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Increased transient receptor potential canonical 3 activity is involved in the pathogenesis of detrusor overactivity by dynamic interaction with Na^+/Ca^{2+} exchanger 1

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Transient receptor potential canonical 3 (TRPC3) is a nonselective cation channel, and its dysfunction is the basis of many clinical diseases. However, little is known about its possible role in the bladder. The purpose of this study was to explore the function and mechanism of TRPC3 in partial bladder outlet obstruction (PBOO)-induced detrusor overactivity (DO). We studied 31 adult female rats with DO induced by PBOO (the DO group) and 40 sham-operated rats (the control group). Here we report that the expression of TRPC3 in the bladder of DO rats increased significantly. Furthermore, PYR10, which can selectively inhibit the TRPC3 channel, significantly reduced bladder excitability in DO and control rats, but the decrease of the bladder excitability of DO rats was more obvious. PYR10 significantly reduced the intracellular calcium concentration in smooth muscle cells (SMCs) in DO and control rats. Finally, Na^+/Ca^{2+} exchanger 1 (NCX1) colocalizes with TRPC3 and affects its expression and function. Collectively, these results indicate that TRPC3 plays an important role in the pathogenesis of DO through a synergistic effect with NCX1. TRPC3 and NCX1 may be new therapeutic targets for DO.

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INTRODUCTION

Overactive bladder (OAB) syndrome is a symptom complex, with the chief complaint being urgency, with or without frequent urination or incontinence. OAB seriously affects quality of life, especially among the elderly. According to statistics, OAB affects nearly 455 million people, and the annual healthcare burden is $82.6 billion[1,2]. Although the etiology of OAB is unclear, many studies have shown that detrusor overactivity (DO) is one of the main structural and functional mechanisms of the disease[3].

The canonical transient receptor potential (TRPC) channel family includes seven members (TRPC1–7), which are nonselective cation channels activated by a variety of stimuli and play an important role in cellular Ca^{2+} homeostasis[4,5]. Dysfunction of TRPC3, a member of the TRPC family, has been associated with many diseases[6]. Previous studies have demonstrated that the expression of TRPC3 is significantly increased in the airway smooth muscle cells (SMCs) of patients with asthma and in a mouse model of allergic asthma. Inhibition of transient receptor potential canonical 3 (TRPC3) expression can reduce airway hyperresponsiveness in mice[7]. The expression of TRPC3 in human myometrial SMCs can also be increased by stretch[8]. In rodent cardiomyocytes, TRPC3 acts as a pivotal mediator of pathological hypertrophy, and TRPC3 deletion can inhibit cardiac remodeling in response to pressure overload[9]. Noorani et al[10]. reported that upregulated TRPC3 channel expression during hypertension is associated with increased vascular contractility in rats. However, the role of TRPC3 in bladder excitability has not been investigated.

NCX is a nine-transmembrane protein that transports three Na^+ ions in exchange for one Ca^{2+} ion. It is thought to be a vital molecule in the regulation of Ca^{2+} homeostasis and to be involved in various physiological processes[11]. Na^+/Ca^{2+} exchanger 1 (NCX1) has been shown to couple to TRPC3 in cardiac muscle, neurons and endocrine cells[12]. Therefore, the present study was designed to explore the functions and mechanisms of TRPC3 and NCX1 in DO.

MATERIALS AND METHODS

Animals

All animal experimental and handling protocols were performed according to the Guide for the Care and Use of Laboratory Animals and approved by the Research Council and Animal Care and Use Committee of the Army Medical University (Chongqing, China). All efforts were made to minimize animal suffering and reduce the number of animals used. Eighty adult female Sprague-Dawley rats (180–230 g) were used in our study. All rats were assigned to two groups, i.e., the control group and DO group, with a random number table. Some rats were sacrificed by cervical dislocation for the measurement of detrusor strip contraction, and the other rats were euthanized by pentobarbital injection.

Animal model establishment

Rats were anesthetized by intraperitoneal injection of phenobarbital (40 mg/kg). DO was induced in female rats using partial bladder outlet

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obstruction (PBOO) as previously described by our group[13,14]. In brief, a PE-50 polyethylene catheter tube (0.965 mm outer diameter) was placed in the urinary bladder via the urethral orifice. After a small incision was made in the lower abdomen, the bladder neck of each PBOO rat was tied around the catheter with a 3-0 silk ligature. Subsequently, the catheter was removed, and the incision was closed. The sham-operated rats underwent the same procedure without ligation. All animals received intraperitoneal injection of 20,000 units of penicillin after the operation.

Cystometry and group classification

Cystometry was conducted 6 weeks after surgery. The rats were anesthetized by intraperitoneal injection of urethane (1.2 g/kg). A PE-50 catheter was inserted into the rat bladder via the urethral orifice, and it was connected to urodynamic equipment (AVI 270, 3 M, Minnesota, USA) and an electrophysiological signal acquisition system (RM6480C, Chengyi, Chengdu, China) via a three-way connector for both infusion and pressure recording. Cystometry was performed by infusing saline containing N-(4-(3,5-Bis(trifluoromethyl)-1H-pyrazol-1-yl)phenyl)-4-methyl- benzenesulfonamide (PYR10, 100 nM, MedChemExpress, Shanghai, China), 2-(4-((2,5-difluorophenyl)methoxy) -phenoxy)-5-ethoxyaniline (SEA0400, 10 μM, Sigma, St Louis, MO, USA) or dimethyl sulfoxide (DMSO, Beyotime Institute of Biotechnology, Shanghai, China) at a rate of 10 ml/h at room temperature. Continuous urodynamic curves were recorded. PBOO rats that exhibited nonvoiding detrusor contractions before the onset of micturition during bladder filling were classified as the DO group. PBOO rats with a stable detrusor before the onset of micturition contractions were excluded from this study. The sham-operated rats were classified as the control group.

Quantitative RT-PCR (qRT-PCR)

The expression levels of TRPC3 and NCX1 mRNA in the bladder were analyzed using real-time quantitative PCR. Total RNA was extracted from rat bladders using TRIzol reagent (Beyotime, Shanghai, China) according to the manufacturer's protocol. The extracted RNA was dissolved in RNAse-free water. Complementary DNA was synthesized using the PrimeScript RT reagent kit (TaKaRa Bio, Tokyo, Japan) and 2 × Taq MasterMix (CWbio, Beijing, China). The sequences of the primers were as follows: TRPC3 forward, 5′-CCTGAGCGAAGTCACACTCCCAC-3′; TRPC3 reverse, 5′-CCACTC-TACATCACTGTCATCC-3′; NCX1 forward, 5′-TGCGGCCAAC GGGGAACAG-3′; NCX1 reverse, 5′-CACAGGAGCACAAACAGGGAAGA-3′; GAPDH forward, 5′-ACGGGAAGCTCACTGGCATGG-3′; and GAPDH reverse, 5′-GCCGCCTGCT TCACCACCTTCT-3′. qRT-PCR was performed using SYBR Green Master Mix (Toyobo, Osaka, Japan) on the StepOnePlus RT-PCR system (Life Technologies, Carlsbad, CA). GAPDH was selected as the housekeeping gene for normalization.

