Knockout of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in smooth muscle attenuates vasoconstriction and L-type Ca\textsuperscript{2+} channel current and lowers blood pressure

Jin Zhang,1 Chongyang Ren,1 Ling Chen,2 Manuel F. Navedo,3 Laura K. Antos,4 Stephen P. Kinsey,1 Takahiro Iwamoto,4 Kenneth D. Philipson,5 Michael I. Kotlikoff,6 Luis F. Santana,3 W. Gil Wier,1 Donald R. Matteson,1* and Mordecai P. Blaustein1,2*

Departments of 1Physiology and 2Medicine, University of Maryland School of Medicine, Baltimore, Maryland; 3Department of Physiology and Biophysics, University of Washington, Seattle, Washington; 4Department of Pharmacology, Fukuoka University School of Medicine, Fukuoka, Japan; 5Department of Physiology, David Geffen School of Medicine at the University of California, Los Angeles, California; and 6Department of Biomedical Sciences, Cornell University Veterinary College, Ithaca, New York

Submitted 13 October 2009; accepted in final form 15 February 2010

Zhang J, Ren C, Chen L, Navedo MF, Antos LK, Kinsey SP, Iwamoto T, Philipson KD, Kotlikoff MI, Santana LF, Wier WG, Matteson DR, Blaustein MP. Knockout of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in smooth muscle attenuates vasoconstriction and L-type Ca\textsuperscript{2+} channel current and lowers blood pressure. Am J Physiol Heart Circ Physiol 298: H1472–H1483, 2010. First published February 19, 2010; doi:10.1152/ajpheart.00964.2009.—Mice with a low cytosolic Ca\textsuperscript{2+} concentration are protected from high extracellular Na\textsuperscript{+} concentration-induced vasoconstriction and L-type Ca\textsuperscript{2+} channel (LVGC) activity in mesenteric arteries. We conclude that, under physiological conditions, when ASMC relative activation of the exchanger, governed by cytosolic Na\textsuperscript{+} and Ca\textsuperscript{2+} entry contributes significantly to the maintenance of vascular tone and BP.

Three isoforms of NCX have been identified: NCX1 is abundant in the heart and is ubiquitously expressed, whereas NCX2 and NCX3 are both restricted to brain and skeletal muscle (34). NCX1 is the only isoform present in ASMCs, where two splice variants, NCX1.3 and NCX1.7, are expressed (27, 35). In cardiac myocytes, NCX1 makes a major contribution to Ca\textsuperscript{2+} extrusion during diastole (3, 11). Nevertheless, cardiac-specific NCX1 knockout mice thrive: the loss of NCX1 is compensated by a reduced L-type voltage-gated Ca\textsuperscript{2+} channel (LVGC) current and a shortened action potential because of accelerated Ca\textsuperscript{2+}-dependent LVGC inactivation and augmented transient outward K\textsuperscript{+} current (18, 31, 32). Importantly, cardiac NCX1-mediated Ca\textsuperscript{2+} flux must be inward during depolarization and systole (5). The situation is complicated, however, because exchanger-mediated fluxes depend on the relative activation of the exchanger, governed by cytosolic Na\textsuperscript{+} and Ca\textsuperscript{2+} (26), as well as on the net driving force, governed by the membrane potential (V\textsubscript{m}) and the Na\textsuperscript{+} and Ca\textsuperscript{2+} electrochemical gradients (5).

ASMNCX1, too, can play an important role in Ca\textsuperscript{2+} extrusion when [Ca\textsuperscript{2+}]\textsubscript{cys} is suddenly elevated (23, 40, 41). Nevertheless, under near steady-state conditions, when ASMNCX activation and the V\textsubscript{m} are both relatively constant, NCX1

* D. R. Matteson and M. P. Blaustein made equal contributions to this study.

Address for reprint requests and other correspondence: J. Zhang, Dept. of Physiology, Univ. of Maryland School of Medicine, 655 W. Baltimore St., Baltimore, MD 21201 (e-mail: jzhan002@umaryland.edu).
should tend to operate close to its reversal potential \( (E_{Na/Ca}) \). This is determined by the Na\(^+\) and Ca\(^{2+}\) electrochemical potentials, \( E_{Na} \) and \( E_{Ca} \), respectively, and the 3 Na\(^+\)::Ca\(^{2+}\)-coupling ratio: i.e., \( E_{Na/Ca} = 3E_{Na} - 2E_{Ca} \) (5). The driving force on Ca\(^{2+}\) \( (V_{m} - E_{Na/Ca}) \) is governed by changes in \( V_{m} \) and cytosolic Na\(^+\) and Ca\(^{2+}\). The exchanger can move (net) Ca\(^+\) either outward when \( V_{m} - E_{Na/Ca} \) is negative or inward when \( V_{m} - E_{Na/Ca} \) is positive.

The inhibitory effects of SEA0400 on \([Ca^{2+}]_{cyt}\) and myogenic tone (MT) suggested that NCX1 mediates (net) Ca\(^{2+}\) entry in ASMCs within pressurized, tonically constricted small arteries (21, 36, 48). The present report shows that MT is also reduced, as is BP, in SM-specific knockout of NCX1 \((NCX1^{SM/-/-})\) mice. This is consistent with the view (21) that NCX1 normally mediates Ca\(^{2+}\) entry in tonically constricted small arteries. Reduced ASMC NCX1 expression is, however, as in heart (32), accompanied by a functional reduction in Ca\(^{2+}\) entry via LGVCs and, thus, reduced LGVC activation in ASMCs (37).

### METHODS

#### Ethical approval.

All mouse protocols were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

#### Generation of NCX1\(^{SM/-/-}\) mice.

NCX1\(^{SM/-/-}\) mice were generated by crossing homozygous NCX1 LoxP [floxed (Fx)] mice \((NCX1^{Fx/Fx})\) (18) with hemizygous SM-specific Cre recombinase mice \((Cre^{SM})\) (46). These genetically altered mice are on a C57BL/6J background keeping protein, as indicated.

