Role of \(Na^+\)/\(Ca^{2+}\) exchange in regulating cytosolic \(Ca^{2+}\) 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 \(Na^+\)/\(Ca^{2+}\) exchange in regulating cytosolic \(Ca^{2+}\) 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 \(Ca^{2+}\)-concentration ([\(Ca^{2+}\]cyt) in pulmonary artery smooth muscle cells (PASMC) is an important stimulus for cell contraction, migration, and proliferation. Depletion of intracellular \(Ca^{2+}\) stores opens store-operated \(Ca^{2+}\) channels (SOC) and causes \(Ca^{2+}\) entry. Transient receptor potential (TRP) cation channels that are permeable to \(Na^+\) and \(Ca^{2+}\) are believed to form functional SOC. Because sarcenomal \(Na^+\)/\(Ca^{2+}\) exchanger has also been implicated in regulating [\(Ca^{2+}\]cyt], this study was designed to test the hypothesis that the \(Na^+\)/\(Ca^{2+}\) exchanger (NCX) in cultured human PASMC is functionally involved in regulating [\(Ca^{2+}\]cyt] by contributing to store depletion-mediated \(Ca^{2+}\) entry. RT-PCR and Western blot analyses revealed mRNA and protein expression for NCX1 and NCKX3 in cultured human PASMC. Removal of extracellular \(Na^+\), which switches the \(Na^+\)/\(Ca^{2+}\) exchanger from the forward (\(Ca^{2+}\) exit) to reverse (\(Ca^{2+}\) entry) mode, significantly increased [\(Ca^{2+}\]cyt], whereas inhibition of the \(Na^+\)/\(Ca^{2+}\) exchanger with KB-R7943 (10 \(\mu\)M) markedly attenuated the increase in [\(Ca^{2+}\]cyt] via the reverse mode of \(Na^+\)/\(Ca^{2+}\) exchange. Store depletion also induced a rise in [\(Ca^{2+}\]cyt] via the reverse mode of \(Na^+\)/\(Ca^{2+}\) exchange. Removal of extracellular \(Na^+\) or inhibition of the \(Na^+\)/\(Ca^{2+}\) exchanger with KB-R7943 attenuated the store depletion-mediated \(Ca^{2+}\) 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 \(Ca^{2+}\) entry via the reverse mode of \(Na^+\)/\(Ca^{2+}\) exchange contributes to store depletion-mediated increase in [\(Ca^{2+}\]cyt], and that blockade of the \(Na^+\)/\(Ca^{2+}\) 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 \(Ca^{2+}\) is a critical second messenger that links external stimuli to cell contraction, proliferation, migration, and gene expression (3, 4). An increase in cytosolic free \(Ca^{2+}\) concentration ([\(Ca^{2+}\]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^{2+}\]cyt] in PASMC can be increased by \(Ca^{2+}\) release from intracellular stores, such as the sarcoplasmic (or endoplasmic) reticulum (SR), and by \(Ca^{2+}\) influx through \(Ca^{2+}\)-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\(_3\)) and membrane-bound diacylglycerol. IP\(_3\) induces a rapid \(Ca^{2+}\) release through IP\(_3\) receptors at the SR membrane (14, 51), and the subsequent depletion of \(Ca^{2+}\) from the stores (i.e., the SR) opens a special family of \(Ca^{2+}\)-permeable channels, namely, store-operated \(Ca^{2+}\) channels (SOC), and elicits capacitative \(Ca^{2+}\) 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 \(Ca^{2+}\) and \(Na^+\) to go through (29, 50, 64). Therefore, opening of TRP channels would increase both [\(Ca^{2+}\]cyt] and cytosolic [\(Na^+\)] as a result of \(Ca^{2+}\) and \(Na^+\) influx via the channels.

