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Original Paper

Generation and Role of Oscillatory Contractions in Mouse Airway Smooth Muscle

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Key Words
Oscillatory contractions • Airway smooth muscle • Ca^{2+} • Oscillations • Ion channels

Abstract
Background/Aims: Tetraethylammonium chloride (TEA) induces oscillatory contractions in mouse airway smooth muscle (ASM); however, the generation and maintenance of oscillatory contractions and their role in ASM are unclear. ***Methods:*** In this study, oscillations of ASM contraction and intracellular Ca^{2+} were measured using force measuring and Ca^{2+} imaging technique, respectively. TEA, nifedipine, niflumic acid, acetylcholine chloride, lithium chloride, KB-R7943, ouabain, 2-Aminoethoxydiphenyl borate, thapsigargin, tetrodotoxin, and ryanodine were used to assess the mechanism of oscillatory contractions. ***Results:*** TEA induced depolarization, resulting in activation of L-type voltage-dependent Ca^{2+} channels (LVDCCs) and voltage-dependent Na^+ (V_{Na}) channels. The former mediated Ca^{2+} influx to trigger a contraction and the latter mediated Na^+ entry to enhance the contraction via activating LVDCCs. Meanwhile, increased Ca^{2+}-activated Cl^- channels, inducing depolarization that resulted in contraction through LVDCCs. In addition, the contraction was enhanced by intracellular Ca^{2+} release from Ca^{2+} stores mediated by inositol (1,4,5)-trisphosphate receptors (IP_3Rs). These pathways together produce the contractile phase of the oscillatory contractions. Furthermore, the increased Ca^{2+} activated the Na^+-Ca^{2+} exchanger (NCX), which transferred Ca^{2+} out of and Na^+ into the cells. The former induced relaxation and the latter activated Na^+/K^+-ATPase that induced hypopolarization to inactivate LVDCCs causing further relaxation. This can also explain the relaxant phase of the oscillatory contractions. Moreover, the depolarization induced by V_{Na} channels and NCX might be greater than the hypopolarization caused by Na^+/K^+-ATPase

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alone, inducing LVDCC activation and resulting in further contraction. ***Conclusions:*** These data indicate that the TEA-induced oscillatory contractions were cooperatively produced by LVDCCs, V_{Na} channels, Ca^{2+}-activated Cl^- channels, NCX, Na^+/K^+ ATPase, IP_3Rs-mediated Ca^{2+} release, and extracellular Ca^{2+}.

Introduction

Deep inspiration and tidal breathing in humans inhibit subsequent bronchocontraction (termed bronchoprotection), which is due to an increased muscle length due to stretching of the lung parenchyma [1, 2]. The mechanism might be disruption of the cross-linking of actomyosin and other proteins such as zyxin; however, muscle stretch may have less or even reverse effects on the subsequent contraction in asthmatic airway smooth muscle (ASM) [3-9]. Consistent with these observations, oscillatory muscle stretch enhanced and attenuated electrical field stimulation-induced contraction in tracheal and bronchial ASM, respectively [10]. Thus, the effect of oscillatory contractions on the subsequent contraction of ASM is still unclear.

Oscillatory contractions in ASM can also spontaneously occur [11]; they were observed in cultured airways from human fetuses at 37°C but were reduceed as the temperature was lowered to 32°C. Moreover, oscillatory contractions can be abolished by nitric oxide (NO)-releasing drugs but are enhanced by carbachol [11]. Temperature decrease-induced abolition of oscillatory contractions has also been observed in precontracted canine ASM induced by cyclopiazonic acid (a selective blocker of the sarcoplasmic reticulum (SR) calcium-pump). This was because the Na^+/K^+ ATPase was inhibited at low temperature, which resulted in a sustained increase in intracellular Ca^{2+} and then led to the disappearance of the oscillatory contraction [12, 13]. The effect of NO-releasing drugs on the oscillatory contractions was due to the finding that the increased NO inhibited release of calcium through inositol (1, 4,5)-trisphosphate receptors (IP_3Rs), leading to abolition of the contractions [14]. Carbachol is a known agonist of muscarinic (M) receptors and stimulates M receptors to increase calcium. Therefore, the spontaneous oscillatory contractions would result from changes in intracellular Ca^{2+} concentration.