When Cre is expressed in a tissue-specific manner in mice that are transiently during male gametogenesis (9, 10, 16), resulting in the recombination of NCX1 exon 11 in meiotic cells. Thus crosses of female NCX1\(^{Fx/Fx}\) mice with male NCX1\(^{Fx/+}\),Cre\(^{SM+}\) mice \((i.e., \text{floxed heterozygotes with a SM-specific Cre gene})\) produced either NCX1\(^{Fx/-}\),(-Cre\(^{-}\)) or NCX1\(^{Fx/-}\),Cre\(^{-}\) \((=NCX1^{SM/-/-})\) mice. The latter contain one globally deleted allele and one SM-specific-deleted allele; i.e., they were SM-specific NCX1 knockout mice. Controls for NCX1\(^{SM/-/-}\) mice were NCX1\(^{Fx/-}\) littermates as well as C57BL/6J WT mice. Genomic DNA, obtained from tail biopsies, was used for genotyping by PCR. Primers for the floxed exon 11 were designed to distinguish the WT, floxed, and deleted alleles (Fig. 1B).

#### BP Measurements

**Telemetric BP measurement.** WT, NCX1\(^{Fx/-}\), and NCX1\(^{SM/-/-}\) mice \((~16\text{ wk old})\) were anesthetized with isoflurane supplemented with 100% O\(_2\). The right common carotid artery was exposed and ligated via an anterior neck midline incision. Telemetric BP sensors...
Arterial Myocyte Isolation

Male WT, NCX1Fx/−, and NCX1SM/−/− mice (12–16 wk of age) were euthanized with CO2 followed by cervical dislocation. The main mesenteric artery was removed, cleaned, and used for myocyte isolation. The artery was digested for 35 min at 37°C in low-Ca2+/PSS containing (in mg/ml) 1.5 collagenase type XI, 0.16 elastase type IV, and 1 bovine serum albumin, and individual myocytes were dissociated by trituration (2, 47). Only cells with elongated morphology (i.e., relaxed cells) were studied.

Patch-Clamp Recording

Whole cell, patch-clamp configuration was applied to record membrane currents using an Axopatch 200 patch-clamp amplifier (Axon Instruments, Union City, CA), as described (47). Fire-polished micropipettes (1–3 MΩ resistance) were manufactured from borosilicate capillary tubing (Garner Glass, Claremont, CA) using a flame-pipette puller (model P-97, Sutter Instruments, Novato, CA). An Ag-AgCl reference electrode was connected to the bath using an agar salt bridge containing 1 M KCl. Before the pipette approached the cell, the chamber was perfused with Ca2+-free PSS containing 1.4 mM extra- cellular Mg2+ concentration to prevent cell contraction caused by ATP diffusing from the pipette. After seal formation, the cells were superfused with normal PSS. The holding potential was −70 mV in all experiments. The leak and capacity transients were subtracted using a P/4 protocol. The series resistance was compensated to give the fastest possible capacity transient without producing oscillations. The membrane currents were recorded with 12-bit analog-to-digital converters (Digidata 1322A, Axon Instruments). Data were sampled at 500 kHz (unless otherwise stated), filtered at 5 kHz with a 902LPF low-pass Bessel filter (Frequency Devices, Haverhill, MA), and stored for subsequent analysis. All records were obtained at room temperature (25–26°C).

Macroscopic currents were recorded using 1-ms voltage clamp steps to a range of potentials from −50 to +90 mV. Preliminary experiments showed that LVGC currents carried by Ca2+ were small and difficult to measure accurately; therefore, we used Ba2+ as the current carrier. To construct current-voltage curves, the current during the pulse (I_ho) is plotted as a function of pulse voltage. Because the amplitude of the tail current following the pulse (−I_mem) is a measure of the conductance activated during the pulse, conductance-voltage (G–V) curves are obtained by plotting −I_mem as a function of pulse voltage.

Recording of Ca2+ Sparklets

Detailed methods for recording Ca2+ sparklets are published (28, 29). Briefly, Ca2+ sparklets were recorded using a total internal reflection fluorescence microscope (Olympus, South Windsor, CT). Cells were loaded with the Ca2+ indicator Fluo-5F. Images were acquired at 30–90 Hz. Background-subtracted fluorescence signals were converted to concentration units using the F_max equation (28, 47). Ca2+ sparklets were detected and defined for analysis using an automated algorithm written in Interactive Data Language software (IDL, Research Systems, Boulder, CO). Ca2+ sparklets had an amplitude equal to or larger than the mean basal [Ca2+]cyt plus three times its standard deviation. For a [Ca2+]cyt elevation to be considered a sparklet, a grid of 3 × 3 contiguous pixels had to have a [Ca2+]cyt value at or above the amplitude threshold.

Reagents and Solutions

The artery dissection solution contained (in mM) 145 NaCl, 4.7 KCl, 1.2 MgSO4·7H2O, 2 MOPS, 0.02 EDTA, 1.2 NaH2PO4, 2 CaCl2·2H2O, 5 glucose, and 2.0 pyruvate and 1% albumin (pH 7.4 at 5°C). The PSS perfusion solution contained (in mM) 112 NaCl, 25.7 NaHCO3, 4.9 KCl, 2.5 CaCl2, 1.2 MgSO4·7H2O, 1.2 K2HPO4, 11.5 glucose, and 10 HEPES (adjusted pH to 7.3–7.4 with NaOH). High (10–75 mM) extracellular K+ concentration ([K+]o) solution was made by replacing NaCl with an equimolar KCl of normal PSS. Ca2+-free solution was made by omitting CaCl2 and adding 0.5 mM EGTA. Low (25.7 mM) extracellular Na+ concentration ([Na+]o)
solution was made by replacing 112 mM NaCl with equimolar LiCl; 25.7 mM NaHCO3 was retained to minimize the perturbation of the acid-base balance. Solutions were gassed with 5% O2-5% CO2-90% N2 (48).

