Overactive bladder mediated by accelerated \(\text{Ca}^{2+}\) influx mode of \(\text{Na}^+/\text{Ca}^{2+}\) exchanger in smooth muscle

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†Department of Molecular and Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan; ‡The Smooth Muscle Research Group, Department of Physiology and Pharmacology, Faculty of Medicine, University of Calgary, Calgary, Canada; §Department of Pharmacology, Faculty of Medicine, Fukuoka University, Fukuoka, Japan; and ⊥Department of Pharmacology, Division of Pathological Sciences, Kyoto Pharmaceutical University, Kyoto, Japan

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Yamamura H, Cole WC, Kita S, Hotta S, Murata H, Suzuki Y, Ohya S, Iwamoto T, Imaizumi Y. Overactive bladder mediated by accelerated \(\text{Ca}^{2+}\) influx mode of \(\text{Na}^+/\text{Ca}^{2+}\) exchanger in smooth muscle. Am J Physiol Cell Physiol 305: C299–C308, 2013. First published May 22, 2013; doi:10.1152/ajpcell.00065.2013.—The \(\text{Na}^+/\text{Ca}^{2+}\) exchanger (NCX) is thought to be a key molecule in the regulation of cytosolic \(\text{Ca}^{2+}\) dynamics. The relative importance of the two \(\text{Ca}^{2+}\) transport modes of NCX activity leading to \(\text{Ca}^{2+}\) efflux (forward) and influx (reverse) in smooth muscle, however, remains unclear. Unexpectedly, spontaneous contractions of urinary bladder smooth muscle (UBSM) in transgenic mice specifically overexpressing NCX1.3 (NCX1.3tg/tg) were enhanced in transgenic mice overexpressing NCX1.3tg/tg. The enhanced activity was attenuated by KB-R7943 or SN-6. Whole cell outward NCX current sensitive to inwardly directed electrochemical gradient for \(\text{Na}^+\) was readily detected in UBSM cells from NCX1.3tg/tg mice. We conclude that NCX1.3 overexpression is associated with abnormal urination owing to enhanced \(\text{Ca}^{2+}\) influx via reverse mode NCX leading to \(\text{Ca}^{2+}\) overload and associated electrical and mechanical dysfunction (arrhythmias; Refs. 4, 6, 41). Moreover, suppression of NCX activity with KB-R7943, a benzylpheryl NCX inhibitor, or heterozygous deletion improves ischemia/reperfusion-induced dysfunction in the heart (28) and kidney (52).

In contrast, the physiological role(s) of NCX in smooth muscle appears to be more complicated, as forward and reverse modes have both been postulated to be functionally relevant in controlling cytosolic \(\text{Ca}^{2+}\) dynamics in different preparations (3, 9, 40). In mammals, three NCX isoforms have been identified as products of the SLC8 gene family (37). NCX1 is abundant in the heart and brain and also expressed at much lower levels in other tissues, whereas the expression of NCX2 and NCX3 is restricted mainly to brain and skeletal muscle. Among alternative splicing variants of NCX1, NCX1.3 and NCX1.7 are the predominant isoforms expressed in smooth muscle tissues (38). NCX1 was suggested to contribute to \(\text{Ca}^{2+}\) extrusion when cytosolic \(\text{Ca}^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)) is suddenly elevated (9, 42, 51). For example, we previously reported that \(\text{Ca}^{2+}\) extrusion was enhanced after stimulation in urinary bladder smooth muscle (UBSM) of transgenic mice overexpressing NCX1.3 (NCX1.3tg/tg), whereas increased reverse mode activity was evident when the transmembrane \(\text{Na}^+\) gradient was reduced by reduction of extracellular \([\text{Na}^+]_0\) (Ref. 32). Significantly, reverse mode NCX activity was shown to predominate under resting conditions in vascular smooth muscle of NCX1.3tg/tg mice leading to elevated systemic blood pressure and salt-sensitive hypertension, whereas a selective NCX inhibitor, SEA0400, was found to lower blood pressure in salt-dependent hypertensive animal models (23). Moreover, upregulation of NCX and enhanced function in the reverse mode were also previously

Na\(^{+}/\text{Ca}^{2+}\) exchangers (NCXs) are expressed in a variety of cell types and participate in the regulation of cytosolic \(\text{Ca}^{2+}\) mobilization. For example, the physiological and pathological roles of NCX are well understood in cardiac myocytes (6, 41). NCX is a bidirectional antiporter that transports three \(\text{Na}^+\) per \(\text{Ca}^{2+}\) ion in an electrogenic manner. Normally, the large inwardly directed electrochemical gradient for \(\text{Na}^+\) results in forward mode NCX activity and \(\text{Ca}^{2+}\) efflux to maintain \(\text{Ca}^{2+}\) homeostasis following influx through voltage-dependent \(\text{Ca}^{2+}\) channels (VDCCs) and \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release (CICR).