Oscillatory contractions can also be produced by agents such as the non-selective blocker of K^+ channels tetraethylammonium chloride (TEA) and Ca^{2+}-activated K^+ (Kca) channel blockers charybdotoxin and iberiotoxin [15-18]. On the other hand, the oscillatory contractions can be inhibited by the removal of external Ca^{2+} and by addition of Ca^{2+} channel blockers [15, 16, 18] or a protein kinase C (PKC) inhibitor, but are enhanced by a PKC activator [18]. In addition, PKC can activate L-type voltage-dependent Ca^{2+} channels (LVDCCs) [19]. Therefore, oscillatory contractions would depend on cytosolic Ca^{2+} increases. Consistent with this, ryanodine (a selective blocker of ryanodine receptors, RyRs) and thapsigargin (TG, an inhibitor of the calcium-pump) can also inhibit the oscillatory contractions [18]. Furthermore, the oscillatory contractions were abolished by an inhibitor of prostaglandin E_2 (PGE_2) receptor subtype EP1 [17], which mediated intracellular Ca^{2+} increases [20]. Overall, oscillatory contractions likely result from alternations of intracellular Ca^{2+} concentration.

Oscillations of cytosolic Ca^{2+} have been observed in ASM cells, induced by various agents such as acetylcholine (ACH), carbachol, methacholine, ATP, 5-hydroxytryptamine, leukotriene 4, phorbol myristate acetate, and high K^+ [14, 21-34]. Ca^{2+} concentration increased via release from the SR through IP_3Rs [23, 26, 30, 32] and RyRs [30, 31], as well as via influx through ion channels such as, LVDCCs [31, 33, 34] and store-operated Ca^{2+} entry [30, 33, 34]. Moreover, Ca^{2+} oscillations were regulated by many other factors such as M_3 receptor [21], bitter taste receptor TAS2R10 [32], PLC [23], cAMP [26], and PI3Kγ [28].

However, these Ca^{2+} oscillations resulted in a sustained contraction, not the above-mentioned oscillatory contractions [14, 23, 25-28, 32, 33]. Why did the oscillatory contractions not occur? The reason could be that their frequency was too high. It has been reported that ACH induced electrical oscillatory waves in pig tracheal smooth muscle in the

presence of TEA, which then resulted in rhythmic activation of LVDCCs, leading to Ca^{2+} influx to cause the oscillatory contractions. However, when the frequency of the electrical waves >40/min, the triggered oscillatory contractions would become a sustained contraction [16]. We estimated the frequencies of Ca^{2+} oscillations in these studies and found that they were >40/min. Thus, oscillatory contractions were not observed. Therefore, the mechanism underlying the oscillatory contractions remains unclear.

TEA can induce oscillatory contractions allowing us to investigate the underlying mechanism [15]. Thus, in this study, TEA was used to induce oscillatory contractions in mouse ASM and then investigated the mechanism. We found that the oscillatory contractions were cooperatively produced by LVDCCs, V_{Na} channels, Ca^{2+}-activated Cl^- (Clca) channels, Na^+-Ca^{2+} exchanger (NCX), Na^+/K^+ ATPase, IP_3Rs, Ca^{2+} pump, and external Ca^{2+}.

Materials and Methods

Reagents

TEA, nifedipine, niflumic acid, ACH, lithium chloride, KB-R7943, ouabain, 2-Aminoethoxydiphenyl borate (2-APB), thapsigargin (TG), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Ryanodine was purchased from Alomone Labs (Jerusalem, Israel), and tetrodotoxin (TTX) was purchased from Aladdin (Shanghai, China). Nifedipine, niflumic acid, 2-APB, TG, and ryanodine were dissolved in DMSO, TTX was dissolved in acetic acid, and all others were dissolved in the vehicle used in the experiments.