The tissue homogenization medium contained (in mM) 140 NaCl, 10 NaH2PO4, 2 EDTA, and 10 Na2S and 2.5x concentrated Roche (Hoffmann-La Roche, Nutley, NJ) complete protease inhibitor cocktail.

To isolate Ba2+ currents (Iba) in electrophysiological experiments, the pipette was filled with high Cs+ solution of the following composition: (in mM) 130 CsCl, 2.5 MgCl2, 10 HEPES, 10 EGTA, and 2 Na2ATP (pH was adjusted to 7.2 with CsOH). The bath solution contained (in mM) 140 NaCl, 2.7 KCl, 10 BaCl2, and 10 HEPES (pH was adjusted to 7.4 with NaOH). All experiments were performed in the presence of 200 nM tetrodotoxin (TTX) to block the Na+ currents previously described in these cells (2). The osmolarity of all internal and external solutions was maintained at 271 and 296 ± 1 mosmol/l, respectively. Osmolarity was measured with a model 5500 vapor pressure osmometer (Wescor, Logan, UT).

Reagents and sources were as follows: ouabain, phenylephrine, nifedipine, collagenase, elastase, and bovine serum albumin (Sigma-Aldrich, St. Louis, MO); SEAO4000 (Taisho Pharmaceutical, Tokyo, Japan); and TTX and Bay K 8644 (Calbiochem, San Diego, CA). Other reagents were reagent grade or the highest grade available. TTX was dissolved in deionized water; nifedipine was dissolved in DMSO.

Data Analysis and Statistics

The data are expressed as means ± SE; n denotes the number of animals, the number of arteries studied (1 artery per animal), or the number of cells studied in electrophysiological experiments. Comparisons of data were made using Student's paired or unpaired t-test, as appropriate; one-way or two-way ANOVA was used where indicated. Differences were considered significant at P < 0.05. Electrophysiological data analysis was performed using pClamp software (version 9.0; Axon Instruments). Iba amplitudes were measured relative to the current level before the pulse.

To characterize the voltage dependence of LVGC activation, we fit G–V curves with a Boltzman equation of the following form:

\[-I_{\text{tail}}(V_m) = \frac{-I_{\text{tail max}}}{1 + \exp\left(\frac{V_m - V_{0.5}}{k}\right)}\]

where \(-I_{\text{tail}}(V_m)\) is the magnitude of \(I_{\text{tail}}\) following a step to \(V_m\), \(-I_{\text{tail max}}\) is the maximum tail current, \(V_{0.5}\) is the voltage at which the conductance (i.e., the \(-I_{\text{tail}}\) magnitude) is half-maximal, and \(k\) is the slope factor.

RESULTS

NCX1 Protein Expression Is Reduced in NCX1SM+-/- Mouse Arteries

The expression of Cre recombinase, and thus the excision of NCX1 exon 11 in arterial myocytes of NCX1SM+-/- mice, was verified by the simultaneous expression of eGFP. GFP fluorescence was observed in about 75–90% of the myocytes in the NCX1SM+-/- mouse mesenteric small arteries employed in physiological experiments (Fig. 1C). There was, however, substantial variability not only from artery to artery but also from region to region in a single artery. The absence of a GFP signal in some myocytes (Fig. 1C) may indicate that Cre was not expressed in these cells or that it was only transiently expressed, in which case, NCX1 exon 11 should already have been excised.

The downregulation of normal NCX1 expression in these arteries was confirmed by immunoblot. In cardiac muscle, NCX1 appears as two bands, at 160 and 120 kDa. The relative expression of these bands varies, however, depending on the redox conditions used for membrane preparation (14); the 120-kDa band predominates under our conditions (Fig. 1D). In cardiac-specific knockout of NCX1, the intensities of both bands are reduced and a new, truncated NCX1 band appears at ~110 kDa (18). As illustrated in Fig. 1D, comparable results are obtained in the arteries of WT and NCX1SM+-/- mice, although some bands appear slightly smaller (~140, ~120, and ~90 kDa, respectively). Only the two larger bands are observed in arteries from WT mice. The intensity of the 120-kDa band, which predominates in WT arteries, is reduced by about half in NCX1Fx/- mouse arteries and by ~80–90% in NCX1SM+-/- arteries; a new, truncated, ~90-kDa band is then observed. The NCX1 expression is similarly reduced in urinary bladders from NCX1Fx/- and NCX1SM+-/- mice (Fig. 1D). Quantification is difficult, however, because of the change in band size and the apparent degradation of the truncated, non-functional protein. In contrast, both hearts (Fig. 1D) and brains (not shown) from NCX1SM+-/- as well as NCX1Fx/- mice express about half the normal (WT) level of NCX1, because both genotypes have one global, null-mutant NCX1 allele, but the SM-specific Cre recombinase is not activated in these tissues. In sum, these data demonstrate that the marked knockdown of NCX1 expression in NCX1SM+-/- mouse is SM specific; moreover, homozygous global knockout is embryonic lethal (39).