from internal stores during excitation-contraction coupling (4). Genetic deletion of NCX (i.e., NCX1-deficient mice, NCX1\(^{-/-}\)) is an embryonically lethal knockout, presumably because of abnormal \(\text{Ca}^{2+}\) homeostasis and associated cardiac development and dysfunction (45). Whether reverse mode NCX activity is associated with \(\text{Na}^+\) accumulation in the subplasmalemmal space during the cardiac action potential to provide additional trigger \(\text{Ca}^{2+}\) influx remains controversial (33). However, it is well-established that elevated cytosolic \(\text{Na}^+\) concentration ([\(\text{Na}^+]_i\]) owing to decreased intracellular pH and increased \(\text{Na}^+/\text{H}^+\) exchange activity, combined with membrane potential depolarization in cardiac ischemia/reperfusion injury provides an appropriate electrogentic environment for enhanced \(\text{Ca}^{2+}\) influx via reverse mode NCX leading to \(\text{Ca}^{2+}\) overload and associated electrical and mechanical dysfunction (arythmias; Refs. 4, 6, 41). Moreover, suppression of NCX activity with KB-R7943, a benzylpheryl NCX inhibitor, or heterozygous deletion improves ischemia/reperfusion-induced dysfunction in the heart (28) and kidney (52).

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reported to be associated with ouabain-induced hypertension (36) and pulmonary arterial hypertension (55).

In preliminary experiments, we unexpectedly found that spontaneous contractions of UBSM were enhanced in NCX1.3<supwendung</sup>tg/tg compared with wild-type (WT) mice. For this reason, the present study was undertaken to elucidate the contribution of Ca<sup2+</sup> influx via reverse mode NCX activity to the control of membrane electrical excitability, cytosolic Ca<sup2+</sup> mobilization in UBSM myocytes, UBSM contractility, and urinary bladder function.

MATERIALS AND METHODS

Experimental animals. All experiments were approved by the Ethics Committee of the Nagoya City University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society. The generations of NCX1.3<supwendung</sup>tg/tg and G833C-NCX1.3<supwendung</sup>tg/tg mice were described previously (22, 23). Data from NCX1.3<supwendung</sup>tg/tg and G833C-NCX1.3<supwendung</sup>tg/tg mice were compared with age-matched WT mice (C57BL/6; Japan SLC, Hamamatsu, Japan).

Contractility measurements. Measurements of UBSM contractility were carried out as reported previously (31). In brief, urinary bladders were removed from male mice (8–12 wk old) and placed in aerated Krebs solution. A small strip (1 mm in wide and 5 mm in length) of detrusor muscle was prepared and placed in a tissue bath containing aerated Krebs solution at 37°C. One end of the segment was pinned to a rubber plate at the bottom of the bath, and the other end was connected to an isometric transducer. Strips were stretched to ~1 mN of tension. The Krebs solution had an ionic composition of 112 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl<sub2</sub>, 1.2 mM MgCl<sub2</sub>, 25 mM NaHCO<sub3</sub>, 1.2 mM KH<sub>2</sub>PO<sub4</sub>, and 14 mM glucose. The pH was adjusted to 7.4 by gassing with a mixture of 95% O<sub2</sub>-5% CO<sub2</sub>. To suppress the effects of transmitter release from nerve endings in the preparations, all contractility experiments were performed in the presence of the following neurotransmitter antagonists: 1 μM atropine, 1 μM phentolamine, 1 μM propranolol, 1 μM tetrodotoxin, and 10 μM suramin.

Cell isolation. UBSMs of male mice (7- to 13-wk-old) were immersed in Ca<sup2+</sup>/Mg<sup2+</sup>-free Krebs solution for 15 min at 37°C. Subsequently, the solution was replaced with Ca<sup2+</sup>/Mg<sup2+</sup>-free Krebs solution containing 0.25% collagenase (Amano, Nagoya, Japan) and 0.01% papain (Sigma-Aldrich, St. Louis, MO), and then the tissue was incubated for 30 min at 37°C. After incubation, the solution was replaced with Ca<sup2+</sup>/Mg<sup2+</sup>-free Krebs solution. Thereafter, individual myocytes were mechanically dispersed from the tissue fragments by gentle trituration using a glass pipette. All experiments using cells were carried out at room temperature (25°C).

Electrophysiological recording. Electrophysiological studies were performed using a whole cell patch-clamp technique with EPC-7 (List, Darmstadt, Germany) or CEZ-2400 (Nihon Kohden, Tokyo, Japan) amplifiers, an analog-digital converter (Digidata 1440A; Axon, Foster City, CA), and a pCLAMP software (version 10.2; Axon, Foster City, CA). Neuronal recordings were done using an Axopatch 1D patch-clamp amplifier and a Digidata 1440A analog-digital converter (Axon, Foster City, CA), and a pCLAMP software (version 10.2; Axon, Foster City, CA) amplifiers. The extracellular solution contained 20 mM NaCl, 40 mM NaCl, 10 mM BAPTA, 70 mM CsOH, 30 mM aspartate, 5 mM MgCl<sub2</sub>, 5 mM MgATP, and 10 mM HEPES. The pH was adjusted to 7.2 with NaOH. Myocytes were clamped at a holding potential of ~60 mV, and a descending ramp protocol from +15 mV to ~100 mV for 500 ms was performed every 10 s. When spontaneous transient outward currents (STOCs) were recorded at a holding potential of ~30 mV, normal HEPES-buffered saline solution was used with a composition of 137 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl<sub2</sub>, 1.2 mM MgCl<sub2</sub>, 14 mM glucose, and 10 mM HEPES and a pH of 7.4 adjusted with NaOH. The pipette solution contained 20 mM NaCl, 120 mM KCl, 1 mM MgCl<sub2</sub>, 10 mM HEPES, and 2 mM MgATP and a pH of 7.2 adjusted with KOH.