Western blot analysis

Protein was extracted for western blot analysis as reported previously[15]. Proteins (30 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked with 5% bovine serum albumin (BSA) for 2 h at room temperature and incubated overnight at 4 °C with the following primary antibodies: TRPC3 (ab241343, 1:1000), NCX1 (ab177952, 1:5000) and GAPDH (ab8245, 1:5000) (all from Abcam, Cambridge, MA, USA). Then, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase (1:2000, Zhongshan Inc., Beijing, China). The immunolabeled proteins were detected with the Azure c300 Imaging System (Azure Biosystems, CA, USA). GAPDH was used as an internal control for total protein content.

Immunohistochemistry (IHC)

The expression and distribution of TRPC3 and NCX1 in the rat bladder were assessed using conventional IHC. Briefly, the bladders were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). The specimens were dehydrated in alcohol solutions and xylene, embedded in paraffin, and cut into 5-μm sections for further experiments. The paraffin sections were then dewaxed and washed with PBS. Antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6.0) at 95 °C for 20 min, 0.3% H_2O_2 solution was added and the sections were incubated at room temperature for 20 min. The sections were incubated in blocking buffer (0.2% Tween 20 and 1% BSA) for 2 h at room temperature and incubated overnight at 4 °C with primary antibody (TRPC3, Abcam, ab188802, 1:200; NCX1, Abcam, ab2869, 1:100). The tissues were then incubated with streptavidin-biotin peroxidase (SP)-conjugated secondary goat anti-rabbit IgG and standard SP kit reagents (Zhongshan Co., SPN-9002) for 30 min at 37 °C. Finally, the sections were stained with diaminobenzidine (Zhongshan Co., ZLI-9019) and counterstained with hematoxylin. All sections were visualized and photographed with an optical microscope (IX73, Olympus Corp., Tokyo, Japan) and were evaluated by two individuals in a double-blind manner.

Contractility studies

As previously described[15], the bladder was carefully acquired from each euthanized rat and placed in ice-cold Krebs solution composed of 119.0 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 11.0 mM glucose (pH = 7.4). The bladders were longitudinally cut into 2 × 3 × 7-mm strips. Each sample was vertically suspended in a 15-ml organ bath filled with 37 °C Krebs solution and bubbled with a mixture of 95% O_2 and 5% CO_2. After equilibration for 30 min, each strip was stretched with 0.75 g tension, and continuous curves were recorded with an isometric force transducer (Chengyi Co., Chengdu, China). Pharmaceuticals including an inhibitor of TRPC3 (PYR10, 100 nM), an inhibitor of NCX1 (SEA0400, 10 μM) and vehicle (DMSO) were added to the bath.

Coimmunoprecipitation (co-IP)

The bladder lysates were precleared with magnetic beads (Thermo Fisher Scientific) at 4 °C for 15 min and then incubated with an anti-TRPC3 primary antibody (Abcam), an anti-NCX1 primary antibody (Abcam) or IgG overnight at 4 °C. The magnetic beads were precipitated at 4 °C (1000 × g for 5 min). Sequentially, the immunocomplexes were washed with lysis buffer (Beyotime) four times at 4 °C. Finally, the precipitates were analyzed using western blot analysis.

Immunofluorescence (IF) staining

The isolated bladders were cut into 5-μm sections. These sections were then fixed in 4% paraformaldehyde (pH = 7.4) for 1 h at room temperature and incubated overnight with a rabbit anti-TRPC3 antibody (Abcam, ab188802, 1:200) and a mouse anti-NCX1 antibody (Abcam, ab2869, 1:200) at 4 °C. The samples were washed with PBS and incubated with appropriate secondary fluorescent antibodies conjugated to TRITC (1:200) and FITC (1:200) for 90 min at room temperature. The samples were then washed with PBS and incubated with 4, 6-diamidino-2-phenylindole dihydrochloride (Sigma, St Louis, MO, USA) for 10 min at room temperature for nuclear staining. Finally, the samples were viewed and photographed using a laser confocal microscope (Leica, Solms, Germany).

Isolation of bladder SMCs

Primary SMCs were isolated using a one-step enzymatic digestion method. Bladder tissues were placed into 5 ml digestion solution (2.0 mg/mL type II collagenase, 2.0 mg/mL type IV collagenase, and 2.0 mg/mL BSA, dissolved in D-hank's) (all from Sigma) and cut into small pieces. After being digested for approximately 20 min at 37 °C, 10 ml Dulbecco's modified eagle medium (DMEM, Gibco-Life Technologies, Grand Island, NY, USA) composed of 10% fetal bovine serum (Gibco-Life Technologies, Grand Island, NY, USA) was applied to terminate digestion. After centrifugation (1000 rpm for 5 min), the supernatant was discarded. DMEM composed of 10% fetal bovine serum and 1% antibiotics/antimycotics (Beyotime Institute of Biotechnology, Shanghai, China) was added to the precipitate. The sample was beaten approximately 100 times. The SMCs were filtered using a cell strainer (20 μm) and then cultured in an incubator (37 °C, 95% O2 and 5% CO2) for 48 h.

Measurement of the intracellular calcium concentration ($[Ca^{2+}]_i$)

The SMCs were washed with Hank's solution for 5 min and stained with Fluo-4 AM (10 μM, Abcam, Cambridge, MA, USA) for 30 min at 37 °C. After incubation, the Fluo-4/AM-loaded SMCs were washed with Hank's solution 3 times, and live Ca^{2+} imaging was conducted under a laser-scanning confocal microscope (Leica, Solms, Germany) at an emission wavelength of 488 nm. The final data are presented as the relative fluorescence intensity (RF): RF = F1/F0, where F1 is the mean RF after drug administration and F0 is the mean baseline fluorescence intensity before drug administration.