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

Many functional studies have demonstrated that the plasma membrane \(Na^+\)/\(Ca^{2+}\) exchanger is involved in regulating \(Ca^{2+}\) homeostasis of blood vessels (1, 8, 44, 45). The functional evidence for the sarcenomal \(Na^+\)/\(Ca^{2+}\) exchanger in vessels is also supported by direct demonstration that the \(Na^+\)/\(Ca^{2+}\) exchanger is expressed in vascular smooth muscle and endothelial cells (23). However, the expression and function of the \(Na^+\)/\(Ca^{2+}\) 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, 42, 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 ng/ml 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 were stored at 4°C and then homogenized in a glass tube with a Teflon Dounce pestle three times. Rat brain and pulmonary artery tissues were used in some experiments. Left and right branches of the main pulmonary artery along with intrapulmonary 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 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 assay reagent (Pierce Biotechnology, Rockford, IL) with BSA as a 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% bromophenol blue). Protein suspensions were electrophoretically separated on an 8% acrylamide gel, and protein bands were transferred to nitrocellulose membranes by electroblothing 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 RNasy 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 were 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+]cyt. [Ca2+]cyt 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 fluorescent 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+]cyt 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>
</tr>
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<tr>
<td>rNCX1</td>
<td>AF109166</td>
<td>231</td>
<td>5'−GCCATGGCTTGGCTCCGGTTGCGT−3'</td>
<td>1540–1559</td>
</tr>
<tr>
<td>hNCX1</td>
<td>NM_021097</td>
<td>230</td>
<td>5'−GCCAGGTTCCTGCTTCAAGTCC−3'</td>
<td>1770–1749</td>
</tr>
<tr>
<td>rNCKX3</td>
<td>Ay009158</td>
<td>213</td>
<td>5'−TTTCTCGACGCTCCAGTGT−3'</td>
<td>1556–1575</td>
</tr>
<tr>
<td>hNCKX3</td>
<td>Af169257</td>
<td>188</td>
<td>5'−AACGACGGACAGGCTGAG−3'</td>
<td>1467–1448</td>
</tr>
</tbody>
</table>

Accession numbers are GenBank accession numbers for the sequences used in designing the primers. r, Rat; h, human.
380 nm (F_F340/F380) using the ratio method based on the equation 
\[ [Ca^{2+}]_{cyt} = K_d \times \frac{(S/F_{Sb2})}{(R - R_{min}/(R_{max} - R))} \], where \( K_d \) (225 nM) is the dissociation constant for Ca\(^{2+}\), \( R \) is the measured fluorescence ratio, and \( R_{min} \) and \( R_{max} \) are minimal and maximal ratios, respectively (20).

**Cell cycle analysis.** Human PASMC cell cycle distribution was analyzed using flow cytometry. Cells were first growth arrested in SmBM for 24 h and then cultured in 5% FBS-SmBM with or without KB-R7943 for 24 h. The cells were trypsinized, washed once with PBS, and fixed with 70% ethanol for at least 30 min at room temperature. The fixed cells were washed with PBS and incubated with a solution containing 50 \( \mu \)g/ml propidium iodine (Sigma) and 50 \( \mu \)g/ml RNase A (Sigma) for 30 min at room temperature in the dark. The stained cells were analyzed by FACS-Calibur with excitation at 488 nm and emission at 560–640 nm (FL2 mode) using CellQuest software (Becton Dickinson, Mountain View, CA).

**Measurement of cell number.** Human PASMC were cultured in growth-arresting medium (SmBM) for 24 h and then switched to culture in 5% FBS-SmBM with or without KB-R7943 for 48 h. Cells were pelleted, washed twice with cold PBS, and resuspended in PBS for counting. Cell numbers were determined using a Z2 Coulter counter.

**Chemicals.** All chemicals were of analytical grade or better and were obtained from Fisher (Nepean, ON, Canada), BDH (Toronto, ON, Canada), or 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\(^+/Ca\(^{2+}\) exchanger in regulating [Ca\(^{2+}\)]\(_{cyt}\) .** To test whether the Na\(^+/Ca\(^{2+}\) exchanger is functionally involved in regulating [Ca\(^{2+}\)]\(_{cyt}\), we first measured [Ca\(^{2+}\)]\(_{cyt}\) 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 [Ca\(^{2+}\)]\(_{cyt}\) as a result of switching the Na\(^+\)/Ca\(^{2+}\) exchanger from the forward (Ca\(^{2+}\) exit) mode to the reverse (Ca\(^{2+}\) entry) mode (8, 28). Extracellular application of nifedipine (10 \( \mu \)M), a specific blocker of voltage-dependent Ca\(^{2+}\) channels, had no effect on the increase in [Ca\(^{2+}\)]\(_{cyt}\) driven by the reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger. However, extracellular application of KB-R7943, a potent and selective inhibitor of the Na\(^+\)/Ca\(^{2+}\) exchanger (especially in its reverse mode), significantly attenuated the increase in [Ca\(^{2+}\)]\(_{cyt}\) induced by removal of extracellular Na\(^+\) (Fig. 2B). These results suggest that the Na\(^+\)/Ca\(^{2+}\) exchanger can operate actively in the reverse mode in human PASMC.