BP is Modestly Reduced in NCX1SM+-/- Mice

NCX1SM+-/- mice appear normal and grow to adulthood, and there are no obvious behavioral or gross anatomical differences between these mice and either the WT or NCX1Fx/- controls. Nevertheless, the fact that mice which overexpress NCX1 in SM have elevated BP (21) indicates that NCX1 plays a role in BP regulation and suggests that NCX1SM+-/- mice might also have a vascular phenotype. To test this, we compared BP in WT, NCX1Fx/-, and NCX1SM+-/- mice with two invasive methods (Fig. 2). First, the mean arterial BP (MBP) was measured with an intrafemoral catheter in mice under light isoflurane anesthesia. The results revealed that MBP in NCX1Fx/- mice was comparable with WT controls and was significantly reduced in NCX1SM+-/- mice (Fig. 2A). Similar results were obtained with telemetric BP measurements on awake, free-moving mice: MBP was lower in NCX1SM+-/- mice than in NCX1Fx/- or WT mice (Fig. 2B,a). Interestingly, the heart rate was significantly slower in both NCX1Fx/- and NCX1SM+-/- mice than in WT mice (Fig. 2B,b). The similarity of the heart rate in NCX1Fx/- and NCX1SM+-/- mice, despite the difference in BP, implies that the slow heart rate does not account for the low BP in the NCX1SM+-/- mice.

MT and Reactivity Are Attenuated by Reduced NCX1 Activity

To determine the contractile properties of the arteries from NCX1Fx/- and NCX1SM+-/- mice, the diameter changes in isolated, pressurized mesenteric small arteries were measured. These arteries constrict spontaneously (=myogenic reactivity) when intralumenal pressure is increased to more than 40 or 50
we have reported that nanomolar ouabain raises \([\text{Ca}^{2+}]_{\text{Cyt}}\) and augments MT in WT arteries; these effects are markedly inhibited by 1 \(\mu\)M SEA0400, suggesting that they result from increased NCX-mediated \(\text{Ca}^{2+}\) entry (21, 48). To explore this role of NCX further, we compared the effects of ouabain on MT in arteries from WT, NCX1Fx/-, and NCX1SM/- mice. As illustrated in Fig. 5, A and B, the ability of 100 nM ouabain to augment MT is significantly impaired in NCX1Fx/- and, to a greater extent, in NCX1SM/- arteries.

**Agnostist-Induced Vasoconstriction Is Impaired in NCX1SM/- Arteries**

The effect of reduced NCX1 expression on high \([K^+]_{o}\)- and phenylephrine (PE)-evoked vasoconstriction in small arteries are illustrated in Fig. 6. The maximal vasoconstriction induced by elevating \([K^+]_{o}\) from 5 to 75 mM in NCX1Fx/- arteries, \(-60\%\) of PD (which nearly obliterated the lumen), is similar to that in WT arteries (Fig. 6A,a). In contrast, the 75 mM \([K^+]_{o}\)-induced vasoconstriction is significantly reduced in NCX1SM/- arteries (Fig. 6A, a and b). This may indicate that the maximal \(\text{Ca}^{2+}\) entry through LVGCs is reduced in NCX1SM/- arteries because the 75 mM \([K^+]_{o}\)-evoked vasoconstriction is abolished by the LVGC blocker, nifedipine (47).

The maximal constriction to high-dose (3–100 \(\mu\)M) PE also is significantly smaller in NCX1SM/- than in WT arteries; the constriction to 100 \(\mu\)M PE is slightly, but not significantly, reduced in NCX1Fx/- arteries (Fig. 6B, a and b). Furthermore, both the high \([K^+]_{o}\) (Fig. 6A,b) and PE (Fig. 6B,b) dose-response curves are significantly depressed in NCX1SM/- compared with in NCX1Fx/- arteries. Thus, for example, the
PE constriction is reduced at all PE concentrations, but the concentration required for half-maximal constriction, ~0.35 μM, is not significantly different in the NCX1^{SM−/−} and WT mouse arteries (Fig. 6B,a,b). The differences between the NCX1^{SM−/−} and NCX1^{Fx−/−} arteries at the low-dose ends of the respective curves can be attributed, at least in part, to the lower MT in the NCX1^{SM−/−} arteries (i.e., at 5 mM [K+]o, and at 0 mM PE). Some possible mechanisms for the reduced constriction to high [K+]o, and high concentrations of PE are described in DISCUSSION.

LVGC Current Is Reduced in NCX1^{SM−/−} Myocytes

The aforementioned studies on high [K+]o-induced vasoconstriction raise the possibility that LVGC activity might be reduced in NCX1^{SM−/−} myocytes. Therefore, LVGC currents were measured in isolated mesenteric artery myocytes, using Ba^{2+} as the current carrier in the arterial myocytes (47). Indeed, the data reveal that LVGC currents in NCX1^{SM−/−} mouse myocytes are smaller than the currents in myocytes from WT and NCX1^{Fx−/−} mice (Fig. 7A, a–c). This was confirmed by measuring both the current-voltage and G-V curves in myocytes from WT, NCX1^{Fx−/−}, and NCX1^{SM−/−} mice. The amplitude of the LVGC, −I_{cat}, was used as a measure of the conductance activated during the preceding depolarizing pulse (Fig. 7A,d). Both the LVGC current and conductance are significantly smaller in NCX1^{SM−/−} myocytes compared with WT myocytes (Fig. 7B, a and b). LVGC currents and conductances are, however, nearly identical in WT and NCX1^{Fx−/−} myocytes (Fig. 7B, a and b).

Block of NCX1 with SEA0400 Does Not Affect LVGC Current

Because the LVGC current is reduced in NCX1^{SM−/−} mice, we examined the effect of acute block of NCX1 with SEA0400 on LVGC current in dialyzed, patch-clamped WT myocytes. In the experiment illustrated in Fig. 7C, I_{Ba} at +10 mV is plotted as a function of time to control for the rundown of the current. The addition of 0.01% DMSO or 1 μM SEA0400 in 0.01% DMSO had no detectable effect on I_{Ba}. In another type of control experiment performed in a similar manner, −85–90% of I_{Ba} was blocked by 10 μM nifedipine within 30 s (data not shown) (47). These experiments demonstrate that an acute block of NCX1 by SEA0400 in WT myocytes does not reduce the nifedipine-sensitive LVGC current.

