Original Paper

Role of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ Cotransporter 1 in Phenylephrine-Induced Rhythmic Contraction in the Mouse Aorta: Regulation of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ Cotransporter 1 by Ca^{2+} Sparks and K_{Ca} Channels

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Key Words

$\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter 1 • Aorta • Rhythmic contraction • Phenylephrine • K_{Ca} channels • Ca^{2+} sparks • Ryanodine receptor

Abstract

Background/Aims: Vasoconstrictor-induced rhythmic contraction of arteries or veins has been observed both *in vivo* and *in vitro*. Many studies have reported that gap junctions, ryanodine receptors, Na^+ , K^+ -ATPase and other factors are involved in vasoconstrictor-induced rhythmic contraction in vascular smooth muscle. However, the mechanism is still not completely understood. **Methods:** We used vessel tension measurements, intracellular recordings and intracellular Cl^- concentration ($[\text{Cl}^-]_i$) measurements to investigate the mechanism underlying phenylephrine (PE)-induced rhythmic contraction in the mouse aorta. **Results:** We found that $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter 1 (NKCC1) inhibitor bumetanide abolished PE-induced rhythmic contraction. The Cl^- channel blockers DIDS and niflumic acid initially augmented the amplitude of PE-induced rhythmic contraction but later inhibited the rhythmic contraction. The large Ca^{2+} -activated K^+ channel blocker TEA and iberiotoxin increased the amplitude of PE-induced rhythmic contraction. The voltage-dependent Ca^{2+} channel blocker, nifedipine, and a Ca^{2+} -free solution abolished PE-induced rhythmic contraction. The inhibitor of ryanodine receptors in the sarcoplasmic reticulum, ryanodine, inhibited PE-induced rhythmic contraction. Moreover, bumetanide hyperpolarized the membrane potential of vascular smooth muscle cells in a resting state or after PE pre-treatment. Bumetanide, niflumic acid, ryanodine, iberiotoxin, nifedipine and Ca^{2+} -free buffer significantly suppressed the PE-induced $[\text{Cl}^-]_i$ increase. **Conclusion:** These data indicate that NKCC1 is involved in the formation of PE-induced rhythmic contraction, and we also provide a method with which to indirectly observe the NKCC1 activity in isolated intact mouse thoracic aortas.

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Introduction

Vasoconstrictor-induced rhythmic contraction (vasomotion) of arteries or veins has been observed both *in vivo* and *in vitro* for more than one hundred years [1, 2]. According to the literature, rhythmic contraction can be abolished in vascular smooth muscle cells (VSMCs) by heptanol (a putative gap junction inhibitor), Ca^{2+} -free solution, ryanodine (an inhibitor of Ca^{2+} -release channels in sarcoplasmic reticulum (SR)), doxorubicin (an anticancer drug) and ouabain (an inhibitor of Na^+ , K^+ -ATPase) [3-9]. Peng et al. suggested a model in which the rhythmic contraction involved the synchronously intermittent release of Ca^{2+} from the SR, which is initially unsynchronized amongst various VSMCs [10]. Three studies have also demonstrated that rhythmic contraction in vessels results from cyclic GMP-dependent activation of K^+ channels by endothelium-derived nitric oxide [9, 11, 12]. Moreover, in an animal model of atherosclerosis, rhythmic contraction in mouse thoracic aorta is related to Ca^{2+} -activated potassium channels (K_{Ca} channels), but in a normal animal, rhythmic contraction is related to voltage-dependent potassium channels (K_{v} channels) [12]. Additionally, in the early stage of diabetes in a mouse thoracic aorta, the rhythmic contraction also involves prostaglandin I_2 and endothelium-derived hyperpolarizing factor [13]. Mesenteric VSMCs from diabetic rats undergo phenotypic modulations, switching from a contractile to a synthetic-proliferative phenotype [14]. Gustafsson et al. thoroughly studied noradrenaline-induced rhythmic activity in the mesenteric arteries of rats and demonstrated that endothelium Ca^{2+} and K^+ channels and the Na^+ - K^+ -pump were all involved in noradrenaline-induced rhythmic activity [11, 15-17]. However, the role of Na^+ - K^+ -2 Cl^- cotransporter 1 (NKCC1) and Cl^- channels in vasoconstrictor-induced rhythmic contraction has not been evaluated.