Patch-clamp experiments

The NCX current (I_{NCX}) was recorded using the whole-cell patch clamp method. Patch electrodes with a resistance of 3–6 MΩ when filled with the internal solution were pulled from glass capillaries (1.5 mm outer diameter,

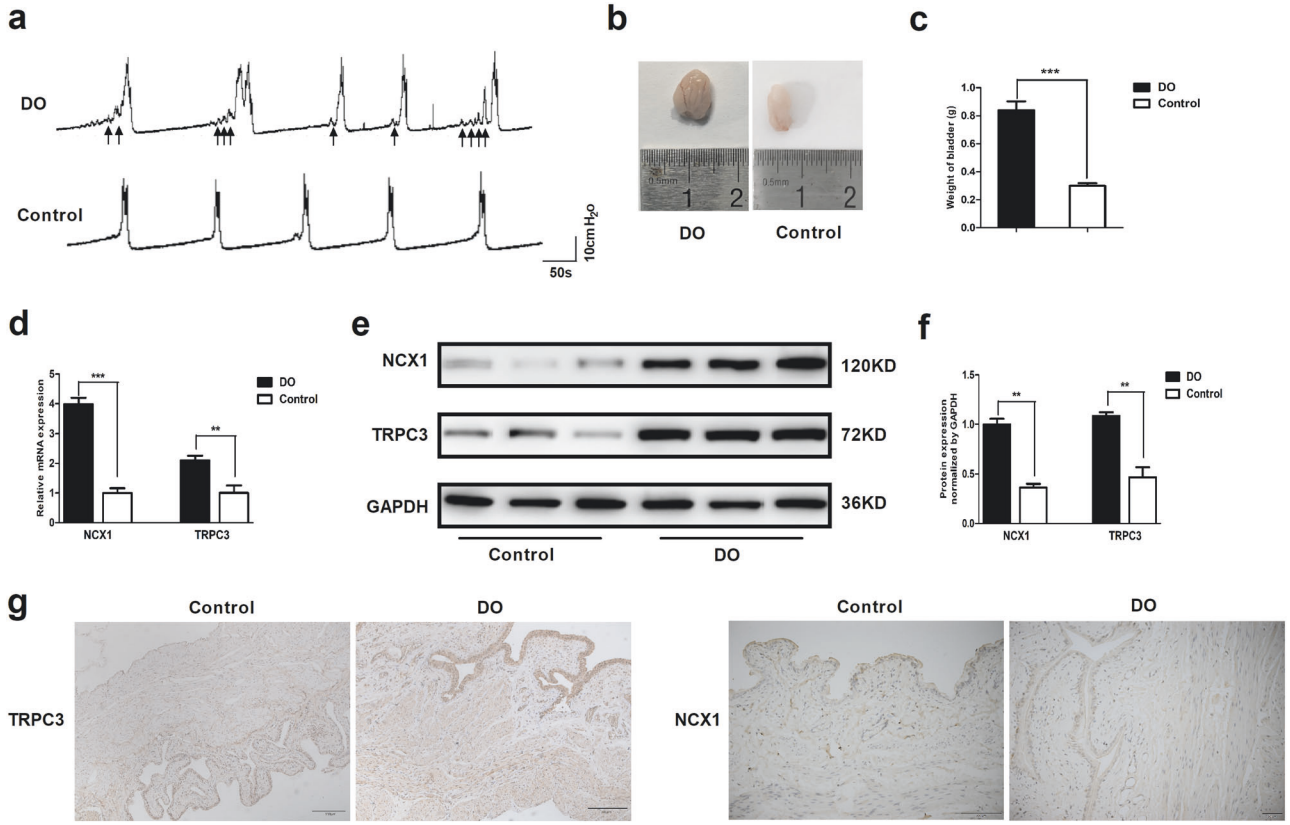


Fig. 1 The expression levels of TRPC3 and NCX1 were significantly increased in DO rat bladders. a Unlike control rats ($n = 40$), DO rats ($n = 31$) exhibited significant nonvoiding detrusor contractions before micturition. The black arrows represent nonvoiding detrusor contractions. **b**, **c** Bladder weight was significantly increased in DO rats ($n = 5$). **d–f** The mRNA ($n = 9$) and protein ($n = 3$) levels of TRPC3 and NCX1 were significantly increased in the DO bladder. **g** The IHC staining results demonstrated that TRPC3 ($n = 3$) and NCX1 ($n = 3$) were distributed in all layers of the bladder). $^{**}P < 0.01$, $^{***}P < 0.001$.

0.9 mm inner diameter; Narishige Scientific Instrument Laboratory, Tokyo, Japan) using a P-97 puller (Sutter Instrument Co., Novato, CA, USA). The intracellular solution comprised 51 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 100 mM CsOH, 4.94 mM $CaCl_2$, 20 mM tetraethylammonium chloride, 1 mM Na_2ATP, 5 mM EGTA, 10 mM HEPES and 8 mM D-glucose (pH = 7.2). The bath solution comprised 137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES and 10 mM D-glucose (pH = 7.4). Cells with a seal < 1 GΩ before rupture of the membranem were discarded and the test seal function was constantly monitored throughout the recording to ensure that the seal was stable. The I_{NCX} was recorded with a patch clamp amplifier filtered at 2.9 kHz bandwidth, and the series resistance was compensated. The I_{NCX} was induced by a step potential ranging from −100 mV to +60 mV in increments of 10 mV for 200 ms with a holding potential of −40 mV. The step pulse interval was 50 ms. The I_{NCX} was recorded using the HEKA EPC10USB amplifier (HEKA Elektronik, Heidelberg, Germany). 10 μM SEA0400, 100 nM PYR10 or DMSO was added to external solution to detect the properties of I_{NCX} currents. The data were analyzed using FitMaster software (HEKA Elektronik), and the density of the I_{NCX} was normalized to the cell capacitance. All experiments were completed at room temperature.

Statistical analyses

The data are presented as the mean ± SD. SPSS 16.0 software (SPSS Inc., Chicago, IL) was used to analyze all data. Statistical analysis was performed using Student's *t* tests. $P < 0.05$ was considered statistically significant.

RESULTS

Establishment of the DO model

During bladder filling, 31 PBOO rats exhibited significant nonvoiding detrusor contractions before the onset of micturition (Fig. 1a) and thus were classified as DO rats. The other 9 rats (death, $n = 3$; stable detrusor, $n = 6$) were excluded from this study. Bladder weight was significantly increased in DO rats compared with control rats (Fig. 1b, c).

The expression levels of TRPC3 and NCX1 were increased in the bladders of DO rats

The expression of TRPC3 and NCX1 was in the bladders of control and DO rats were assessed using qRT-PCR and western blot analysis. The mRNA and protein levels of TRPC3 and NCX1 were significantly increased in the bladders of DO rats compared with those of control rats (Fig. 1d–f). In addition, the distribution of TRPC3 and NCX1 were also analyzed using IHC. The results revealed that TRPC3 and NCX1 were distributed in all layers of the bladder (Fig. 1g).