Ca<sup2+</sup> imaging. Cytosolic Ca<sup2+</sup> dynamics were imaged using a total internal reflection fluorescence (TIRF) microscope (Eclipse TE2000-U; Nikon) equipped with an objective lens (CFI Plan Apo TIRF ×60/1.45, oil immersion; Nikon), an EM-CCD camera (C9100–12; Hamamatsu Photonics, Hamamatsu, Japan), and AQUACOSMOS software (version 2.6; Hamamatsu Photonics), as reported previously (48, 49). Single UBSM cells were loaded with fluorescent Ca<sup2+</sup> indicator, 10 μM fluo-4/AM for 10 min. Ca<sup2+</sup> images were acquired every 27.2 ms and a resolution of 107 nm per pixel. The bath contained HEPES-buffered saline solution as indicated above.

Urination patterns. Urination pattern analysis was carried out as reported previously (18). In brief, female mice (13- to 22-wk-old) were placed in standard cages for 3 h with the bedding replaced by a sheet of filter paper. Food and water were freely given. Urine spots were photographed under ultraviolet light. Test mice were given 10 mg/kg KB-R7943 (dissolved in 5% gum arabic solution) by oral administration an hour before the experiments.

Drugs. Pharmacological reagents were obtained from Sigma-Aldrich except for KB-R7943 and SN-6 (Tocris, Ellisville, MO), BAPTA and HEPES (Dojin, Kumamoto, Japan), and CdCl<sub2</sub>, NiCl<sub2</sub>, and ryanodine (Wako, Osaka, Japan). KB-R7943 and SN-6 were dissolved in dimethyl sulfoxide at the concentration of 10 mM as a stock solution.

Statistics. Pooled data are shown as the means ± SE. Statistical significance between the two groups was determined by Student’s t-test. Significant difference is expressed in the Figs. 1–7 as *<sup</sup>P < 0.05 or **<sup</sup>P < 0.01.

RESULTS

Enhanced spontaneous contractions in NCX1.3<supwendung</sup>tg UBSM. Spontaneous contractions were measured in UBSM tissue strips from WT and NCX1.3<supwendung</sup>tg mice. Spontaneous contractions were observed in WT tissues in the presence of neurotransmitter antagonists (1 μM atropine, 1 μM phentolamine, 1 μM propranolol, 1 μM tetrodotoxin, and 10 μM suramin), but unexpectedly, they were significantly enhanced in UBSM of NCX1.3<supwendung</sup>tg mice (Fig. 1A). The area under the curve (mN/min; AUC) of contractions for a 5-min recording period was markedly larger in NCX1.3<supwendung</sup>tg (208 ± 43; n = 7) compared with WT tissues (22 ± 3; n = 3; P < 0.01; Fig. 1B). Spontaneous contractions of WT tissues were almost completely blocked by application of 300 nM nicardipine (to 11 ± 2%; n = 6; Fig. 1, C and D) but only partially suppressed in NCX1.3<supwendung</sup>tg tissues (to 39 ± 9%; n = 8). Spontaneous contractions in NCX1.3<supwendung</sup>tg strips were inhibited by omitting Ca<sup2+</sup> from the bath solution or by treatment with 50 μM ryanodine, 100 μM tetracaine, or 10 μM cyclopiazonic acid (n = 3–8; P < 0.01), but they were not affected by 10 μM xestospongin C, 10 μM La<sup3+</sup>, or 10 μM Gd<sup3+</sup> (n = 3–11; Fig. 2). Spontaneous contractions of NCX1.3<supwendung</sup>tg (to 67 ± 9%; n = 7; P < 0.01), but not WT tissues (to 97 ± 12%; n = 3), were significantly reduced by 10 μM KB-R7943 (Fig. 3, A and B) or 1 mM Ni<sup2+</sup> (to 11 ± 4%; n = 4; P < 0.01; Fig. 2B), inhibitors of NCX. Significantly, the nicardipine-resistant, spontaneous contractions of NCX1.3<supwendung</sup>tg were reduced by 10 μM KB-R7943 (to 6 ± 1% of control; n = 6; P < 0.01 vs. nicardipine; Fig. 3, C and D). Other blockers of NCX activity, 3 μM SN-6 (n = 4; Fig. 3, E and F) or 1 mM Ni<sup2+</sup> (data not shown), caused a similar attenuation of spontaneous contraction of NCX1.3<supwendung</sup>tg UBSM in the presence of nicardipine.
respectively), the reversal potential was a standard voltage axis). In WT cells (28 ± 3 pA n = 6; Fig. 5B), and there was no significant difference in the frequency of STOCs (Fig. 5C). However, the half duration and area of single STOCs in NCX1.3tg/g cells (73 ± 9 ms and 4,759 ± 1,008 pA·ms, respectively; n = 11) were significantly larger than those of WT cells (39 ± 3 ms and 1,697 ± 243 pA·ms; n = 6; P < 0.05, respectively; Fig. 5, D and E). Figure 5F shows that STOCs with relatively large area (>10^4 pA·ms) were rarely detected in WT cells (<1% of 140 STOCs from 6 cells),