Recent studies show that NKCC1 has important functions in various cell types, including cardiomyocytes, endothelial cells, neurons, glial cells and blood cells [18]. In VSMCs, NKCC1 is a prominent transporter that mediates the transmembrane movement of four ions, including Na^+ , K^+ , and 2 Cl^- in an electrically neutral pathway [18, 19], and several studies indicate that NKCC1 plays a pivotal role in the regulation of vascular smooth muscle contraction by modulating membrane potentials [20, 21]. This mechanism is an important method for the accumulation of intracellular Cl^- in smooth muscle cells [19, 22] and the maintenance of $[\text{Cl}^-]_i$ at higher than equilibrium values. Decreasing $[\text{Cl}^-]_i$ stimulates NKCC1 and increases in $[\text{Cl}^-]_i$ inhibit it [23, 24]. NKCC1 also plays a direct role in cell volume regulation and is activated by cell shrinkage and inhibited by cell swelling [25, 26]. Additionally, NKCC1 is also regulated by protein kinase C (PKC), cAMP, high $[\text{K}^+]_o$, cGMP, and Ca^{2+} /calmodulin [27-30]. Although studies of NKCC1 have been documented in many cell types, its function and regulation in VSMCs have not been well understood.

The influx of $^{86}\text{Rb}^+$, a tracer for K^+ , is an extensive method to measure NKCC1 activity. Limited by technical considerations, few studies of NKCC1 in intact vascular smooth muscle have been performed. In the present study, we not only investigated the role of NKCC1 in PE-induced rhythmic contraction but provided a method with which to indirectly observe NKCC1 activity in isolated intact mouse thoracic aortas.

Materials and Methods

Mice and diets

Male and female Kunming mice at 7-8 weeks of age were used in the experiments. The mice were housed with a 12-hour light/dark cycle and had access to food and water ad libitum. All animal experiments were conducted in accordance with NIH publication no. 8523 and approved by the Animal Experimentation Ethics Committee of Anhui Medical University.

Tissue preparation

The mice were sacrificed via an overdose of CO_2 . The thoracic aorta was quickly dissected and placed in Krebs Henseleit solution at room temperature (22-23°C). Using a dissecting microscope, adhered perivascular tissue was carefully removed, and the descending thoracic aorta was cut into 3 mm long rings.

Tension measurement

Vessel tension measurements were performed as previously described [31]. The vessels were mounted onto two thin stainless steel holders, one of which was connected to a force displacement transducer and the other to a movable device that allowed for the application of a passive tension of 500–550 mg, which was determined to be the optimal resting tension for obtaining the maximal active tension induced by a 60 mmol/l K⁺ solution. The mounted rings were kept in 2 ml organ baths containing Krebs Henseleit solution, kept at 37°C and continuously bubbled with a gas mixture of 95% O₂ and 5% CO₂ to maintain a pH of 7.4. The isometric tension was recorded on a polygraph (BL-420E+, Chengdu Technology & Market Corp). After an equilibration period of 1 h, the contractile function of the vessel was tested twice by replacing the Krebs Henseleit solution with a 60 mmol/l K⁺ solution, and the second contraction was used as the reference contraction. Following a washout, the vessel was contracted once with 10 μmol/l phenylephrine (PE) for 10 min and then relaxed with 10 μmol/l acetylcholine (ACh) for 4 min. Prolonged exposure to 10 μmol/l PE evoked spontaneous rhythmic activity, which was recorded for 15 min. The amplitude was measured as the mean of the last five oscillations. Each drug was investigated using thoracic aorta segments from at least four mice.

Measurement of [Cl]_i

Segments of the isolated mice thoracic aortas were cut open. The endothelial layer was rubbed off; then, the endothelium-denuded aortic strips were incubated with 500 μmol/l 6-methoxy-N-ethylquinolinium iodide (MEQ) at 37°C for 1 h in a Krebs–HEPES buffer solution, which contained the following (in mM): NaCl 128, KCl 2.5, CaCl₂ 2.7, MgCl₂ 1, glucose 16, HEPES 20 at pH 7.4. After removing the loading buffer, the tissues were washed three times in the Krebs–HEPES solution. Intracellular trapped MEQ is sensitive to Cl[−], and the fluorescence intensity of MEQ is inversely related to [Cl]_i. The [Cl]_i fluorescence signal was recorded using a FlexStation 3 multi-mode microplate reader (Molecular Devices) with excitation and emission wavelengths set to 355 and 460 nm, respectively. Changes in [Cl]_i were displayed as the percentage of fluorescence change relative to the intensity before the application of PE.