Voltage-Dependent Inactivation of LVGC Channels Is Unchanged in NCX1^{SM−/−} Myocytes

In cardiac-specific NCX1 knockout mice, too, LVGC current is decreased. In those myocytes, the current declines as a result of an increase in the rate of Ca^{2+}-induced inactivation and increased Ca^{2+}-dependent tonic inactivation of LVGCs, presumably because of the elevation of subsarcolemmal [Ca^{2+}] (32). Such a mechanism cannot explain our results because Ba^{2+} was used as the current carrier in the arterial myocytes and because Ba^{2+} does not support Ca^{2+}-dependent inactivation (15). Because LVGCs also exhibit voltage-dependent inactivation, however, we tested whether a shift in voltage dependence or an increase in inactivation could explain the reduction of LVGC current in NCX1^{SM−/−} mice. The results reveal that voltage-dependent inactivation is nearly identical in WT, NCX1^{Fx−/−}, and NCX1^{SM−/−} myocytes (Fig. 8A). Thus a change in inactivation cannot explain the reduction of LVGC current in NCX1^{SM−/−} myocytes.

Bay K 8644 Eliminates the Difference in LVGC Current Between Control and NCX1^{SM−/−} Myocytes

Another possible explanation for the reduced LVGC current in NCX1^{SM−/−} myocytes is that the expression of the channels is reduced. To get an indication of LVGC expression, we applied Bay K 8644, a potent activator of LVGCs (19). In both WT and NCX1^{SM−/−} myocytes, 1 μM Bay K 8644 increased

**Fig. 3.** Effect of reduced NCX1 activity on myogenic reactivity (MR) and tone (MT) in pressurized mouse mesenteric small arteries. A: MR in WT (n = 7) and NCX1^{SM−/−} (n = 5) mouse arteries. The data are shown as the mean steady-state arterial diameter of arteries superfused with PSS and the passive diameter (PD; in Ca^{2+}-free PSS) at each pressure indicated on the abscissa. ***P < 0.001 vs. WT (2-way ANOVA). B: MT in WT (n = 9), NCX1^{Fx−/−} (n = 5), and NCX1^{SM−/−} (n = 11) mouse arteries treated with 1 μM SEA0400. The data are normalized to the WT control (Ctrl) myogenic tone (MT_{Ctrl}). *P < 0.05; ***P < 0.001. C,a: fluorescent images of a longitudinal section through the left-hand wall of an artery loaded with fluo-4 before (i), during (ii), and after (iii) treatment with 1 μM SEA0400 (see C,b). Bright areas (see left-hand image) are individual myocytes in cross-section. C,b: time course of changes in cytosolic Ca^{2+} concentration ([Ca^{2+}]_{cyt}; i.e., fluo-4 fluorescence, in arbitrary units, AU) for the entire wall (blue line) and for the two bright cells in the small boxes in the left-hand image (green and red lines). Simultaneous diameter changes are indicated by the black line. The times when images C,a, i–iii, were captured are shown on the graph. Results are representative of data from 5 arteries.
LVGC current and maximum conductance and shifted the G–V curve to more negative potentials (Fig. 8B). Interestingly, the current and conductance increases were greater in NCX1SM myocytes than in WT myocytes. In fact, Bay K 8644 eliminated the difference between the current, as well as the conductance, in WT and NCX1SM−/− myocytes (Fig. 8B). This implies that LVGC channel expression is comparable in WT and NCX1SM−/− myocytes.

Fig. 4. Effect of reducing extracellular Na+ concentration ([Na+]o) on MT in WT, NCX1^Fx/−, and NCX1SM^Fx/− mouse arteries and in WT arteries treated with SEA0400 (SEA). A: representative experiments show the effects of acute inhibition of NCX1 by lowering [Na+]o from 137.7 to 25.7 mM on MT in a WT, a NCX1Fx/−, and a NCX1SM^Fx/− mouse artery. B: representative experiment shows the effect of 1 μM SEA0400 on low [Na+]o-induced vasodilation and vasoconstriction in a WT artery. C: summary of effects of lowering [Na+]o on the initial relaxation and delayed vasoconstriction in WT (n = 7), NCX1Fx/− (n = 9), and NCX1SM^Fx/− (n = 16) arteries and WT arteries treated with 1 μM SEA0400 (n = 4). *P < 0.05; ***P < 0.001 vs. WT.

Fig. 5. Effect of ouabain (Ouab) on MT in WT, NCX1^Fx/−, and NCX1SM^Fx/− mouse arteries. A: effect of 100 nM Ouab on MT in 8 WT, 3 NCX1Fx/−, and 12 NCX1SM^Fx/− mouse arteries. **P < 0.01; ***P < 0.001 for the indicated pairs. B: Ouab-induced increase in MT. *P < 0.05; **P < 0.01 vs. WT.

Fig. 6. High extracellular K+ concentration ([K+]o)- and phenylephrine (PE)-induced vasoconstriction in WT, NCX1^Fx/−, and NCX1SM^Fx/− mouse arteries. A:a: vasoconstriction (as a percentage of PD) induced by elevating [K+]o from 5 to 75 mM in 8 WT, 11 NCX1Fx/−, and 7 NCX1SM^Fx/− arteries; *P < 0.05. A,b: [K+]o concentration-vasoconstriction curves for 7 NCX1Fx/− and 5 NCX1SM^Fx/− arteries; P < 0.01 (2-way ANOVA). B:a: PE (100 μM)-induced vasoconstriction in 5 WT, 5 NCX1Fx/−, and 5 NCX1SM^Fx/− arteries; *P < 0.05. B,b: PE dose-response curves for 5 NCX1Fx/− and 5 NCX1SM^Fx/− arteries; P < 0.001 (2-way ANOVA).