PYR10 significantly inhibited bladder hyperactivity by inhibiting TRPC3

Pyrazole derivatives were initially considered as selective blockers of TRPC, among which PYR3 and PYR10 were considered as inhibitors of TRPC3 channels[16]. But the selectivity of PYR3 for TRPC3 has been questioned[16,17]. PYR3 showed inhibitory effects in several channels, such as TRPC3, ORAI1, TWIK-related K^+ channels and TWIK-related acid-sensitive K^+ channel 2[18]. In contrast, PYR10 exhibited improved selectivity for TRPC3[16,17,19]. Previous study in the heart also has shown that using PYR10 can achieve the same inhibitory effect as gene deletion (TRPC3−/−) in the activation of ventricular cardiac fibroblasts. Therefore, in our experiment, PYR10 was used instead of PYR3 as the inhibitor of TRPC3. PYR10 was administered to determine whether the increase in TRPC3 expression was involved in bladder hyperactivity in DO rats. Administration of PYR10 significantly prolonged the micturition interval (MI) of control and DO rats (Fig. 2a,

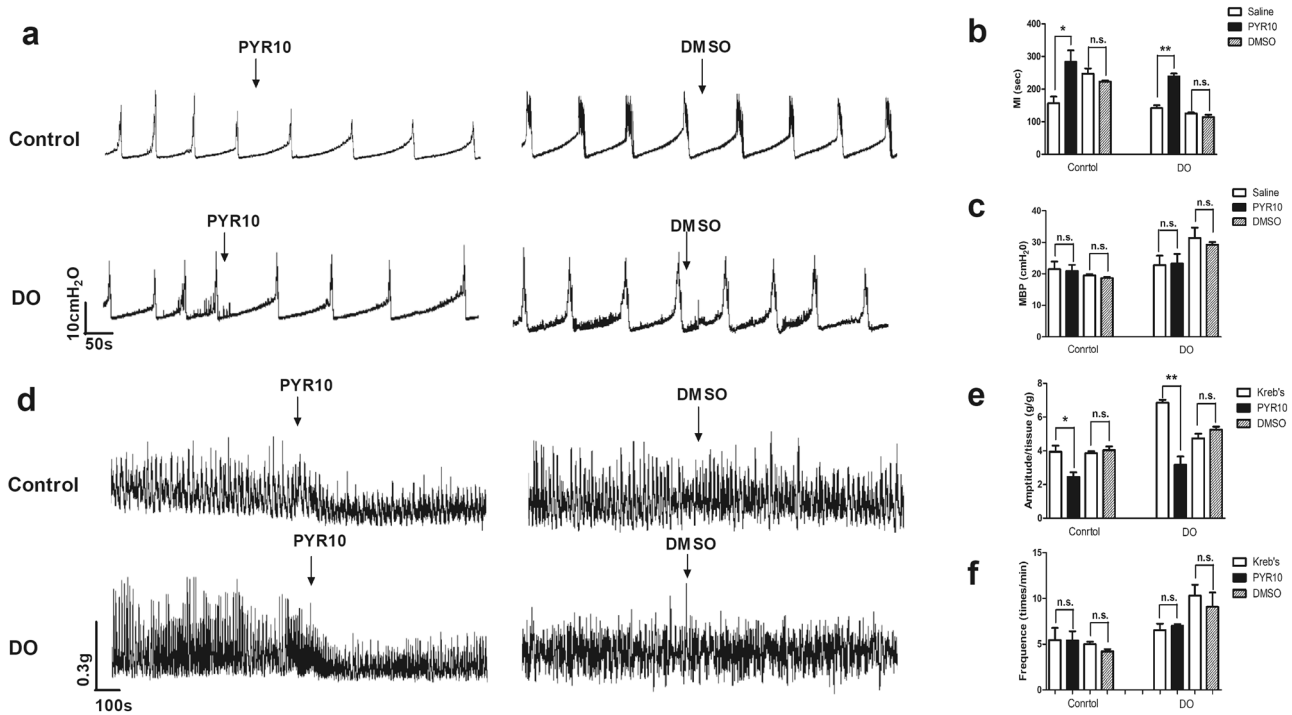


Fig. 2 PYR10 significantly inhibited bladder hyperactivity. a–c PYR10 (100 nM) significantly prolonged the MI in control ($n = 4$) and DO ($n = 3$) rats. The MBP was not changed in control ($n = 4$) or DO ($n = 3$) rats. The effect of PYR10 on the MI was more significant in DO rats than in control rats. DMSO had no significant effect on the MI or MBP in control ($n = 3$) and DO ($n = 3$) rats. The effect of PYR10 on the MI was more significant in DO rats than in control rats. **d–f** PYR10 (100 nM) significantly reduced the contraction amplitudes of isolated strips from control ($n = 3$) and DO ($n = 3$) rats. The contraction frequencies of isolated strips were not changed in control ($n = 3$) or DO ($n = 3$) rats. The effect of PYR10 on the contraction amplitude was more significant in DO rats than in control rats. DMSO had no significant effect on the contraction amplitude or contraction frequencies in control ($n = 3$) and DO ($n = 3$) rats. *$P < 0.05$, **$P < 0.01$, n.s. no significance.

b). However, the maximum bladder pressure (MBP) of control and DO rats was not changed (Fig. 2a, c). In addition, the effect of PYR10 on the MI rats was more significant in DO than in control rats (Fig. 2b). In the detrusor strip contractility studies, the contraction amplitude was significantly decreased in control and DO rats upon PYR10 administration (Fig. 2d, e). The contraction frequency was not changed in control or DO rats (Fig. 2d, f). Similarly, the effect of PYR10 on contraction amplitude was more significant in DO rats than in control rats (Fig. 2e). DMSO had no significant effect on either contractility or cystometric parameters in control or DO rats (Fig. 2a–f).

Roles of NCX1 in TRPC3-mediated changes in bladder excitation

To determine whether NCX1 is involved in TRPC3-mediated changes in bladder excitation, we studied the role of NCX1 in bladder excitation. The results showed that upon administration of a blocker of NCX1 (SEA0400), the MI was significantly prolonged in DO rats but not in control rats (Fig. 3a, c). However, SEA0400 had no influence on the MBP in either control or DO rats (Fig. 3a, d). In the detrusor strip contractility studies, the contraction amplitude was significantly decreased in DO rats but not in control rats upon SEA0400 administration (Fig. 3b, e). The contraction frequency was not changed in control or DO rats (Fig. 3b, f). After the bladders were pumped with SEA0400 for 5 min, neither the MI nor the MBP was significantly changed in either the two groups of rats upon PYR10 application (Fig. 3a, c, d). Similarly, upon administration of SEA0400 for 5 min, neither the contraction amplitudes nor contraction frequencies of the detrusor strips were significantly changed in either of the two groups of rats upon PYR10 application (Fig. 3b, e, f).

Colocalization of TRPC3 and NCX1 in the rat bladder

To further explore the relationships between TRPC3 and NCX1, co-IP analysis and IF staining of the rat bladder tissues were performed. We found that TRPC3 and NCX1 can be combined with each other (Fig. 4a). In addition, IF staining of rat bladder tissue showed that TRPC3 was colocalized with NCX1 (Fig. 4b).

Changes in the $[Ca^{2+}]_i$ in SMCs induced by NCX1 and TRPC3

Ca^{2+} is one of the most important ions for regulating cell excitation. To further explore the mechanisms of TRPC3 in DO rats, the $[Ca^{2+}]_i$ in bladder SMCs was measure via live imaging. The results showed that upon administration of SEA0400, the $[Ca^{2+}]_i$ was significantly reduced in the SMCs of DO rats but not in the SMCs of control rats (Fig. 5a, b, d). In addition, PYR10 decreased the $[Ca^{2+}]_i$ in SMCs in DO and control rats (Fig. 5a, b, d). After administration of SEA0400, PYR10 no longer reduced the $[Ca^{2+}]_i$ in SMCs in either group (Fig. 5c, d). DMSO had no significant effect on the $[Ca^{2+}]_i$ in bladder SMCs in either group (Fig. 5a, b, d).