Membrane potential measurement

Intracellular recording via sharp microelectrode was used to measure the membrane potential [32]. Briefly, the segments of the isolated mice thoracic aortas were cut open. After tearing off the endothelial layer, a conventional sharp microelectrode, filled with 3 mol/l KCl (tip resistance 20 MΩ), was inserted into smooth muscle cells from the lumen side. After the membrane potential was stabilized for several minutes, the drugs were perfused into the bath.

Solutions

The standard Krebs Henseleit solution consisted of the following (in mmol/l): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ (7H₂O) 1.2, NaHCO₃ 25.2, and glucose 11.1 (pH 7.4). It was titrated to the appropriate temperature-corrected pH with 10 mol/l NaOH. The high K⁺ solution (60 mmol/l) was prepared by exchanging NaCl with an equimolar amount of KCl. Ca²⁺-free solution was prepared by omitting Ca²⁺ from the Krebs Henseleit solution and by adding 0.1 mM EGTA.

Drugs

Phenylephrine (PE), acetylcholine (ACh), tetraethylammonium (TEA), niflumic acid, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS) (Sigma Chemical, St Louis, MO, U.S.A.) and ryanodine (Wako Pure Chemical Industries, Ltd. Japan) were dissolved in distilled water. Nifedipine, bumetanide and iberiotoxin (IbTX) (Sigma) were dissolved in DMSO. All subsequent dilutions were made with Krebs Henseleit solution. Similar dilutions of the solvents into the Krebs Henseleit solution were used as controls and had no effect on either the basal tension or the evoked tension of the thoracic aortic rings. All concentrations given are the final molar concentrations in the organ chambers.

Statistics

The results are expressed as the mean ± SEM. The number of observations indicated is the number of vessel segments studied. Because several segments were taken from the same animal, the number of vessels was larger than the number of animals in some experiments. Statistical analyses were always performed

using both the number of vessels and the number of animals. Differences were significant only if significant differences were found using both methods. The independent-sample *t*-test was used to compare the results in treated and untreated aortas from each strain. Differences were considered significant if $P < 0.05$.

Results

Effects of bumetanide, DIDS and niflumic acid on PE-induced rhythmic contraction

10 $\mu\text{mol/l}$ PE induced a sustained thoracic aorta contraction, and after several minutes, the rhythmic contraction appeared (Fig. 1A). After application of the NKCC1 inhibitor 10 $\mu\text{mol/l}$ bumetanide for 10 min, basal tension was not affected, but PE-induced rhythmic contraction was abolished, and ACh-induced vasorelaxation was not affected (Fig. 1B, Table 1). In another group, after 10 $\mu\text{mol/l}$ PE induced the rhythmic contraction, 10 $\mu\text{mol/l}$ bumetanide was added to the bath. PE-induced rhythmic contraction was abolished, and the contractions of vessels were reduced (Fig. 1C). In dose-dependent effects of PE-induced vessel contraction and ACh-induced vasorelaxation, bumetanide dramatically suppressed PE-induced vessel contraction but did not affect ACh-induced vasorelaxation (Fig. 1D-E). To investigate Cl^- channels, DIDS (0.3 mmol/l) and niflumic acid (0.1 mmol/l), inhibitors of Cl^- channels, were added, and the amplitude of PE-induced rhythmic contractions initially