Animals

Six-week-old male BALB/c mice were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China) and were housed in a standard animal facility. Experiments were approved by the Institutional Animal Care and Use Committee of the South-Central University for Nationalities.

Contraction measurements of ASM

ASM contraction was measured in tracheal rings (TRs) as previously described [35-37]. In brief, after the mice were killed by an intraperitoneal injection of sodium pentobarbital (150 mg/kg) and then the tracheae were cut and placed in ice cold physiological salt solution (PSS) (mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, and glucose 10 (pH = 7.4). Distal TRs (5mm) were cut and mounted in 10-mL organ baths containing PSS bubbled with 95% O_2 and 5% CO_2 at 37°C. The TRs with 0.3 g preload were equilibrated for 60 min. Precontraction with 10^{-4} M ACH was was performed three times, and experiments were performed after 30 min.

Measurements of intracellular Ca^{2+}

Single mouse tracheal smooth muscle cells were isolated as previously described with some modifications [36, 38]. Briefly, mice were killed by intraperitoneal injection of sodium pentobarbital (150 mg/kg) and the tracheae were removed and transferred to ice-cold PSS containing 0.1 mM $CaCl_2$. The trachealis tissues were isolated and minced and incubated for 22 min at 35°C in the above solution supplemented with 2 mg/mL papain, 1 mg/mL dithioerythritol, and 1 mg/mL BSA. The tissues were transferred to the above solution supplemented with 1 mg/mL collagenase H, 0.15 mg/mL dithiothreitol, and 1mg/mL BSA and incubated for a further 8 min. Then, the tissues were washed and gently triturated to release single smooth muscle cells.

Cells were loaded with 2 μM fluo-4AM for 20 min and intracellular Ca^{2+} was measured and analyzed using an LSM 700 laser scanning confocal system and Zen 2010 software (Carl Zeiss, Jena, Germany) as previously described [35].

Statistical Analysis

The results are expressed as means ± SEM, Student's *t*-test was used for comparisons, and the $P < 0.05$ was considered significant.

Results

TEA triggers oscillatory contractions

TEA dose-dependently induced oscillatory contractions in quiescent mouse TRs (Fig. 1A). ASM has a tone *in vivo*, therefore, to mimic this tone *in vitro*, we precontracted TRs using a low concentration of ACH (1μM) and then observed the TEA-induced oscillatory contractions (Fig. 1B). We did not investigate the relaxation induced by 1 mM TEA in this study (Fig. 1B). The results, summarized in Fig. 1C, show that TEA (from 10 μM) induced oscillatory contractions in ACH-precontracted TRs, but their frequency (at 20 and 30 μM) was lower and amplitude (at 30 μM) was larger compared with resting TRs.