Changes in I_{NCX} in SMCs

Whole-cell patch clamp experiments were conducted to record the I_{NCX} of bladder SMCs. To verify the specificity of the I_{NCX}, a specific inhibitor of NCX1 (SEA0400) was administered. The results demonstrated that SEA0400 significantly reduced the absolute value of the I_{NCX} density in SMCs (The mean value of cell capacitance (MC) was 3.27 ± 0.23 pF) (Fig. 6a, b). DMSO had no significant influence on the absolute value of the I_{NCX} in SMCs from control (MC: 3.64 ± 0.56 pF) and DO (MC: 3.66 ± 0.44 pF) rats (Fig. 6c–f). When normalized to cell capacitance, the absolute value of the I_{NCX} density was significantly enhanced over a voltage range of -100 to -50 mV (forward mode) and at 0 to $+60$ mV (reverse mode) in the SMCs of DO rats (MC:4.27 ± 0.66 pF) compared with the SMCs of control rats (MC:3.49 ± 0.48 pF) (Fig. 6g, h). PYR10 significantly reduced the absolute value of the I_{NCX} density in SMCs from control and DO rats (Fig. 6g, i, j). The reduction in the reverse mode of NCX ($+60$ mV) was more

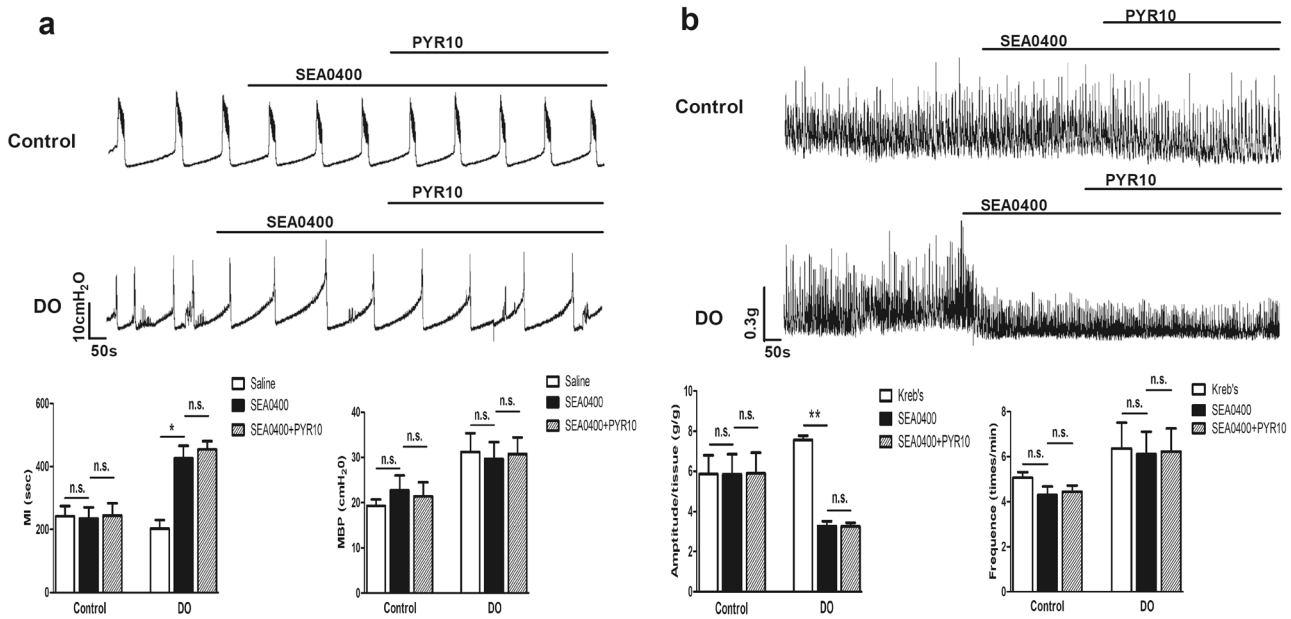


Fig. 3 SEA0400 abolished the changes in bladder excitation mediated by PYR10. a, **c**, **d** SEA0400 (10 μM) significantly prolonged the MI in DO rats ($n = 3$) but not in control rats ($n = 3$). SEA0400 had no influence on the MBP in either control ($n = 3$) or DO ($n = 3$) rats. SEA0400 significantly abolished the effects of PYR10 on the MI and MBP in control ($n = 3$) and DO ($n = 3$) rats. **b**, **e**, **f** SEA0400 (10 μM) significantly decreased the contraction amplitudes of isolated strips from DO ($n = 3$) rats but not control rats ($n = 3$). SEA0400 had no influence on the contraction frequencies of isolated strips from either control ($n = 3$) or DO ($n = 3$) rats. SEA0400 significantly abolished the effects of PYR10 on the contraction amplitudes and contraction frequencies of isolated strips from control ($n = 3$) and DO ($n = 3$) rats. *$P <$ 0.05, **$P < 0.01$, n.s. no significance.

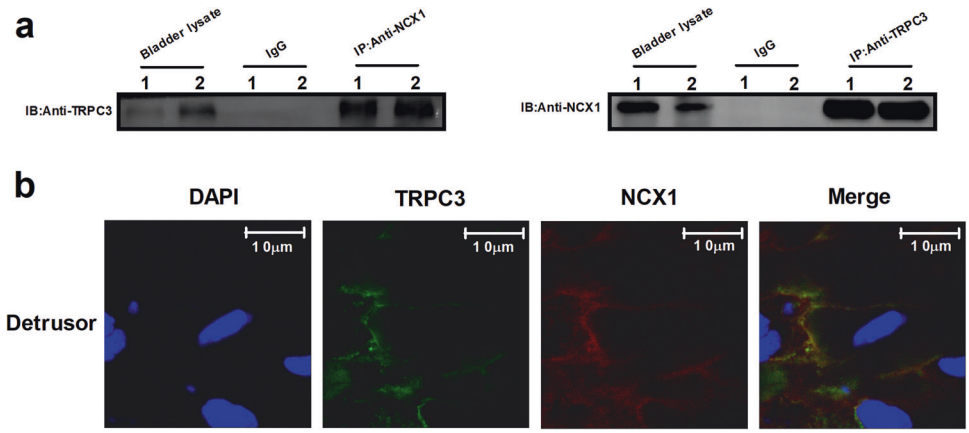


Fig. 4 Colocalization and interaction between TRPC3 and NCX1. a The interaction between TRPC3 and NCX1 was assessed by co-IP ($n = 2$). **b** Colocalization of TRPC3 (green) and NCX1 (red) was evaluated by double immunostaining ($n = 3$).

significant than that in the forward mode of NCX (−100 mV) in DO rats (Fig. 6j).