The activity of the Na\(^+\)/Ca\(^{2+}\) exchanger in human PASMC appeared to be independent of extracellular K\(^+\), because removal of extracellular K\(^+\) had little effect on the rise in [Ca\(^{2+}\)]\(_{cyt}\) due to the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange (Fig. 2). These results suggest that the Ca\(^{2+}\) entry via the reversed mode of Na\(^+\)/Ca\(^{2+}\) exchange is likely due to the function of NCX (rather than NCKX) in human PASMC.
showing the time course of \([\text{Ca}^{2+}]_{\text{cyt}}\) in human PASMCS. A: a representative record (a) showing the time course of \([\text{Ca}^{2+}]_{\text{cyt}}\) 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 \(\mu\)M) was included in all solutions to block voltage-gated Ca\(^{2+}\) channels. Summarized data (means ± SE, b) show the amplitude of increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) 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 \([\text{Ca}^{2+}]_{\text{cyt}}\) changes induced by 0Na\(^+\)+5K solution in the absence or presence of 10 \(\mu\)M KB-R7943 (KB-R). Summarized data (means ± SE, b) show the amplitude of increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) in cells superfused with 0Na\(^+\)+5K solution in the absence (Cont, \(n = 30\)) or presence (KB-R, \(n = 50\)) of 10 \(\mu\)M KB-R7943. ***\(P < 0.001\) vs. Cont.

**Involvement of Na\(^+\)/Ca\(^{2+}\) exchanger in store depletion-induced Ca\(^{2+}\) entry.** Upon activation of GPCR or receptor tyrosine kinases, IP\(_3\)-mediated Ca\(^{2+}\) release (which induces an early transient increase in \([\text{Ca}^{2+}]_{\text{cyt}}\)) depletes Ca\(^{2+}\) from intracellular stores (e.g., the SR). The store depletion-mediated opening of TRP channels would promote not only Ca\(^{2+}\) influx but also Na\(^+\) influx, because TRP channels are permeable to both Ca\(^{2+}\) and Na\(^+\) (35, 54). Because Ca\(^{2+}\) entry via the Na\(^+\)/Ca\(^{2+}\) exchanger depends greatly on Na\(^+\)\(_{\text{cyt}}\), the store depletion-mediated Na\(^+\) influx through TRP channels would increase Na\(^+\)\(_{\text{cyt}}\), activate the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange, and enhance Ca\(^{2+}\) entry. The next step of experiments was designed to test the hypothesis that in addition to triggering capacitative Ca\(^{2+}\) entry, the passive store depletion mediated by cyclopiazonic acid (CPA; 10 \(\mu\)M) induces Ca\(^{2+}\) entry as well via the Na\(^+\)/Ca\(^{2+}\) exchanger.