Fig. 1. The effect of bumetanide on phenylephrine (PE)-induced rhythmic contraction. (A) Original traces showing the contractile effect and rhythmic activity induced by PE (10 $\mu\text{mol/l}$) and relaxation induced by acetylcholine (ACh, 10 $\mu\text{mol/l}$) in thoracic aortic rings. (B) Pre-treatment of the vessels with 10 $\mu\text{mol/l}$ bumetanide for 10 min did not affect basal tension but abolished PE-induced rhythmic contraction and enhanced ACh-induced vasorelaxation. (C) PE-induced rhythmic activity was abolished by 10 $\mu\text{mol/l}$ bumetanide. (D) Phenylephrine-induced mouse aortic contraction. 10 μM bumetanide significantly inhibited phenylephrine-induced contraction. The mean $\pm$ SE ($n=3-5$); $*P < 0.05$ compared to control. (E) Acetylcholine-induced mouse aortic relaxation. 10 μM bumetanide did not significantly affect acetylcholine-induced relaxation when the vessel was pre-treated with 10 μM phenylephrine. The mean $\pm$ SE ($n=3-4$).

Table 1. Effects of bumetanide, DIDS and niflumic acid on phenylephrine (PE)-induced rhythmic contraction (RC) and acetylcholine (ACh)-induced vasorelaxation. Values are presented as the mean $\pm$ SE. n = 6-10 from 6 mice. Significant differences from control responses are shown, *P < 0.05, **P < 0.01

Group	n	Maximal tension 1 (MT, mg)	Maximal tension 2 (MT, mg)	Amplitude of RC (% of MT)	ACh-induced relaxation (% of MT)
Control	7	522 $\pm$ 106		9.2 $\pm$ 3.9	59.6 $\pm$ 11.5
Bumetanide (10 μ mol/L)	6	401 $\pm$ 96*		0 $\pm$ 0**	60.2 $\pm$ 5.3
Control	6	478 $\pm$ 106		10.3 $\pm$ 3.9	62.2 $\pm$ 9.8
DIDS	6	468 $\pm$ 146	268 $\pm$ 153**	21.8 $\pm$ 4**	84.3 $\pm$ 12.2*
Control	6	542 $\pm$ 108		9.1 $\pm$ 4.2	67.3 $\pm$ 11.5
Niflumic acid	6	485 $\pm$ 109	285 $\pm$ 142**	22.8 $\pm$ 2.4**	81.2 $\pm$ 7.8*

Fig. 2. The effects of DIDS and niflumic acid on phenylephrine (PE)-induced rhythmic contraction. (A) Original traces showing the contractile effect and rhythmic activity induced by PE (10 μ mol/l) and relaxation induced by acetylcholine (ACh, 10 μ mol/l) in thoracic aortic rings. Pre-treatment of the vessels with Cl⁻ channel inhibitors 0.3 mmol/l DIDS (B) or (C) niflumic acid (0.1 mmol/l) for 10 min initially increased the amplitude of PE-induced rhythmic contractions but inhibited after a few minutes and significantly increased ACh-induced vasorelaxation.

Fig. 3. The effects of TEA and iberiotoxin (IbTX) on phenylephrine (PE)-induced rhythmic contraction. (A) Original traces showing the contractile effect and rhythmic activity induced by PE (10 μ mol/l) and relaxation induced by acetylcholine (ACh, 10 μ mol/l) in thoracic aortic rings. (B) Pre-treatment of the vessels with 1 mmol/l TEA for 10 min increased the amplitude of PE-induced rhythmic contractions but significantly decreased ACh-induced vasorelaxation. (C) Pre-treatment of the vessels with 50 nmol/l IbTX enhanced the amplitude of PE-induced rhythmic contractions but significantly decreased ACh-induced vasorelaxation.

increased but disappeared after a few minutes. ACh-induced vasorelaxation significantly increased (Fig. 2A-C and Table 1).

Effects of IbTX and TEA on PE-induced rhythmic contraction

Incubating the vessels in 1 mmol/l TEA, a concentration that selectively blocks K_{Ca} channels [33], did not affect basal tension. In the presence of TEA, the amplitude of PE-induced rhythmic contraction significantly increased, but ACh-induced vasorelaxation was markedly decreased (Fig. 3A and B, Table 2). In another group, incubating the vessels in a selective large conductance Ca²⁺-sensitive potassium channel (BK_{Ca}) channel inhibitor 50 nmol/l IbTX, also caused a dramatic increase in PE-induced rhythmic contraction, but ACh-induced vasorelaxation was markedly suppressed (Fig. 3C, Table 2).