**Occurrence of long-lasting STOCs in NCX1.3tg/g cells.** At a holding potential of −30 mV, STOCs sensitive to 100 nM iberiotoxin or 1 μM paxilline (n = 3 for each) and due to large-conductance Ca^{2+}-activated K⁺ (BKCa) channel activity were detected in all UBSM cells, but cells of NCX1.3tg/g mice frequently showed STOCs with different kinetics (Fig. 5A). The peak amplitude of STOCs in NCX1.3tg/g cells (39 ± 5 pA; n = 11) was slightly, but not significantly, larger than that in WT cells (28 ± 3 pA n = 6; Fig. 5B), and there was no significant difference in the frequency of STOCs (Fig. 5C).

NCX current in NCX1.3tg/g smooth muscle cells. Whole cell current recordings were made by standard patch-clamp method to detect NCX activity in freshly isolated UBSM cells from WT and NCX1.3tg/g mice. To facilitate detection of NCX current, the bath and pipette solutions employed were adjusted to favor Na⁺ extrusion and Ca^{2+} influx. Specifically, external Na⁺ and K⁺ were substituted by NMDG in the bathing solution that also contained 1 mM Ca^{2+}, 20 μM ouabain, and 10 μM nifedipine, and the pipette solution contained Cs⁺ to block outward K⁺ currents, 40 mM Na⁺ and 10 mM BAPTA (16). Figure 4 shows the current-voltage relationship for membrane current in WT and NCX1.3tg/g myocytes in response to 500-ms descending ramp protocols applied between +15 and −100 mV (0.24 V/s, 10-kHz sampling rate) from a holding potential of −60 mV every 10 s (for clarity all recordings are shown on a standard voltage axis). In WT cells (n = 4), an inwardly directed 1 mM Ni^{2+}-sensitive current was detected at negative potentials (−9.6 ± 2.1 and 0.6 ± 3.4 pA at −100 and 0 mV, respectively), the reversal potential was −5.9 ± 6.3 mV, and the Ni^{2+}-sensitive holding current was inwardly directed at −60 mV (Fig. 4A). In contrast, the Ni^{2+}-sensitive current of NCX1.3tg/g cells (n = 4) was outwardly directed (−1.8 ± 3.0 and 27.9 ± 3.6 pA at −100 and 0 mV, respectively), the reversal potential was −100.7 ± 10.5 mV (P < 0.01), and the holding current was outward at −60 mV (Fig. 4B). A similar whole cell NCX current with a reversal potential of −89.9 ± 23.0 mV was detected in NCX1.3tg/g myocytes using the selective NCX inhibitor KB-R7943 at 10 μM (n = 5; P < 0.01; Fig. 4C).

![Fig. 1. Enhanced spontaneous contractions in NCX1.3tg/g urinary bladder smooth muscle (UBSM) tissues. The contribution of Na⁺/Ca^{2+} exchanger (NCX) to spontaneous contractions of UBSM was determined in presence of 1 μM atropine, 1 μM phenolamine, 1 μM propranolol, 1 μM tetrodotoxin, and 10 μM suramin to block neuronal modulation. The number of tissue preparations examined is given in parentheses. A and B: representative recordings of isometric tension development due to spontaneous contractions in wild-type (WT) and NCX1.3tg/g [transgenic (TG)] UBSM (A) and integrated areas of contraction above resting tension level for a 5-min period [area under the curve (AUC); B]. C and D: representative recordings of spontaneous contractions in 300 nM nifedipine (C) in WT and NCX1.3tg/g strips and summarized data of AUC for 5 min (D). Note that the nicardipine-insensitive component in NCX1.3tg/g was significantly larger than WT UBSM. *P < 0.05, **P < 0.01 vs. WT; ***P < 0.01 vs. before application of nicardipine.](http://ajpcell.physiology.org/)