In the absence of extracellular Ca\(^{2+}\) and presence of nifedipine (10 \(\mu\)M), extracellular application of CPA, a blocker of the Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in the SR (SERCA), induced a transient increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) due to Ca\(^{2+}\) leakage from the SR to the cytosol. Approximately 5–10 min later when the store was depleted (i.e., when the \([\text{Ca}^{2+}]_{\text{cyt}}\) transients declined back to the basal level), restoration of extracellular \([\text{Ca}^{2+}]_{\text{cyt}}\) (to 1.8 nM) induced a large increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) due to Ca\(^{2+}\) entry (Fig. 3A). Extracellular application of KB-R7943 (10 \(\mu\)M; an inhibitor of the reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger) significantly attenuated the sustained phase of the store depletion-mediated Ca\(^{2+}\) entry (Fig. 3). Furthermore, removal of extracellular Na\(^+\) (by replacing external Na\(^+\) with equimolar NMDG\(^+\) or Li\(^+\)) also markedly inhibited the store depletion-mediated Ca\(^{2+}\) entry (Fig. 4, A and C). In the absence of extracellular Na\(^+\) (i.e., when Na\(^+\)/Ca\(^{2+}\) exchange was in the reverse mode or Ca\(^{2+}\) entry mode), the CPA-induced transient \([\text{Ca}^{2+}]_{\text{cyt}}\) rise and baseline \([\text{Ca}^{2+}]_{\text{cyt}}\) were both enhanced as a result of inhibited Ca\(^{2+}\) extrusion via the forward mode (or Ca\(^{2+}\) exit mode) of Na\(^+\)/Ca\(^{2+}\) exchange (Fig. 4, A and B). These results indicate that the Ca\(^{2+}\) entry via the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange is involved in store depletion-mediated Ca\(^{2+}\) entry in human PASMCS.

**Involvement of Na\(^+\)/Ca\(^{2+}\) exchangers in human PASMCS proliferation.** Store depletion-mediated elevation of \([\text{Ca}^{2+}]_{\text{cyt}}\) is associated with human PASMCS proliferation in response to serum and growth factors (19, 48, 60). As mentioned earlier, the store depletion-mediated increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) is partially due to the KB-R7943-sensitive Ca\(^{2+}\) entry via the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange. To examine the possible involvement of the Na\(^+\)/Ca\(^{2+}\) exchanger in human PASMCS 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 PASMCS with KB-R7943 (10–30 \(\mu\)M for 24 h) significantly reduced the counts of cells in S/G2/M phases. Furthermore, inhibition of the Na\(^+\)/Ca\(^{2+}\) exchanger with KB-R7943 (3–30 \(\mu\)M for 48 h) also markedly inhibited the increase in cell numbers when human PASMCS 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 \(\mu\)M), KB-R7943 inhibits only the forward mode of Na\(^+\)/Ca\(^{2+}\) exchange, whereas at high

![Image](http://ajpcell.physiology.org/)

**Fig. 3.** Inhibition of Na\(^+\)/Ca\(^{2+}\) exchanger with KB-R7943 attenuates store depletion-mediated Ca\(^{2+}\) entry. A: representative records showing the time course of \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in human PASMCS in response to cyclopiazonic acid (CPA; 10 \(\mu\)M) in the presence or absence (0Ca) of extracellular Ca\(^{2+}\). KB-R7943 (10 \(\mu\)M) or vehicle (DMSO) was applied to the cells when the CPA-mediated \([\text{Ca}^{2+}]_{\text{cyt}}\) increase reached the maximal level in the presence of extracellular Ca\(^{2+}\). Nifedipine (10 \(\mu\)M) was included in all solutions to eliminate the contribution of voltage-gated Ca\(^{2+}\) channels to the \([\text{Ca}^{2+}]_{\text{cyt}}\) changes. B: summarized data (means ± SE) showing the amplitude of CPA-mediated increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) after treatment with vehicle (\(n = 36\)) or KB-R7943 (\(n = 48\)). ***\(P < 0.001\) vs. vehicle control.
the NaPASMC is likely due to its inhibition of the reverse mode of KB-R7943-mediated antiproliferative effect on human PASMC. Indeed, the resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) is greater in PASMC from normal subjects and patients without pulmonary hypertension (19, 61). Therefore, understanding the cellular mechanisms involved in regulating [Ca\(^{2+}\)]\(_{\text{cyt}}\) in human PASMC would help develop new therapeutic approaches for patients with pulmonary arterial hypertension.