Table 2. Effects of TEA and IbTX on phenylephrine (PE)-induced rhythmic contraction (RC) and acetylcholine (ACh)-induced vasorelaxation. Values are presented as the mean $\pm$ SE. n = 6-9 from 6 mice. Significant differences from control responses are shown, *P < 0.05, **P < 0.01

Group	n	Maximal tension (MT, mg)	Amplitude of RC (% of MT)	ACh-induced relaxation (% of MT)
Control	6	487 $\pm$ 89	9.2 $\pm$ 2.9	65.6 $\pm$ 9.1
TEA (1 mmol/L)	9	623 $\pm$ 68*	21.4 $\pm$ 3**	54.4 $\pm$ 7.9*
Control	6	471 $\pm$ 44	9.4 $\pm$ 3.9	64.6 $\pm$ 13.3
IbTX (50 nmol/L)	6	537 $\pm$ 110	18.1 $\pm$ 3.5	52.5 $\pm$ 7.1*

Fig. 4. The effects of Ca^{2+} -free solution and nifedipine on phenylephrine (PE)-induced rhythmic contraction. (A) Original traces showing the contractile effect and rhythmic activity induced by PE (10 $\mu\text{mol/l}$) and relaxation induced by acetylcholine (ACh, 10 $\mu\text{mol/l}$) in thoracic aortic rings. (B) Pre-treatment of the vessels with Ca^{2+} -free solution abolished PE-induced rhythmic contraction and notably reduced the phasic contractions of vessels. (C) Pre-treatment of the vessels with 3 $\mu\text{mol/l}$ nifedipine inhibited PE-induced rhythmic contraction and dramatically reduced PE-induced phasic contraction.

Fig. 5. The effects of ryanodine on phenylephrine (PE)-induced rhythmic contraction. (A) Original traces showing the contractile effect and rhythmic activity induced by PE (10 $\mu\text{mol/l}$) and relaxation induced by acetylcholine (ACh, 10 $\mu\text{mol/l}$) in thoracic aortic rings. (B) Pre-treatment of the vessels with 10 $\mu\text{mol/l}$ ryanodine abolished PE-induced rhythmic contraction, notably increased the phasic contractions of vessels and reduced ACh-induced vasorelaxation. (C) After PE-induced rhythmic contraction appeared, the addition of 10 $\mu\text{mol/l}$ ryanodine enhanced PE-induced rhythmic contraction and decreased maximal tension in the initial stage but then abolished PE-induced rhythmic contraction.

Effects of Ca^{2+} -free solution and nifedipine on PE-induced rhythmic contraction

After 10 $\mu\text{mol/l}$ PE evoked the rhythmic contraction in thoracic aortic rings, the organ chamber was rinsed with Krebs-Henseleit solution. Before the next application of 10 $\mu\text{mol/l}$ PE, the organ chamber was rinsed with Ca^{2+} -free Krebs-Henseleit solution three times, and the vessel rings were equilibrated for 20 min in Ca^{2+} -free Krebs-Henseleit solution. PE-induced rhythmic contraction was abolished, and the phasic contractions of vessels were markedly reduced, from 528 ± 94 to 302 ± 42 mg (n = 7 from 5 mice, $P < 0.01$) (Fig. 4A and B). Incubation in 3 $\mu\text{mol/l}$ nifedipine also reduced PE-induced phasic contraction and inhibited rhythmic contraction (Fig. 4C, Table 3).

Table 3. Effects of nifedipine and ryanodine on phenylephrine (PE)-induced rhythmic contraction (RC) and acetylcholine (ACh)-induced vasorelaxation. Values are presented as the mean $\pm$ SE. n = 5 from 5 mice. Significant differences from control responses are shown, *P < 0.05, **P < 0.01

Group	Maximal tension (MT, mg)	Amplitude of RC (% of MT)	ACh-induced relaxation (% of MT)
Control	529 $\pm$ 43	10.5 $\pm$ 3.3	69.9 $\pm$ 10.3
Nifedipine	62 $\pm$ 21**	0 $\pm$ 0**	89.7 $\pm$ 6.3**
Control	512 $\pm$ 24	8.9 $\pm$ 4.3	62.3 $\pm$ 14.5
Ryanodine	648 $\pm$ 76*	0 $\pm$ 0**	15.7 $\pm$ 6.4**