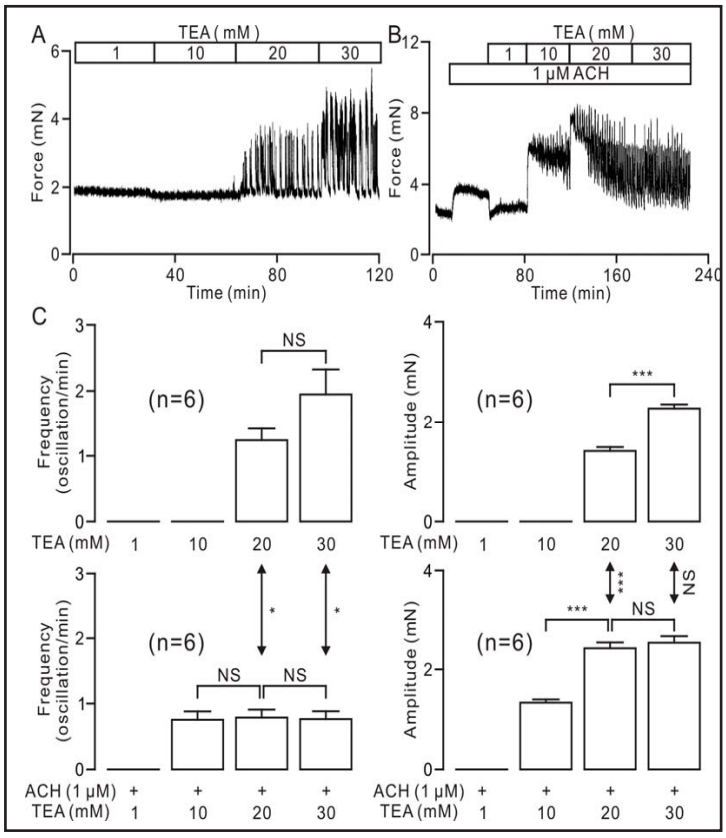

Fig. 1. TEA induces oscillatory contractions. (A) TEA induced oscillatory contractions in a concentration-dependent manner in a mouse TR. (B) Oscillatory contractions were also induced by TEA in a TR precontracted by 1μM ACH. However, at 1 mM, TEA caused relaxation. (C) Summary of the results is shown. NS: not significant; *: P<0.05; ***: P<0.001. These results indicate that TEA easily induces oscillatory contractions in precontracted ASM, but, the frequency is lower and the amplitude higher in precontracted ASM than in the quiescent ASM.

Mechanism of the oscillatory contractions

We then used 10 mM TEA to induce the oscillatory contractions in 1μM ACH-precontracted TRs (Fig. 2, n = 8) and investigated the underlying mechanism. TEA is a non-selective blocker of K^+ channels. It can induce depolarization of approximately 28 mV in human ASM cells [39]. This depolarization would activate LVDCCs and V_{Na} channels expressed in ASMCs [35, 40], mediating Ca^{2+} and Na^+ influx. The former will induce cytosolic Ca^{2+} elevations, causing a contraction; the latter will lead to an additional depolarization to activate LVDCCs, resulting in further contraction. Thus, we investigated the effect of nifedipine (a selective blocker of LVDCCs) and TTX (a selective blocker of V_{Na} channels) on the oscillatory contractions. The results show that both blockers inhibited the oscillatory contractions (Fig. 3, n = 6 TRs for both groups).

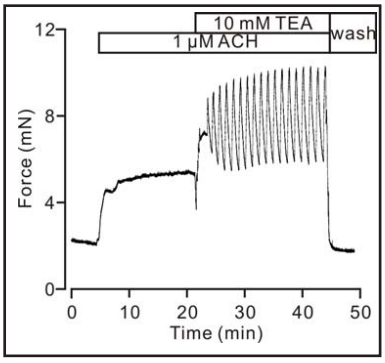

Fig. 2. TEA is required for generation and maintenance of oscillatory contractions. TEA triggered oscillatory contractions in a TR precontracted by 1μM ACH, and the contractions disappeared following TEA washout.

The increased Ca^{2+} will activate Ca^{2+}-activated channels such as Clca and Kca channels. However, the latter would have been blocked by TEA. Thus, we blocked the former using niflumic acid, which abolished oscillatory contractions in the TRs (Fig. 4, n = 6). These data