The results from this study demonstrate that 1) human PASMC express NCX1 and NCKX3; 2) removal of extracellular Na\(^{+}\) activates the operation of the Na\(^+\)/Ca\(^{2+}\) exchanger dependent function occurring in the cytosol (e.g., contraction) but also activates Ca\(^{2+}\)-sensitive events in the nucleus (e.g., expression of the nuclear proteins that are related to the cell cycle) (4, 9). KB-R7943 is more potent in inhibiting the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange by removing extracellular Na\(^{+}\) inhibits store depletion-mediated Ca\(^{2+}\) entry. A: representative records showing the time course of [Ca\(^{2+}\)]\(_{\text{cyt}}\) changes in response to CPA (10 \(\mu\)M) in the presence or absence (0Ca) of extracellular Ca\(^{2+}\). Cells were superfused with solutions containing 140 (140Na) or 0 mM Na\(^{+}\) (0Na). In these experiments, extracellular Na\(^{+}\) was replaced with equimolar Li\(^{+}\), and nifedipine (10 \(\mu\)M) was included in all solutions to eliminate the contribution of voltage-gated Ca\(^{2+}\) channels to the [Ca\(^{2+}\)]\(_{\text{cyt}}\) changes. B: summarized data (means ± SE) showing the amplitudes of CPA-induced increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) due to Ca\(^{2+}\) leakage or release in the absence of extracellular Ca\(^{2+}\) (shaded bars) and the basal levels of [Ca\(^{2+}\)]\(_{\text{cyt}}\) after CPA-induced Ca\(^{2+}\) release (solid bars) in cells bathed in Na\(^{+}\)-containing (140Na, \(n = 38\)) or Na\(^{+}\)-free (0Na-) solution. C: summarized data (means ± SE) showing the amplitudes of CPA-induced increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) due to Ca\(^{2+}\) entry or influx in the presence of extracellular Ca\(^{2+}\) in cells bathed in Na\(^{+}\)-containing (140Na, \(n = 38\)) or Na\(^{+}\)-free (0Na-) solution. Extracellular Na\(^{+}\) was replaced with NMDG (\(n = 63\)) or LiCl (\(n = 53\)). ***P < 0.001 vs. 140Na.

**DISCUSSION**

Pulmonary vasoconstriction and vascular medial hypertrophy (due to inappropriate PASMC hyperplasia and hypertrophy) greatly contribute to the elevated pulmonary vascular resistance and pulmonary arterial pressure in patients with pulmonary arterial hypertension (41, 47). Intracellular Ca\(^{2+}\) is a critical signal transduction element in regulating PASMC contraction (46) and proliferation (3, 18), as well as gene expression (4, 9). A rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) not only activates Ca\(^{2+}\)-
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\(^{+}/Ca^{2+}\) exchanger is a critical mechanism that regulates intracellular Ca\(^{2+}\) homeostasis in human PASM C. The NCX1 is probably the major Na\(^{+}/Ca^{2+}\) exchanger isoform in human PASM C 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 PASM C 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 PASM C (Fig. 1C). There are several possibilities that explain the discrepancies: 1) expression level of NCX1 may be significantly different between human and rat PASM C, and 2) expression level of NCX1 in human pulmonary arteries may be changed (e.g., downregulated) in cultured PASM C 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:

\[
[\text{Ca}^{2+}]_{\text{cyt}} = [\text{Ca}^{2+}]_{\text{out}} \times ([\text{Na}^{+}]_{\text{cyt}}/[\text{Na}^{+}]_{\text{out}}) \times e^{(E_{\text{rev}}/RT)}
\]

where \(F\) is the Faraday constant, \(R\) is the gas constant, and \(T\) is absolute temperature. The equation indicates that for a constant [Ca\(^{2+}\)\(_{\text{out}}\)] and [Na\(^{+}\)\(_{\text{out}}\)], [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 PASM C, 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 NCX 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\(^{+}/Ca^{2+}\) exchangers in the reverse mode and subsequently increases [Ca\(^{2+}\)\(_{\text{cyt}}\)] (1, 2).

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 PASM C 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 PASM C 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 PASM C 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 PASM C 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 PASM C 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 PASM C, 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 PASM C proliferation. Whether TRPC isoforms, as well as which ones, are functionally or physically interacted with NCX proteins in human PASM C 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 PASM C. Ca\(^{2+}\) entry via the reverse mode of Na\(^{+}/Ca^{2+}\) exchange is a critical pathway for increasing [Ca\(^{2+}\)\(_{\text{cyt}}\)] in human PASM C, inducing pulmonary vasoconstriction, and stimulating PASM C proliferation. In patients with pulmonary arterial hypertension, sustained pulmonary vasoconstriction and pulmonary vascular medial hypertrophy (due to excessive PASM C 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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