Collectively, our results suggested that increased TRPC3 expression facilitated the influx of Na^+ and Ca^{2+} into bladder SMCs in DO rats. Furthermore, TRPC3 functionally interacted with NCX1 in bladder SMCs. TRPC3 activated the reverse mode of NCX1 by elevating Na^+ levels in the subplasmalemmal space, thereby promoting Ca^{2+} entry into bladder SMCs in DO rats. As a consequence, three subplasmalemmal Na^+ ions were transported out of the cells via TRPC3 to allow the entry of one Ca^{2+} ion into the cytoplasm through the reverse mode of NCX1 in bladder SMCs. The synergistic effects of TRPC3 and NCX1 significantly increased the $[Ca^{2+}]_i$ in bladder SMCs, which may have consequently contributed to DO (Fig. 7).

DISCUSSION

In this study, we demonstrated that increased TRPC3 activity in SMCs plays an important role in bladder hyperactivity in DO rats. The potential mechanism involves Ca^{2+} overload in SMCs induced by TRPC3 via activation of the reverse mode of NCX1.

PBOO was used to establish a DO model in female rats to explore variations in bladder excitability. The results of cystometric investigation showed that the number of nonvoiding detrusor contractions was significantly increased in the DO rats, suggesting that the excitability of the rat's bladder increased. In addition, the weight of the bladder was significantly increased in DO rats, which is consistent with previous studies[20]. Therefore, we confirmed the successful establishment of the DO rat model in this study.

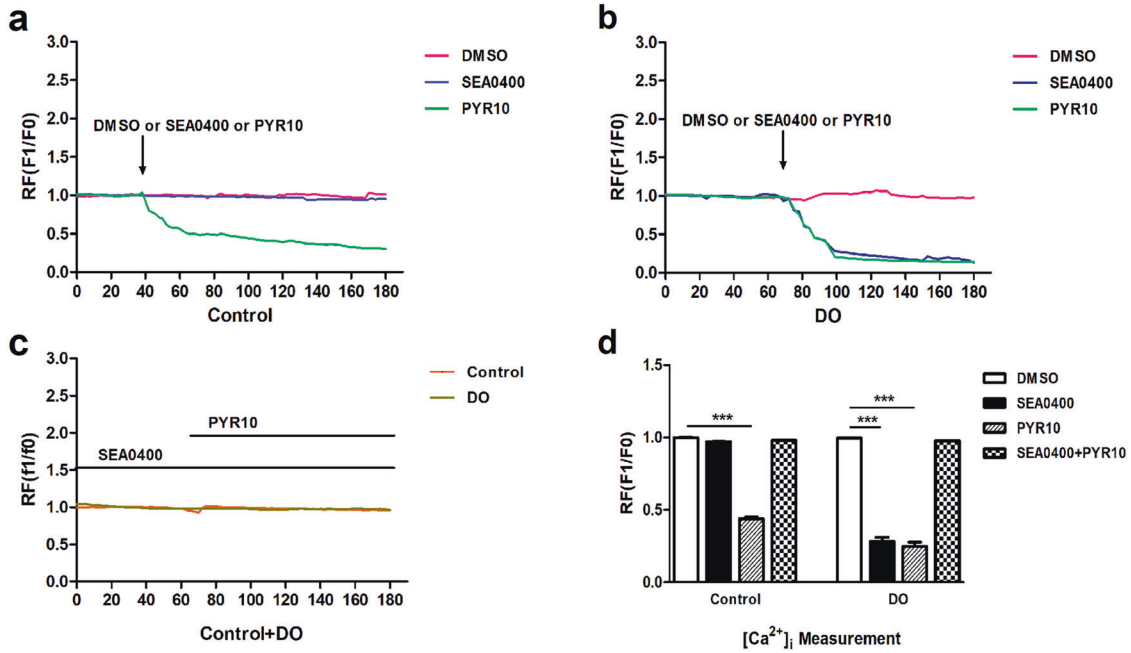


Fig. 5 SEA0400 abolished the reduction in the $[Ca^{2+}]_i$ in bladder SMCs mediated by PYR10. a, **d** PYR10 decreased the $[Ca^{2+}]_i$ in the bladder SMCs of control rats (100 nM, $n = 29$). SEA0400 (10 μM, $n = 11$) and DMSO ($n = 15$) had no effect on the $[Ca^{2+}]_i$ in the bladder SMCs of control rats. **b**, **d** PYR10 (100 nM, $n = 22$) and SEA0400 (10 μM, $n = 14$) decreased the $[Ca^{2+}]_i$ of bladder SMCs in DO rats. DMSO ($n = 21$) had no effect on the $[Ca^{2+}]_i$ in the bladder SMCs of DO rats ($n = 3$). **c**, **d** SEA0400 (10 μM) abolished the reduction in the $[Ca^{2+}]$i of bladder SMCs mediated by PYR10 (100 nM) ($n = 18$). ***$P < 0.001$.