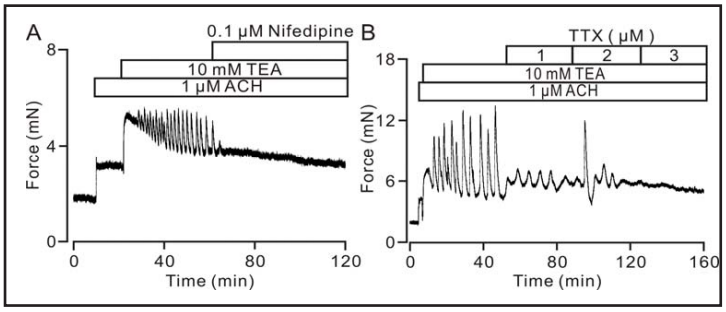

Fig. 3. LVDCCs and V_{Na} channels play a role in TEA-induced oscillatory contractions. TEA-induced oscillatory contractions in a TR were blocked by LVDCC blocker nifedipine (A) and V_{Na} channel blocker TTX (B).

suggest that Clca channels play a role in the oscillatory contractions.

The increased intracellular Ca^{2+} will also activate NCX expressed in ASM [41, 42]. We found that when NCX was inhibited by replacing Na^+ with Li^+, the TEA-induced oscillatory contractions were transiently enhanced and then disappeared (Fig. 5A, n = 6 TRs). Moreover, the TEA-induced oscillatory contractions were also abolished by the selective NCX blocker KB-R7943 (Fig. 5B, n = 6 TRs). These experiments reveal that the oscillatory contractions require NCX.

In addition, NCX will induce cytosolic Na^+ increases, which will then activate Na^+/K^+-ATPase [42, 43]. Following the inhibition of this enzyme by ouabain, a larger contraction was observed and the oscillatory contractions then vanished (Fig. 6, n = 6 TRs). This suggests that Na^+/K^+-ATPase is essential for the occurrence of the oscillatory contractions.

The above results indicate that the oscillatory contractions likely result from oscillatory rises in intracellular Ca^{2+} concentration. To test this hypothesis, cytosolic Ca^{2+} was measured. The results show that TEA induced fast Ca^{2+} oscillations, as well as another relatively slow rhythmicity.. The frequency of the rhythmicity was 0.32 ± 0.05/min (n = 25) (Fig. 7A), which was lower than that of the oscillatory contractions induced by TEA (0.55 ± 0.05 oscillation/min, n = 38, $P < 0.001$). These results suggest that in addition to the lower rhythmicity, the larger, faster Ca^{2+} oscillations might also trigger a single contraction.

ACH causes Ca^{2+} release from intracellular Ca^{2+} store, which would affect the TEA-induced Ca^{2+} oscillations and contraction. TG, an inhibitor of the SR calcium pump, abolished the oscillatory contractions (Fig. 8A, n = 6 TRs), which

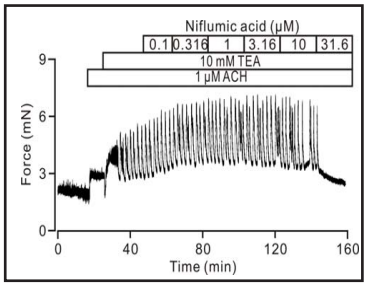

Fig. 4. Niflumic acid inhibits its oscillatory contractions. TEA-induced oscillatory contractions in a TR were inhibited by niflumic acid.

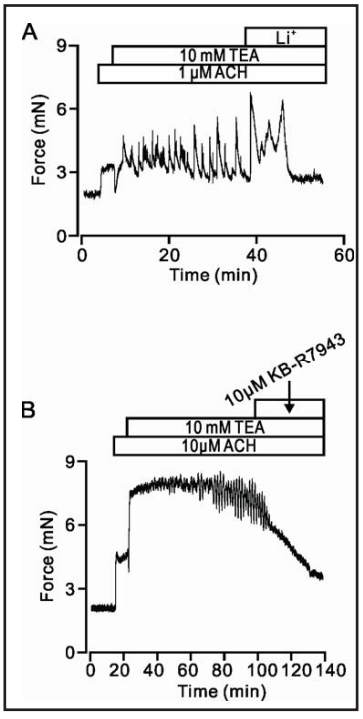

Fig. 5. NCX inhibition abolishes oscillatory contractions. TEA-induced oscillatory contractions in a TR were transiently enhanced and then disappeared following substitution of Na^+ with Li^+ (A) and the addition of selective NCX blocker KB-R7943 (B).

