Ca\(^{2+}\) entry, and 2) removal of extracellular Na\(^+\) or extracellular application of KB-R7943 (an inhibitor of the Na\(^+/Ca\(^{2+}\) exchanger) (8, 16, 21, 43) significantly attenuates the store depletion-mediated Ca\(^{2+}\) entry. These data suggest that the reverse mode of Na\(^+/Ca\(^{2+}\) exchange participates in store depletion-mediated Ca\(^{2+}\) entry. In other words, the store depletion-mediated Ca\(^{2+}\) entry in human PASMC is composed of two components: 1) capacitative Ca\(^{2+}\) entry through Ca\(^{2+}\)-permeable SOC (or TRPC) channels and 2) Ca\(^{2+}\) entry via the reverse model of the Na\(^+/Ca\(^{2+}\) exchanger.

Store depletion-mediated Ca\(^{2+}\) entry is an important stimulus for mitogen-mediated PASMC proliferation (5, 9, 34, 39). Our previous studies demonstrated that functional blockade of SOC with La\(^{3+}\), Ni\(^{2+}\), or SKF-96365 as well as downregulation of TRPC channels with siRNA or antisense oligonucleotides all inhibited PASMC growth in the presence of serum and growth factors (19, 26, 48, 60). Consistent with these observations, we show in this study that inhibition of the reverse mode of Na\(^+/Ca\(^{2+}\) exchange with KB-R7943, in addition to reducing the amplitude of store depletion-mediated Ca\(^{2+}\) entry, significantly inhibits PASMC proliferation. These results suggest that Ca\(^{2+}\) entry via the reverse mode of Na\(^+/Ca\(^{2+}\) exchange is, at least in part, involved in inducing the rise in [Ca\(^{2+}\)\(_{\text{cyt}}\)] required for PASMC proliferation.

Increasing evidence suggests that there is a functional interaction between the Na\(^+/Ca\(^{2+}\) exchanger and SOC (40) or TRPC channels that are activated by store depletion (34). Under physiological conditions, opening of these nonselective cation channels not only results in Na\(^+\) influx to the restricted plasma membrane-SR junctional space but also causes membrane depolarization as a result of inward cationic currents. Both the increase in [Na\(^{+}\)\(_{\text{cyt}}\)] and membrane depolarization drive the plasma membrane Na\(^+/Ca\(^{2+}\) exchanger to its reversed mode of operation, thereby transporting more Ca\(^{2+}\) into cell (1, 7, 28). In human PASMC, our data support the contention that store depletion-mediated Ca\(^{2+}\) entry is caused by both capacitative Ca\(^{2+}\) entry and NCX-mediated Ca\(^{2+}\) entry, and the latter is also involved in serum- and growth factor-mediated PASMC proliferation. Whether TRPC isoforms, as well as which ones, are functionally or physically interacted with NCX proteins in human PASMC remains unclear.

Together, our results indicate that function of the Na\(^+/Ca\(^{2+}\) exchanger plays an important role in regulating [Ca\(^{2+}\)\(_{\text{cyt}}\)] in human PASMC. Ca\(^{2+}\) entry via the reverse mode of Na\(^+/Ca\(^{2+}\) exchange is a critical pathway for increasing [Ca\(^{2+}\)\(_{\text{cyt}}\)], inducing pulmonary vasoconstriction, and stimulating PASMC proliferation. In patients with pulmonary arterial hypertension, sustained pulmonary vasoconstriction and pulmonary vascular medial hypertrophy (due to excessive PASMC proliferation) are the major causes of increased pulmonary arterial pressure (41, 47). Therefore, better understanding the functional role of the Na\(^+/Ca\(^{2+}\) exchanger in the pulmonary vasculature may lead to development of novel therapeutic approaches for treatment of pulmonary vascular diseases such as idiopathic pulmonary arterial hypertension.

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