Fig. 6. The effect of bumetanide on the membrane potential of mouse thoracic aortic smooth muscle cells. (A) 10 μ mol/l bumetanide hyperpolarized the resting membrane potential of mouse thoracic aortic smooth muscle cells. (B) 10 μ mol/l phenylephrine (PE) induced membrane potential depolarization and decreased the depolarization following the application of 10 μ mol/l bumetanide. (C) Summarized data showing bumetanide-induced membrane potential change with or without PE-pre-treatment. The mean $\pm$ SE (n=4-5); *P < 0.05 compared to bumetanide only.

Effects of ryanodine on PE-induced rhythmic contraction

10 μ mol/l ryanodine, an inhibitor of Ca^{2+} -release channels (RyR channels) in SR [34], was added 10 min before the administration of 10 μ mol/l PE. PE-induced rhythmic contraction was abolished, and ACh-induced vasorelaxation was decreased (Fig. 5A and B, Table 3). In another group, 10 μ mol/l ryanodine was added 10 min after the appearance of PE-induced rhythmic contraction. The amplitude of PE-induced rhythmic contraction was enhanced (from 10.9 $\pm$ 2.7 to 19.1 $\pm$ 5.1% of maximal tension, n = 5 from 5 mice, P < 0.05), and maximal tension was decreased (from 537 $\pm$ 79 to 425 $\pm$ 68 mg, n = 5 from 5 mice, P < 0.01) in the initial stage of the application of ryanodine. Later rhythmic contractions were then abolished (Fig. 5C).

Fig. 7. The effect of inhibitors on phenylephrine (PE)-induced intracellular Cl^- concentration ($[\text{Cl}^-]_i$) change. (A) 10 $\mu\text{mol/l}$ PE decreased MEQ fluorescence intensity, which indicates a $[\text{Cl}^-]_i$ increase. After several minutes, 10 $\mu\text{mol/l}$ bumetanide application increased MEQ fluorescence intensity, which represents a $[\text{Cl}^-]_i$ decrease. (B) Summarized data showing that 10 $\mu\text{mol/l}$ bumetanide, 0.1 mmol/l niflumic acid, 10 $\mu\text{mol/l}$ ryanodine, 50 nmol/l ibe-riotoxin (IbTX), nifedipine and Ca^{2+} -free solution pre-treatment significantly inhibited the PE-induced $[\text{Cl}^-]_i$ increase. The mean $\pm$ SE (n=3-8); *P < 0.05 compared to control.

Effect of bumetanide on resting membrane potential and PE-induced membrane potential depolarization

Because membrane potential is a crucial factor for the regulation of vascular smooth muscle tone, we employed intracellular recording to examine the effect of bumetanide on resting membrane potential and PE-induced membrane potential depolarization. 10 $\mu\text{mol/l}$ bumetanide obviously induced resting membrane potential hyperpolarization in VSMCs (-15.6 ± 3.2 mV, n = 5) (Fig. 6A and C). In another group, 10 $\mu\text{mol/l}$ PE first evoked a membrane potential depolarization. Then, 10 $\mu\text{mol/l}$ bumetanide application still hyperpolarized the membrane potential, although the hyperpolarized effect was largely decreased (-5.9 ± 1.1 mV, n = 4) (Fig. 6B and C).

Change of $[\text{Cl}^-]_i$ in mouse aortic VSMC

NKCC1 mediates Na^+ , K^+ , and 2Cl^- influx into the cell. Therefore, $[\text{Cl}^-]_i$ will reflect the activity of NKCC1. MEQ fluorescence dye was used to indicate $[\text{Cl}^-]_i$. The fluorescence intensity increase represents the decrease of $[\text{Cl}^-]_i$. The results showed that 10 $\mu\text{mol/l}$ PE notably induced a $[\text{Cl}^-]_i$ increase, but the 10 $\mu\text{mol/l}$ bumetanide application decreased $[\text{Cl}^-]_i$ (Fig. 7A). Additionally, we utilized several inhibitors including 10 $\mu\text{mol/l}$ bumetanide, 0.1 mmol/l niflumic acid, 10 $\mu\text{mol/l}$ ryanodine, 50 nmol/l IbTX, 3 $\mu\text{mol/l}$ nifedipine and Ca^{2+} -free buffer to treat mouse aortas for 10 min. All inhibitors strongly suppressed the 10 $\mu\text{mol/l}$ PE-induced $[\text{Cl}^-]_i$ increase (Fig. 7B). Moreover, niflumic acid markedly enhanced $[\text{Cl}^-]_i$ of mouse aortic smooth muscle cells (the fluorescence intensity decreased $21.5\% \pm 2.8$, n = 5).