![Fig. 2. Ca^{2+} sources of nicardipine-resistant component of spontaneous contractions in NCX1.3tg/g UBSM tissues. Ca^{2+} sources for the residual spontaneous contractions observed in the presence of 1 μM nicardipine were examined in UBSM strips from NCX1.3tg/g (TG). All experiments were performed in the presence of 1 μM nicardipine. To avoid effects of transmitters released from nerve endings, 1 μM atropine, 1 μM phenolamine, 1 μM propranolol, 1 μM tetrodotoxin, and 10 μM suramin were also added to the bathing solution. A: representative effects of Ca^{2+}-free bath solution, 50 μM ryanodine, 100 μM tetracaine, 10 μM La^{3+}, and 10 μM Gd^{3+} on nicardipine-resistant, spontaneous activity of NCX1.3tg/g UBSM tissues are shown. B: relative AUC above the resting level for a 5-min period was measured after the application of varied solutions and/or blockers (Ca^{2+}-free solution, 50 μM ryanodine, 100 μM tetracaine, 10 μM cyclopiazonic acid, 10 μM xestospongin C, 10 μM La^{3+}, or 10 μM Gd^{3+}) and expressed as a percent of the AUC for nicardipine-resistant component of spontaneous contractions. **P < 0.01 vs. control.](http://ajpcell.physiology.org/)
whereas they were frequently observed in NCX1.3tg/tg cells (~10% of 254 STOCs from 11 cells).

Global propagation of local Ca2+ transients in NCX1.3tg/tg myocytes. Local Ca2+ transients were imaged using a TIRF microscope and UBSM myocytes loaded with the fluorescent Ca2+ indicator fluo-4 acetoxyethyl ester (fluoro-4/AM, 10 μM). Spatiotemporally restricted elevations in [Ca2+]i in NCX1.3tg/tg UBSM. B: average level of suppression of spontaneous contractility indicated by AUC above the resting level for a 5-min period in the presence of KB-R7943 compared with control period for WT and NCX1.3tg/tg UBSM tissues. Note that KB-R7943 did not affect the spontaneous contractions in WT but significantly suppressed activity in NCX1.3tg/tg strips. C and D: effects of 10 μM KB-R7943 on spontaneous contraction in the presence of 1 μM nicardipine in NCX1.3tg/tg UBSM. E and F: effects of 3 μM SN-6 on spontaneous contraction in the presence of nicardipine in NCX1.3tg/tg UBSM. *P < 0.05 vs. WT; **P < 0.01 vs. before application of KB-R7943; $$P < 0.01$$ vs. nicardipine alone.

Overactive bladder in NCX1.3tg/tg mice. The possibility that NCX overexpression affected the pattern of urination was examined using WT and NCX1.3tg/tg mice. When the urination

the TIRF visualization area were rarely observed in WT cells, but Ca2+ spark-triggered propagating elevations in [Ca2+]i, leading to a global increase were frequently observed in NCX1.3tg/tg cells. Based on the distribution histogram of maximum area shown in Fig. 6F, it is clear that Ca2+ transients with relatively large propagation covering >20 μm2 and reminiscent of propagating Ca2+ waves were rare in WT cells (<1% of 168 events from 5 cells) but were ~20% of the events in NCX1.3tg/tg cells (469 events from 9 cells). To address the role of enhanced NCX activity as a cause of the abnormal Ca2+ events in NCX1.3tg/tg myocytes, the effects of KB-R7943 were examined under identical recording conditions. Following treatment with 10 μM KB-R7943, the large propagating Ca2+ transients disappeared, but Ca2+ sparks still remained (n = 7). Exposure to 10 μM ryanodine plus 100 μM Cd2+ abolished all changes in [Ca2+]i in NCX1.3tg/tg cells (n = 4).

Overactive bladder in NCX1.3tg/tg mice. The possibility that NCX overexpression affected the pattern of urination was examined using WT and NCX1.3tg/tg mice. When the urination...
pattern of freely moving mice was recorded for 3 h, the number of spots of urine created by NCX1.3tg/tg mice (5.3 ± 1.4; n = 14) was larger than that of WT mice (1.4 ± 0.4; n = 8; P < 0.05; Fig. 7A and B). There was no significant difference in the number of large urine spots (>1 cm²) or in the total area of spots between the groups of mice, but the number of small spots (<1 cm²) produced by NCX1.3tg/tg mice was larger (4.1 ± 1.3; n = 14), and the average area of individual spots (3.5 ± 0.8 cm²; n = 74) was significantly smaller than those of WT mice (0.4 ± 0.2; n = 8; P < 0.05; and 12.2 ± 3.5 cm²; n = 11; P < 0.01 respectively; Fig. 7C). The effect of KB-R7943 on urination was examined to determine whether the pattern of urination in freely moving mice consistent with overactive bladder (OAB) syndrome. These findings have important implications concerning the role of Ca²⁺ influx via reverse mode NCX in control of UBSM contractility.

**DISCUSSION**

The study addresses the effect of overexpression of NCX1.3 on membrane excitability and cytosolic Ca²⁺ mobilization in isolated UBSM myocytes, spontaneous contractions of UBSM tissues, and regulation of urinary bladder function. Here, we report that smooth muscle-specific NCX overexpression was associated with 1) enhanced spontaneous UBSM contractions; 2) an outwardly directed KB-R7943- and Ni²⁺-sensitive NCX current with a reversal potential (NCXrev) of between −90 and −100 mV; 3) STOCs and Ca²⁺ sparks of prolonged duration, with the latter frequently exhibiting propagation in the form of Ca²⁺ wave-like events; and 4) alterations in the pattern of urination in freely moving mice consistent with overactive bladder (OAB) syndrome. These findings have important implications concerning the role of Ca²⁺ influx via reverse mode NCX in control of UBSM contractility.