Bay K 8644 Eliminates the Difference in Ca2+ Sparklet Activity Between Control and NCX1SM^Fx/− Myocytes

Ca2+ sparklets are local Ca2+ signals produced by the opening of single or small clusters of LVGCs in arterial myocytes (28, 29). Accordingly, Ca2+ sparklets are the elementary events underlying dihydropyridine-sensitive Ca2+ influx in these cells. Therefore, in view of the smaller LVGC currents in NCX1SM^Fx/− than in WT or NCX1Fx/− myocytes, we examined Ca2+ sparklet activity in NCX1SM^Fx/− and sibling NCX1Fx/− mouse myocytes to test the hypothesis that sparklet activity is reduced in the NCX1SM^Fx/− myocytes.

The images in Fig. 9A show a representative Ca2+ sparklet recorded from an NCX1SM^Fx/− myocyte and one from an NCX1Fx/− myocyte. Summarized data indicate that the mean peak amplitude of the sparklets, as well as the width at 50% of peak amplitude, is comparable in NCX1Fx/− and NCX1SM^Fx/− myocytes: 41 ± 1 vs. 40 ± 1 nM, and 0.73 ± 0.03 vs. 0.82 ± 0.05 μm (n = 7 and 9 cells, respectively; P > 0.05 for both comparisons). These results suggest that NCX1-mediated Ca2+ transport does not significantly alter the amplitude or spatial spread of quantal Ca2+ sparklets.

Despite these similarities in the quantal events, Ca2+ sparklet density (in sites/mm2) and the overall level of sparklet activity (in nP2s, where n is the number of quantal levels and P2 is the probability that a sparklet site is active) (29) are significantly reduced in NCX1SM^Fx/− myocytes compared with NCX1Fx/− myocytes (Fig. 9, A and B). Bay K 8644 (500 nM), however, abolishes this difference (Fig. 9, B and C). These
results are consistent with the effect of Bay K 8644 on LVGC currents in NCX1SM−/− and control (WT) myocytes (Fig. 8B).

Bay K 8644 Does Not Eliminate the Difference in Myogenic Reactivity Between WT and NCX1SM−/− Arteries

Because LVGCs play a major role in myogenic constriction (8, 20), one possibility is that the reduced myogenic reactivity observed in NCX1SM−/− arteries (Fig. 3A) might be the result of the reduced LVGC activity in these arteries. Bay K 8644 normalizes LVGC current (Fig. 8) and Ca2+ sparklet activity (Fig. 9) in NCX1SM−/− arteries. Therefore, we reexamined the myogenic reactivity curves in WT and NCX1SM−/− arteries in the presence of 1 μM Bay K 8644. Figure 10 shows that Bay K 8644 constricts WT and NCX1SM−/− arteries at 20 mmHg. The WT arteries then exhibit slight additional constriction when the intralumenal pressure is increased to 40 mmHg or more. In contrast, raising intraluminal pressure from 20 to 40 mmHg dilates the Bay K 8644-treated NCX1SM−/− arteries, albeit, not as much as in the absence of Bay K 8644, increasing intraluminal pressure further, and then causes only slight constriction. Importantly, Bay K 8644 did not affect the PD in either WT or NCX1SM−/− arteries (not shown). Clearly, the NCX1SM−/− arteries exhibit significantly less myogenic constriction than WT arteries at intraluminal pressures of 40–120

Fig. 7. Effects of genetic and pharmacological reduction of NCX activity on L-type voltage-gated Ca2+ channel (LVGC) current and conductance in WT, NCX1Fx−/−, and NCX1SM−/− mouse mesenteric artery myocytes. A: representative of LVGC current records. A, a–c: families of macroscopic LVGC-mediated Ba2+ currents (I_Ba) recorded in response to voltage-clamp steps to −50, −30, −10, +10, and +30 mV in a myocyte from a WT (a), a NCX1Fx−/− (b), or a NCX1SM−/− (c) mouse, respectively. A,d: illustration of current measurements used to construct current-voltage (I-V) and conductance-voltage (G-V) curves. The maximum current during the voltage-clamp step, I_na, is plotted as a function of the voltage during the step to construct I-V curves; the amplitude of the tail current (I_tail) is a measure of the conductance (G) activated during the preceding step. G-V curves are constructed by plotting −I_tail as a function of the V_mem during the preceding step. Scale bars (in A, b and d) represent 100 pA, 5 ms. B: whole cell LVGC currents were measured during voltage-clamp steps to −50 to +50 mV. B,a: I-V curves. The currents from WT (38 cells from 24 mice) and NCX1Fx−/− (35 cells from 11 mice) are similar, whereas the currents from NCX1SM−/− (40 cells from 8 mice) are significantly smaller. P < 0.01 at +10 mV. B,b: G-V curves from the same myocytes as in B,a. The amplitude of I_tail is plotted vs. V_mem. The maximum LVGC conductance is significantly smaller in NCX1SM−/− myocytes than in either WT or NCX1Fx−/− myocytes (P < 0.001; 2-way ANOVA). C: effect of SEA0400 on I_Ba in dialyzed WT myocytes. I_Ba at +10 mV is plotted as a function of time. Addition of 0.01% DMSO or 1 μM SEA0400 in 0.01% DMSO at the times indicated had no significant effect on the amplitude of I_Ba. Similar results were obtained in 12 cells from 5 WT mice.