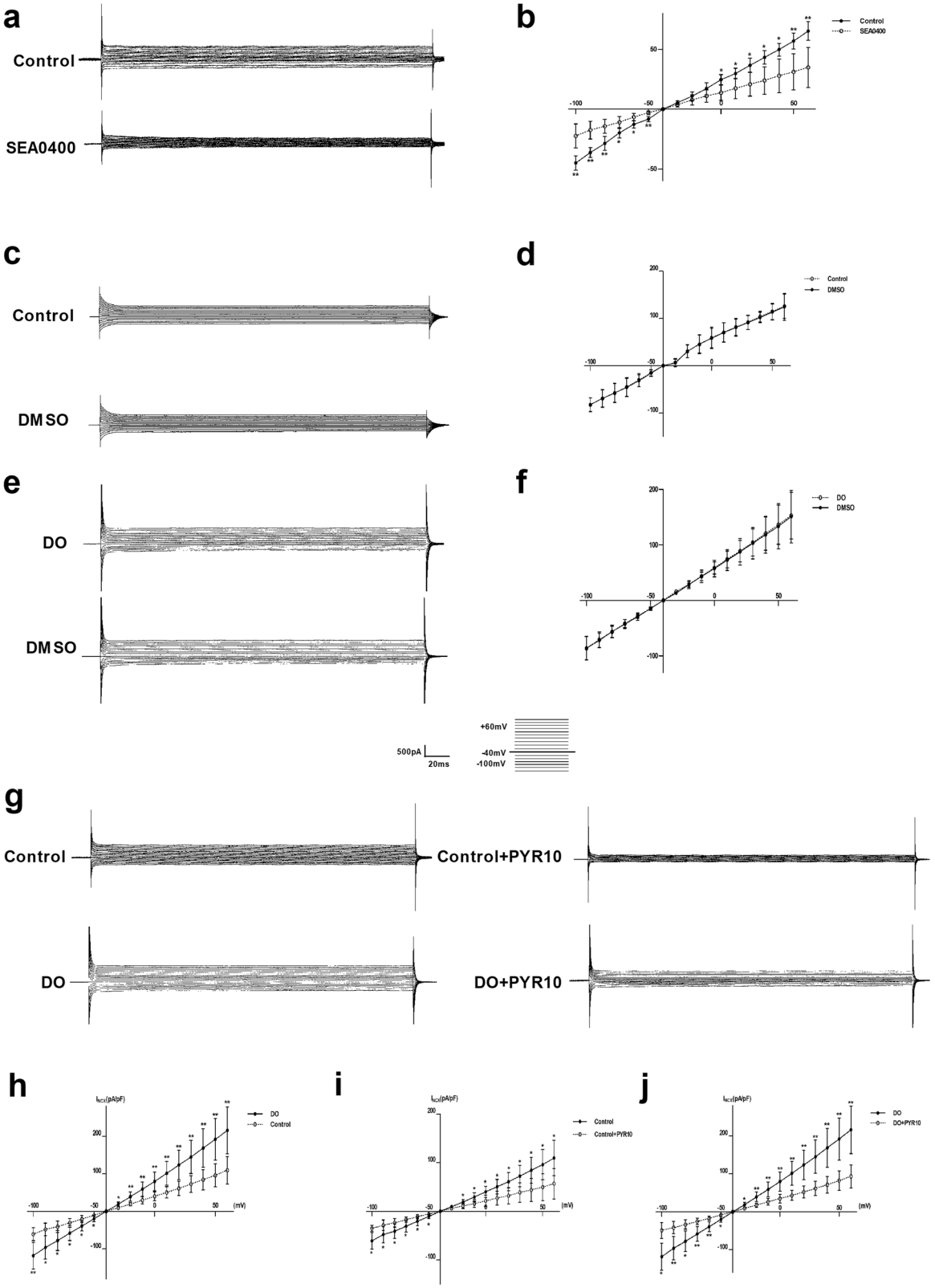
TRPC3 is a nonselective cation channel and belongs to the classical TRP family. TRPC3 regulates homeostasis of many ions, such as Ca^{2+} and Na^{+}, in numerous cell types[21]. Previous studies have demonstrated that TRPC3 plays an important role in contractility and excitability of the airway, uterus, vasculature and heart[6]. However, the function of TRPC3 in the bladder is still unclear. In the present study, we found that TRPC3 was expressed in all layers of the bladder. In addition, the mRNA and protein expression levels of TRPC3 were significantly increased in the bladders of rats with PBOO-induced DO. These findings indicate that TRPC3 may be related to overactive bladder.

Therefore, we further explored the function of TRPC3 in the DO bladder via contractility and cystometric studies. As we expected, the TRPC3 inhibitor (PYR10) significantly reduced the contractility of detrusor strips and the frequency of micturition in the control and DO groups. Furthermore, the effect of PYR10 on bladder excitability was more significant in DO group than in control group. These results indicate that TRPC3 is involved in the development of DO. However, the specific mechanism of TRPC3 is still unclear.

Interestingly, PYR10 did not significantly decrease the MBP in control and DO rats. Previous studies have also shown that some reagents can affect bladder contractility and cause changes in MI, but there is no significant change in BMP[22,23]. The mechanism of PYR10 on the MBP may be multifactorial. TRPC3 is widely expressed in the body, and this channel may also be expressed in urethral tissue[24]. Therefore, similar to detrusor, urethral contraction may be decreased upon PYR10 administration, there is no significant change in MBP[23]. In addition, in in vivo animal models, urination behavior is regulated by many factors, such as nerve, endocrine and so on[25,26]. These factors may affect the changes of MBP after PYR10 administration.

The NCX family, which is composed of nine-transmembrane proteins, including the proteins encoded by the NCX1, NCX2 and NCX3 genes, is thought to be crucial for the regulation of the homeostatic balance of Ca^{2+}[,27]. NCX has two working modes, i.e., the forward mode (Ca^{2+} exit mode) and the reverse mode (Ca^{2+} entry mode), which bidirectionally regulated the $[Ca^{2+}]$ depending on the electrochemical gradient of Na^{+}[,28,29]. The forward mode of NCX is the dominant mode under physiological conditions. NCX can regulates the motility of smooth muscle in many tissues, including arterial[30], venous[31], tracheal[32], ileal[33] and cardiac[25] tissues and participates in various pathological processes[11]. NCX1 is abundant in the bladder, while the expression of NCX2 and NCX3 is mainly restricted to the brain and bone tissues[34]. Yamamura and colleagues demonstrated that bladder smooth muscle contraction is increased in transgenic mice overexpressing NCX 1.3[35]. Our previous studies have also shown that increased NCX1 levels contribute to the hyperexcitability of the bladder by elevating the $[Ca^{2+}]_i$ in the reverse mode of NCX1[13]. In this study, we first evaluated the expression of NCX1 in the rat bladder. The results showed that the expression of NCX1 was significantly increased in the bladders of DO rats. In addition, the effects of NCX1 on bladder excitability in control and DO rats were evaluated by administering SEA0400. In DO rats but not in control rats, the MI was prolonged and the contraction amplitude was decreased when SEA0400 was applied. Based on these findings, we believe that NCX1 also plays an important role in bladder hyperexcitability in DO. What is the relationship between TRPC3 and NCX1 in DO?

We studied the relationship between TRPC3 and NCX1 in the rat bladder by co-IP analysis and IF staining. The results showed that TRPC3 was colocalized and interacted with NCX1 in the bladder. These data provide a structural foundation for the functional interaction between TRPC3 and NCX1. The association between TRPC3 and NCX1 has also been previously confirmed in cardiovascular cell[5,25]. We further explored the relationship between the two channels based on functional experiments. The results showed that after SEA0400 was administered, the results of both the contractility and cystometric tests were not significantly influenced in either of the two groups of rats when PYR10 was applied. Taken together, these findings indicate that TRPC3 might regulate bladder excitability in DO rats by changing the mode of NCX1.