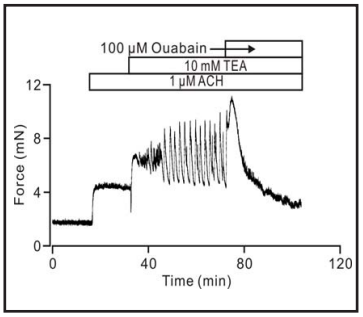

Fig. 6. Ouabain blocks oscillatory contractions. Ouabain induced a transient contraction and then abolished the TEA-induced oscillatory contractions in a TR.

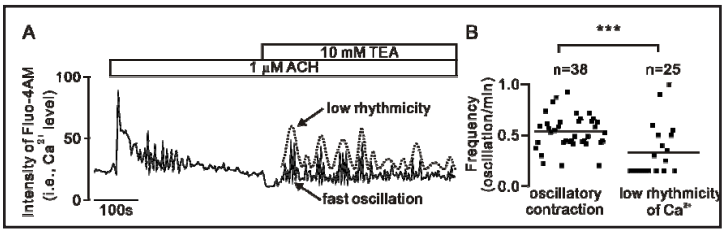

Fig. 7. TEA induces Ca^{2+} oscillations in ASMCs cells. (A) ACH induced an increase in Ca^{2+} concentration in ASM cells, and TEA then induced fast oscillations. In addition, a low-frequency rhythmicity was observed as indicated by the dashed line. (B) Comparison of the frequency of the oscillatory contractions and the rhythmicity. ***: P<0.01.

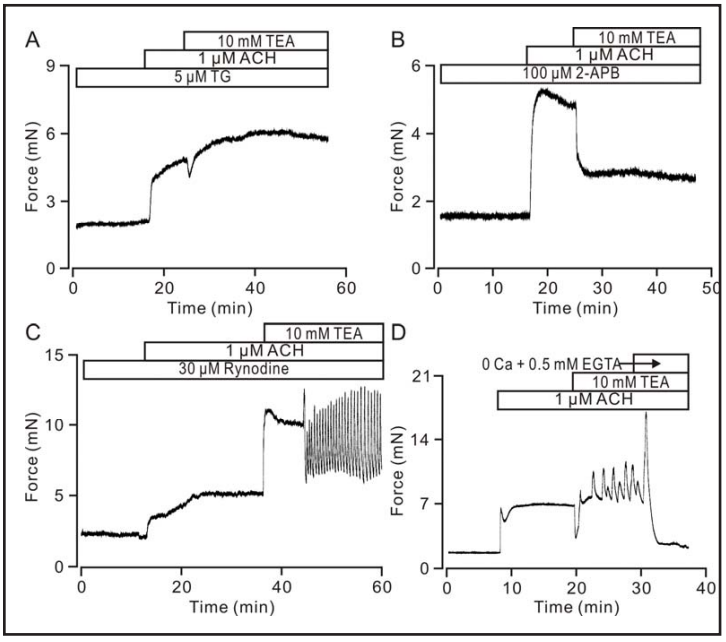

Fig. 8. Effect of intracellular Ca^{2+} store release and Ca^{2+} influx on the TEA-induced oscillatory contractions. (A) The incubation of TG abolished TEA-induced oscillatory contractions. (B) Oscillatory contractions were blocked by 2-APB, (C) but not by ryanodine. (D) Oscillatory contractions were abolished following the removal of the external Ca^{2+}.

were abrogated by 2-APB, a blocker of IP_3Rs (Fig. 8B, n = 6) but not by ryanodine, a selective blocker of RyRs (Fig. 8C, n = 6). In addition, the oscillatory contractions were inhibited by the removal of extracellular Ca^{2+} (Fig. 8D, n = 4). These results suggest that IP_3Rs-mediated Ca^{2+} release and external Ca^{2+} influx (mediated by LVDCCs) are required for oscillatory contractions.