It is widely accepted that NCX plays an important role as a mechanism for Ca²⁺ extrusion following global increases [Ca²⁺]i in smooth muscle myocytes, despite the fact that NCX expression in these cells is much lower than that in cardiac muscle (9, 27, 51). For example, the duration of Ca²⁺ transients evoked by serotonin stimulation is increased after pretreatment of cultured vascular myocytes with antisense oligonucleotides against NCX1 (42), and Na⁺-dependent vascular dilation is reduced in heterozygous NCX1 knock-out (NCX1+/−) mice (45). Based on these results, it was suggested that NCX principally operates in the forward mode to extrude Ca²⁺ in smooth muscle cells under normal physiological conditions. Accordingly, our previous study demonstrated that NCX1.3tg/µg UBSM expressed a fourfold greater level of NCX1 protein compared with WT UBSM and that NCX1.3 overexpression potentiated the ability of forward mode Ca²⁺ efflux to reduce the amplitude of the plateau phase of contractions induced by acetylcholine, high K⁺, or direct electrical stimulation (32).

However, reverse mode activity of the NCX was also proposed to be important for smooth muscle function (3, 12, 17, 39, 40). Inhibition of NCX with KB-R7943 decreased [Ca²⁺]i, responses evoked by agonists or store depletion in smooth muscles (11, 17, 56), and SEA0400 decreased [Ca²⁺]i and evoked vasodilation of arteries preconstricted with ouabain or following a reduction in [Na⁺]. (23, 39). More recently, Ca²⁺
entry via NCX1 and [Ca\(^{2+}\)], were shown to be reduced and associated with a reduction in arterial myogenic tone and blood pressure in smooth muscle-specific NCX1-null mice (NCX1\(^{-/-}\); Ref. 54). These findings suggest that the reverse mode of NCX is also important in the regulation of smooth muscle function under physiological conditions.

The present study was initiated by the finding that NCX overexpression enhanced spontaneous contractions of UBSM. In UBSM cells, membrane excitability and Ca\(^{2+}\) influx through VDCC and CICR play a crucial role in the regulation of myogenic contraction (26). Consistent with this view, we found that spontaneous contractions of UBSM from WT mice were almost completely suppressed by nicardipine or ryanodine, as was reported previously (18, 31). However, nicardipine only partially suppressed spontaneous activity of NCX1.3\(^{tg/tg}\) tissues, and the residual, nicardipine-resistant contractions of NCX1.3\(^{tg/tg}\) tissues were not affected by La\(^{3+}\) or Gd\(^{3+}\), but they were dependent on extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_o\)), suggesting the participation of an additional Ca\(^{2+}\) entry pathway not involving VDCC or nonselective cation channels. The nicardipine-insensitive spontaneous contractions in NCX1.3\(^{tg/tg}\) UBSM were susceptible to inhibition by KB-R7943, SN-6, and Ni\(^{2+}\) implied the possibility that this Ca\(^{2+}\) entry pathway was dependent on the NCX operating in the reverse mode (24, 47).

The presence of a detectable KB-R7943- and Ni\(^{2+}\)-sensitive outward current in conditions favoring reverse mode NCX activity in myocytes isolated from NCX1.3\(^{tg/tg}\) mice was susceptible to inhibition by KB-R7943, SN-6, and Ni\(^{2+}\) implied the possibility that this Ca\(^{2+}\) entry pathway was dependent on the NCX operating in the reverse mode (24, 47).

The presence of a detectable KB-R7943- and Ni\(^{2+}\)-sensitive outward current in conditions favoring reverse mode NCX activity in myocytes isolated from NCX1.3\(^{tg/tg}\) but not WT mice, is consistent with the view that Ca\(^{2+}\) influx due to reverse mode NCX is greater in UBSM of the genetically modified mice. To our knowledge, the data presented in Fig. 4 represent the first quantitative recordings of NCX current in smooth muscle. Detection of NCX currents in smooth muscle cells has presented difficulties in the past, presumably owing to the relatively low level of NCX protein expression compared