Discussion

The present study provided the first evidence that a Cl^- flux is involved in PE-induced rhythmic contraction in the mouse aorta. Simultaneously, we provided the first hypothesis that the activity of NKCC1 is the key to PE-induced rhythmic contraction and can be regulated by Ca^{2+} sparks.

These results indicate that the NKCC1 inhibitor, bumetanide, abolished PE-induced rhythmic contraction. However, the Cl^- channel blockers DIDS and niflumic acid enhanced the

amplitude of the rhythmic contraction initially and later inhibited the rhythmic contraction. The BK_{Ca} channel blockers TEA and IbTX enhanced the amplitude of PE-induced rhythmic contraction.

The voltage-dependent Ca^{2+} channel blockers nifedipine and Ca^{2+} -free solution abolished PE-induced rhythmic contraction. Additionally, the inhibitor of RyR channels in the SR, ryanodine, abolished PE-induced rhythmic contraction.

The NKCC1 inhibitor bumetanide hyperpolarized the membrane potential of VSMCs in a resting status or after PE pre-treatment. Additionally, inhibitors including bumetanide, niflumic acid, ryanodine, IbTX, nifedipine and Ca^{2+} -free buffer significantly suppressed the PE-induced $[Cl^-]_i$ increase. However, niflumic acid, an inhibitor of Cl^- channels, markedly increased $[Cl^-]_i$ in VSMCs.

Our finding provides evidence of the role of NKCC1 in PE-induced rhythmic contraction and the regulatory relationship between NKCC1, Ca^{2+} sparks and BK_{Ca} channels, which may be helpful for understanding the effects of NKCC1 on vessel tension regulation.

Effects of Cl^- flux to regulate NKCC1

The coupling of NKCC1, which can be inhibited by bumetanide and furosemide, is an important method of accumulation of intracellular Cl^- in smooth muscle cells [19-22]. Additionally, transport occurs via an electrically silent pathway, moving Na^+ , K^+ , and Cl^- across the plasma membrane [18, 35]. A furosemide-sensitive, Cl^- -dependent Na^+ - K^+ cotransporter in intact smooth muscle cells was first observed by Deth et al. [36] in rat and rabbit aortas. In smooth muscle cells, Davis et al. simultaneously measured membrane potential and $[Cl^-]_i$ and found that noradrenaline was able to stimulate NKCC1 and Na^+ -independent inward Cl^- pumping to cause a $[Cl^-]_i$ increase and membrane potential depolarization [37]. In addition to noradrenaline, angiotensin II, histamine, KCl, PE and endothelin can acutely stimulate the activity of NKCC1, but nitroprusside inhibited the activity of NKCC1 [19, 35, 37, 38].

In the present study, PE-induced rhythmic contraction and the $[Cl^-]_i$ increase were inhibited by 10 μ mol/l bumetanide, which indicates that NKCC1 is involved in the rhythmic contraction. Simultaneously, the inhibitors of Cl^- channels DIDS and niflumic acid initially enhanced the amplitudes of PE-induced rhythmic contraction but later inhibited rhythmic contraction, which suggests that rhythmic contraction is inhibited indirectly by the blockers of Cl^- channels. Additionally, niflumic acid dramatically increased the $[Cl^-]_i$ of VSMCs but inhibited the PE-induced $[Cl^-]_i$ increase. Taken together, these results indicated that NKCC1 is possibly a promoter of PE-induced rhythmic contraction. When Cl^- channels were inhibited by the inhibitor of Cl^- channels, niflumic acid, $[Cl^-]_i$ strongly increased. Thus, NKCC1 was inhibited by high $[Cl^-]_i$, which is consistent with previous studies [39, 40], and the rhythmic contraction disappeared.