It is well-known that Ca^{2+}, as an important intracellular signal, plays a central role in excitation and contraction of SMCs[36]. Both TRPC3 and NCX1 can regulate the homeostatic balance of Ca^{2+} in SMCs[25,37]. TRPC has been proposed to affect the function of NCX by elevating Na^{+} cellular levels, thereby reducing Ca^{2+} extrusion or even promoting Ca^{2+} entry at positive potentials[25,38,39]. A previous study in the heart found that reverse-mode NCX activity is associated with Na^{+} accumulation in the subplasmalemmal space, which provides an additional trigger for Ca^{2+} influx[40]. When the transmembrane Na^{+} gradient is reduced by increasing the intracellular sodium concentration ($[Na^{+}]_i$) in the subplasmalemmal space, reverse-mode NCX activity is increased[41]. In the

Fig. 6 PYR10 significantly reduced the absolute value of the I_{NCX} in bladder SMCs. a, **b** SEA0400 significantly inhibited the absolute value of the I_{NCX} density in bladder SMCs (10 μM, $n = 5$). **c**, **d** DMSO had no significant influence on the absolute value of the I_{NCX} in SMCs from control rats ($n = 4$). **e**, **f** DMSO had no significant influence on the absolute value of the I_{NCX} in SMCs from DO rats ($n = 5$). **g**, **h** The absolute value of the I_{NCX} density was significantly enhanced over a voltage range of −100 to −50 mV (forward mode) and at 0 to +60 mV (reverse mode) in SMCs from DO rats ($n = 5$) compared with SMCs from control rats ($n = 6$). The enhancement in the absolute value of the I_{NCX} density in SMCs in the reverse mode of NCX (+60 mV) was more significant than that in the forward mode of NCX (−100 mV) in DO rats. **g**, **i**, **j** PYR10 (100 nM) significantly reduced the absolute value of the I_{NCX} density in SMCs from control ($n = 6$) and DO rats ($n = 5$). The reduction in the absolute value of the I_{NCX} density in SMCs in the reverse mode of NCX (+60 mV) was more significant than that in the forward mode of NCX (−100 mV) in DO rats. $^*P < 0.05$, $^{**}P < 0.01$.

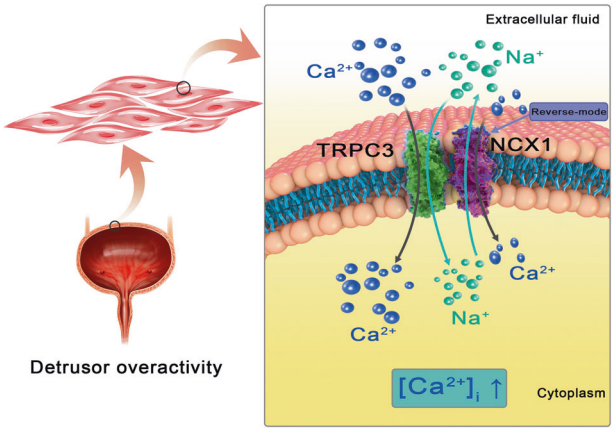


Fig. 7 Schematic representation of the functional roles of TRPC3 and NCX1 in bladder SMCs. The influx of Na^+ and Ca^{2+} into bladder SMCs was increased through TRPC3 in DO rats. Furthermore, TRPC3 functionally interacted with NCX1 in bladder SMCs. TRPC3 activated the reverse mode of NCX1 by elevating Na^+ levels in the subplasmalemmal space, thereby promoting Ca^{2+} entry into bladder SMCs in DO rats. As a consequence, three subplasmalemmal Na^+ ions were transported out of the cell via TRPC3 to allow the entry of one Ca^{2+} ion into the cytoplasm through the reverse mode of NCX1 in bladder SMCs. The synergistic effects of TRPC3 and NCX1 significantly increased the $[Ca^{2+}]_i$ in bladder SMCs, which may have consequently contributed to DO.

present study, the change in $[Ca^{2+}]_i$ was measured to evaluate the effects of TRPC3 and NCX1 channels on the excitability of SMCs using the fluorescent calcium indicator Fluo-4 AM[42]. When SEA0400 was administered, the $[Ca^{2+}]$i in SMCs was reduced in DO rats. In addition, when PYR10 was administered, the $[Ca^{2+}]^i$ in SMCs was reduced in both DO and control rats. However, after SEA0400 was administered, the decrease in the $[Ca^{2+}]_i$ in SMCs induced by PYR10 was abolished. We speculate that the decrease in $[Na^+]_i$ in the subplasmalemmal region reduces reverse-mode NCX1 activity when TRPC3 is inhibited using PYR10, mediating the decrease in $[Ca^{2+}]_i$ in SMCs and inhibiting the excitability of the bladder. Taken together, these findings indicate that increased TRPC3 activity induces bladder hyperactivity in DO rats via the reverse mode of NCX1.

To further test this idea, a whole-cell patch clamp experiment was conducted to record the I_{NCX} in isolated SMCs. To verify the specificity of the I_{NCX}, a specific inhibitor of NCX1 (SEA0400) was administered. The results showed that SEA0400 significantly inhibited the absolute value of the I_{NCX} density in SMCs. Based on these results, we demonstrated that the current we recorded reflected the electrical activity of NCX. The I_{NCX} was recorded from the control and DO groups, and the results showed that the absolute value of the I_{NCX} density in SMCs was increased in DO rats and that changes in the I_{NCX} might be involved in the pathogenesis of DO. PYR10 reduced the absolute the I_{NCX} density in SMCs from DO and control rats. The reduction in reverse-mode NCX activity (+60 mV) was more significant than that in forward-mode NCX activity (−100 mV). These results further indicate that reverse-mode NCX1 is the main target of TRPC3.

SEA0400 used in this study is a compound synthesized to block the NCX. But, SEA0400 has also been shown to inhibit L-type Ca^{2+} channel[43]. Low concentrations of SEA0400 displayed relatively good selectivity for NCX over L-type Ca^{2+} channel[43]. In order to eliminate the interference of L-type Ca^{2+} channel in our study, Nifedipine, a specific blocker of L-type Ca^{2+} channel, is administrated in the detrusor strip contractility studies. The results showed that upon administration of Nifedipine (5 μmol/L), the contraction amplitude was decreased in control rats upon PYR10 application (data not shown). These results suggest that L-type Ca^{2+} channel does not play a major role in the decrease of bladder excitability induced by PYR10. Combined with the results of this study, we have reason to think that TRPC3 may play an important role in the pathogenesis of DO through synergism with NCX1.

In conclusion, this study demonstrates that the expression and functional levels of TRPC3 and NCX1 were increased in the bladders of rats with PBOO-induced DO. The synergistic effects of TRPC3 and NCX1 significantly increased the $[Ca^{2+}]_i$ in SMCs, which induced bladder hyperactivity in DO rats. To our knowledge, the present study is the first to demonstrate that NCX1 may be regulated by TRPC3 in the bladder and may be responsible for the development of DO. Understanding the exact link between TRPC3 and NCX1 in the DO bladder will present new opportunities for the development of pharmacological strategies based on TRPC3 and NCX1 as targets.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

J.Z. performed the major experiments and drafted the manuscript. X.L., Z.Y. and L.L. analyzed the data. Y.F., Q.L. and Y.Y. were involved in the model establishment, quantitative PCR, western blotting and Immunohistochemistry. H.L., H.Z., Q.Liu and J. Zhao participated in the Cystometry, contractility studies, [Ca^{2+}]i measurements and patch clamp experiments. B.S. drew the schematic representation. J.X. and H.F. designed the study and edited the paper. All authors read and approved the final paper.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

All animal studies were approved by Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (project identification code: AMUWEC2019416).

ADDITIONAL INFORMATION

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