Fig. 6. Abnormal Ca\(^{2+}\) mobilization in NCX1.3\(^{tg/tg}\) myocytes. Local Ca\(^{2+}\) transients of WT and NCX1.3\(^{tg/tg}\) (TG) UBSM cells were imaged using a total internal reflection fluorescence (TIRF) microscope and 10 μM fluo-4/AM. A: representative Ca\(^{2+}\) images in WT and NCX1.3\(^{tg/tg}\) cells within TIRF zone are sequentially shown (left). Traces of local [Ca\(^{2+}\)] changes corresponding indicated as “1, 2, 3” in images are plotted against time (right). Ca\(^{2+}\) changes in global area are plotted as “W.” Note that Ca\(^{2+}\) sparks occurred in both types of cells (indicated by 1st * in WT and TG), but Ca\(^{2+}\) propagation triggered by Ca\(^{2+}\) spark (2nd * in TG) was occasionally observed in NCX1.3\(^{tg/tg}\) cells. B–E: data of peak amplitude (B), frequency (C), number of spark sites (D), and area (maximum area of Ca\(^{2+}\) propagation; E) of Ca\(^{2+}\) transients in WT and NCX1.3\(^{tg/tg}\) cells. F: distribution histogram of Ca\(^{2+}\) events is plotted against area. Inset: enlarged histogram for y-axis. Note that Ca\(^{2+}\) transients with large area propagating as Ca\(^{2+}\) waves (>20 μm\(^2\)) were rare in WT cells but ~20% of events in NCX1.3\(^{tg/tg}\) cells. *p < 0.05, **p < 0.01 vs. WT.

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with other excitable cells, such as cardiac myocytes. Here, we detected a KB-R7943- and Ni²⁺-sensitive outward current that reversed approximately −95 mV in NCX1.3tg/tg cells, but a similar conductance was not apparent in WT cells. Rather, the Ni²⁺-sensitive difference current of WT myocytes was inwardly directed at negative voltages, reversed at 0 mV, and was likely due to gating of Ca²⁺ permeable nonselective cation channels. The reversal potential of the KB-R7943- and Ni²⁺-sensitive current of NCX1.3tg/tg cells does not correspond to that expected for a nonselective cation conductance, but it was also positive to the predicted value for NCXrev based on the bath and pipette solutions employed and a Na⁺-Ca²⁺ transport ratio of 3:1 (i.e., negative to −150 mV assuming a contaminating level of [Na⁺]o, [Na⁺]i of 40 mM, [Ca²⁺]o of 1 mM, [Ca²⁺]i of 1–10 nM with 10 mM BAPTA and no added Ca²⁺; Ref. 16). We attribute this discrepancy to the labile nature of NCXrev and that it can deviate considerably from predicted values when cells are maintained in nonequilibrium conditions (5). In the present experiments, UBSM myocytes were held at −60 mV (500-ms ramp protocols were applied every 10 s); this is considerably positive to the predicted value of NCXrev, and an outwardly directed KB-R7943- and Ni²⁺-sensitive holding current was detected indicative of steady-state Na⁺ efflux and Ca²⁺ influx, i.e., steady-state reverse mode NCX activity. Given the dissipative nature of NCX activity, this steady-state current would be expected to alter [Na⁺]o and [Ca²⁺]i and, thereby, NCXrev due to local accumulation within diffusion-restricted microdomains, such as within caveolae (extracellularly) and sites of close apposition between the junctional sarcoplasmic reticulum (SR) and the plasma membrane (intracellularly; Refs 5, 7).

The present findings are consistent with the view that overexpression of NCX in smooth muscle cells leads to abnormal [Ca²⁺]i dynamics and increased spontaneous contractions as a result of enhanced reverse mode NCX activity. Specifically, that enhanced reverse mode activity increases spontaneous Ca²⁺ release from SR stores leading to propagating Ca²⁺ wave-like events that initiate spontaneous contractions. We do
not attribute the enhanced spontaneous contractility of NCX1.3tg/tg tissues to an increase in membrane excitability. This view is based on the following line of reasoning: BKca channels in UBSMs are known to be activated by l) membrane depolarization and the rise in [Ca2+]i associated with action potential firing (13, 21, 31, 49); and 2) local subplasmalemmal elevations in [Ca2+]i, referred to as Ca2+ sparks, owing to release from ryanodine receptors (RyRs) in the SR (15, 31, 35, 53). STOCs arising from BKca channel activity evoked by Ca2+ sparks are thought to maintain resting membrane potential at a hyperpolarized level to suppress VDCC activity, lower [Ca2+]i, and inhibit contractility; i.e., Ca2+ spark/BKca channel coupling plays a crucial role in the negative feedback regulation of membrane excitability, resting [Ca2+]i, and contraction of UBSM (20, 25). Accordingly, elevated UBSM contractility and OAB syndrome are observed following BKca channel inhibition with ibiotoxin and in genetic BKca channel null mice (14, 29). Here, we found that Ca2+ transients of longer duration and increased propagation were associated with STOCs of increased duration in NCX1.3tg/tg UBSM myocytes. This change in STOC duration would be expected to reduce membrane excitability, but we observed enhanced spontaneous contractility in NCX1.3tg/tg UBSM tissues. STOCs are occasionally influenced by global Ca2+ release (e.g., Ca2+ release from SR stores by caffeine or agonists), which often results in smooth muscle contraction (8, 34, 50). Furthermore, VDCC block with nicardipine completely suppressed spontaneous contractions in WT but not NCX1.3tg/tg UBSM tissues, implying that a change in membrane excitability was not involved. Also, we found no differences in the expression of mRNA and protein for VDCC (α1C-subunit), BKca channel (α-subunit), transient receptor potential canonical channel (TRPC3), RyRs (RyR2 and RyR3), and inositol 1,4,5-trisphosphate receptor (IP3/R1) in WT and NCX1.3tg/tg UBSM (data not shown), and the current-voltage relationships of VDCC (100 µM Cd2+ sensitive at 0 mV: WT, 5.6 ± 1.1 pA/pF, n = 5; NCX1.3tg/tg, 5.4 ± 1.3 pA/pF, n = 5) and BKca (1 µM paulline-sensitive pCa 6.5 at +60 mV: WT, 34 ± 12 pA/pF, n = 4; NCX1.3tg/tg, 38 ± 11 pA/pF, n = 5) currents were similar (P > 0.05).