Fig. 8. Voltage-dependent inactivation of I_Ba and effects of Bay K 8644 (BayK) on I_Ba in WT and NCX1 mutant arterial myocytes. A: voltage-dependent inactivation of LVGCs. I_Ba at +10 mV was measured following 10-s inactivating prepulses to voltages from −70 to +30 mV. I_Ba, normalized relative to its maximum value, is plotted vs. prepulse voltage. As prepulse voltage becomes more positive, I_Ba decreases because of voltage-dependent inactivation. These inactivation curves are nearly identical in WT (24 cells from 12 mice), NCX1Fx−/− (15 cells from 5 mice), and NCX1SM−/− (22 cells from 7 mice) myocytes. B,a: I-V curves from WT (13 cells from 9 mice) and NCX1SM−/− (11 cells from 6 mice) myocytes with and without 1 μM Bay K 8644, the LVGC activator. B,b: G-V curves from the same cells as in B,a. In the absence of Bay K 8644, the LVGC current and conductance are smaller in NCX1SM−/− compared with WT myocytes (also see Fig. 7B). Bay K 8644 increases the current and conductance more in NCX1SM−/− than in WT myocytes and thereby eliminates the difference in current and conductance amplitude between WT and NCX1SM−/− myocytes.
mmHg, even in the presence of Bay K 8644. This difference apparently cannot be attributed to the reduced LVGC activity in NCX1SM−/− arterial myocytes.

**DISCUSSION**

This study on the functional consequences of SM-specific knockout of NCX1 complements our earlier report that SM-specific overexpression of NCX1 elevates BP in mice. The results we describe here strengthen the view that NCX1 plays an important role in vascular function.

**NCX1Fx−/− Mice vs. WT Mice**

A complication encountered in this study was the discovery that the Cre recombinase gene, under the control of the SM-MHC promoter, is activated during gametogenesis. Thus both the CreSM+ and Cre− mice used in this study all carried one floxed NCX1 allele and one null-mutant allele, in which NCX1 exon 11 was permanently deleted in all cells (METHODS). In other words, both Cre− and CreSM+ mice are, effectively, NCX1 heterozygotes; then, upon activation of the Cre gene in the SM cells of CreSM+ mice, exon 11 in the remaining floxed NCX1 allele is excised in these cells. Therefore, in many experiments, we employed two controls for the NCX1SM−/− mice: WT mice and NCX1Fx−/− mice. It is noteworthy that the homozygous global null mutation of NCX1 is embryonic lethal (39).

Relative to WT controls, the NCX1Fx−/− mice have a reduced expression of full-length NCX1 in all tissues tested (see Fig. 1D). As a result, the NCX1Fx−/− arteries exhibit a reduced vasodilator response to SEA0400 (Fig. 3B), a reduced low [Na+]o-induced vasodilation and vasoconstriction (Fig. 4B), and a reduced vasoconstrictor response to low-dose ouabain (Fig. 5). In many other respects, however, the function of NCX1Fx−/− arteries is comparable with that of WT controls. For example, MT, vasoconstrictor responses to high [K+]o, and the function of LVGCs are all normal; moreover, the NCX1Fx−/− mice have normal BP. The implication is that approximately half of the normal level of NCX1 in ASMC is sufficient to maintain normal arterial function.

**Similarity of Effects of Genetic and Pharmacological Knockout of NCX1 on Arterial Function**

In contrast to the situation in NCX1Fx−/− mice, vascular function is substantially impaired in NCX1SM−/− mice. This is exemplified by the reduced myogenic reactivity and MT (Fig. 3), attenuated vasconstrictor responses to PE and high [K+]o, (Fig. 6), and reduced LVGC activity (Fig. 7). Moreover, SM-specific knockout of NCX1 and an acute SEA0400 application to WT mouse arteries, i.e., a genetic and pharmacological reduction of NCX activity, respectively, have comparable effects on MT (Fig. 3) and on the responses to low [Na+]o, (Fig. 4) and low-dose ouabain (Fig. 5 and Ref. 48). In rat small arteries, too, pharmacological blockade or antisense oligonucleotide knockdown of NCX1 reduces myogenic reactivity and tone (36). As expected for a NCX-specific blocker (albeit somewhat controversial (38)), SEA0400 has no effect on MT in NCX1SM−/− mouse arteries (Fig. 3) or on LVGC current in WT artery myocytes (Fig. 7C). These findings emphasize the importance of NCX1-mediated Ca2+ entry in arterial function.

Clearly, this mode of NCX operation is a normal mode and, perhaps, the dominant mode in ASMCs in arteries with tone.

The NCX1-mediated, low [Na+]o-induced initial vasodilation and, later, sustained vasoconstriction (Fig. 4A) raise the...
interesting question of mechanism(s). The vasoconstriction appears to be explicable by the decline in the inwardly directed $E_{\text{Na}}$; this is expected to promote net $Ca^{2+}$ gain via NCX1 (Introduction) and direct activation of myosin light chain kinase and, therefore, ASMC contraction. The explanation for the early vasodilation is less clear. About half of the dilatation is blocked by bath-applied SEA0400 or by SM-specific NCX1 knockout and therefore appears to be attributable to SM NCX1 (Fig. 4C). Two possible mechanisms that could also be triggered by NCX1-mediated $Ca^{2+}$ entry and a rise in $Ca^{2+}_{\text{Cyt}}$ are (1) the rapid activation of large-conductance $Ca^{2+}$-activated $K^+$ channels in ASMCs (7) and (2) the $Ca^{2+}$-induced activation of nitric oxide synthase in endothelial cells (43). The latter possibility is consistent with the view that NCX1 activity in ASMCs and endothelial cells may have opposing effects on arterial constriction.

Reduced LVGC Activation: Cause of Reduced MT or Consequence of Reduced NCX1 Activity?

An unexpected observation was that LVGC activity is reduced in arterial myocytes from NCX1SM−/− mice (Fig. 7). Three possible explanations are (1) a reduction in the number of expressed LVGCs in NCX1SM−/− myocytes or (2) a decrease in the magnitude of the LVGC-mediated elementary $Ca^{2+}$ influx events ($Ca^{2+}$ sparklets) in NCX1SM−/− myocytes (i.e., a decrease in quantal size), or (3) a reduction in the number of open channels under the physiological conditions that prevail in NCX1SM−/− myocytes. The maximal activation of LVGCs (both whole cell currents and $Ca^{2+}$ sparklets) by Bay K 8644 reveals that the reduced channel activity is functional and is not due to reduced LVGC expression.