Effects of K_v , BK_{Ca} channels to regulate NKCC1

Four types of vascular K^+ channels have been found to exit the same VSMC membrane. As one of these types, K_v channels, which are inhibited by 4-AP, are a major K^+ channel that controls the resting membrane potential and regulates $[Ca^{2+}]_i$ in VSMCs. However, the physiological activation of K_{Ca} channels, especially BK_{Ca} channels, is an important buffer to counteract vessel depolarization following increased $[Ca^{2+}]_i$. Jiang et al. found that 5 mmol/l 4-AP abolished PE-induced rhythmic contraction in normal C57BL/6J mouse thoracic aortas, but TEA enhanced the amplitude of rhythmic contraction [12]. However, Jiang et al. did not explain why TEA could enhance the amplitudes of rhythmic contraction. In VSMCs, Owen et al. found that increasing $[Ca^{2+}]_i$ markedly stimulated NKCC1 [38], and in the femoral artery of the rat, $[Cl^-]_i$ was also regulated by the membrane potential [37]. In this study, we found that TEA and IbTX enhanced the amplitude of PE-induced rhythmic contraction. After BK_{Ca} channels were inhibited by TEA or IbTX, the membrane potential increased to depolarization, and the activity of voltage-dependent Ca^{2+} channels was enhanced. Thus, $[Ca^{2+}]_i$ increased in VSMCs after the vessel was agitated by PE, and this high $[Ca^{2+}]_i$ thereby stimulated NKCC1. Additionally, NKCC1 activity contributes to the membrane potential of

VSMCs. Our finding showed that bumetanide hyperpolarized the resting membrane potential or PE-induced depolarization. This hyperpolarization suppressed the activity of voltage-dependent Ca^{2+} channels. In rat mesenteric arteries, Gustafsson et al. also reported that TEA increased the frequency, and 4-AP increased the amplitude of norepinephrine-induced rhythmic contraction. These phenomena are similar to our findings. However, in Jiang's experiments, PE-induced rhythmic contraction was abolished by 4-AP, which is probably due to the difference of distribution and role of K_v and K_{ca} channels in various species [12]. IbTX significantly inhibited the PE-induced $[\text{Cl}^-]_i$ increase, which was mainly caused by activating NKCC1, as observed by $[\text{Cl}^-]_i$ measurement. This result indirectly suggests that TEA and IbTX enhanced the amplitude of the rhythmic contraction possibly by stimulating the NKCC1 activity, which further proved that the activity of NKCC1 is essential for rhythmic contraction.

Effects of influx of extracellular Ca^{2+} and release of intracellular Ca^{2+} to regulate NKCC1

In VSMCs, intracellular Ca^{2+} can be accumulated by the influx of extracellular Ca^{2+} and the release of intracellular Ca^{2+} . There are some reports of activation of NKCC1 fluxes by treatments that increase $[\text{Ca}^{2+}]_i$ in various cells such as VSMCs, mouse fibroblasts, Ehrlich ascites tumour cells and rat cortical astrocytes [38, 41, 42]. However, the mechanism by which a rise in $[\text{Ca}^{2+}]_i$ stimulates NKCC1 is still unknown. In this experiment, Ca^{2+} -free solution and nifedipine abolished PE-induced rhythmic contraction in isolated mouse thoracic aorta. Because PE-induced rhythmic contraction shows the activity of NKCC1, we also demonstrated that $[\text{Ca}^{2+}]_i$ can regulate the activity of NKCC1. Additionally, an inhibitor of RyR channels, ryanodine, abolished the rhythmic contraction. More importantly, nifedipine and ryanodine and Ca^{2+} -free solution significantly inhibited the PE-induced $[\text{Cl}^-]_i$ increase induced by NKCC1 activity. These results indirectly indicate that Ca^{2+} sparks mediated by RyR channels from SR were also able to activate NKCC1.

In conclusion, we demonstrated that NKCC1 is involved in the formation of PE-induced rhythmic contraction. Additionally, we provide a study model to indirectly observe the NKCC1 activity in isolated intact thoracic aortas.

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Disclosure Statement

There is no conflict of interest.

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