On the other hand, we did detect the presence of abnormal [Ca2+]i dynamics involving enhanced SR Ca2+ release leading to propagating Ca2+ wave-like events and global elevations in [Ca2+]i within the TIRF visualization area in NCX1.3tg/tg myocytes that can be attributed to enhanced NCX reverse mode activity. The frequency of Ca2+ sparks and number of spark sites per cell were similar in myocytes of NCX1.3tg/tg and WT mice, but the amplitude and duration of the transients were larger, and propagation of Ca2+ sparks leading to a global rise in [Ca2+]i over the TIRF visualization area occurred more frequently in NCX1.3tg/tg cells. These differences in [Ca2+]i, dynamics were abolished by inhibition of NCX with KB-R7943 or SR Ca2+ release with ryanodine or tetracaine, as were the nicardipine-resistant spontaneous contractions of NCX1.3tg/tg UBSM tissues. The enhanced Ca2+ release detected in NCX1.3tg/tg UBSM myocytes is presumably mediated by overfilling of the SR owing to enhanced Ca2+ influx through NCX reverse mode, as CICR triggered by NCX reverse mode activity was previously reported in Na+-loaded coronary arterial smooth muscle cells (12). Transgenic mice with doxycycline withdrawal-induced cardiac NCX1 overexpression exhibit increased SR Ca2+ content and larger Ca2+ transients (46), but SR Ca2+ content measured by caffeine-induced Ca2+ transients was not changed in NCX1.3tg/tg UBSM myocytes (32). However, the possibility that Ca2+ content in superficial, subplasmalemmal SR, which is preferentially filled by Ca2+ influx via reverse mode NCX (7, 44), is larger in NCX1.3tg/tg cells is indicated by the increased amplitude and duration of the Ca2+ transients/sparks.

The most impressive finding of the present study was that NCX1.3tg/tg mice exhibited frequent urination and the phenotype was greatly improved by the oral administration of KB-R7943. Urination pattern analysis showed that smaller volumes of urine were excreted more frequently in NCX1.3tg/tg mice than WT mice, while the total urine volume was comparable. OAB is a syndrome characterized by urinary urgency and is usually associated with increased daytime frequency and/or nocturia (2) that affects ~17% of adults in the United States and Europe (30, 43). OAB symptoms are often associated with UBSM overactivity, and there is currently a lack of effective therapeutic agents to correct this disorder. Muscarinic receptor antagonists that impair UBSM contractility are the main option, but they have limited effectiveness and undesirable side effects (1, 23). In recent years, β3-adrenoceptor agonists, purinergic receptor antagonists, phosphodiesterase inhibitors, and K+ channel openers have all been considered to be potential candidates for the treatment of OAB syndrome (10). In this study, urinary pattern analysis using G833C-NCX1.3tg/tg mice, in which the G883C mutation confers a resistance to KB-R7943-mediated inhibition (22), failed to reproduce the inhibitory effect of KB-R7943 on frequent urination detected in NCX1.3tg/tg mice. These data are consistent with the view that the effect of KB-R7943 on urination in the NCX1.3tg/tg mice may be attributed to suppression of NCX activity, rather than a nonselective effect on another mechanism relevant to the regulation of Ca2+.
homeostasis. Our findings suggest, therefore, that selective, potent inhibitors of NCX1 could also be considered as a potential therapy for OAB syndrome due to enhanced UBSM contractility, whereas the relevance between NCX overexpression and human OAB remains to be determined.

In conclusion, NCX in UBSM can work in both forward and reverse modes under physiological conditions. Overexpression of NCX impairs the central role of Ca\(^{2+}\) sparks to reduce membrane excitability and global [Ca\(^{2+}\)], via generation of superficial stores due to enhanced Ca\(^{2+}\) influx via reverse mode NCX results in Ca\(^{2+}\) sparks of increased amplitude and duration that propagate, triggering further Ca\(^{2+}\) release by CICR to elevate global [Ca\(^{2+}\)] and evoke spontaneous contractions in NCX1.3\(\text{tg/kg}\) cells. The enhanced spontaneous contractions of UBSM provoke the OAB symptom of frequent, small volume urination that is effectively suppressed by NCX inhibition with KB-R7943.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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