To distinguish between the second and third possibilities, we employed sparklet analysis. The evidence that the magnitude and spatial spread of individual sparklets do not differ in NCX1SM−/− and NCX1Fx−/− myocytes indicates that the individual quanta are similar; this also implies that the single-channel conductance of the LVGCs is not altered. Thus the reduced LVGC current must be due to a decreased open-channel probability. This fits with the observed reduction in sparklet activity (number of active sites and probability of activation) in NCX1SM−/− myocytes (Fig. 9).

LVGC activity plays a critical role in the maintenance of MT (8, 20, 47), and the reduced LVGC activity might be the cause of the reduced MT and low BP in NCX1SM−/− mice. Figure 10 shows, however, that myogenic constriction is not reduced in NCX1SM−/− arteries, compared with WT arteries, even in the presence of $1 \mu M$ Bay K 8644, which abolishes the difference between LVGC activity in these arteries (Fig. 8). Furthermore, evidence presented in the accompanying article (37) implies that the depressed LVGC activity is a consequence of the low $Ca^{2+}_{\text{Cyt}}$ and reduced $Ca^{2+}$-dependent protein kinase C activity that likely results from a marked knockdown of NCX1 expression. Indeed, an acute inhibition of NCX by SEA0400, which does not block LVGCs (Fig. 7C), lowers $Ca^{2+}_{\text{Cyt}}$ and reduces MT in WT arteries (Fig. 3, B and C). The fact that NCX1SM−/− mouse arteries have significantly reduced MT therefore implies that $Ca^{2+}_{\text{Cyt}}$ may also be low in these ASMCs. Thus the attenuation of MT (Fig. 3A), LVGC activity (Fig. 7), and high $K^+$-induced vasoconstriction (Fig. 6A) in NCX1SM−/− arteries may all be the consequence of a low $[Ca^{2+}]_{\text{Cyt}}$ as a result of reduced NCX1-mediated $Ca^{2+}$ entry.

The mechanism(s) responsible for the attenuated high-dose PE-induced vasoconstriction (Fig. 6B) is (are) uncertain. Preliminary data imply that the mobilization of stored $Ca^{2+}$ (42) is not impaired in arteries from NCX1SM−/− mice because high PE-induced vasoconstriction in $Ca^{2+}$-free media is not attenuated (data not shown). A likely possibility is that receptor-operated channels (ROCs), which are an important source of $Ca^{2+}$ during receptor activation (44), especially at high catecholamine concentrations (30), may be downregulated in NCX1SM−/− arteries. In primary cultured arterial myocytes, a knockout of NCX1 markedly decreases the expression of transient receptor potential canonical 6 channel protein (a component of ROCs) and reduces ROC activity (33).

Correlation Between BP and In Vitro MT

Davis and Hill (8) observed that the level of tone in isolated arteries "is often comparable to that observed in the same vessels in vivo." This implies that MT in isolated arteries predicts not only in vivo vascular tone but BP as well, as exemplified by the increased MT and elevated BP in $\alpha_2 Na^+$-pump heterozygous null mutant mice (48). The present report extends this concept by showing that, at the other end of the spectrum, SM-specific knockout of NCX1 not only reduces MT but lowers BP as well. Conversely, SM-specific overexpression of NCX1 is associated with elevated BP (21); an increase in MT is anticipated, but this has not yet been tested in arteries from these mice.

Most important, the present report adds to the growing body of evidence that $\alpha_2 Na^+$ pumps and adjacent NCX1 are critical mechanisms that link $Na^+$ metabolism to BP regulation (6). Recently, Wirth and colleagues (45) suggested that salt-dependent hypertension is specifically mediated by a $G_{12/13}$ protein-coupled receptor-mediated pathway, but our findings do not support their conclusion. On the contrary, our results suggest that the modulation of NCX1 activity directly affects $Ca^{2+}$ metabolism and the level of $[Ca^{2+}]_{\text{Cyt}}$ and thus likely acts via myosin light chain kinase to modulate vasconstriction. In conjunction with earlier reports (12, 13, 21, 48), the present study is consistent with the view that retained salt can act via the endogenous ouabain-$\alpha_2 Na^+$ pump-NCX1 pathway to elevate BP without directly involving G protein-coupled receptor pathways (6). Of course, the downstream effects are likely to be modulated by the G protein-coupled receptor pathways that regulate the $Ca^{2+}$ sensitivity of the contractile apparatus (24).

We conclude that the arterial SM NCX1 is needed to maintain the normal $[Ca^{2+}]_{\text{Cyt}}, MT$, and BP. This direct link to NaCl metabolism is often overlooked in studies of the mechanisms that influence the BP in salt-dependent hypertension.

ACKNOWLEDGMENTS

We thank Michelle Izuka for technical support and transgenic mouse genotyping and Katherine Frankel for assistance with the manuscript. We also thank Taisho Pharmaceutical (Tokyo, Japan) for a gift of SEA0400.

Present address of C. Ren: Neuroscience program, Otolaryngology/Department of Surgery, University of Utah, Salt Lake City, UT.

GRANTS

This work was supported by a National Scientist Development grant from the American Heart Association (to J. Zhang); an International Society of
Hypertension-Pfizer award (to J. Zhang); an National Institute of Arthritis and Musculoskeletal and Skin Diseases training grant (to L. K. Antos); and National Heart, Lung, and Blood Institute research grants (to M. P. Blaustein, K. D. Philipson, M. I. Kotlikoff, L. F. Santana, and W. G. Wier).

DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