Discussion

In this study, we have shown that TEA induced oscillatory contractions in resting and precontracted mouse tracheal ASM. In addition, TEA induced fast Ca^{2+} oscillations in the presence of ACH, as well as another rhythmicity. The frequency of the rhythmicity was close to that of the oscillatory contractions, but was significantly lower than the fast Ca^{2+} oscillations in the presence of ACH. This suggests that the oscillatory contractions may be triggered by the rhythmicity of cytosolic Ca^{2+} and some single fast Ca^{2+} oscillations. Moreover, the oscillatory contractions were inhibited by nifedipine, TTX, niflumic acid, oubain, KB-R7943, TG, 2-APB, the replacement of Na^+ with Li^+, and the removal of external Ca^{2+}, but not by ryanodine. These results suggest that oscillatory contractions are coordinately produced by LVDCCs, V_{Na} channels, Clca channels, Na^+, K^+-ATPase, NCX, intracellular Ca^{2+} release mediated by IP_3Rs and extracellular Ca^{2+} through changing the level of cytosolic Ca^{2+}.

Mechanism of the contractile phase of the oscillatory contractions

The aim of this study was to investigate the mechanism underlying and the role of the oscillatory contractions. We hypothesized that one oscillatory contraction would be produced by an increase then decrease of cytosolic Ca^{2+}. Since TEA blocks K^+

channels, resulting in a contraction through the pathway: K^+ efflux termination; membrane depolarization; LVDCC activation; and Ca^{2+} influx. This process was evidenced by the fact that nifedipine abolished the oscillatory contractions (Fig. 3A). Thus, in this study, TEA induced oscillatory contractions regardless of the presence of ACH (Figs. 1A, 2). Such a role of LVDCCs in the oscillatory contractions has been reported previously [44, 45]. In addition, the LVDCCs-mediated contraction will be enhanced by depolarization caused by V_{Na} channels-mediated Na^+ influx. Therefore, following V_{Na} channel inhibition by TTX, the oscillatory contractions disappeared (Fig. 3B).

LVDCCs-mediated Ca^{2+} influx will result in increases in cytosolic Ca^{2+} concentration, which will activate Clca channels. The Clca channels mediate Cl^- efflux, resulting in membrane depolarization that leads to activation of LVDCCs to enhance contraction. This was confirmed by the finding that Cl^- channel blocker niflumic acid inhibited the oscillatory contractions (Fig. 4). Consistent with this, knockout of TMEM16A, a Clca channel [46, 47], reduced the oscillatory contractions in mouse stomach smooth muscle [48]. Taken together, the contractile phase of the oscillatory contractions was produced by LVDCCs, V_{Na} channels and Clca channels.

In addition, the TEA-induced oscillatory contractions would be affected by Ca^{2+} release from the ER. This was confirmed by the finding that TEA failed to induce the oscillatory contractions following depletion of intracellular Ca^{2+} stores with TG (Fig. 8A). Thus, the released Ca^{2+} would have a role in producing the contractile phase of the oscillatory contractions. Furthermore, we found that 2-APB (Fig. 8B), but not ryanodine (Fig. 8C), abolished the oscillatory contractions. These data suggest that the release was mediated by IP_3Rs, but not by RyRs. However, previous results indicate that both IP_3Rs and RyRs play a role in the oscillatory contractions in guinea pig ASM [18]. Furthermore, in artery smooth muscle, the TEA-induced oscillatory contractions might not be modulated by the intracellular Ca^{2+} store and RyRs [49]. These discrepancies may be due to species differences, the agents used to induce the oscillatory contractions, and the state of the muscle (i.e., resting or precontracted).

Ca^{2+} release and LVDCCs-mediated Ca^{2+} influx will need extracellular Ca^{2+}. Indeed, this was confirmed by the observation that the oscillatory contractions disappeared following the removal of Ca^{2+} from the bath (Fig. 8D).

Taken together, our results suggest that the contractile phase of oscillatory contractions depends on LVDCCs-mediated Ca^{2+} influx, V_{Na} channels-mediated Na^+ entry, Cl_{Ca} channels-mediated Cl^- efflux, IP_3Rs-mediated Ca^{2+} release.

Mechanism of the relaxant phase of the oscillatory contractions

The above pathways-induced Ca^{2+} increases will activate NCX, which then transfers 1 Ca^{2+} ion to the extracellular space from the cytoplasm in exchange for 3 Na^+ ions into the cytoplasm, leading to a decrease in cytosolic Ca^{2+} to cause relaxation. This was confirmed by the finding that the replacement of Na^+ with Li^+ induced a transient enhancement of the oscillatory contractions (Fig. 5A). Moreover, the oscillatory contractions were inhibited by NCX inhibitor (Fig. 5B). Thus, NCX contributes to the relaxant phase of the oscillatory contractions.

Meanwhile, NCX will transfer Na^+ into cells, which, together with that mediated by V_{Na} channels, will activate Na^+/K^+-ATPase to remove 3 Na^+ ions from and transfer 2 K^+ ions into the cytoplasm. Thus, the result is hypopolarization, which will then cause inactivation of LVDCCs, leading to relaxation. This was supported by the observation that ouabain, an inhibitor of Na^+/K^+-ATPase, first induced a transient contraction and then abolished the oscillatory contractions (Fig. 6). However, it was previous shown that ouabain induced oscillatory contractions in uterine muscle in a concentration-dependent manner [50]. This discrepancy might be due to the different the muscle types.

Overall, the relaxant phase of the oscillatory contractions is induced by NCX-mediated Ca^{2+} efflux and Na^+/K^+-ATPase-induced hypopolarization.

Mechanism of the regeneration of oscillatory contractions

How dose the next cycle start? It could be hypothesized that the Na^+ entry mediated by V_{Na} channels and NCX will be larger than that removed from the cytoplasm by Na^+/K^+-ATPase alone. This will lead to a gradual depolarization, which activate LVDCCs inducing a contraction again. This would explain the regeneration of the oscillatory contractions. In addition, IP_3Rs have been suggested to play a role in the regeneration [51].

Potential role of the oscillatory contractions

What is the role of the oscillatory contractions? It has been suggested that the Ca^{2+} oscillations induce a sustained contraction of ASM [14, 16, 23, 25-28, 32, 33]. Our results showed not only the same fast Ca^{2+} oscillations but also a slow rhythmicity (Fig. 7). We speculated that the slow rhythmicity might underlie the TEA-induced oscillatory contractions, based on the finding that the frequency of the rhythmicity was close to that of the oscillatory contractions. However, our results indicate that the former was significantly lower than the latter. We reasoned that each cycle of rhythmicity would trigger one oscillatory contraction. The remaining contractions would be evoked by single larger Ca^{2+} oscillations.

In summary, our results indicate that TEA induces two types of Ca^{2+} changes: high frequency oscillation and low-frequency rhythmicity. All of the low-frequency rhythmicities would trigger one contraction and some of the high-frequency oscillations could also respectively trigger a contraction. Thus, the oscillatory contractions persisted. Moreover, the contractile phase of the oscillatory contractions would be induced by LVDCCs, V_{Na} channels, Cl_{Ca} channels and IP_3Rs; the relaxant phase would be induced by NCX and Na^+/K^+-ATPase; the regeneration of the oscillatory contractions would be dependent on V_{Na} channels, NCX and Na^+/K^+-ATPase.

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Disclosure Statement

All authors declare that they have no conflict of interests pertaining to this study.

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